Merkel cell polyomavirus infection and host defence in patients with Merkel cell carcinoma

HARRI SIHTO

Department of Oncology,

and Molecular Cancer Biology Program,

University of Helsinki,

Helsinki, Finland

ACADEMIC DISSERTATION

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SUPERVISED BY:

Professor Heikki Joensuu, M.D., Ph.D
Department of Oncology
University of Helsinki and
Helsinki University Central Hospital
Helsinki, Finland

REVIEWED BY:

Professor Klaus Hedman, M.D., Ph.D.
Department of Virology
Haartman Institute
University of Helsinki, and HUSLAB
Helsinki, Finland

Professor Veli-Matti Kähäri, M.D., Ph.D.
Department of Dermatology
University of Turku, and Turku University Hospital
Turku, Finland

OPPONENT:

Professor and Chair Stina Syrjänen, D.D.S., Ph.D. FDSRCSEd (hon)
Department of Oral Pathology and Radiology,
Institute of Dentistry, Faculty of Medicine
University of Turku
and
Department of Pathology (Chief Physician),
Turku University Hospital
Turku, Finland

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ABBREVIATIONS

4E-BP1	4E-binding protein 1
AIDS	acquired immune deficiency syndrome
AKT	v-AKT murine thymoma viral oncogene homolog
AMBRA1	activated molecule in beclin 1-regulated autophagy
BCL-2	B-cell chronic lymphocytic leukemia/lymphoma 2
BKPyV	BK polyomavirus
cDNA	complementary deoxyribonucleic acid
CK20	cytokeratin 20
CLL	chronic lymphocytic leukaemia
CTLA-4	cytotoxic lymphocyte-associated 4
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
FoxP3	forkhead box protein 3
HIV	human immunodeficiency virus
HPV	human papillomavirus
HPyV	human polyomavirus
HRP	horseradish peroxidise
Ig	immunoglobulin
JCPyV	JC polyomavirus
KIPyV	KI polyomavirus
LNA	locked nucleic acid
LT	large T antigen
MCC	Merkel cell carcinoma
MCPyV	Merkel cell polyomavirus
MCV	Merkel cell polyomavirus (equals MCPyV)
MECP2	methyl CpG-binding protein 2
MHC	major histocompatibility complex

mRNA	messenger ribonucleic acid
MUR	MCPyV T antigen unique region
NK	natural killer cell
PCR	polymerase chain reaction
PD-1	programmed death 1
PDGF (A)	plateled-derived growth factor (alpha)
PDGFR (A)	platelet-derived growth factor receptor (alpha)
PD-L1	programmed death ligand 1
PIK3CA	phosphatidylinositol 3-kinase, catalytic, alpha
PIK3CD	phosphatidylinositol 3-kinase, catalytic, delta
PP2A	protein phosphatase 2A
PSME3	proteasome activator subunit 3
PTPRG	protein-tyrosine phosphatase receptor gamma
qPCR	quantitative polymerase chain reaction
RASSF1A	RAS-associated domain family protein 1A
RB	retinoblastoma protein
RUNX1	RUNT-related transcription factor 1
shRNA	small hairpin ribonucleic acid
sT	small T antigen
SV40	simian virus 40
TIL	tumor infiltrating lymphocyte
TMA	tissue microarray
TP53	tumor suppressor gene TP53
TSPyV	thricodysplasia spinulosa-associated polyomavirus
TTF-1	thyroid transcription factor-1
UV	ultraviolet
WUPyV	WU polyomavirus

LIST OF ORGINAL PUBLICATIONS

This thesis is based on the following publications that are referred to in the text by their Roman numerals.

(I)   Sihto H*, Kukko H*, Koljonen V, Sankila R, Böhling T, Joensuu H. 2009 "Clinical factors associated with Merkel cell polyomavirus infection in Merkel cell carcinoma", *Journal of National Cancer Institute*, vol. 101, no. 13, pp. 938-45.

        * equal contribution

(II)   Sihto H, Kukko H, Koljonen V, Sankila R, Böhling T, Joensuu H. 2011 "Merkel cell polyomavirus infection, large T antigen, retinoblastoma protein and outcome in Merkel cell carcinoma", *Clinical Cancer Research,* vol 17, no. 14, pp. 4806-13.

(III)   Sihto H, Böhling T, Kavola H, Koljonen V, Salmi M, Jalkanen S, Joensuu H. 2012 "Tumor infiltrating immune cells and outcome of Merkel cell carcinoma: A population-based study", *Clinical Cancer Research*, vol. 18, no. 10, pp. 2872-81.

(IV)   Waltari M, Sihto H, Kukko H, Koljonen V, Sankila R, Böhling T, Joensuu H. 2011 "Association of Merkel cell polyomavirus infection with tumor p53, KIT, stem cell factor, PDGFR-alpha and survival in Merkel cell carcinoma", *International Journal of Cancer,* vol. 129, no. 3, pp. 619-28.

The publication (I) has appeared in the thesis of Heli Kukko.

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ABSTRACT

Background and purpose: Merkel cell carcinoma (MCC) is a rare and often aggressive skin cancer that usually arises in elderly individuals. MCC is frequently associated with ultraviolet radiation exposure and immunosuppressive conditions. The majority of MCCs harbor Merkel cell polyomavirus (MCPyV), a newly discovered human cancer virus. Several studies have indicated its importance in MCC tumorigenesis.

The aim of the thesis was to evaluate the frequency of MCPyV infection in MCC and to investigate its associations with patient and tumor characteristics, and with survival. In addition, we aimed to investigate the associations between MCPyV infection with cell cycle regulatory protein expression, platelet-derived growth factor receptor (PDGFR) family protein expression, *TP53, KIT* and *PDGFRA* mutations, and their associations with clinicopathological factors. We also determined the frequency and type of leukocytes that infiltrate MCC, and their associations with the presence of MCPyV DNA in MCC and clinicopathological factors including disease outcome.

Experimental design: The study was based on a population-wide MCC patient series from Finland, with the MCCs diagnosed in 1979 to 2004, and the corresponding archival formalin-fixed paraffin-embedded tumor tissue samples. The patients were identified from the files of the Finnish Cancer Registry. MCPyV DNA was detected using polymerase chain reaction (PCR) and quantitative PCR, tumor infiltrating immune cells were identified and the expression of MCPyV large T (LT) antigen and other proteins was assessed by immunohistochemistry. Gene mutations were investigated using PCR and DNA sequencing. Protein expression was usually assessed from tissue microarray (TMA) sections, while the numbers of tumor infiltrating leukocytes were counted from full tumor tissue sections. The associations between the molecular and host response factors studied and the clinicopathological factors including survival were investigated using conventional statistical tests, such as Kaplan-Meier survival analyses and Cox's proportional hazards models.

Results: We found that most (approximately 80%) MCCs harbor MCPyV DNA and that the MCPyV LT antigen was expressed in 67% of the tumors. The presence of MCPyV DNA in tumor was associated with better disease-specific (5-year survival: 75.9% vs. 41.1%, p < 0.001) and overall survival (5-year survival: 45.0% vs. 13.0%, p < 0.001) as compared to MCPyV-negative MCCs. The MCPyV DNA-positive MCCs were located more often in a limb than in a trunk or head and neck region, they had less often metastasized to regional lymph nodes at the time of the diagnosis, and less often expressed p53 and KIT than MCPyV-negative tumors (p-values < 0.05). LT antigen expression in tumor cells was associated with the female gender, location of the tumor in a limb, low cell proliferation rate, and absence of p53 expression in tumor (p-values < 0.05). Retinoblastoma protein (RB) expression was almost invariably associated with presence of MCPyV DNA and LT expression in MCC (p-values < 0.0001), whereas *TP53* mutations were found exclusively in MCPyV-negative tumors (p=0.001). The presence of MCPyV DNA and LT antigen expression in tumor were independent prognostic factors for favorable overall survival in a Cox multivariable analysis, when gender and the nodal status, or the post-surgical stage were included as covariables in the analyses.

No *KIT* or *PDGFRA* mutations were found in MCC. Tumors with p53 expression were associated with worse MCC-specific and overall survival as compared to p53-negative tumors, whereas tumor RB expression was associated with favorable survival. MCPyV DNA-positive MCCs contained significantly higher numbers of tumor infiltrating CD3+, CD8+, CD16+, FoxP3+, and CD68+ cells in comparison to MCPyV DNA-negative MCCs. A higher than the median number of CD3+, CD8+ and FoxP3+ T lymphocytes, and high CD8+/CD4+ and FoxP3+/CD4+ cell ratios in tumor were associated with favorable overall survival. Both a higher than the median number of intratumoral CD3+ cells and the presence of MCPyV DNA in tumor were independently associated with favorable overall survival in a Cox multivariable analysis that included also the nodal status and gender as covariables.

Conclusions: MCPyV-positive and -negative MCCs differ in molecular features. They show important differences also in their clinical outcomes and associations with several clinicopathological factors, as well as in host immune response. A high number of tumor infiltrating T lymphocytes and presence of MCPyV DNA in tumor were identified as novel independent prognostic factors in MCC.

REVIEW OF THE LITERATURE

1. Merkel cell carcinoma

Merkel cells are neuroendocrine cells of the skin that are essential for generation of light-touch responses (Maricich et al. 2009). They differentiate from the epidermal progenitor cells during the embryonic development. In adults, Merkel cells are replaced from the epidermal stem cells and not from differentiated Merkel cells (Van Keymeulen et al. 2009). In 1972 Toker introduced for the first time the term "trabecular carcinoma of the skin" to describe a malignant skin cancer that is now known as Merkel cell carcinoma (MCC) (Toker 1972). Six years later Tang and Toker suggested that trabecular carcinoma of skin is derived from cells of the neural crest origin, probably from Merkel cells, due to the close structural similarities found between the Merkel cells and MCC cells in electron microscopic analyses (Tang, Toker 1978). The demonstration that Merkel cells differentiate from the epidermal stem cells (Van Keymeulen et al. 2009), rare basal cell carcinoma progression to MCC (Patel, Adsay & Andea 2010) and co-existence of MCC, basal cell and squamous cell carcinomas in the same skin lesion (Cerroni, Kerl 1997), and several studies that report MCCs with squamous or sarcomatous differentiation (Walsh 2001, Hwang et al. 2008, Cooper et al. 2000), favor the hypothesis that MCC has a stem cell origin.

1.1 Epidemiology

MCC is a rare cancer that usually manifests in elderly white people. In the National Cancer Data Base of the United States, consisting of 10,020 patients diagnosed with MCC from 1986 to 2004, the majority (96.2%) of MCC patients were found to be white, whereas only 1.3% were African American and 2.5% belonged to other races (Lemos et al. 2010). Men were reported to be more frequently affected than females in Scotland (Mills, Durrani & Watson 2006), Western Australia (Girschik et al. 2011), the Netherlands (Reichgelt, Visser 2011) and the United States (Albores-Saavedra et al. 2010), whereas in Denmark (Kaae et al. 2010) and Finland (Kukko et al. 2012) the age-adjusted incidence rate is similar for both genders (Mills,

Durrani & Watson 2006, Girschik et al. 2011, Reichgelt, Visser 2011, Albores-Saavedra et al. 2010, Kaae et al. 2010, Kukko et al. 2012).

The incidence of MCC increases after the age of 60, and the mean and the median age at the time of MCC diagnosis is approximately 75 years in several series (Lemos et al. 2010, Mills, Durrani & Watson 2006, Girschik et al. 2011, Reichgelt, Visser 2011, Albores-Saavedra et al. 2010, Kaae et al. 2010, Kukko et al. 2012, Bzhalava et al. 2011, Agelli, Clegg 2003). Only less than 4% of the patients are under 50 at the time of the diagnosis (Albores-Saavedra et al. 2010, Kaae et al. 2010, Kukko et al. 2012).

The reported age-adjusted incidence rates has varied between different populations, being the lowest in Finland (1.2 cases per million per annum)(Kukko et al. 2012), followed by France (Riou-Gotta et al. 2009), Scotland (Mills, Durrani & Watson 2006), and Denmark (Kaae et al. 2010) (1.3 to 2.2 cases per million per year), and the Netherlands (Reichgelt, Visser 2011) and the United States (3.5 to 4.4) (Hodgson 2005). The highest rate has been reported form Western Australia (8.2) (Girschik et al. 2011). These aged-adjusted incidence rates may not be directly comparable, since different standard populations were used as the reference population in these analyses. The annual incidence rate has been reported to increase in the Netherlands (Reichgelt, Visser 2011), in the United States (Albores-Saavedra et al. 2010), whereas in Australia (Girschik et al. 2011) and the Nordic countries (Kaae et al. 2010, Kukko et al. 2012) the incidence rate has been relatively stable. A few studies have reported an increase in the incidence prior to its stabilization during the last few decades, which may in part reflect improved awareness of MCC among clinicians and advances in the diagnostic methods (Agelli, Clegg 2003).

1.2 Clinical features

Most of the MCC lesions are asymptomatic and may resemble benign tumors in appearance (Heath et al. 2008). In 2008 Heath and colleagues reviewed the tumor characteristics and proposed the AEIOU features (*A*symptomatic/lack of tenderness, *E*xpanding rapidly, *I*mmune suppression, *O*lder than 50 years, and *U*ltraviolet-exposed site on a person with fair

skin) to help the clinicians to recognize a lesion suspicious for MCC that warrants performing a biopsy. Heath et al. found that the majority of MCCs were asymptomatic and not tender, appeared on sun-exposed skin areas, the lesions were violaceous, pink or red in color, and the tumors typically grew rapidly during a few weeks or months. In addition, 90% of the patients were over 50 years old, 98% were white and 8% were immunosuppressed due to human immunodeficiency virus (HIV) infection, a solid organ transplant or chronic lymphocytic leukemia. Most tumors measured less than 2 cm in diameter.

Approximately 70% to 80% of MCC lesions are located in the head and neck region or in the extremities, approximately 10% manifest in the trunk, and less than 1% occur in the genitals (Lemos et al. 2010, Girschik et al. 2011, Reichgelt, Visser 2011, Albores-Saavedra et al. 2010, Kaae et al. 2010, Kukko et al. 2012, Heath et al. 2008). In a Western Australia cohort, the tumors were located on an extremity or in the head and neck region in 88% of the cases (Girschik et al. 2011). MCC may rarely occur at an unusual site such as the parotid gland or the oral mucosa (Prabhu, Smitha & Punnya 2010, Ghaderi et al. 2010). Sometimes only metastases are found without an identifiable primary tumor; the proportion of such cases is reported to vary between 0.8% and 14% of all MCC cases (Reichgelt, Visser 2011, Albores-Saavedra et al. 2010, Kukko et al. 2012, Heath et al. 2008). MCC metastases with an unknown primary tumor are found most often in men and in younger individuals, often manifesting in the lymph nodes of the head and neck region (Foote et al. 2011).

A majority of the MCC patients have local disease at presentation. In Australia and Europe the disease is local in 71% to 90% and has spread only to the regional lymph nodes in 10% to 21% of the cases, respectively (Girschik et al. 2011, Reichgelt, Visser 2011, Kaae et al. 2010, Kukko et al. 2012), whereas in the Surveillance, Epidemiology, and End Results and the National Cancer Data Base databases of the United States these figures are 56% to 66% and 27% to 35%, respectively (Lemos et al. 2010, Girschik et al. 2011, Reichgelt, Visser 2011, Albores-Saavedra et al. 2010, Kaae et al. 2010, Kukko et al. 2012). The most common sites for distant metastases are non-regional lymph nodes (27% to 60%), the skin (28% to 30%), the lungs (10% to 23%), the central nervous system (6% to 18%), bone (10% to 15%), and the liver (13%) (Medina-Franco et al. 2001, Voog et al. 1999).

1.3 Histopathological diagnosis

The diagnosis is confirmed by histopathological assessment of the tumor tissue. In hematoxylin & eosin stained tissue sections MCC cells are basophilic (purple-blue), small and monomorphic, and usually have a high nucleus-to-cytoplasm ratio. MCC cells have pale round or oval-shaped nuclei, which contain finely dispersed chromatin, and nucleoli are rarely present. The mitotic rate of the tumor cells is usually high (Bickle et al. 2004).

MCC can be divided into three histological subtypes. The most common subtype, *the intermediate cell type*, displays a solid, diffuse growth pattern (Figure 1, panel A); *the small cell type* resembles small cell lung carcinoma at histological examination and is composed of solid sheets of cells and clusters of cells that lack glandular differentiation (panel B); and the least common subtype, *the trabecular type*, contains cells growing in ribbon-like clusters and can exhibit gland-like formations (panel C) (Bickle et al. 2004). The tumors are frequently necrotic and invade into the subcutaneous adipose tissue, and a dense lymphocytic infiltration is often present within and around the tumor (Bickle et al. 2004, Mott, Smoller & Morgan 2004). Perineural (48%) or vascular invasion (44% up to 93%) is frequent in MCC, albeit the majority (66%) of tumors show only lymphovascular invasion (Mott, Smoller & Morgan 2004, Kukko et al. 2010).

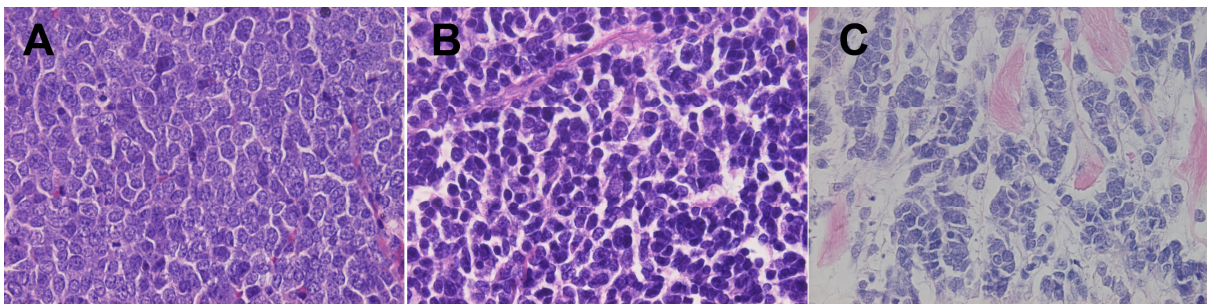Figure 1. The representative figures of histological MCC subtypes; (A) the intermediate type, (B) the small cell type, and (C) the trabecular type.

Immunohistochemical stainings are required to confirm the diagnosis and to distinguish MCC from other cancers. The World Health Organization (WHO) Classification of Tumors recommends using immunostainings to detect cytokeratin 20 (CK20), the thyroid

transcription factor-1 (TTF-1), S-100, the leukocyte common antigen and several neuroendocrine differentiation markers such as chromogranin, synaptophysin, neuron-specific enolase and bombesin in the differential diagnosis (Kohler, Kerl 2006). CK20 positivity and TTF-1 negativity differentiate MCCs effectively from other small cell carcinomas (Cheuk et al. 2001).

1.4 Etiology

A causal role of ultraviolet light (UV) exposure is supported by several findings in the pathogenesis of MCC. MCC arises frequently in sun-exposed skin areas (Lemos et al. 2010, Girschik et al. 2011, Reichgelt, Visser 2011, Albores-Saavedra et al. 2010, Kaae et al. 2010, Kukko et al. 2012, Heath et al. 2008). Geographically MCC incidence has been higher in areas with high UV-B solar index, being the highest in Australia and the lowest in the Nordic countries and Scotland (Mills, Durrani & Watson 2006, Girschik et al. 2011, Kukko et al. 2012). The incidence rates of MCC correlated with the UV-B index within the U.S., being the highest in Hawaii and the lowest in Detroit (Agelli, Clegg 2003). UV-radiation induces mutagenesis by damaging DNA, and UV-associated mutations can be found in known tumor suppressor genes such as *TP53* in cancer (Pfeifer, You & Besaratinia 2005). UV radiation induces local immunosuppression on the skin, and a number of different immune cell types such as T and B lymphocytes and mast cells are involved in this process (Fisher, Kripke 1982, Toda et al. 2011, Krasteva et al. 2002, Byrne, Limon-Flores & Ullrich 2008). MCC patients have an increased risk for non-melanoma and melanoma skin cancers before and after the diagnosis of MCC (Kaae et al. 2010, Bzhalava et al. 2011, Koljonen et al. 2010, Howard et al. 2006). UV-exposure may thus be a common risk factor for these skin cancers, and some of them may share the epidermal stem cell origin.

Host immunosuppression is also strongly associated with the genesis of MCC. A population-based study from the U.S. found that HIV/AIDS patients had an 11-fold risk for MCC in comparison to the general population (Lanoy et al. 2009). Patients with a solid organ transplant are also at a higher risk for MCC due to the long-term immunosuppression treatments (Koljonen et al. 2009, Buell et al. 2002, Penn, First 1999, Lanoy, Costagliola & Engels 2010). Interestingly, both patients with an organ transplant and HIV had the mean

age at the time of MCC diagnosis less than 50 years as compared to 75 years in patients without one of these risk factors (Buell et al. 2002, Penn, First 1999). Other immunosuppressive conditions may also predispose to MCC. In one study, five out of six young MCC patients diagnosed at the age of less than 50 had altered immunocompetence either due to pregnancy, sarcoidosis or medications such as psoralen administered for UV-A treatment of psoriasis (Sahi et al. 2010).

Chronic lymphocytic leukemia (CLL) and some other hematological malignancies are associated with MCC (Kaae et al. 2010, Koljonen et al. 2010, Howard et al. 2006, Tadmor et al. 2012, Ascoli et al. 2011). Several immune aberrations are linked with CLL, such as B cell anergy and hypogammaglobulinemia, decreased T cell responses to mitogens and aberrant T cell receptor expression, increased numbers of regulatory T cells and atypical T cell subtype ratios, disturbances in natural killer (NK) cell activity, and aberrations in granulocyte and monocyte function, cytokine balance and complement levels and activity (Tadmor, Aviv & Polliack 2011).

In 2008 Feng and colleagues found a new human polyomavirus, Merkel cell polyomavirus (MCPyV, also known as MCV) in 80% of MCCs, and other studies have later confirmed this finding (Feng et al. 2008, Kassem et al. 2008, Garneski et al. 2009). MCPyV will be discussed more thoroughly in Chapter 2.

1.5 Risk factors for recurrence and death

Men with MCC have a higher risk for death compared to women (Reichgelt, Visser 2011, Albores-Saavedra et al. 2010, Kaae et al. 2010, Kukko et al. 2012, Agelli, Clegg 2003). Tumor location in the trunk or areas other than the head and neck region is associated with worse outcome as compared to the head and neck region (Albores-Saavedra et al. 2010, Agelli, Clegg 2003, Yiengpruksawan et al. 1991, Allen, Zhang & Coit 1999), although some studies have not detected such a survival difference (Girschik et al. 2011, Reichgelt, Visser 2011, Kaae et al. 2010, Kukko et al. 2012). Results regarding a potential correlation between high age and unfavorable survival have been controversial (Reichgelt, Visser 2011, Albores-Saavedra

et al. 2010, Kaae et al. 2010, Kukko et al. 2012, Agelli, Clegg 2003, Foote et al. 2011, Allen et al. 2005). Tumor diameter smaller than 2 cm and absence of nodal or distant metastases are generally considered to be associated with favorable survival (Lemos et al. 2010, Girschik et al. 2011, Reichgelt, Visser 2011, Albores-Saavedra et al. 2010, Kaae et al. 2010, Kukko et al. 2012, Agelli, Clegg 2003, Allen, Zhang & Coit 1999). Chronic lymphocytic leukemia in history has been reported to be an adverse prognostic factor for death (Brewer et al. 2012).

The 5-year MCC-specific or relative survival rates are reported to range from 54% to 75% (Girschik et al. 2011, Reichgelt, Visser 2011, Kukko et al. 2012, Agelli, Clegg 2003, Allen et al. 2005). Several risk factors for MCC-specific survival have been identified. The risk of MCC recurrence is increased in patients who have clinically detectable lymph node metastases, the tumor shows lymphovascular invasion or the patient has leukemia or lymphoma in history (Fields et al. 2011). Presence of histopathologically verified nodal metastases is considered a strong prognostic factor for recurrence (Lemos et al. 2010, Allen et al. 2005). It has been reported that 23% to 32% of nodal metastases are not detected at clinical examination, supporting the importance of pathological examination (Allen et al. 2005, Gupta et al. 2006). In addition, in a large study consisting of 5823 patients, a pathologically verified negative nodal status had significantly better 5-year relative survival compared to patients with clinically negative nodal status (76% vs. 59%, $p < 0.0001$) (Lemos et al. 2010). In one study patients presenting with regional nodal metastases (stage III disease) from MCC and who had an occult primary tumor had better relapse-free survival compared to patients with regional nodal metastases and a known primary tumor (Foote et al. 2011).

Historically, many different staging systems for MCC were used until the American Joint Committee on Cancer (AJCC) adopted a new consensus staging system based on a large study of 5823 MCCs as proposed by Lemos and colleagues (Lemos et al. 2010). In the consensus staging system cancers without pathologically detected nodal metastases or clinically detected distant metastases are divided into stage I (diameter ≤ 2 cm) and stage II (diameter > 2 cm), tumors of any size with nodal metastases or with in-transit metastasis into stage III, and tumors with distant metastases into stage IV regardless of their size or the nodal status (Table 1).

Table 1. American Joint Committee on Cancer staging system for Merkel cell carcinoma

Stage	Tumor size	Regional nodal metastasis	Distant metastases
0	In situ primary tumor	No regional node metastasis	No distant metastasis
IA	Primary tumor ≤ 2 cm	Nodes negative in pathologic examination	No distant metastasis
IB	Primary tumor ≤ 2 cm	Nodes negative in clinical examination	No distant metastasis
IIA	Primary tumor > 2 cm	Nodes negative in pathologic examination	No distant metastasis
IIB	Primary tumor > 2 cm	Nodes negative in clinical examination	No distant metastasis
IIC	Primary tumor invades bones, muscle, fascia, or cartilage	Nodes negative in clinical and or pathologic examination	No distant metastasis
IIIA	Any primary tumor size	Micrometastasis diagnosed in sentinel or elective lymphadenectomy	No distant metastasis
IIIB	Any primary tumor size	Macrometastasis or in-transit metastasis[†]	No distant metastasis
IV	Any primary tumor size	Any nodal metastasis status	Distant metastasis

[†] Clinically detected macrometastasis that is confirmed in pathological examination of biopsy or therapeutic lymphadenectomy or in-transit metastasis that is distal to primary lesion or is located between primary tumor and draining lymph nodes.

1.6 Treatment and outcome

In the absence of randomized trials, the optimal treatment of MCC is not well known. The standard treatment for localized MCC is surgical excision of the primary tumor followed by postoperative radiation therapy. Clear surgical margins are associated with a lower risk for local recurrence as compared to involved margins (Kukko et al. 2012, Allen et al. 2005). Lateral surgical margins of 1-2 cm are usually recommended in surgical treatment protocols, and margins wider than these (> 2 cm) may not add any benefit (Kukko et al. 2012). As compared to patients treated with surgery only, postoperative radiotherapy improved the

median 5-year local relapse-free survival (93% vs. 64%, $p < 0.001$), regional relapse-free survival (76% vs. 27%, $p < 0.001$), distant metastasis-free survival (70% vs. 40%, $p=0.01$), disease-free survival (59% vs. 4%, $p<0.001$) and MCC-specific survival (65% vs. 49%, $p=0.03$) in patients with local or regional disease in a large retrospective multicenter study (Ghadjar et al. 2011). Several other studies have also suggested reduction of the local recurrence rate after radiotherapy (Medina-Franco et al. 2001, Gillenwater et al. 2001). Some investigators have suggested that locally advanced MCC considered not suitable for surgery can be treated with radiotherapy only leading to outcomes similar to those achieved with surgery and postoperative radiotherapy (Mortier et al. 2003, Pape et al. 2011).

When sentinel lymph node metastases are found, surgical excision of the primary tumor and excision of the involved nodes is recommended followed by locoregional radiotherapy (Poulsen 2004). Lymph node dissection has been found to improve relapse-free survival and decrease the risk of nodal recurrence (Allen, Zhang & Coit 1999, Allen et al. 2005). Adjuvant chemotherapy has been linked with improved outcome as compared to local therapy only in some retrospective studies and has been suggested to be given after surgery and radiotherapy (Poulsen 2004). On the other hand, Voog and colleagues concluded after a review of the literature that although MCC was chemosensitive, it was not chemocurable, and chemotherapy was associated with a risk of severe toxicity (Voog et al. 1999). Patients with overt distant metastases may be treated with palliative radiotherapy, chemotherapy or surgery.

In an analysis of 2856 MCC patients classified by the consensus staging system, the 5-year relative survival rate of patients with primary tumor ≤2 cm (T1) and who were node negative at clinical examination (cN0, stage Ib) or at pathological examination (pN0, stage Ia) was 60% and 79%, respectively. The corresponding figures for patients with tumor larger than 2 cm in diameter (>2 cm but ≤ 5 cm, T2; or >5 cm, T3) with pathologically negative nodes (T2/3pN0, stage IIb) was 58% and with clinically negative nodes (T2/T3cN0, stage IIb) 49%, and the 5-year relative survival rate was 47% when the primary tumor invaded the surrounding tissues (T4), but the regional nodes were not involved (stage IIc). Patients with micrometastases in the regional lymph nodes or with an unknown regional nodal status (stage IIIa) had a 42% 5-year relative survival rate, those with macrometastases in the regional nodes (IIIb) 26%, and patients with overtly metastatic disease (M1, stage IV) 18%

(Lemos et al. 2010). Another study reported the 5-year relapse-free survival in MCC to be 48% and the median relapse-free time 9 months (Allen et al. 2005).

2. Merkel cell polyomavirus (MCPyV)

Viruses that belong to the *polyomaviridae* family are small, non-enveloped icosahedral viruses that contain a single, circular and approximately 5000 base pair (bp) long double-stranded DNA genome (Johne et al. 2011). Polyomaviruses are widespread in the human population. They cause persistent latent infections that are usually asymptomatic, and polyomavirus-associated diseases occur mainly after reactivation of the virus in immunocompromised inviduals (Delbue, Comar & Ferrante 2012). At present, ten different spieces of human polyomaviruses are known (Table 2). The BK polyoavirus (BKPyV) was isolated from the urine of a patient with a renal transplant (Gardner et al. 1971), and the JC polyomavirus (JCPyV) from the brain of a patient with Hodgkin's disease and progressive multifocal leukoencephalopathy already in 1971 (Padgett et al. 1971). The other seven human polyomaviruses were identified recently, the KIPyV and the WUPyV from respiratory track samples (Allander et al. 2007, Gaynor et al. 2007), human polyomavirus 6 and 7 (HPyV6 and HPyV7) from skin swabs of healthy people (Schowalter et al. 2010), *trichodysplasia spinulosa*-associated polyomavirus (TSPyV) from papules of the nose of an adolescent heart transplant patient diagnosed with *trichodysplasia spinulosa* (van der Meijden et al. 2010), the HPyV9 from the serum of a kidney transplant patient (Scuda et al. 2011), and HPyV10 from stool samples of children with diarrhea and from condyloma sample of a patient with warts, hypogammaglobulinemia, infections, and myelokathexis syndrome (Siebrasse et al. 2012, Buck et al. 2012).

MCPyV was the fifth human polyomavirus identified. It was detected from mRNA libraries generated from four MCCs using digital transcriptome substraction, a method that enables detection of foreign viral transcripts from human high-throughput cDNA sequencing data (Feng et al. 2008). The presence of MCPyV DNA in a majority of MCCs was soon verified by several other studies (Kassem et al. 2008, Garneski et al. 2009, Becker et al. 2009, Foulongne et al. 2008). MCPyV turned out to be a ubiquitous virus. Circulating serum IgG antibodies against MCPyV has been found in 9% of 1- to 4-year-old children, and their

incidence has increased to 80% in people older than 50 (Chen et al. 2011, Tolstov et al. 2009). Low amounts of MCPyV DNA are detected in the normal human tissues at several different body sites, in other skin diseases, and in healthy skin flora (Kantola et al. 2009, Foulongne et al. 2010, Loyo et al. 2010). Two studies indicates that the percentage of MCPyV-positive MCCs might be lower in Australia than in countries with a lower exposure to UV radiation (Garneski et al. 2009, Paik et al. 2011), although in one study as many as 97% of Australian MCCs were MCPyV-positive (Schrama et al. 2011).

Table 2. Known human polyomaviruses

Virus	Time of finding	Disease caused by polyomavirus
BKPyV	1971	BKPyV nephropathy in renal transplant patients
JCPyV	1971	Progressive multifocal leukoencephalopathy
KIPyV	2007	
WUPyV	2007	
MCPyV	2008	Merkel cell carcinoma
HPyV6	2010	
HPyV7	2010	
TSPyV	2010	Thricodylsplasia spinulosa
HPyV9	2011	
HPyV10	2012	

2.1 MCPyV genome, structure and function

In the study where MCPyV was first identified, Feng and colleagues found that MCPyV contains a typical circular and approximately 5.4 kbp long polyomavirus genome (Figure 2) that showed the highest sequence homology with the African green monkey lymphotropic polyomavirus (also known as B-lymphotropic polyomavirus, LPyV) (Feng et al. 2008). MCPyV is known to express the small T antigen (sT), the large T antigen (LT), the 57kT antigen and capsid proteins VP1 and VP2, whereas the VP3 capsid protein may not be

functional (Shuda et al. 2008, Feng et al. 2011, Pastrana et al. 2009, Shuda et al. 2009). LT, 57kT and sT antigens are produced from overlapping genes due to alternative splicing (Shuda et al. 2008). The MCPyV genome was found to be integrated into the MCC tumor cell genome, and eight (80%) out of the ten MCCs investigated contained MCPyV DNA, whereas only five (8.5%) out of 59 of tissue samples from various body sites and four (16%) out of the 25 samples from other skin cancers were MCPyV DNA-positive with only a low viral genome copy number (Feng et al. 2008). A phylogenetic analysis of the polyomavirus genome sequences indicates that MCPyV, TSPyV and HPyV9 genomes are distinct from other human polyomaviruses and that they belong to a branch of a phylogenetic tree that includes also several non-human primate polyomaviruses and two murine polyomaviruses (Van Ghelue et al. 2012).

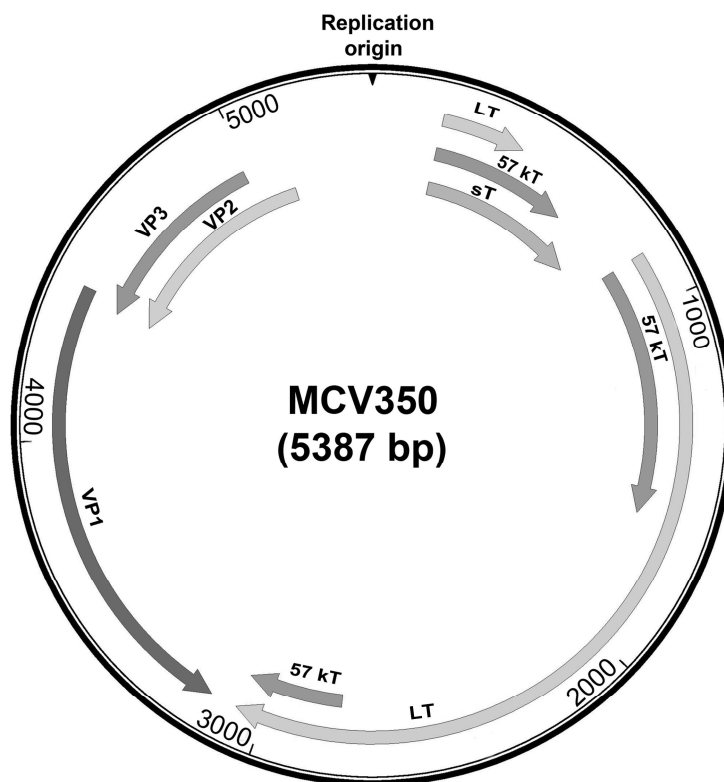
Figure 2. The organization of MCPyV genome.

The importance of the 57kT antigen for viral or cellular functions is not yet known, but it might be analogous to the simian virus 40 (SV40) 17kT antigen. The 57kT antigen contains DnaJ and the retinoblastoma (RB) protein binding domains (Shuda et al. 2008). The SV40 17kT antigen complements RB binding of the LT antigen, and its DnaJ domain promotes

transcription of cyclin A; therefore, it can promote cell cycle progression and cellular transformation (Boyapati et al. 2003, Skoczylas, Henglein & Rundell 2005). The 57kT antigen expression is detectable early after MCPyV genome transfection to human embryonic kidney 293 cells, and its splicing might depend on viral DNA replication (Feng et al. 2011).

Several molecular mechanisms for the sT and the LT antigen function have already been identified. The MCPyV sT antigen contains binding domains for the protein phosphatase 2A (PP2A) and DnaJ (Shuda et al. 2008). The promoter of the sT antigen, located in a non-coding control region of the MCPyV genome, is UV-inducible *in vitro* and the sT antigen transcription is regulated by a UV-dependent manner in the skin, whereas the LT antigen expression or the VP1 expression is not affected by the UV light (Mogha et al. 2010). MCPyV sT antigen enhances LT antigen-mediated MCPyV replication by targeting PP2A (Feng et al. 2011, Kwun et al. 2009), and its expression might be required for the production of the capsid proteins from the late gene region of the genome (Feng et al. 2011). In addition, the sT antigen promotes mitogenesis, cell proliferation and cellular transformation by reducing the hyperphosphorylated eukaryotic transcription initiation factor 4E-binding protein 1 (4E-BP1) turnover in a PP2A or DnaJ domain independent manner (Shuda et al. 2011). Its inhibition halts the cell cycle progression in MCC cell lines but does not cause cell death (Shuda et al. 2011).

The LT antigen contains DnaJ and RB binding domains, a replication origin binding domain, an ATPase domain and a helicase domain (Shuda et al. 2008). Several of these LT antigen domains function in polyomavirus genome replication (Khopde, Roy & Simmons 2008). The DnaJ domain is conserved in polyomaviruses (Khopde, Roy & Simmons 2008) and it is essential for viral DNA replication, cellular transformation, transcriptional activation, and virion assembly (Chromy, Pipas & Garcea 2003, Sullivan, Pipas 2002, Srinivasan et al. 1997, Fewell, Pipas & Brodsky 2002, Campbell et al. 1997). An intact DnaJ region is also required for MCPyV replication (Kwun et al. 2009). The polyomavirus DnaJ domain is known to recruit and stimulate the heat shock protein 70 (Hsc70), a chaperone protein that induces disruption of the RB-E2F complex, and therefore induces E2F driven expression of the genes required for the G1 to S phase entry of the cell cycle (Srinivasan et al. 1997). The origin binding domain recognizes and binds to the replication origin and induces an unwinding of

the double-stranded DNA by the action of helicase domain (Dean et al. 1987, Foster, Simmons 2010). DNA unwinding and translocation is powered by the LT ATPase domain (Shi et al. 2009). In addition, the SV40 LT domain recruits cellular proteins such as topoisomerase I, replication protein A, and DNA polymerase α that are required for DNA replication (Khopde, Roy & Simmons 2008, Braun et al. 1997).

Polyomavirus LT antigens are known to bind also two important tumor cell cycle suppressor proteins, p53 and RB (Sullivan, Pipas 2002). The MCPyV LT antigen targets RB, a key regulator of the cell cycle G1 to S phase progression, whereas the p53 binding coding region is often missing from the mature LT antigen due to MCC-specific mutations in the MCPyV genome (Shuda et al. 2008). However, both wild-type and truncated LT antigen are able to reduce p53 expression in the UISO MCC cell line with a yet unidentified mechanism (Demetriou et al. 2012). A functional LT RB binding domain is required to induce gene expressions of survivin, an apoptosis inhibitor protein, and the E2F1 transcription factor and cyclin E, promoters of G1 to S phase progression (Arora et al. 2012).

The LT antigen contains also an MCV (MCPyV) T antigen unique region (MUR) adjacent to the RB-binding site (Liu et al. 2011). The MUR region binds to the clathrin heavy chain repeat domain in the hVam6p protein and translocates hVam6p to the nucleus (Liu et al. 2011). Surprisingly, hVam6p is able to inhibit MCPyV virion production suggesting that it may have a role in self-regulatory viral replication repression that might sustain viral latency (Feng et al. 2011) Nuclear sequestration inhibits hVam6p-induced lysosomal clustering, and has been suggested to contribute also to viral uncoating or virus egress (Liu et al. 2011).

 In addition to viral protein expression, the MCPyV genome encodes MCV-mir-M1-5p, a microRNA that is expressed at low levels in 50% of MCPyV-positive MCCs (Seo, Chen & Sullivan 2009, Lee et al. 2011). The MCV-mir-M1 sequence shows antisense orientation to the LT antigen and likely downregulates LT antigen expression (Lee et al. 2011). Similarly, SV40 expresses SV-microRNA that downregulates T antigen expression and CD8 cell interferon-γ release in late infection, but does not affect the VP protein expression and infectious virus assembly. As a consequence of T antigen downregulation, the infected cells are less susceptible to lysis induced by CD8 cells (Sullivan et al. 2005).

Other predicted targets of MCV-mir-M1 are cellular genes *AMBRA1*, *FOX2*, *MECP2*, *PIK3CD*, *PSME3* and *RUNX1*, which have roles in neural and immune system function, and in mRNA splicing (Lee et al. 2011). AMBRA1 has an important role in regulation of autophagy and in the nervous system development (Fimia et al. 2007), and a chromatin structure regulating protein MeCP2 that is expressed predominantly in neuronal nuclei, may have a crucial role in neuronal maintenance (Skene et al. 2010). FOX2 is an alternative exon splicing regulator, associated with some cell types and cancer-specific mRNA splicing (Lapuk et al. 2010). RUNX1 is a transcription factor that stimulates polyomavirus DNA replication and regulates T cell differentiation and function (Murakami et al. 2007, Wong et al. 2011). *PIK3CD* encodes the subunit protein p110δ of the PI3K that is important for antigen receptor signaling in B and T cells and for cytotoxicity of natural killer cells against cancer cells or virally infected cells (Zebedin et al. 2008, Okkenhaug et al. 2002, Guo et al. 2008). *PSME3* encodes a subunit of the immunoproteasome activator PA28 that enhances MHC I class antigen processing and epitope presentation in infection (de Graaf et al. 2011).

2.2 MCPyV in tumorigenesis

Several studies indicate that MCPyV is not just a passenger virus but contributes to tumorigenesis in the majority of MCCs. Most MCCs contain MCPyV DNA (Feng et al. 2008, Kassem et al. 2008). The MCPyV DNA copy numbers are significantly higher in MCC samples than in samples from other tissues of healthy or immunocompromised individuals (Garneski et al. 2009, Becker et al. 2009, Loyo et al. 2010). The MCPyV genome is clonally integrated into the MCC tumor cell genome suggesting that viral integration is an early event in MCC tumorigenesis (Feng et al. 2008). The integrated viral genome contains frequently MCC-specific truncating mutations that prevent virus replication within the tumor cells (Feng et al. 2008, Kassem et al. 2008, Shuda et al. 2008, Laude et al. 2010, Martel-Jantin et al. 2012, Schmitt et al. 2012). Like MCPyV, other cancer viruses may become replication incompetent by number of different molecular mechanisms (zur Hausen 2008). Viral replication incompetence prevents MCPyV reactivation and replication that might prevent the host cell survival (Shuda et al. 2008). Mutations may increase the transforming properties of MCPyV as with SV40 polyomavirus (zur Hausen 2008, Roberge, Bastin 1988).

Houben and colleagues showed that expression of the T antigen is necessary for the growth and survival of MCPyV-positive MCC cell lines (Houben et al. 2010). Silencing of T antigen expression with exon 1 targeting shRNA caused cell cycle arrest or apoptosis of MCPyV-positive cell lines, but did not affect a MCPyV-negative cell line (Houben et al. 2010). In a subsequent study the same authors found that a silenced T antigen expression by shRNA induces MCC regression in a mouse xeno-transplantation model (Houben et al. 2012a). Release of RB from the interaction with the T antigen caused accumulation of MCC cells into the G1 phase and cell cycle arrest (Houben et al. 2012a). Although the LT antigen targets RB, sT antigen expression may also be required for tumor cell proliferation (Shuda et al. 2011). Binding of RB by the LT antigen induces expression of survivin in addition to cell cycle dysregulation (Arora et al. 2012). Survivin expression is critical for sustained growth of MCPyV-positive MCCs, and its selective inhibition by a small-molecule inhibitor YM155 halts tumor growth of xenograft MCCs in mice (Arora et al. 2012).

Expression of MCPyV sT antigen induces anchorage- and contact-independent growth in a rodent Rat-1 fibroblast cell line, whereas LT antigen expression alone is incapable of cell transformation (Shuda et al. 2011). Expression of sT antigen sustains also serum-independent growth of human fibroblasts (Shuda et al. 2011). In UISO MCC cell lines, ectopically expressed mutant LT antigen impairs repair of UV-induced DNA damage and reduces expression of XPC, a protein that functions in DNA damage recognition, and expression of p53 and p21, proteins that induce cell cycle arrest after DNA damage (Demetriou et al. 2012).

3. Molecular biology of Merkel cell carcinoma

Besides to MCPyV, relatively little is known about other factors that contribute to MCC molecular pathogenesis. Commonly occuring genomic aberrations found in comparative genomic hybridization analysis of MCCs are gains of 1q, 3q, 5p, 8q and within chromosome 6, and losses of 3p, 13q and within chromosome 4 (Larramendy et al. 2004, Van Gele et al. 2002, Popp et al. 2002, Paulson et al. 2009). In general, MCPyV-positive MCCs contain

fewer genetic aberrations than MCPyV-negative MCCs (Paulson et al. 2009). Amplification of the L-Myc proto-oncogene in 1p34 is found in 39% of MCCs (Paulson et al. 2009). Approximately 15% of MCCs contain p53 tumor suppressor protein mutations (Van Gele et al. 2000, Lassacher et al. 2008), and expression of p63, another p53 family protein, is associated with unfavorable outcome (Asioli et al. 2011). *PIK3CA* mutations are also found in MCC, and the mutated tumor cells might thus be sensitive for PI3K/AKT-pathway inhibitors (Nardi et al. 2012, Hafner et al. 2012). Epigenetic inactivation of tumor suppressor gene *RASSF1A* expression by hypermethylation of the promoter region is found in 51% of MCCs (Helmbold et al. 2009).

Some studies have assessed expression of an anti-apoptotic protein Bcl-2 and found it to be expressed frequently in MCC (Plettenberg, Pammer & Tschachler 1996, Feinmesser et al. 1999, Tucci et al. 2006, Sahi et al. 2012). However, in a multicenter phase II trial carried out in a series of 12 patients with advanced MCC and treated with a Bcl-2 targeting agent (G3139, Genasense), no responses were observed, suggesting that Bcl-2 may not have a key role in MCC tumorigenesis (Shah et al. 2009). A small-molecule survivin inhibitor, YM155, has been reported to have a cytostatic effect on MCC xenograph tumors in mice (Arora et al. 2012).

Immunohistochemical studies on cell adhesion and migration linked proteins showed that P-cadherin expression is associated with prolonged recurrence-free survival and the presence of MCPyV DNA in tumor (Vlahova et al. 2012). In the normal tissues, Merkel cells are connected to the surrounding keratinocytes by E- and P-cadherins, whereas in MCC this linkage is switched to N-cadherin, which might facilitate tumor cell invasion and migration (Werling et al. 2011). Tetraspanins CD9 and CD151 are also associated with outcome of MCC patients (Woegerbauer et al. 2010). Tumor CD151 expression is associated with poor overall survival, in line with its known role as a tumor invasion promoting factor (Woegerbauer et al. 2010, Wang et al. 2011). On the other hand, tumor CD9 expression is associated with favorable disease-free and overall survival in agreement with its function as a tumor and cell invasion suppressive factor (Woegerbauer et al. 2010, Wang et al. 2011).

Expression of the KIT receptor tyrosine kinase has been studied by many investigators of MCC. The proportion of tumors considered KIT-positive has varied widely in different studies, ranging from 7% to 89% (Su et al. 2002, Feinmesser et al. 2004, Swick et al. 2007, Brunner et al. 2008, Krasagakis et al. 2009, Andea et al. 2010, Kartha, Sundram 2008). The variation in the proportion of KIT-positive tumors reported might be explained by use of different antibodies and epitope retrieval methods in immunohistochemistry (Yang et al. 2009). The interest to study KIT in MCC was fuelled by the effectiveness of small molecule tyrosine kinase inhibitors in other human tumor types such as gastrointestinal stromal tumor and some melanomas, where *KIT* mutations activate downstream survival signaling routes important for cancer pathogenesis (Carvajal et al. 2011, Joensuu et al. 2001, Joensuu et al. 2011, George et al. 2009). However, *KIT* mutations have not been found in MCC (Andea et al. 2010, Kartha, Sundram 2008). PDGFRA and PDGFA expression is frequent in MCCs (Kartha, Sundram 2008, Swick et al. 2008), and single nucleotide substitutions are present within the *PDGFRA* gene (Kartha, Sundram 2008, Swick et al. 2008), but these are likely single nucleotide polymorphisms that are found at a high frequency also in healthy human populations. One study suggested that KIT expression in MCC tends be associated with an unfavorable clinical outcome (Andea et al. 2010), but other studies have not confirmed this (Su et al. 2002, Feinmesser et al. 2004, Llombart et al. 2005). Growth of the KIT-positive MCC-1 cell line could be inhibited with small molecule KIT inhibitors imatinib and nilotinib, but in a clinical trial imatinib turned out to be ineffective in treatment of patients with advanced MCC (Samlowski et al. 2010). However, a single patient response to pazopanib (an inhibitor of KIT and several other kinases) has been reported (Davids et al. 2009).

4. Host defence and MCC

Already in 1909 Paul Ehrlich proposed that the immune system can repress growth of cancers that occur spontaneously and that cancers would otherwise manifest at an overwhelming frequency (Manjili 2011). This idea was refined in the 1950s to a hypothesis of cancer immunosurveillance by Burnet, who suggested that small tumors may develop and express antigens that provoke immunological reaction and tumor regression without causing clinical symptoms (Burnet 1957), and by Thomas who suggested that the immune system is evolutional necessity to protect multicellular organisms from malignancy (Dunn et al. 2002,

Manjili 2011). The concept of cancer immunosurveillance is supported by the observation that people with an immune deficiency such as organ transplant recipients, and HIV or AIDS patients are more susceptible to cancer, especially to cancers with a known infectious cause (Grulich et al. 2007). On the other hand, many cancers may evolve at sites of infection, chronic irritation or inflammation (Coussens, Werb 2002). At present, it is understood better that the immune system has a complex role in tumorigenesis, it can either limit or enhance tumor growth and progression depending on the type of immune cells that may be activated in tumor tissue.

4.1 Tumor infiltrating immune cells

4.1.1 Lymphocytes

B and T lymphocytes are cells of the adaptive immune system, and their immune response against neoplastic or infected cells is awakened after recognition of a specific foreign antigen by cell type-specific receptors. A high number of tumor infiltrating lymphocytes (TILs) is associated with a favorable outcome in many types of human cancer, such as melanoma, small cell lung carcinoma and carcinomas of the ovary, colorectum, esophagus and breast (Clemente et al. 1996, Erdag et al. 2012, Hwang et al. 2012, Pages et al. 2005, Pages et al. 2005, Zhang et al. 2003, Galon et al. 2006, Schumacher et al. 2001, Mahmoud et al. 2011, Eerola, Soini & Paakko 2000). In particular, the presence of cytotoxic CD8-positive T cells was associated with a favorable outcome in a meta-analysis that compared the prognostic significance of different types of tumor infiltrating T cells in several types of malignancies (Gooden et al. 2011). This is supported by a finding that T helper (CD4+) cell and CD8+ cell deficient mice were more prone to 3'-methylcholantrene-induced sarcoma than wild type mice (Koebel et al. 2007), and in a spontaneous melanoma mouse model CD8+ cells were able to prevent growth of disseminated cells, but soon after CD8+ cell depletion the mice rapidly developed metastases (Eyles et al. 2010).

Immunoregulatory CD4-positive T helper cells and their subtype, CD25+/FoxP3+ T regulatory (Treg) cells are generally thought to suppress anti-tumoral effects of cytotoxic T cells (Terabe, Berzofsky 2004). Depletion of CD4+/CD25+ cells with anti-CD25 (anti-interleukin-2 receptor alpha) antibody in mice caused regression of tumors in four of five

cancer types examined in syngenic mice (Onizuka et al. 1999), and depletion of CD25+ cells together with administration of an antibody against a CTLA-4 antigen, the CD8+ cell responses downregulating antigen, resulted in a synergistic effect on B16 melanoma regression in mice (Sutmuller et al. 2001). Of note, overall survival of patients with metastatic melanoma improved when treated with an anti-CTLA-4 antibody ipilimumab plus dacarbazine as compared to dacarbazine alone in a large clinical trial (Robert et al. 2011). A recent meta-analysis demonstrated that tumor infiltrating FoxP3+ cells are associated with favorable prognosis in colon carcinoma, whereas they were associated with poor survival in hepatocellular cancer, and inconsistently with prognosis of several other tumor types (Deleeuw et al. 2012). These results suggest that Treg cells may have a different role depending on the tumor type.

The functional role of B cells as tumor infiltrating lymphocytes (TILs) has not been studied extensively. B cells function as antigen presenting cells and produce antibodies against tumor-associated antigens, such as overexpressed or mutated proteins (Reuschenbach, von Knebel Doeberitz & Wentzensen 2009). Studies comparing B cell deficient and wild type mice showed that B cells may inhibit Th cell-mediated activation of anti-tumorigenic CD8+ cells (Qin et al. 1998, Shah et al. 2005), whereas DeLillo and colleagues found that depletion of mature B cells in wild type mice using a CD20-antibody increased melanoma growth and impaired CD4+ and CD8+ cell activation (DiLillo, Yanaba & Tedder 2010). B cell tumor infiltration is associated with favorable disease-specific survival in soft tissue sarcomas treated surgically with wide resection margins (Sorbye et al. 2011), and was recently found to be associated with favorable disease-specific and disease-free survival in a large breast cancer cohort (n=1470) (Mahmoud et al. 2012).

Natural killer (NK) cells are cytotoxic cells of the innate immune system that recognize and kill neoplastic cells without need of prior sensitization, and attract other immune cells into their vicinity by releasing interferon-gamma (IFN-γ) (Schoenborn, Wilson 2007). Their pivotal role in spontaneous resistance against tumor growth and metastasis has been shown in NK cell deficient or NK reactivity suppressed mice (Talmadge et al. 1980, Gorelik et al. 1982). In humans, low NK cell activity is associated with familial melanoma (Hersey et al. 1979) and increased risk of cancer (Imai et al. 2000).

4.1.2 Macrophages

Macrophages are phagocytic cells of the innate immune system that can identify and kill tumor cells, engulf and digest cell debris, and induce anti-tumoral responses of the immune system by expressing immunostimulatory cytokines and by presenting digested tumor antigens for Th cells on their cell membrane MHC class II molecules (Jadus et al. 1996, Bingle, Brown & Lewis 2002). Several studies have found that presence of a high number of tumor-associated macrophages correlates with poor prognosis of cancer patients (Bingle, Brown & Lewis 2002, Lewis, Pollard 2006). The dual role of macrophages in cancer may be explained by their heterogeneity. Depending on the microenvironmental stimuli at the target tissue, monocytes can differentiate towards one of the two extremes of the macrophage lineage continuum; either to M1 macrophages, which activate immune responses against malignant cells or to M2 macrophages, which down-regulate M1-mediated immune responses, express less antigen-presenting MHC II protein, and promote angiogenesis, tumor cell invasion and metastases (Mantovani et al. 2002, Lewis, Pollard 2006, Movahedi et al. 2010). Tumor-associated macrophages are considered to be mainly M2 macrophages.

4.2 Merkel cell carcinoma and immune cells

Spontaneous regressions of MCC occur in up to 1.5% of patients, usually after a tumor biopsy (Inoue et al. 2000, Burack, Altschuler 2003, Herrmann et al. 2004, Kubo et al. 2007, Richetta et al. 2008, Vesely et al. 2008, Turk et al. 2009, Ciudad et al. 2010). At least two likely immune function-related complete remissions have been reported, one in MCC of the scalp with multiple local and regional metastases after topical treatment with the immune system-stimulating agent dinitrochlorobenzene (Herrmann et al. 2004), and the other one in a patient with metastatic MCC with HIV after antiretroviral therapy (Burack, Altschuler 2003). Spontaneously regressing MCCs have more TILs compared to non-regressing tumors, suggesting that the immune system may sometimes restrict tumor growth (Inoue et al. 2000).

A high number of tumor infiltrating mast cells and lymphocyte infiltrates are associated with favorable prognosis in MCC (Llombart et al. 2005, Beer, Ng & Murray 2008, Andea et al. 2008). In a more detailed study, Paulson and colleagues first identified a cluster of immune response-related genes from a gene expression profile, and the cluster was associated with good prognosis in a series of MCC patients. CD8 cell-associated genes turned out to be overexpressed in this gene cluster, and immunohistochemical staining of intratumoral CD8 cells confirmed that a high number of cytotoxic T cells in tumor was an independent prognostic factor for favorable MCC-specific survival in a multivariate analysis (Paulson et al. 2011).

The humoral immune response to MCPyV might also affect the outcome in MCC. Interestingly, the blood IgG antibody levels against the MCPyV VP1 capsid protein were higher in general in MCC patients than individuals without MCC (Tolstov et al. 2009, Pastrana et al. 2009). In one study, high blood VP1 antibody levels were associated with a low risk for MCC recurrence (Touze et al. 2011). The blood levels of anti-T antigen antibodies correlate with the tumor burden in MCC patients (Paulson et al. 2010).

AIMS OF THE STUDY

1. To investigate the frequency of MCPyV infection in MCC and its association with patient and tumor characteristics, and survival.

2. To investigate the associations between presence of MCPyV DNA and LT antigen expression in MCC, PDGFR family protein and cell cycle regulatory protein expression, *TP53*, *KIT* and *PDGFRA* mutations, and their associations with patient and tumor characteristics, and survival.

3. To evaluate the frequency and type of tumor infiltrating immune cells in MCC, and to investigate their associations with the presence of MCPyV DNA in MCC and clinicopathological factors including patient outcome.

MATERIALS AND METHODS

1. Patients and tumors

Patients diagnosed with MCC in Finland from January 1, 1979 to October 24, 2004 were identified from files of the Finnish Cancer Registry. A total of 207 individuals with a diagnosis of MCC could be identified. The first case of MCC was diagnosed in Finland in 1983. The Finnish Cancer Registry was founded in 1952, and a systematic survey on the coverage of the registry performed in 1985 to 1988 found the registry to cover 99% of all solid tumors and 92% of hematologic malignancies diagnosed in Finland. The coverage of the registry thus approaches 100% (Teppo, Pukkala & Lehtonen 1994, Korhonen et al. 2002).

From the 207 patients, thirty-seven were excluded due to lack of tumor tissue for histological review, 13 because the MCC diagnosis was not confirmed at histopathological review, and eight since the site of the primary tumor was not known; in 16 cases clinical data were not available. A flow chart considering patient exclusion criteria for studies I to IV is provided in Figure 3.

MCC diagnosis was confirmed when tumor morphology was compatible with MCC on hematoxylin-eosin-stained slides and the tumor cells were CK20-positive in immunohistochemistry. Three cases in which the tumor did not express CK20 were included in the series, because these tumors did express both synaptophysin and chromatogranin A. To exclude metastatic small cell lung carcinomas, negative immunostaining for TTF-1 was also required. Tumor histology was classified either as small cell, trabecular cell or intermediate cell type, following the World Health Organization criteria (Kohler, Kerl 2006).
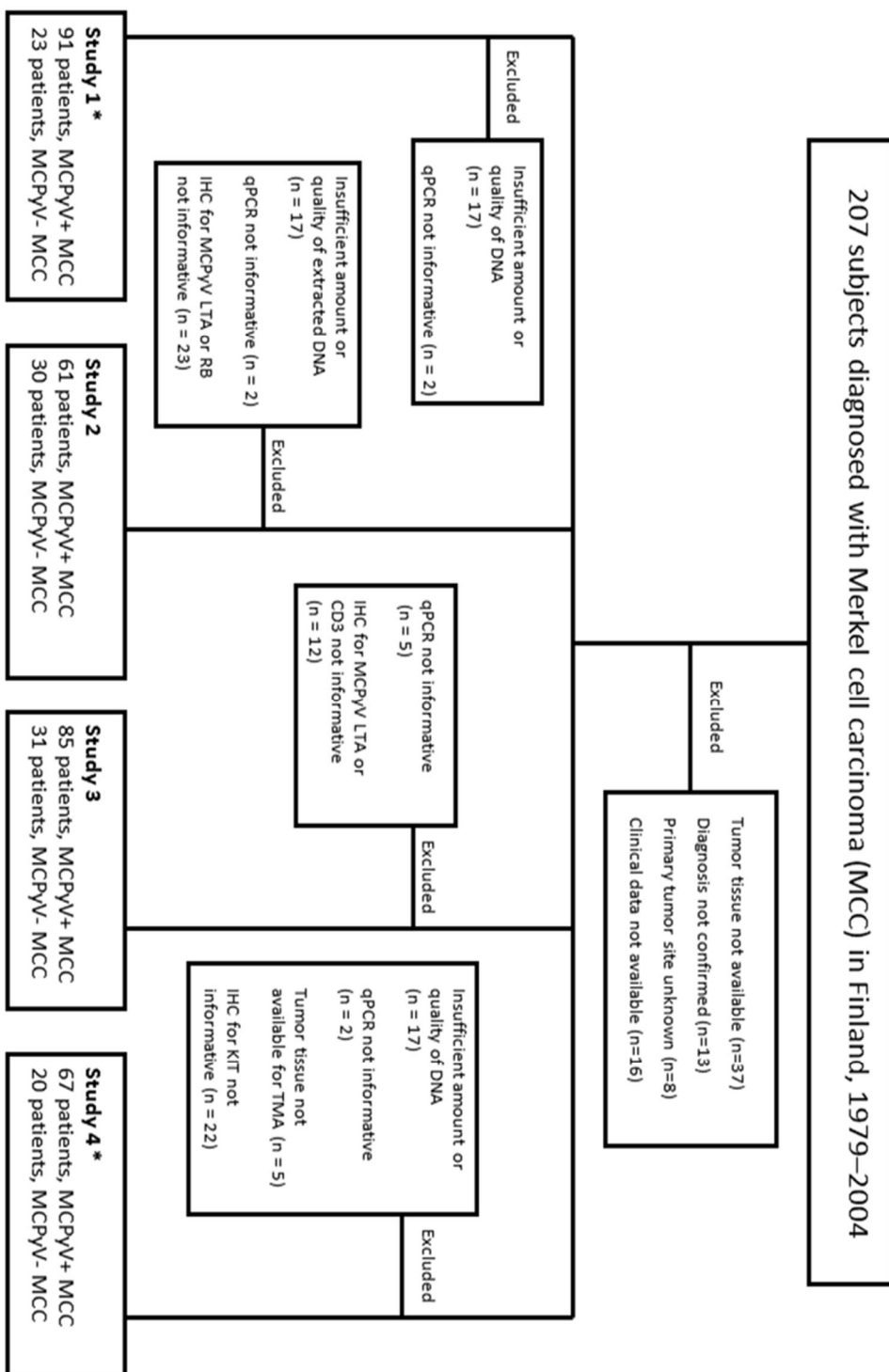
Figure 3. Consort diagram. * MCC was considered positive if qPCR result > 0 in studies 1 and 4, whereas in studies 2 and 3, cut-off for positivity was MCPyV DNA to *PTPRG* ratio > 0.1.

Tumors containing mixed histological MCC subtypes were classified as trabecular cell subtype if the trabecular cell morphology was present, whereas tumors with mixed small cell and intermediate cell types without trabecular cell morphology features were classified as intermediate cell subtype. The tumor diameter was measured from hematoxylin-eosin stained slides whenever feasible. When the size could not be estimated from tissue sections, the diameter provided in case records was accepted.

Clinical data were extracted from the hospital case records and from the case records of the primary care centers. The date and cause of death was obtained from the files of the Finnish Cancer Registry and the Register Office of Helsinki.

2. Tissue microarray (II-IV)

A representative region of MCC was identified from hematoxylin-eosin slides, and 0.6 mm diameter tumor tissue biopsy cores were used for construction of a tissue microarray (TMA). Two parallel sample cores were taken for each TMA block whenever feasible, and two parallel TMA blocks were made.

3. Immunohistochemistry (II-IV)

For all immunohistochemical stainings, a 5 µm tissue section was cut either from a TMA or a MCC tissue block on a SuperFrost+ slide (Menzel-Gläser, Braunschweig, Germany). The tissue sections were deparaffinised in xylene and rehydrated through a decreasing alcohol gradient. Endogenous peroxidase activity was blocked by incubating the sections in 3% hydrogen peroxide for 30 minutes, except for CD4 immunostaining, where blocking was performed with 0.5% hydrogen peroxide in methanol for ten minutes. The antibodies, their dilutions, incubation times and temperatures, and the methods used for antigen retrieval are given in Table 3. Binding of the primary antibody was detected by using a PowerVision+ Poly-HRP Histostaining kit (Immunovision Technologies Co, Daly City, California), except for CD3 and CD68 stainings, where detection was carried out using a double-labeling

method (impress Anti-mouse and Anti-rabbit Ig Polymer detection kits, Vector Laboratories Inc, Burlingame, California). Tissue samples with known antigen expression were used as positive and negative controls. The immunostainings were scored either based on the staining intensity or the percentage of positive cells.

Table 3. A list of antibodies, their manufacturers, dilutions, pre-treatment buffers and methods used.

Protein	Antibody clone	Dilution	Incubation time and temperatute	Pre-treatment buffer☐	Manufacturer
Cyclin D1	SP4	1:50	30 min at RT	SC + Autoclave	Thermo Fisher Scientific, Fremont, CA
Cyclin E	HE-12	1:100	30 min at RT	SC + Autoclave	Thermo Fisher Scientific
Ki-67	MM1	1:500	overnight at 4°C	SC + Autoclave	Novocastra Laboratories Ltd, Newcastle Upon Tyne, UK
MCPyV LTA	CM2B4	1:100	1h at RT	SC + wb	Santa Cruz Biotechnology, Inc, Santa Cruz, CA
MDM-2	MDM2	1:100	overnight at 4°C	SC + Autoclave	Novocastra Laboratories Ltd
p16	JC8	1:600	overnight at 4°C	SC + Autoclave	Santa Cruz Biotechnology, Inc
p21	DCS-60.2	1:30	1h at RT	SC + Autoclave	Thermo Fisher Scientific
p27	DCS-72.F6	1:150	1h at RT	SC + Autoclave	Thermo Fisher Scientific
p53	DO7	1:500	overnight at 4°C	SC + Autoclave	Novocastra Laboratories Ltd
RB	1F8	1:200	30 min at RT	SC + Autoclave	Thermo Fisher Scientific
Phospho-RB	Ser807/811	1:400	overnight at 4°C	SC + Autoclave	Cell Signalling Technology, Inc, Danvers, MA
CD3	SP7	1:100	30 min at RT	SC + Autoclave	Thermo Fisher Scientific
CD4	NCL-CD4-1F6	1:50	1h at RT	EDTA + wb	Novocastra Laboratories Ltd
CD8	SP16	1:100	20 min at RT	SC + Autoclave	Thermo Fisher Scientific
CD16	2H7	1:20	20 min at RT	SC + Autoclave	Thermo Fisher Scientific
CD68	PG-M1	1:50	30 min at RT	SC + Autoclave	Thermo Fisher Scientific
CD163	10D6	1:70	20 min at RT	SC + Autoclave	Thermo Fisher Scientific
CLEVER-1		1:10	overnight at 4°C	Proteinase K	Self produced (Palani et al. 2011)
FoxP3	236A/E7	1:300	1h at RT	SC + Autoclave	Abcam, Cambridge, UK
KIT	A4502	1:300	overnight at 4°C	SC + wb	DakoCytomation, Glostrup, Denmark
PDGFRA	C-20	1:1000	overnight at 4°C	SC + Autoclave	Santa Cruz Biotechnology, Inc
SCF	#2273	1:200	overnight at 4°C	SC + wb	Cell Signalling Technology, Inc
Phospho-KIT	Tyr719/#3391	1:35	overnight at 4°C	SC + wb	Cell Signalling Technology, Inc

* wb = water bath (at 98°C for 20 to 30 minutes); SC = sodiumcitrate (10 μmol/L, pH 6.0), EDTA = Ethylenediaminetetraacetic acid (1 mmol/L, pH 9.0).

4. Polymerase chain reaction (I, II, IV)

Genomic DNA was extracted from the tissue samples after deparaffinization using a QIAamp DNA Mini kit (Qiagen GmbH, Hilden, Germany), and polymerase chain reaction (PCR) was performed using a FastStart Taq DNA Polymerase dNTPack kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturers' instructions. The PCR reaction contained 50 ng of DNA, 1x PCR buffer, 2 mmol/L of $MgCl_2$, 200 µmol/L of dNTP mix, 1 U of FastStart DNA polymerase and 0.3 µmol/L of forward and reverse primers for a 20 µL reaction. The primer sequences are shown in Table 4. The PCR cycling conditions consisted of an initial denaturation step (95 °C, four minutes) followed by 40 to 60 cycles of denaturation (95 °C, 30 seconds), annealing (at 60 °C in study I and II, and at 56 °C in study IV, 30 seconds), and elongation (72°C, 45 seconds). The final elongation was performed at 72 °C for seven minutes. The PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining, and sequenced. To prevent contamination DNA samples and PCR products were handled in different rooms. In addition, a reaction without template DNA was amplified in PCR, to detect any possible contaminations on PCR reagents.

5. Quantitative PCR (I-IV)

MCPyV DNA was detected and its copy numbers measured by real-time quantitative PCR (qPCR) with hydrolysis probes. DNA (50 ng) was amplified in 20 µL using a LightCycler 480 probe master kit (Roche Diagnostics GmbH) and a reaction mix containing 1x PCR buffer, 100 nmol/L of probe for the target gene, 200 nmol/L of each primer specific for the MCPyV LT3 antigen gene sequence or for a protein-tyrosine phosphatase receptor gamma (*PTPRG*) exon 19 sequence used as a reference. The primers and probes were designed using the ProbeFinder program (www.universalprobelibrary.com, Roche Diagnostics GmbH), and the locked nucleic acid (LNA) probes (LNA probe 65 for *PTPRG* and LNA probe 6 for *LT3*) were obtained from the Universal ProbeLibrary Set (Roche Diagnostics GmbH). Primer sequences are given in Table 4. LT3 products were sequenced to verify specificity of qPCR.

 Table 4. Genes and exons analyzed, primer sequences and the

PCR annealing temperatures.

Gene and Exon	Primer sequence (5'->3')	
	Forward	Reverse
Study I		
LT1*	tacaagcactccaccaaagc	tccaattacagctggcctct
LT3*	ttgtctcgccagcattgtag	atatagggggcctcgtcaacc
VP1*	tttgccagcttacagtgtgg	tggatctaggccctgattttt
PTPRG intron 2	tatgggagtgtgggatggtt	taagctgggaggatcgctta
qPCR		
LT 3	gcatctgcaccttttctagactc	tttttgccttatagacttttccatatct
PTPRG exon 19	ctacattgccacccaagga	tccagttgttttgttcccaaat
Study II		
TP53 exon 4.1	cctggtcctctgactgctct	ggtgtaggagctgctggtg
TP53 exon 4.2	gtccagatgaagctcccaga	gccaggcattgaagtctcat
TP53 exon 5	tctgtctccttcctcttcctaca	aaccagccctgtcgtctct
TP53 exon 6	caggcctctgattcctcact	cttaacccctcctcccagag
TP53 exon 7	cttgggcctgtgttatctcc	gggtcagaggcaagcaga
TP53 exon 8	gcttcttgtcctgcttgctt	gggagtagatggagcctggt
TP53 exon 9	ggagaccaagggtgcagtta	ccccaattgcaggtaaaca
Study IV		
KIT exon 9	gtatgccacatcccaagtgt	catgactgatatggtagaca
KIT exon 11	ccagagtgctctaatgactg	ggaagccactggagttcctt
KIT exon 13	gacatcagtttgccagttgt	tgttttgataacctgacagac
KIT exon 17	gcaacactatagttattaaaaag	cctttgcaggactgtcaagca
PDGFRA exon 10	cccaactccttgccatctta	tcagctgatgagttgtcctga
PDGFRA exon 12	atgtggagtgaacgttgttgg	ctagttcttactaagcacaagc
PDGFRA exon 14	gctcagctggactgatatgtga	ccagtgaaaatcctcactcca
PDGFRA exon 18	caggggtgatgatgctattcagc	ttaaagtgaaggaaggatgagcc

* Primer sequences provided in Feng et al. (Feng et al, 2008)

The PCR cycling consisted of an initial denaturation step (at 95 °C, for ten minutes), followed by 45 cycles with denaturation (95 °C, ten seconds), annealing (60 °C, 30 seconds) and elongation (72 °C, 45 seconds) steps. The fluorescence at the end of the annealing step of each cycle was measured, and the collected data were analyzed using the Advanced Relative Quantification software (Roche Diagnostics GmbH). The MCPyV DNA content of each tissue sample was expressed in relation to that of MCPyV DNA to *PTPRG* DNA. A calibrator sample was chosen from a randomly selected set of 88 MCC samples analyzed in a preliminary qPCR run based on a criterion that the sample had a crossing point (Cp) value near the median Cp

value of the series. The calibrator sample was subsequently used also for construction of the standard curve for the target and reference genes.

6. DNA sequencing and sequence analyses (I, II, IV)

PCR products were subjected to treatment with an ExoSAP IT enzyme mix (USB Corboration, Cleveland, Ohio) prior to sequencing. Sequencing of the template was performed in both directions using BigDye3 termination chemistry and an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, California). The acquired sequences were compared with the reference sequences of MCPyV isolates MCC350 (gb/EU375803.1) and MCC339 (gb/EU375804.1) obtained from the Entrez Nucleotide database of National Center for Biotechnology Information, and with a human gene sequence database obtained from the Ensembl database. The sequences were analyzed by using a LaserGene 7.2 softaware (DNASTAR, Inc, Madison, Wisconsin).

7. Statistical analysis (I-IV)

Frequency tables were analyzed using the chi^2 test or Fisher's exact test. Continuous distributions between categorical groups were compared using the Mann-Whitney U test or the Kruskal-Wallis test. Correlation of continuous variables was analyzed using the Spearman's rank correlation test.

Cumulative survival was analyzed with the Kaplan-Meyer method, and survival between groups was compared using the log-rank test or Breslow's test. The independence of variables for survival was assessed using Cox's proportional hazard model. Overall survival was measured from the time of diagnosis to death from any cause, censoring patients alive on the last date of follow-up. MCC-specific survival was computed from the date of diagnosis to death caused by MCC, censoring patients alive at the time of data collection and those who died from another cause. Locoregional recurrence-free survival was analyzed from the date of diagnosis to the date of locoregional recurrence, censoring patients who did not have

locoregional recurrence on the date of last follow-up and those who died without locoregional recurrence on the date of death. Proportional hazard assumptions for covariates were evaluated using log-minus-log plots before covariate entry into the Cox's proportional hazard model. All *P*-values are 2-sided and not adjusted for multiple testing.

8. Ethical aspects (I-IV)

The tumor tissue samples were originally collected for diagnostic purposes, and leftover tissue was collected from the archives of pathological departments. The Institutional Review Board of the Helsinki University Helsinki Central Hospital approved the studies, and the Ministry of Social Affairs and Health granted a permission to collect clinical data and the National Authority for Medicolegal Affairs a permission to collect tissue samples for the studies.

RESULTS

The findings of the thesis are summarized here. More details can be found in the four articles that follow the reference list.

1. Presence of MCPyV DNA, MCPyV LT antigen expression, clinical factors and disease outcome (I and II)

MCPyV DNA was detected using qPCR in 91 (79.8%) of the 114 MCC tumors studied. The MCPyV LT antigen was expressed in 61 (67.0%) of the 91 tumors analyzed by immunohistochemistry. The presence of MCPyV DNA and LT antigen expression were strongly associated (p<0.0001), although 12 (40.0%) of the 30 LT antigen-negative MCCs contained viral DNA. Five (41.7%) out of these 12 LT antigen-negative tumors contained MCPyV DNA in low levels (MCPyV DNA to *PTPRG* ratio less than 0.02), suggesting that MCPyV might be a passenger virus in these cases and that such tumors may not present true MCPyV-associated MCC. We found little of MCPyV DNA also in samples of skin tumors other than MCC, and the viral LT antigen was not expressed in these tissues (unpublished data). The quantitative PCR was more sensitive in detection of MCPyV DNA than the qualitative PCR with amplicon detection by agarose gel electrophoresis and ethidium bromide staining. Due to the close association between the qPCR results and viral LT antigen immunostaining, the latter appears to be a useful marker for the presence of MCPyV infection in tumor.

Neither presence of MCPyV DNA nor the LT antigen expression were associated with patient age at the time of diagnosis (p>0.05), while both factors were associated with MCC location in the limbs compared to the head and neck region or the trunk (both p-values ≤0.015). Tumor LT antigen expression was significantly associated with the female gender (p=0.021) and with tumor intermediate morphology as compared to the trabecular or small cell types (p=0.023), but these comparisons were not significant when the presence of MCPyV DNA in tumor was examined in place of tumor LT expression (p=0.109 and 0.327, respectively). Tumors that harbored MCPyV DNA gave less often rise to regional nodal metastases at the

time of diagnosis than MCPyV-negative MCCs (6.6% vs. 21.7%, respectively; p=0.043), and in line with this, MCCs that expressed LT were less often stage III or stage IV than tumors lacking LT expression (6.5% vs. 30.0%, respectively; p=0.006).

Presence of MCPyV DNA in tumor was associated with favorable disease-specific and overall survival rates compared to MCPyV DNA-negative cases (hazard ratio [HR] 0.37; 95% confidence interval [CI], 0.15 to 0.89; p=0.022; and HR 0.39; 95% CI, 0.24 to 0.65; p<0.001, respectively). Similarly, tumor LT antigen expression was associated with favorable MCC-specific survival (HR 0.22; 95% CI, 0.09 to 0.52; p=0.0005) and overall survival (HR, 0.40; 95% CI, 0.24 to 0.65; p=0.0002) compared to LT-negative cases. Presence of MCPyV DNA was an independent prognostic factor for favorable overall survival in a Cox multivariable analysis exluding patients with distant metastases (n=4), and with gender and presence of regional lymph node metastases at the time of the diagnosis entered as covariables in analysis (HR 0.42; 95% CI, 0.25 to 0.71; p=0.001). Similarly, tumor LT antigen expression was associated with favorable overall survival when entered as a covariable in a Cox multivariate analysis together with tumor stage (HR, 0.49; 95% CI, 0.29 to 0.83; p=0.008).

2. Cell cycle regulatory proteins and MCPyV in MCC (II)

Retinoblastoma protein (RB) expression was strongly associated with tumor LT antigen expression in MCC. All LT antigen expressing tumors co-expressed also RB (n=61) and only four (13.3%) of the 30 LT antigen negative tumors expressed RB (p<0.0001). The MCPyV DNA to *PTPRG* ratio was higher in RB-positive than RB-negative tumors (median, 2.03; range, 0 to 4224 vs. median, 0; range 0 to 1149, respectively; p<0.0001). Among the 90 cases in which data on tumor LT expression, RB expression and MCPyV DNA status were available, only 11 (12.1%) tumors showed discordance between any of the three factors. As presence of MCPyV DNA and LT expression, as well as tumor RB expression were associated with favorable MCC-specific and overall survival (HR, 0.63; 95% CI, 0.47 to 0.84; p=0.0016; and HR 0.36; 95% CI, 0.21 to 0.60; p<0.0001, respectively). Phosphorylated RB was expressed in 54 (61.4%) out of the 88 tumors available for this analysis, but tumor RB phosphorylation status was not associated with the presence of MCPyV DNA in tumor or with the tumor LT antigen status.

The MCCs examined were highly proliferating tumors, and 97.8% of them expressed the Ki-67 protein. The median percentage of Ki-67-positive tumor cells was lower in LT antigen expressing tumors than in the LT antigen-negative ones (52.3% vs. 61.4%, p=0.004). The cell cycle promoting protein, cyclin D1, was absent in almost all tumors (97.6%), whereas cyclin E was expressed in 78 (94.0%) of the 83 tumors investigated. Cyclin E expression was more often seen in LT antigen-positive than in -negative tumors (98.2% vs. 85.2%, p=0.037). The cell cycle inhibitor protein p16 was expressed in 97.7% of the tumors, and p21 and p27 in 50.6% and in 70.0% of them, respectively. Expression of none of these cyclin-dependent kinase inhibitors was significantly associated with tumor MCPyV LT antigen expression, although expression of p27 tended to be higher in MCPyV LT-positive tumors (p=0.051).

3. Tumor suppressor gene *TP53* mutations and p53 expression in MCC (II, IV)

Expression of p53 was investigated in Studies II and IV. It was found to be uncommon in MCPyV DNA-positive (15.3%) and LT antigen-positive (7.0%) tumors, whereas MCPyV DNA-negative and LT antigen-negative tumors frequently expressed p53 (45.0% and 43.3%; p=0.01 and p<0.0001, respectively). p53 expressing tumors contained also lower MCPyV DNA copy numbers compared to p53-negative tumors. The murine double minute 2 protein, a negative regulator of p53, was rarely expressed in MCCs (5.8%).

TP53 mutations were highly significantly associated with tumor p53 expression (p=0.002), and eight (61.5%) of 13 p53-expressing tumors contained a *TP53* mutation in gene exons 5 to 9, which were sequenced in 39 MCCs. Only three (11.5%) of 26 p53 expression-negative tumors harbored *TP53* mutation. In two of these cases the mutation caused a premature stop codon in the *TP53* reading frame and in one case p53 expression was cytoplasmic instead of nuclear, wherby this tumor was considered p53-negative. *TP53* mutations were found exclusively among the LT antigen-negative MCCs. The *TP53* mutations are listed in Table 5.

Table 5. *TP53* mutations found in Merkel cell carcinoma, and tumor p53, RB and LT antigen expression

Case	TP53 mutation type	Mutated exon	p53 expression	RB expression	LT antigen expression	MCPyV status (copy no.)
1.	Substitution (c.298C>T; Q100X)	4	-	-	-	-
2.	Substitution (c.488A>G; Y163C)	5	+	-	-	-
3.	Substitution (c.517G>A; V173M)	5	N.A.	N.A.	-	-
4.	Substitution (c.535C>A; H178N)	5	+	-	-	+ (0.066)
5.	Substitution (c.536A>G; H178R)	5	+	-	-	+ (0.014)
6.	Substitution (c.580C>T; L194F)	6	+	-	-	+ (0.286)
7.	Substitution (c.586C>T; R196X)	6	-	-	-	-
8.	Substitution (c.844C>T; R282W)	8	+	-	-	-
	Substitution (c.856G>A; E286K)	8				
9.	Substitution (c.845G>C; R282P)	8	+	-	-	-
10.	Deletion (c.938_966del)	9	-	-	-	-
11.	Deletion (c.940_941del)	9	+	-	-	-
12.	Deletion (c.951_954de)	9	+	-	-	-

Patients whose tumor expressed p53 had poorer MCC-specific survival than those with p53-negative cancer (5-year survival 44.0% vs. 76.4%, respectively; p=0.021), and they also had poorer overall survival (5-year survival 22.2% vs. 39.3%, respectively; p=0.046). When the tumors were stratified according to the presence of MCPyV DNA and p53 expression, those patients whose tumor contained MCPyV DNA but did not express p53 had the best outcome as compared to the other groups.

4. KIT and platelet-derived growth factor receptor alpha in MCC (IV)

A total of 59 (67.8%) out of the 87 MCCs investigated showed KIT protein expression of some degree in immunohistochemistry. The expression was classified as negative, faint, moderate or strong in 28 (32.2%), 22 (25.3%), 23 (26.4%) and 14 (16.1%) of the tumors, respectively. The MCCs with moderate or strong expression were considered KIT-positive in

further analyses. KIT expression was found more frequently in MCPyV DNA-negative than in DNA-positive tumors (34.3% vs. 70.0%, p=0.009). Phosphorylated KIT was detected only in four (4.8%) of the 83 MCCs studied, suggesting that KIT is rarely activated in MCC. The KIT ligand (SCF) expression was also rarely found (seven [8.6%] of 81), suggesting that ligand-dependent activation of KIT occurs infrequently in MCC. PDGFRA expression was detected in 23 (31.9%) out of the 72 tumors investigated.

KIT exons 9, 11, 13 and 17, and the corresponding *PDGFRA* exons 10, 12, 14 and 18, were investigated by DNA sequencing in 50 MCCs. All eight exons were successfully sequenced in 34 cases, seven exons in 11 cases, and six exons in five cases. No activating mutations in *KIT* or *PDGFRA* were found. Eight tumors contained a 1432T>C single nucleotide substitution in *PDGFRA* exon 10. Thirty additional MCCs were investigated for this *PDGFRA* exon 10 alteration and a total of ten (12.5%) of the 80 tumors contained the same S478P single nucleotide polymorphism. The S478P alteration was not associated with PDGFRA expression or with the presence of MCPyV DNA, but it was found more commonly in KIT-positive than KIT-negative tumors (23.5% vs. 4.3%, p=0.015).

Patients whose tumor expressed KIT tended to have less favorable overall survival compared to patients with KIT-negative MCC (5-year survival 42.0% vs. 27.0%; p=0.050 by the Breslow test). When the MCCs were stratified based on the presence of MCPyV DNA and KIT expression, those patients whose tumor was KIT-positive and MCPyV DNA-negative had the worst overall survival and tended to have also the worst disease-specific survival.

5. Tumor infiltrating immune cells in MCC (III)

The numbers of tumor infiltrating T lymphocytes (CD3+) and their subsets, cytotoxic T lymphocytes (CD8+), helper T cells (CD4+) and regulatory T cells (FoxP3+), and natural killer cells (CD16+) and macrophages (CD68+) including M2 macrophages (Clever-1+ and CD163+) were investigated in 116 MCCs using immunohistochemistry. The median numbers of tumor infiltrating CD3+, CD8+, CD16+ , FoxP3+ and CD68+ cells were higher in MCPyV DNA-positive MCCs than in MCPyV DNA-negative cancers (all p-values <0.05). Similarly,

the median numbers of CD3+, CD4+, CD8+, FoxP3+ and CD68+ cells were higher in LT antigen-positive than LT antigen-negative MCCs (all p-values <0.05). Intratumoral Clever-1-positive cells were rarely found, although they were frequently present in the stroma surrounding the tumors.

MCCs with a high number of intratumoral CD3+ lymphocytes were infrequently p53-positive (p<0.001) or presented with regional or distant metastases at the time of diagnosis (p=0.006) compared to MCCs with a low number of CD3+ cells, but they were more often RB-positive (p=0.012) and p27-positive (p=0.030). MCCs with a high tumor CD8+ or FoxP3+ cell count expressed less often p53 than MCCs with a low count, and the RB protein was often expressed when the tumor CD4+ or FoxP3+ cell count was high (all p-values <0.05). A high number of intratumoral CD8+, FoxP3+ and CD163+ cells was associated with a larger than the median tumor diameter (16 mm) at the time of the diagnosis (all p-values <0.05). None of the tumor infiltrating immune cell types investigated was associated with gender, age at the time of diagnosis or tumor proliferation rate as assessed with Ki-67 immunostaining.

High number of CD3+ lymphocytes in tumor associated with favorable MCC-specific and overall survival (both p-values ≤ 0.002). Similarly, a high number of CD8+ and FoxP3 cells was significantly associated with favorable survival, whereas the CD4+ counts were not. Interestingly, a low CD8+/CD4+ ratio (<0.38) and a low FoxP3+/CD4+ cell ratio (<0.25) were associated with poor survival (p=0.007 and p=0.002, respectively), whereas the CD8+/FoxP3+ ratio was not significantly associated with survival (p=0.743). A number of natural killer cells or macrophages were not associated with overall survival (Table 6).

A higher than median tumor CD3+ cell count was significantly associated with favorable overall survival in the subset of MCPyV DNA-negative MCC (HR, 0.33; 95% CI, 0.13 to 0.85; p=0.022) and tended to be associated with favorable survival also in MCPyV DNA-positive cancer (HR, 0.64; 95% CI, 0.39 to 1.05; p=0.074). A high tumor CD8+ count was associated with favorable survival in MCPyV DNA-positive MCC (HR, 0.46; 95% CI, 0.23 to 0.92; p=0.028). When the MCCs were stratified into four subgroups based on the presence or absence of MCPyV DNA and the tumor CD3+ or CD8+ cell counts (high vs. low), patients

with MCPyV DNA-positive tumor and with a high CD3+ or CD8+ count had the best outcomes, whereas patients with a low CD3+ count and MCPyV DNA-negative tumor had the worst survival. In Cox multivariable proportional hazard model, lower than median tumor CD3+ cell count was an independent adverse prognostic factor for overall survival when entered in analysis as a covariable together with gender, the regional nodal status (patients with distant metastases at the time of the diagnosis were excluded from the analysis) and the tumor MCPyV DNA status (Table 7).

Table 6. Univariate Cox proportional hazard analysis for overall survival

Covariate[†]	Hazard ratio of death	95% confidence interval	p-value
CD3	0.51	0.34 to 0.78	0.002
CD4	0.94	0.70 to 1.26	0.937
CD8[±]	0.45	0.26 to 0.77	0.004
CD16	0.72	0.46 to 1.14	0.163
FoxP3	0.57	0.36 to 0.91	0.019
CD68	0.88	0.58 to 1.32	0.529
CD163	1.22	0.80 to 1.86	0.357
CD8/CD4 ratio*	2.07	1.22 to 3.51	0.007
FoxP3/CD4 ratio*	2.29	1.35 to 3.88	0.002

[†]Patients with higher than median cell count were compared to rest of the patients.
[±]The quartiles of second to fourth vs. the lowest were compared.
*The lowest quartile vs. the rest were compared.

Table 7. Multivariable Cox proportional hazard analysis of overall survival

Covariate	Hazard ratio of death	95% confidence interval	p-value
CD3+ count (median ≤vs. >)	1.91	1.21 to 3.01	0.005
Nodal metastases Presence vs. absence	2.96	1.46 to 6.01	0.003
Sex Male vs. female	1.78	1.10 to 2.87	0.018
MCPyV DNA Absent vs. present	1.93	1.20 to 3.10	0.007

DISCUSSION

In the present thesis, we investigated the associations between several clinical and molecular factors and MCPyV infection in a large Finnish population-based MCC series. We showed that MCC patients with MCPyV-positive cancers generally have better outcome in comparison to patients with MCPyV-negative tumor. The reasons for this are not yet known, but we found that the MCPyV-positive and -negative tumors differ in several aspects at the molecular level. For example, MCPyV-positive tumors express frequently RB, whereas in MCPyV-negative cancers RB expression is usually absent, whereas p53- and KIT-expression is more common, and *TP53* mutations may be found exclusively in MCPyV-negative tumors. Tumor microenvironment also differs in MCPyV-positive and -negative MCCs with higher numbers of tumor infiltrating leukocytes being present in virus-positive tumors.

1. MCPyV-positive and -negative tumors as two distinct molecular subtypes

At present, the prognostic significance of tumor MCPyV status is controversial in MCC. Although some studies have found an association between favorable disease outcome and MCPyV-positivity (Laude et al. 2010, Bhatia et al. 2010a, Andres et al. 2009, Nardi et al. 2012), other studies have not found such an association (Schrama et al. 2011, Asioli et al. 2011, Handschel et al. 2010). The different results may in part be explained by the sensitivity of the methodology used to detect MCPyV DNA. Three out of seven studies cited above used qPCR for viral DNA detection, and found MCPyV in 74% to 94% of the cases (Schrama et al. 2011, Laude et al. 2010, Bhatia et al. 2010b), while three other studies used PCRs with relative short amplicons (109bp to 195bp), and detected MCPyV DNA in only 66% to 76% of the cases (Andres et al. 2009, Nardi et al. 2012, Asioli et al. 2011). Handschel and colleagues found the lowest percentage of MCPyV DNA-positivity in the tumors examined (58%) (Handschel et al. 2010), probably because they used a PCR employing primer pairs for LT1, LT3 and VP1 coding regions, which we had found less sensitive than the qPCR used here (49.5% vs. 79.8% of the tumors were found MCPyV-positive).

Differences in the comprehensiveness of the patient series studied might also explain the differences in results on the association between MCPyV infection and patient outcome. At present, our series is the only population-based one probably involving the least selection bias. The numerically largest series (n=174) was obtained from Europe and Australia (Schrama et al. 2011). Furthermore, the tumor materials in some studies included both formalin-fixed paraffin-embedded samples and frozen tissue samples, as well as samples from the primary tumors, recurrent tumors and metastatic tissues adding further heterogeneity. These factors probably reflect the difficulties in obtaining adequate numbers of tissue samples from a rare tumor type.

Schrama and colleagues suggested that the favorable outcome of MCPyV DNA-positive MCC patients in the present series might be explained by an exceptional gender distribution with a female predominance (Schrama et al. 2011). Such gender distributions in MCC series are typical for the Nordic countries (Kaae et al. 2010, Kukko et al. 2012), but a female predominance was also present in three out of the seven studies referred to above (Laude et al. 2010, Andres et al. 2009, Handschel et al. 2010). Of note, men were predominant in the series of Bhatia et al. and Nardi et al. in which MCPyV-positivity was associated with favorable outcome, indicating that the gender distribution does not explain the effect of MCPyV infection on survival (Bhatia et al. 2010a, Nardi et al. 2012).

Another striking difference between the series is that regional lymph node and distant metastases were found at the time of diagnosis less frequently in our series and those of Laude et al. and Andres et al. (14%, 25% and 17%, respectively), whereas in the series of Asioli et al. and Schrama et al. metastases were found more often (43% and 39%, respectively). The reasons why the MCCs appear to be more advanced at the time of the diagnosis in some series remain speculative, but this may impact the results. The patient- and doctor-related delays might vary between countries leading to some tumors being diagnosed at a more advanced stage at the time of the diagnosis (Blendon et al. 2002). A large proportion of metastatic MCCs in the series of Nardi et al. (53%) may be explained the fact that the patients originated from an institute where more advanced cases of MCCs are being treated (Nardi et al. 2012). However, we found in a Cox multivariable analysis that absence of MCPyV DNA in tumor was an independent adverse prognostic factor besides the male gender and the nodal status.

In addition to the association with patient outcome, the tumor viral status correlates with some clinical factors, suggesting that MCPyV-positive and -negative tumors might be two distinct molecular subtypes of MCC. As compared with MCPyV LT antigen-negative tumors, LT-positive tumors are more common in females than males (75% vs. 50%), and they are located more often in the limbs than in the trunk (54% vs. 8%). This observation has been confirmed by two independent studies on relatively large MCC series (n=174 and n=60; Schrama et al. 2011, Nardi et al. 2012). It is possible that MCPyV-driven tumorigenesis takes place more often in sun exposed skin areas, since UV radiation activates MCPyV sT antigen expression (Mogha et al. 2010) that is required for cell transformation and promotes cell proliferation (Shuda et al. 2011). The differences in MCC gender distribution might result from yet unknown sex-related biological or hormonal mechanisms that may affect Merkel cells.

In addition to the clinical observations discussed above, several molecular findings support the hypothesis that MCCs could be divided into two distinct molecular subtypes based on the MCPyV status. We found that tumor RB expression is almost invariably associated with MCPyV-positivity and that *TP53* mutations were exclusively found in LT antigen-negative MCC. In line with this, Bhatia and his colleagues reported RB and terminal deoxyribonucleotidyl transferase expression to be more frequent in MCPyV-positive tumors, whereas p53 expression was less common (Bhatia et al. 2010b). In another study that addressed the correlation between *TP53* mutations and the MCC MCPyV status, the authors reported *TP53* mutations in only 3 (5%) of 60 MCCs studied (Nardi et al. 2012), a substantially lower figure than ours (28%). The lower number of mutations detected may reflect the differences between the series or technical factors such as the genotyping assay used (Dias-Santagata et al. 2010). Interestingly, two of the mutations were found in MCPyV DNA-positive tumor samples (Nardi et al. 2012). Three of the eleven MCCs that contained a *TP53* mutation in the present series harbored a low copy number of MCPyV DNA, and LT antigen expression was absent, suggesting that these tumors may not have been MCPyV infection-associated (Table 5).

We found changes also in the expression of other cell cycle-related proteins. Cyclin E expression was more common in MCPyV-positive than in MCPyV-negative MCCs, whereas

the cell proliferation rate assessed by Ki-67 staining was higher in MCPyV-negative cancers. Studies performed in MCC cell lines support these findings. RB sequestration by MCPyV LT antigen activates expression of the G1 to S phase transition promoting proteins cyclin E and E2F1 in a BJ fibroblast cell line, whereas mutant, RB-binding inefficient LT antigen does not (Arora et al. 2012). In one study, MCPyV-negative cell lines had shorter doubling times than MCPyV-positive ones (Houben et al. 2010), which is in line with the higher cell proliferation rate found in the present series in MCPyV-negative MCCs. In addition, KIT expression was more common in MCPyV-positive than MCPyV-negative tumors, although KIT was rarely activated in the current series and, therefore, might not be a suitable target for therapeutic agents such as pazopanib, a multitarget tyrosine kinase inhibitor with which a response was achieved in a single MCC patient in one study (Davids et al. 2009). Further differences between MCPyV-positive and MCPyV-negative MCCs have been reported. MCPyV-negative MCCs contain more genomic aberrations than MCPyV-positive counterparts (Paulson et al. 2009), and most *PIK3CA* mutations are found in MCPyV-negative cancer (Nardi et al. 2012).

It has been also suggested that instead of two etiologically different oncogenic pathways leading to MCPyV-positive and –negative MCC, MCPyV-negative cancers might have lost the virus (Houben et al. 2012b). Houben et al. have studied MCPyV-positive cell line (LoKe), which genome contains integrated virus genome. LT antigen is expressed in LoKe cells and MCPyV-genome contains LT antigen truncating mutation characteristic for cancer, although including also exceptional deletion in RB bindind region (Houben et al. 2012b). T antigen silencing with shRNA didn't inhibited cell growth as with other MCPyV-positive cell lines (Houben et al. 2012a, Houben et al. 2012b). Authors suggested that in some cases MCPyV is effective in tumor initiation, and after additional mutations accumulate in cell, T antigen becomes dispensable; therefore, MCPyV-genome might be lost from cells (Houben et al. 2012b). We can't rule out that MCPyV-genome is lost in our samples, although several different aberrations are found in MCPyV-positive and –negative MCCs suggesting different different oncogenic pathways in these tumors.

Interestingly, MCC shows features similar to those of another virus infection-related cancer, oropharyngeal squamous cell carcinoma. Up to 72% of oropharyngeal head and neck cancers in the United States and Europe are infected with the human papillomavirus (HPV) (Mehanna et al. 2012). The majority of HPV-positive head and neck cancers harbor the high-risk HPV-16 strain (D'Souza et al. 2007). In analogy with the polyomavirus T antigens, the

double-stranded DNA genome of HPV encodes E6 and E7 proteins, which target the p53 and RB tumor suppressor proteins; and suppression of E6 and E7 proteins by shRNA induced cancer cell apoptosis in a oropharyngeal squamous carcinoma cell line (Rampias et al. 2009). In line with our results in MCPyV-positive MCCs, patients with HPV-positive head and neck cancers show better disease outcome than those with HPV-negative cancers (Arora et al. 2012, Ang et al. 2010, Gillison et al. 2000), and HPV-positive head and neck cancers harbor *TP53* mutations infrequently (Gillison et al. 2000, Kumar et al. 2008, Perrone et al. 2006). These features in common with HPV-positive head and neck cancers and MCPyV-positive MCCs suggest that the two virus families function alike in tumorigenesis and that the viral status of the tumor associates with distinct molecular subtypes with different oncogenic pathways.

2. Immune cell infiltration and outcome in MCPyV-positive and MCPyV-negative MCCs

The observation that MCPyV-positive MCCs contain a higher number of tumor infiltrating immune cells than virus-negative tumors might explain in part the generally more favorable outcome of patients with MCPyV-positive cancers along with the molecular differences discussed above. Interestingly, a high number of tumor infiltrating T lymphocytes is associated with favorable survival in both MCPyV-positive and MCPyV-negative MCC. However, those patients whose tumor was MCPyV-positive and contained a high number of CD3+ or CD8+ cells had the best overall survival, indicating that intratumoral T cell infiltration is beneficial for survival regardless of the tumor MCPyV status, although MCPyV-positivity also had independent influence on survival as a single factor. Thus far one other study has found a high intratumoral CD8 count to be associated with a favorable outcome in MCC (Paulson et al. 2011). MCC is associated with immunocompromised conditions such as HIV/AIDS, chronic lymphocytic leukemia, immunosuppressive therapies, UV-exposure (Agelli, Clegg 2003, Heath et al. 2008) and old age (Vukmanovic-Stejic et al. 2011). Therefore, it may not be surprising that immune cells have tumor growth restricting function in MCC, in a virus-associated skin tumor that appears in elderly person. Similarly, the longer known polyomaviruses BKPyV and CJPyV persisting latently can be reactivated in immunosuppressive conditions and cause diseases such as nephropathy and progressive multifocal leukoencephalopathy, respectively (Delbue, Comar & Ferrante 2012).

Several case studies have reported spontaneous regressions of MCCs that are likely immune cell-mediated (Inoue et al. 2000, Burack, Altschuler 2003, Burack, Altschuler 2003, Herrmann et al. 2004, Herrmann et al. 2004, Kubo et al. 2007, Richetta et al. 2008, Vesely et al. 2008, Turk et al. 2009, Ciudad et al. 2010). In one study, peripherial blood mononuclear cells drawn from MCPyV-seropositive healthy adults showed significantly stronger CD4+ cell responses than in samples of seronegative subjects when stimulated MCPyV VP1 virus-like particles (Kumar et al. 2011). Cell proliferation increased and especially secretion of interferon-γ, an antiviral and tumor suppressing cytokine strengthened (Kumar et al. 2011). Therefore, drugs boosting the immune system might be beneficial in treatment of MCC. However, the interplay between the immune cell types is complex, and better understanding of the UV-related and age-related changes in the immune cell defense in the skin is necessary. For example, studies in mice have shown that UV irradiation enhances the genesis of suppressor T lymphocytes in the lymphoid organs and that these cells may support tumor growth (Fisher, Kripke 1982). In mice immunized with tumor antigens UV irradiation activates CD4+/CD25+/FoxP3- T cells that can impair the anti-tumor immune responses of cytotoxic CD8+ T cells (Toda et al. 2011), and in the absence of CD4+ cells, UV irradiation does not have an immunosuppressive effect on the CD8+ cell activity (Krasteva et al. 2002). UV irradiation enhances also mast cell migration from the skin to the draining lymph nodes, where they probably activate immunosuppressive B cells, and blocking of the UV-induced mast cell migration prevents immunosuppression in the skin of mice (Byrne, Limon-Flores & Ullrich 2008). We found that MCC patients whose CD8+ to CD4+ or FoxP3+ to CD4+ cell ratios were low had poorer survival than patients with high ratios, suggesting that the treatments aiming at immune cell activation should be selective.

Elderly people may have decreased immune function in the skin predisposing to cutaneous infections and malignancies. The impaired immunity in old people likely results from many alterations, such as reduced production of cytokines, impaired immune cell function, changes in the balance between different immune cell types and exhaustion of cytotoxic T cells (reviewed in Vukmanovic-Stejic et al. 2011). MCPyV occurs latently in the majority of humans, and repeated antigen stimulations through of the life and during tumor growth might cause exhaustion of MCPyV-specific cytotoxic T cells and reduced functional activity. Exhausted CD8+ cells have been found in many chronic viral infections, such as lymphocytic choriomeningitis virus infections (Barber et al. 2006) and hepatitis B infections in mice (Tzeng et al. 2012), and HIV (Day et al. 2006), and hepatitis B and C virus infections in

humans (Urbani et al. 2006, Boni et al. 2007). CD8+ cell exhaustion in a chronic infection is regulated by multiple inhibitory receptors, the coexpression of which synergistically increases the severity of T cell exhaustion and infection (Blackburn et al. 2009). One such receptor is the programmed cell death 1 (PD-1), and inhibition of its ligand (PD ligand 1, PD-L1) by an antibody restores the ability of CD8+ cells to proliferate, secrete cytokines, kill infected cells and diminish the viral load in chronically infected mice (Barber et al. 2006, Blackburn et al. 2009). An anti-PD-L1 antibody produced durable tumor regression and prolonged stabilization of cancer in patients with advanced melanoma, non-small cell lung carcinoma and renal cell carcinoma in a recent clinical trial (Brahmer et al. 2012). Hypothetically, cytotoxic T cell activation might be effective also in MCC, in which CD8+ cell function is likely to be of clinical relevance.

3. Study limitations

Selection bias is a potential limitation of the current study. Up to 58.0% (study IV) of the samples were excluded from analysis for various reasons, such as unavailability of clinical data or tumor tissue samples. However, we did not find statistically significant differences between the patients who were included and those who were excluded from the study in median age at time of diagnosis, gender distribution or survival times (study I) suggesting that exclusion did not bring about major bias. Since the current series were a population-based series unlike those in the other corresponding studies that we are aware of, selection bias may remain relatively limited in the current study.

The gender distribution in the present series, which differs from that of some others, could also have impacted the results. Yet, a predominance of females has been reported also in some other MCC series (Laude et al. 2010, Andres et al. 2009, Handschel et al. 2010), and the association between presence of MCPyV in MCC and favorable survival has been reported also in series with a male predominance (Bhatia et al. 2010a, Nardi et al. 2012).

Due to the rarity of MCC, the present series remains numerically small despite being probably the second largest clinical series on MCC published thus far. Therefore, we may not have been able to detect some weak, but yet important associations between the parameters studied due to lacking statistical power (type II error). On the other hand, we may have found significant associations that occurred by chance (type I error), and repeat studies are warranted to confirm many of the results in other patient populations. It is possible that not all results we obtained in a Finnish patient population can be generalized to other patient populations.

The type of tumor tissue used in this thesis work material may also have influenced the results. Formalin-fixed paraffin-embedded tissue samples contain fragmented DNA, which might hinder MCPyV DNA detection by qPCR in some cases. A primer set for another conserved MCPyV genome region might help to verify our results. However, the associations between the presence of MCPyV DNA, LT antigen and RB expression were strong, indicating that both quantitative PCR and immunohistochemistry can identify the majority of virus-positive tumors and that the number of false negative results might be low. Tissue fixation also influences antigen preservation and, the results of immunohistochemistry. However, we found *TP53* mutations to be associated with p53 expression, and the presence of MCPyV DNA, LT antigen and RB expression. In addition, we earlier verified immunohistochemistry for the KIT protein to be reliable using mRNA *in situ* hybridization (Sihto et al. 2007).

4. Future studies

Because of the rarity of the disease, large clinical series of MCC are not generally available, and clinical trials are also challenging to conduct. Fortunately, the discovery of MCPyV, a new human cancer virus, generated substantial interest in MCC, and some molecular mechanisms related to MCC tumorigenesis have been revealed within a few years. Yet, further studies addressing the MCC microenvironment, cancer cell activation and cell signaling are needed both in MCPyV-positive and MCPyV-negative cancers to identify potential new therapeutic targets in this highly malignant disease. The methods to identify MCPyV in tumor cells vary between laboratories, affecting the sensitivity and specificity of virus detection, and, therefore, interlaboratory studies comparing the virus detection

methods are required to standardize the findings. Multinational studies to identify potential differences in MCC tumorigenesis and MCPyV incidence at the population level are also warranted. At present, only few large nationwide population-based tumor series are available, and collection of such series would be valuable.

CONCLUSIONS

The aim of this thesis work was to study MCC with respect to 1) the frequency of intratumoral MCPyV and its association with patient and tumor characteristics, disease outcome and cell cycle regulatory protein expression, 2) the presence of *TP53*, *KIT* and *PDGFRA* gene mutations and their protein expression and their associations with the presence of MCPyV, patient and tumor characteristics and survival, and 3) the frequency and type of tumor infiltrating immune cells, and their association with MCPyV, clinicopathological factors and disease outcome.

The main conclusions are the following:

1. MCPyV DNA and LT antigen were found in the majority of MCCs. Tumors that harbor MCPyV DNA usually expressed the viral LT antigen.

2. Patients whose MCC tumor cells express the viral LT antigen or contain MCPyV DNA in general have more favorable survival than patients whose tumor is LT-negative or MCPyV DNA-negative.

3. MCPyV LT expression was seen often in females, in tumors with intermediate cell type and in MCCs that arise in the limbs compared to those that arise in the trunk or in the head and neck region.

4. LT-positive MCCs generally showed a lower cell proliferation rate than LT-negative tumors. LT-positive MCCs expressed frequently the retinoblastoma protein and harbored rarely *TP53, KIT* or *PDGFRA* mutations, whereas KIT and p53 expression and *TP53* mutations were found more often in LT-negative tumors.

5. The numbers of tumor infiltrating CD3+, CD8+, CD16+, FoxP3+ and CD68+ cells were higher in MCPyV DNA-positive MCCs than in MCPyV DNA-negative cancers, and patients with a high number of CD3+, CD8+ or FoxP3+ lymphocytes in tumor had more favorable survival than patients with low numbers. A high tumor CD3+ cell count was an independent favorable prognostic factor in MCC suggesting that host defence influences survival of patients diagnosed with MCC.

Taken together, these findings suggest that the outcome of MCC patients is influenced by several molecular mechanisms where MCPyV infection, different cell signaling routes and the host immune response may all be involved. Further studies on these mechanisms may lead to new therapeutic approaches, such as vaccinations of selected risk groups or use of novel immunomodulatory agents.

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REFERENCES

Agelli, M. & Clegg, L.X. 2003, "Epidemiology of primary Merkel cell carcinoma in the United States", *Journal of the American Academy of Dermatology,* vol. 49, no. 5, pp. 832-841.

Albores-Saavedra, J., Batich, K., Chable-Montero, F., Sagy, N., Schwartz, A.M. & Henson, D.E. 2010, "Merkel cell carcinoma demographics, morphology, and survival based on 3870 cases: a population based study", *Journal of Cutaneous Pathology,* vol. 37, no. 1, pp. 20-27.

Allander, T., Andreasson, K., Gupta, S., Bjerkner, A., Bogdanovic, G., Persson, M.A., Dalianis, T., Ramqvist, T. & Andersson, B. 2007, "Identification of a third human polyomavirus", *Journal of Virology,* vol. 81, no. 8, pp. 4130-4136.

Allen, P.J., Bowne, W.B., Jaques, D.P., Brennan, M.F., Busam, K. & Coit, D.G. 2005, "Merkel cell carcinoma: prognosis and treatment of patients from a single institution", *Journal of Clinical Oncology,* vol. 23, no. 10, pp. 2300-2309.

Allen, P.J., Zhang, Z.F. & Coit, D.G. 1999, "Surgical management of Merkel cell carcinoma", *Annals of Surgery,* vol. 229, no. 1, pp. 97-105.

Andea, A.A., Coit, D.G., Amin, B. & Busam, K.J. 2008, "Merkel cell carcinoma: histologic features and prognosis", *Cancer,* vol. 113, no. 9, pp. 2549-2558.

Andea, A.A., Patel, R., Ponnazhagan, S., Kumar, S., DeVilliers, P., Jhala, D., Eltoum, I.E. & Siegal, G.P. 2010, "Merkel cell carcinoma: correlation of KIT expression with survival and evaluation of KIT gene mutational status", *Human Pathology,* vol. 41, no. 10, pp. 1405-1412.

Andres, C., Belloni, B., Puchta, U., Sander, C.A. & Flaig, M.J. 2009, "Re: Clinical factors associated with Merkel cell polyomavirus infection in Merkel cell carcinoma", *Journal of the National Cancer Institute,* vol. 101, no. 23, pp. 1655-6; author reply 1656-7.

Ang, K.K., Harris, J., Wheeler, R., Weber, R., Rosenthal, D.I., Nguyen-Tan, P.F., Westra, W.H., Chung, C.H., Jordan, R.C., Lu, C., Kim, H., Axelrod, R., Silverman, C.C., Redmond, K.P. & Gillison, M.L. 2010, "Human papillomavirus and survival of patients with oropharyngeal cancer", *The New England Journal of Medicine,* vol. 363, no. 1, pp. 24-35.

Arora, R., Shuda, M., Guastafierro, A., Feng, H., Toptan, T., Tolstov, Y., Normolle, D., Vollmer, L.L., Vogt, A., Domling, A., Brodsky, J.L., Chang, Y. & Moore, P.S. 2012, "Survivin is a therapeutic target in merkel cell carcinoma", *Science Translational Medicine,* vol. 4, no. 133, pp. 133ra56.

Ascoli, V., Minelli, G., Kanieff, M., Frova, L. & Conti, S. 2011, "Merkel cell carcinoma: a population-based study on mortality and the association with other cancers", *Cancer Causes & Control,* vol. 22, no. 11, pp. 1521-1527.

Asioli, S., Righi, A., de Biase, D., Morandi, L., Caliendo, V., Picciotto, F., Macripo, G., Maletta, F., di Cantogno, L.V., Chiusa, L., Eusebi, V. & Bussolati, G. 2011, "Expression of p63 is the sole independent marker of aggressiveness in localised (stage I-II) Merkel cell carcinomas", *Modern Pathology,* vol. 24, no. 11, pp. 1451-1461.

Barber, D.L., Wherry, E.J., Masopust, D., Zhu, B., Allison, J.P., Sharpe, A.H., Freeman, G.J. & Ahmed, R. 2006, "Restoring function in exhausted CD8 T cells during chronic viral infection", *Nature,* vol. 439, no. 7077, pp. 682-687.

Becker, J.C., Houben, R., Ugurel, S., Trefzer, U., Pfohler, C. & Schrama, D. 2009, "MC polyomavirus is frequently present in Merkel cell carcinoma of European patients", *The Journal of Investigative Dermatology,* vol. 129, no. 1, pp. 248-250.

Beer, T.W., Ng, L.B. & Murray, K. 2008, "Mast cells have prognostic value in Merkel cell carcinoma", *The American Journal of Dermatopathology,* vol. 30, no. 1, pp. 27-30.

Bhatia, K., Goedert, J.J., Modali, R., Preiss, L. & Ayers, L.W. 2010a, "Immunological detection of viral large T antigen identifies a subset of Merkel cell carcinoma tumors with higher viral abundance and better clinical outcome", *International Journal of Cancer,* vol. 127, no. 6, pp. 1493-1496.

Bhatia, K., Goedert, J.J., Modali, R., Preiss, L. & Ayers, L.W. 2010b, "Merkel cell carcinoma subgroups by Merkel cell polyomavirus DNA relative abundance and oncogene expression", *International Journal of Cancer,* vol. 126, no. 9, pp. 2240-2246.

Bickle, K., Glass, L.F., Messina, J.L., Fenske, N.A. & Siegrist, K. 2004, "Merkel cell carcinoma: a clinical, histopathologic, and immunohistochemical review", *Seminars in Cutaneous Medicine and Surgery,* vol. 23, no. 1, pp. 46-53.

Bingle, L., Brown, N.J. & Lewis, C.E. 2002, "The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies", *The Journal of Pathology,* vol. 196, no. 3, pp. 254-265.

Blackburn, S.D., Shin, H., Haining, W.N., Zou, T., Workman, C.J., Polley, A., Betts, M.R., Freeman, G.J., Vignali, D.A. & Wherry, E.J. 2009, "Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection", *Nature Immunology,* vol. 10, no. 1, pp. 29-37.

Blendon, R.J., Schoen, C., DesRoches, C.M., Osborn, R., Scoles, K.L. & Zapert, K. 2002, "Inequites in health care: A five-country survey", *Health Affairs,* vol. 21, no. 3, pp. 182-191.

Boni, C., Fisicaro, P., Valdatta, C., Amadei, B., Di Vincenzo, P., Giuberti, T., Laccabue, D., Zerbini, A., Cavalli, A., Missale, G., Bertoletti, A. & Ferrari, C. 2007, "Characterization of hepatitis B virus (HBV)-specific T-cell dysfunction in chronic HBV infection", *Journal of Virology,* vol. 81, no. 8, pp. 4215-4225.

Boyapati, A., Wilson, M., Yu, J. & Rundell, K. 2003, "SV40 17KT antigen complements dnaj mutations in large T antigen to restore transformation of primary human fibroblasts", *Virology,* vol. 315, no. 1, pp. 148-158.

Brahmer, J.R., Tykodi, S.S., Chow, L.Q., Hwu, W.J., Topalian, S.L., Hwu, P., Drake, C.G., Camacho, L.H., Kauh, J., Odunsi, K., Pitot, H.C., Hamid, O., Bhatia, S., Martins, R., Eaton, K., Chen, S., Salay, T.M., Alaparthy, S., Grosso, J.F., Korman, A.J., Parker, S.M., Agrawal, S., Goldberg, S.M., Pardoll, D.M., Gupta, A. & Wigginton, J.M. 2012, "Safety and activity of anti-PD-L1 antibody in patients with advanced cancer", *The New England Journal of Medicine,* vol. 366, no. 26, pp. 2455-2465.

Braun, K.A., Lao, Y., He, Z., Ingles, C.J. & Wold, M.S. 1997, "Role of protein-protein interactions in the function of replication protein A (RPA): RPA modulates the activity of DNA polymerase alpha by multiple mechanisms", *Biochemistry,* vol. 36, no. 28, pp. 8443-8454.

Brewer, J.D., Shanafelt, T.D., Otley, C.C., Roenigk, R.K., Cerhan, J.R., Kay, N.E., Weaver, A.L. & Call, T.G. 2012, "Chronic lymphocytic leukemia is associated with decreased survival of patients with malignant melanoma and Merkel cell carcinoma in a SEER population-based study", *Journal of Clinical Oncology,* vol. 30, no. 8, pp. 843-849.

Brunner, M., Thurnher, D., Pammer, J., Geleff, S., Heiduschka, G., Reinisch, C.M., Petzelbauer, P. & Erovic, B.M. 2008, "Expression of VEGF-A/C, VEGF-R2, PDGF-alpha/beta, c-kit, EGFR, Her-2/Neu, Mcl-1 and Bmi-1 in Merkel cell carcinoma", *Modern Pathology,* vol. 21, no. 7, pp. 876-884.

Buck, C.B., Phan, G.Q., Raiji, M.T., Murphy, P.M., McDermott, D.H. & McBride, A.A. 2012, "Complete genome sequence of a tenth human polyomavirus", *Journal of Virology,* vol.86, no. 19, pp. 10887.

Buell, J.F., Trofe, J., Hanaway, M.J., Beebe, T.M., Gross, T.G., Alloway, R.R., First, M.R. & Woodle, E.S. 2002, "Immunosuppression and Merkel cell cancer", *Transplantation Proceedings,* vol. 34, no. 5, pp. 1780-1781.

Burack, J. & Altschuler, E.L. 2003, "Sustained remission of metastatic Merkel cell carcinoma with treatment of HIV infection", *Journal of the Royal Society of Medicine,* vol. 96, no. 5, pp. 238-239.

Burnet, M. 1957, "Cancer: a biological approach. III. Viruses associated with neoplastic conditions. IV. Practical applications", *British Medical Journal,* vol. 1, no. 5023, pp. 841-847.

Byrne, S.N., Limon-Flores, A.Y. & Ullrich, S.E. 2008, "Mast cell migration from the skin to the draining lymph nodes upon ultraviolet irradiation represents a key step in the induction of immune suppression", *Journal of Immunology,* vol. 180, no. 7, pp. 4648-4655.

Bzhalava, D., Bray, F., Storm, H. & Dillner, J. 2011, "Risk of second cancers after the diagnosis of Merkel cell carcinoma in Scandinavia", *British Journal of Cancer,* vol. 104, no. 1, pp. 178-180.

Campbell, K.S., Mullane, K.P., Aksoy, I.A., Stubdal, H., Zalvide, J., Pipas, J.M., Silver, P.A., Roberts, T.M., Schaffhausen, B.S. & DeCaprio, J.A. 1997, "DnaJ/hsp40 chaperone domain of SV40 large T antigen promotes efficient viral DNA replication", *Genes & Development,* vol. 11, no. 9, pp. 1098-1110.

Carvajal, R.D., Antonescu, C.R., Wolchok, J.D., Chapman, P.B., Roman, R.A., Teitcher, J., Panageas, K.S., Busam, K.J., Chmielowski, B., Lutzky, J., Pavlick, A.C., Fusco, A., Cane, L., Takebe, N., Vemula, S., Bouvier, N.,

Bastian, B.C. & Schwartz, G.K. 2011, "KIT as a therapeutic target in metastatic melanoma", *The Journal of the American Medical Association,* vol. 305, no. 22, pp. 2327-2334.

Cerroni, L. & Kerl, H. 1997, "Primary cutaneous neuroendocrine (Merkel cell) carcinoma in association with squamous- and basal-cell carcinoma", *The American Journal of Dermatopathology,* vol. 19, no. 6, pp. 610-613.

Chen, T., Hedman, L., Mattila, P.S., Jartti, T., Ruuskanen, O., Soderlund-Venermo, M. & Hedman, K. 2011, "Serological evidence of Merkel cell polyomavirus primary infections in childhood", *Journal of Clinical Virology,* vol. 50, no. 2, pp. 125-129.

Cheuk, W., Kwan, M.Y., Suster, S. & Chan, J.K. 2001, "Immunostaining for thyroid transcription factor 1 and cytokeratin 20 aids the distinction of small cell carcinoma from Merkel cell carcinoma, but not pulmonary from extrapulmonary small cell carcinomas", *Archives of Pathology & Laboratory Medicine,* vol. 125, no. 2, pp. 228-231.

Chromy, L.R., Pipas, J.M. & Garcea, R.L. 2003, "Chaperone-mediated in vitro assembly of Polyomavirus capsids", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 100, no. 18, pp. 10477-10482.

Ciudad, C., Aviles, J.A., Alfageme, F., Lecona, M., Suarez, R. & Lazaro, P. 2010, "Spontaneous regression in merkel cell carcinoma: report of two cases with a description of dermoscopic features and review of the literature", *Dermatologic Surgery,* vol. 36, no. 5, pp. 687-693.

Clemente, C.G., Mihm, M.C.,Jr, Bufalino, R., Zurrida, S., Collini, P. & Cascinelli, N. 1996, "Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma", *Cancer,* vol. 77, no. 7, pp. 1303-1310.

Cooper, L., Debono, R., Alsanjari, N. & Al-Nafussi, A. 2000, "Merkel cell tumour with leiomyosarcomatous differentiation", *Histopathology,* vol. 36, no. 6, pp. 540-543.

Coussens, L.M. & Werb, Z. 2002, "Inflammation and cancer", *Nature,* vol. 420, no. 6917, pp. 860-867.

Davids, M.S., Charlton, A., Ng, S.S., Chong, M.L., Laubscher, K., Dar, M., Hodge, J., Soong, R. & Goh, B.C. 2009, "Response to a novel multitargeted tyrosine kinase inhibitor pazopanib in metastatic Merkel cell carcinoma", *Journal of Clinical Oncology,* vol. 27, no. 26, pp. e97-100.

Day, C.L., Kaufmann, D.E., Kiepiela, P., Brown, J.A., Moodley, E.S., Reddy, S., Mackey, E.W., Miller, J.D., Leslie, A.J., DePierres, C., Mncube, Z., Duraiswamy, J., Zhu, B., Eichbaum, Q., Altfeld, M., Wherry, E.J., Coovadia, H.M., Goulder, P.J., Klenerman, P., Ahmed, R., Freeman, G.J. & Walker, B.D. 2006, "PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression", *Nature,* vol. 443, no. 7109, pp. 350-354.

de Graaf, N., van Helden, M.J., Textoris-Taube, K., Chiba, T., Topham, D.J., Kloetzel, P.M., Zaiss, D.M. & Sijts, A.J. 2011, "PA28 and the proteasome immunosubunits play a central and independent role in the

production of MHC class I-binding peptides in vivo", *European Journal of Immunology,* vol. 41, no. 4, pp. 926-935.

Dean, F.B., Bullock, P., Murakami, Y., Wobbe, C.R., Weissbach, L. & Hurwitz, J. 1987, "Simian virus 40 (SV40) DNA replication: SV40 large T antigen unwinds DNA containing the SV40 origin of replication", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 84, no. 1, pp. 16-20.

Delbue, S., Comar, M. & Ferrante, P. 2012, "Review on the relationship between human polyomaviruses-associated tumors and host immune system", *Clinical & Developmental Immunology,* vol. 2012, pp. 542092.

Deleeuw, R.J., Kost, S.E., Kakal, J.A. & Nelson, B.H. 2012, "The prognostic value of FoxP3+ tumor-infiltrating lymphocytes in cancer: A critical review of the literature", *Clinical Cancer Research*, vol. 18, no. 11, pp. 3022-3029.

Demetriou, S.K., Ona-Vu, K., Sullivan, E.M., Dong, T.K., Hsu, S.W. & Oh, D.H. 2012, "Defective DNA repair and cell cycle arrest in cells expressing Merkel cell polyomavirus T antigen", *International Journal of Cancer,* vol. 131, no. 8, pp. 1818-1827.

Dias-Santagata, D., Akhavanfard, S., David, S.S., Vernovsky, K., Kuhlmann, G., Boisvert, S.L., Stubbs, H., McDermott, U., Settleman, J., Kwak, E.L., Clark, J.W., Isakoff, S.J., Sequist, L.V., Engelman, J.A., Lynch, T.J., Haber, D.A., Louis, D.N., Ellisen, L.W., Borger, D.R. & Iafrate, A.J. 2010, "Rapid targeted mutational analysis of human tumours: a clinical platform to guide personalized cancer medicine", *EMBO Molecular Medicine,* vol. 2, no. 5, pp. 146-158.

DiLillo, D.J., Yanaba, K. & Tedder, T.F. 2010, "B cells are required for optimal CD4+ and CD8+ T cell tumor immunity: therapeutic B cell depletion enhances B16 melanoma growth in mice", *Journal of Immunology,* vol. 184, no. 7, pp. 4006-4016.

D'Souza, G., Kreimer, A.R., Viscidi, R., Pawlita, M., Fakhry, C., Koch, W.M., Westra, W.H. & Gillison, M.L. 2007, "Case-control study of human papillomavirus and oropharyngeal cancer", *The New England Journal of Medicine,* vol. 356, no. 19, pp. 1944-1956.

Dunn, G.P., Bruce, A.T., Ikeda, H., Old, L.J. & Schreiber, R.D. 2002, "Cancer immunoediting: from immunosurveillance to tumor escape", *Nature Immunology,* vol. 3, no. 11, pp. 991-998.

Eerola, A.K., Soini, Y. & Paakko, P. 2000, "A high number of tumor-infiltrating lymphocytes are associated with a small tumor size, low tumor stage, and a favorable prognosis in operated small cell lung carcinoma", *Clinical Cancer Research,* vol. 6, no. 5, pp. 1875-1881.

Erdag, G., Schaefer, J.T., Smolkin, M.E., Deacon, D.H., Shea, S.M., Dengel, L.T., Patterson, J.W. & Slingluff, C.L.,Jr 2012, "Immunotype and immunohistologic characteristics of tumor-infiltrating immune cells are associated with clinical outcome in metastatic melanoma", *Cancer Research,* vol. 72, no. 5, pp. 1070-1080.

Eyles, J., Puaux, A.L., Wang, X., Toh, B., Prakash, C., Hong, M., Tan, T.G., Zheng, L., Ong, L.C., Jin, Y., Kato, M., Prevost-Blondel, A., Chow, P., Yang, H. & Abastado, J.P. 2010, "Tumor cells disseminate early, but immunosurveillance limits metastatic outgrowth, in a mouse model of melanoma", *The Journal of Clinical Investigation,* vol. 120, no. 6, pp. 2030-2039.

Feinmesser, M., Halpern, M., Fenig, E., Tsabari, C., Hodak, E., Sulkes, J., Brenner, B. & Okon, E. 1999, "Expression of the apoptosis-related oncogenes bcl-2, bax, and p53 in Merkel cell carcinoma: can they predict treatment response and clinical outcome?", *Human Pathology*, vol. 30, no. 11, pp. 1367-1372.

Feinmesser, M., Halpern, M., Kaganovsky, E., Brenner, B., Fenig, E., Hodak, E., Sulkes, J. & Okon, E. 2004, "C-Kit Expression in Primary and Metastatic Merkel Cell Carcinoma", *The American Journal of Dermatopathology,* vol. 26, no. 6, pp. 458-462.

Feng, H., Kwun, H.J., Liu, X., Gjoerup, O., Stolz, D.B., Chang, Y. & Moore, P.S. 2011, "Cellular and viral factors regulating Merkel cell polyomavirus replication", *PloS One,* vol. 6, no. 7, pp. e22468.

Feng, H., Shuda, M., Chang, Y. & Moore, P.S. 2008, "Clonal integration of a polyomavirus in human Merkel cell carcinoma", *Science,* vol. 319, no. 5866, pp. 1096-1100.

Fewell, S.W., Pipas, J.M. & Brodsky, J.L. 2002, "Mutagenesis of a functional chimeric gene in yeast identifies mutations in the simian virus 40 large T antigen J domain", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 99, no. 4, pp. 2002-2007.

Fields, R.C., Busam, K.J., Chou, J.F., Panageas, K.S., Pulitzer, M.P., Kraus, D.H., Brady, M.S. & Coit, D.G. 2011, "Recurrence and survival in patients undergoing sentinel lymph node biopsy for merkel cell carcinoma: analysis of 153 patients from a single institution", *Annals of Surgical Oncology,* vol. 18, no. 9, pp. 2529-2537.

Fimia, G.M., Stoykova, A., Romagnoli, A., Giunta, L., Di Bartolomeo, S., Nardacci, R., Corazzari, M., Fuoco, C., Ucar, A., Schwartz, P., Gruss, P., Piacentini, M., Chowdhury, K. & Cecconi, F. 2007, "Ambra1 regulates autophagy and development of the nervous system", *Nature,* vol. 447, no. 7148, pp. 1121-1125.

Fisher, M.S. & Kripke, M.L. 1982, "Suppressor T lymphocytes control the development of primary skin cancers in ultraviolet-irradiated mice", *Science,* vol. 216, no. 4550, pp. 1133-1134.

Foote, M., Veness, M., Zarate, D. & Poulsen, M. 2011, "Merkel cell carcinoma: The prognostic implications of an occult primary in stage IIIB (nodal) disease", *Journal of the American Academy of Dermatology,* vol. 67, no. 3, pp. 395-399.

Foster, E.C. & Simmons, D.T. 2010, "The SV40 large T-antigen origin binding domain directly participates in DNA unwinding", *Biochemistry,* vol. 49, no. 10, pp. 2087-2096.

Foulongne, V., Dereure, O., Kluger, N., Moles, J.P., Guillot, B. & Segondy, M. 2010, "Merkel cell polyomavirus DNA detection in lesional and nonlesional skin from patients with Merkel cell carcinoma or other skin diseases", *The British Journal of Dermatology,* vol. 162, no. 1, pp. 59-63.

Foulongne, V., Kluger, N., Dereure, O., Brieu, N., Guillot, B. & Segondy, M. 2008, "Merkel cell polyomavirus and Merkel cell carcinoma, France", *Emerging Infectious Diseases,* vol. 14, no. 9, pp. 1491-1493.

Galon, J., Costes, A., Sanchez-Cabo, F., Kirilovsky, A., Mlecnik, B., Lagorce-Pages, C., Tosolini, M., Camus, M., Berger, A., Wind, P., Zinzindohoue, F., Bruneval, P., Cugnenc, P.H., Trajanoski, Z., Fridman, W.H. & Pages, F. 2006, "Type, density, and location of immune cells within human colorectal tumors predict clinical outcome", *Science,* vol. 313, no. 5795, pp. 1960-1964.

Gardner, S.D., Field, A.M., Coleman, D.V. & Hulme, B. 1971, "New human papovavirus (B.K.) isolated from urine after renal transplantation", *Lancet,* vol. 1, no. 7712, pp. 1253-1257.

Garneski, K.M., Warcola, A.H., Feng, Q., Kiviat, N.B., Leonard, J.H. & Nghiem, P. 2009, "Merkel cell polyomavirus is more frequently present in North American than Australian Merkel cell carcinoma tumors", *The Journal of Investigative Dermatology,* vol. 129, no. 1, pp. 246-248.

Gaynor, A.M., Nissen, M.D., Whiley, D.M., Mackay, I.M., Lambert, S.B., Wu, G., Brennan, D.C., Storch, G.A., Sloots, T.P. & Wang, D. 2007, "Identification of a novel polyomavirus from patients with acute respiratory tract infections", *PLoS Pathogens,* vol. 3, no. 5, pp. e64.

George, S., Blay, J.Y., Casali, P.G., Le Cesne, A., Stephenson, P., Deprimo, S.E., Harmon, C.S., Law, C.N., Morgan, J.A., Ray-Coquard, I., Tassell, V., Cohen, D.P. & Demetri, G.D. 2009, "Clinical evaluation of continuous daily dosing of sunitinib malate in patients with advanced gastrointestinal stromal tumour after imatinib failure", *European Journal of Cancer,* vol. 45, no. 11, pp. 1959-1968.

Ghaderi, M., Coury, J., Oxenberg, J. & Spector, H. 2010, "Primary Merkel cell carcinoma of the parotid gland", *Ear, Nose, & Throat Journal,* vol. 89, no. 7, pp. E24-7.

Ghadjar, P., Kaanders, J.H., Poortmans, P., Zaucha, R., Krengli, M., Lagrange, J.L., Ozsoy, O., Nguyen, T.D., Miralbell, R., Baize, A., Boujelbene, N., Collen, T., Scandolaro, L., Untereiner, M., Goldberg, H., Pesce, G.A., Anacak, Y., Friedrich, E.E., Aebersold, D.M. & Beer, K.T. 2011, "The essential role of radiotherapy in the treatment of Merkel cell carcinoma: a study from the Rare Cancer Network", *International Journal of Radiation Oncology, Biology, Physics,* vol. 81, no. 4, pp. e583-91.

Gillenwater, A.M., Hessel, A.C., Morrison, W.H., Burgess, M., Silva, E.G., Roberts, D. & Goepfert, H. 2001, "Merkel cell carcinoma of the head and neck: effect of surgical excision and radiation on recurrence and survival", *Archives of Otolaryngology--Head & Neck Surgery,* vol. 127, no. 2, pp. 149-154.

Gillison, M.L., Koch, W.M., Capone, R.B., Spafford, M., Westra, W.H., Wu, L., Zahurak, M.L., Daniel, R.W., Viglione, M., Symer, D.E., Shah, K.V. & Sidransky, D. 2000, "Evidence for a causal association between human papillomavirus and a subset of head and neck cancers", *Journal of the National Cancer Institute,* vol. 92, no. 9, pp. 709-720.

Girschik, J., Thorn, K., Beer, T.W., Heenan, P.J. & Fritschi, L. 2011, "Merkel cell carcinoma in Western Australia: a population-based study of incidence and survival", *The British Journal of Dermatology,* vol. 165, no. 5, pp. 1051-1057.

Gooden, M.J., de Bock, G.H., Leffers, N., Daemen, T. & Nijman, H.W. 2011, "The prognostic influence of tumour-infiltrating lymphocytes in cancer: a systematic review with meta-analysis", *British Journal of Cancer,* vol. 105, no. 1, pp. 93-103.

Gorelik, E., Wiltrout, R.H., Okumura, K., Habu, S. & Herberman, R.B. 1982, "Role of NK cells in the control of metastatic spread and growth of tumor cells in mice", *International Journal of Cancer,* vol. 30, no. 1, pp. 107-112.

Grulich, A.E., van Leeuwen, M.T., Falster, M.O. & Vajdic, C.M. 2007, "Incidence of cancers in people with HIV/AIDS compared with immunosuppressed transplant recipients: a meta-analysis", *Lancet,* vol. 370, no. 9581, pp. 59-67.

Guo, H., Samarakoon, A., Vanhaesebroeck, B. & Malarkannan, S. 2008, "The p110 delta of PI3K plays a critical role in NK cell terminal maturation and cytokine/chemokine generation", *The Journal of Experimental Medicine,* vol. 205, no. 10, pp. 2419-2435.

Gupta, S.G., Wang, L.C., Penas, P.F., Gellenthin, M., Lee, S.J. & Nghiem, P. 2006, "Sentinel lymph node biopsy for evaluation and treatment of patients with Merkel cell carcinoma: The Dana-Farber experience and meta-analysis of the literature", *Archives of Dermatology,* vol. 142, no. 6, pp. 685-690.

Handschel, J., Muller, D., Depprich, R.A., Ommerborn, M.A., Kubler, N.R., Naujoks, C., Reifenberger, J., Schafer, K.L. & Braunstein, S. 2010, "The new polyomavirus (MCPyV) does not affect the clinical course in MCCs", *International Journal of Oral and Maxillofacial Surgery,* vol. 39, no. 11, pp. 1086-1090.

Hafner, C., Houben, R., Baeurle, A., Ritter, C., Schrama, D., Landthaler, M. & Becker, J.C. 2012, "Activation of the PI3K/AKT pathway in Merkel cell carcinoma", *PLoS One,* vol. 7, no. 2, pp. e31255.

Heath, M., Jaimes, N., Lemos, B., Mostaghimi, A., Wang, L.C., Penas, P.F. & Nghiem, P. 2008, "Clinical characteristics of Merkel cell carcinoma at diagnosis in 195 patients: the AEIOU features", *Journal of the American Academy of Dermatology,* vol. 58, no. 3, pp. 375-381.

Helmbold, P., Lahtz, C., Enk, A., Herrmann-Trost, P., Marsch, W.Ch., Kutzner, H. & Dammann, R.H. 2009, "Frequent occurrence of RASSF1A promoter hypermethylation and Merkel cell polyomavirus in Merkel cell carcinoma", *Molecular Carcinogenesis,* vol. 48, no. 10, pp. 903-909.

Herrmann, G., Groth, W., Krieg, T. & Mauch, C. 2004, "Complete remission of Merkel cell carcinoma of the scalp with local and regional metastases after topical treatment with dinitrochlorbenzol", *Journal of the American Academy of Dermatology,* vol. 50, no. 6, pp. 965-969.

Hersey, P., Edwards, A., Honeyman, M. & McCarthy, W.H. 1979, "Low natural-killer-cell activity in familial melanoma patients and their relatives", *British Journal of Cancer,* vol. 40, no. 1, pp. 113-122.

Hodgson, N.C. 2005, "Merkel cell carcinoma: changing incidence trends", *Journal of Surgical Oncology,* vol. 89, no. 1, pp. 1-4.

Houben, R., Adam, C., Baeurle, A., Hesbacher, S., Grimm, J., Angermeyer, S., Henzel, K., Hauser, S., Elling, R., Brocker, E.B., Gaubatz, S., Becker, J.C. & Schrama, D. 2012a, "An intact retinoblastoma protein-binding site in Merkel cell polyomavirus large T antigen is required for promoting growth of Merkel cell carcinoma cells", *International Journal of Cancer,* vol. 130, no. 4, pp. 847-856.

Houben, R., Grimm, J., Willmes, C., Weinkam, R., Becker, J.C. & Schrama, D. 2012b, "Merkel cell carcinoma and Merkel cell polyomavirus: Evidence for hit-and-run oncogenesis", *Journal of Investigative Dermatology, vol. 132, no.1, pp. 254-256.*

Houben, R., Shuda, M., Weinkam, R., Schrama, D., Feng, H., Chang, Y., Moore, P.S. & Becker, J.C. 2010, "Merkel cell polyomavirus-infected Merkel cell carcinoma cells require expression of viral T antigens", *Journal of Virology,* vol. 84, no. 14, pp. 7064-7072.

Howard, R.A., Dores, G.M., Curtis, R.E., Anderson, W.F. & Travis, L.B. 2006, "Merkel cell carcinoma and multiple primary cancers", *Cancer Epidemiology, Biomarkers & Prevention,* vol. 15, no. 8, pp. 1545-1549.

Hwang, J.H., Alanen, K., Dabbs, K.D., Danyluk, J. & Silverman, S. 2008, "Merkel cell carcinoma with squamous and sarcomatous differentiation", *Journal of Cutaneous Pathology,* vol. 35, no. 10, pp. 955-959.

Hwang, W.T., Adams, S.F., Tahirovic, E., Hagemann, I.S. & Coukos, G. 2012, "Prognostic significance of tumor-infiltrating T cells in ovarian cancer: a meta-analysis", *Gynecologic Oncology,* vol. 124, no. 2, pp. 192-198.

Imai, K., Matsuyama, S., Miyake, S., Suga, K. & Nakachi, K. 2000, "Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population", *Lancet,* vol. 356, no. 9244, pp. 1795-1799.

Inoue, T., Yoneda, K., Manabe, M. & Demitsu, T. 2000, "Spontaneous regression of merkel cell carcinoma: a comparative study of TUNEL index and tumor-infiltrating lymphocytes between spontaneous regression and non-regression group", *Journal of Dermatological Science,* vol. 24, no. 3, pp. 203-211.

Jadus, M.R., Irwin, M.C., Irwin, M.R., Horansky, R.D., Sekhon, S., Pepper, K.A., Kohn, D.B. & Wepsic, H.T. 1996, "Macrophages can recognize and kill tumor cells bearing the membrane isoform of macrophage colony-stimulating factor", *Blood,* vol. 87, no. 12, pp. 5232-5241.

Joensuu, H., De Braud, F., Grignagni, G., De Pas, T., Spitalieri, G., Coco, P., Spreafico, C., Boselli, S., Toffalorio, F., Bono, P., Jalava, T., Kappeler, C., Aglietta, M., Laurent, D. & Casali, P.G. 2011, "Vatalanib for metastatic gastrointestinal stromal tumour (GIST) resistant to imatinib: final results of a phase II study", *British Journal of Cancer,* vol. 104, no. 11, pp. 1686-1690.

Joensuu, H., Roberts, P.J., Sarlomo-Rikala, M., Andersson, L.C., Tervahartiala, P., Tuveson, D., Silberman, S., Capdeville, R., Dimitrijevic, S., Druker, B. & Demetri, G.D. 2001, "Effect of the tyrosine kinase inhibitor STI571 in a patient with a metastatic gastrointestinal stromal tumor", *The New England Journal of Medicine,* vol. 344, no. 14, pp. 1052-1056.

Johne, R., Buck, C.B., Allander, T., Atwood, W.J., Garcea, R.L., Imperiale, M.J., Major, E.O., Ramqvist, T. & Norkin, L.C. 2011, "Taxonomical developments in the family Polyomaviridae", *Archives of Virology,* vol. 156, no. 9, pp. 1627-1634.

Kaae, J., Hansen, A.V., Biggar, R.J., Boyd, H.A., Moore, P.S., Wohlfahrt, J. & Melbye, M. 2010, "Merkel cell carcinoma: incidence, mortality, and risk of other cancers", *Journal of the National Cancer Institute,* vol. 102, no. 11, pp. 793-801.

Kantola, K., Sadeghi, M., Lahtinen, A., Koskenvuo, M., Aaltonen, LM., Möttönen, M., Rahiala, J., Saarinen-Pihkala, U., Riikkonen, P., Jartti, T., Ruuskanen, O., Söderlund-Venermo, M. & Hedaman, K. 2009, "Merkel cell polyomavirus DNA in tumor-free tonsillar tissue and upper respiratory tract samples: Implications for respiratory transmission and latency", *Journal of Clinical Virology*, vol. 45, no. 4, pp. 292-295.

Kartha, R.V. & Sundram, U.N. 2008, "Silent mutations in KIT and PDGFRA and coexpression of receptors with SCF and PDGFA in Merkel cell carcinoma: implications for tyrosine kinase-based tumorigenesis", *Modern Pathology,* vol. 21, no. 2, pp. 96-104.

Kassem, A., Schopflin, A., Diaz, C., Weyers, W., Stickeler, E., Werner, M. & Zur Hausen, A. 2008, "Frequent detection of Merkel cell polyomavirus in human Merkel cell carcinomas and identification of a unique deletion in the VP1 gene", *Cancer Research,* vol. 68, no. 13, pp. 5009-5013.

Khopde, S., Roy, R. & Simmons, D.T. 2008, "The binding of topoisomerase I to T antigen enhances the synthesis of RNA-DNA primers during simian virus 40 DNA replication", *Biochemistry,* vol. 47, no. 36, pp. 9653-9660.

Koebel, C.M., Vermi, W., Swann, J.B., Zerafa, N., Rodig, S.J., Old, L.J., Smyth, M.J. & Schreiber, R.D. 2007, "Adaptive immunity maintains occult cancer in an equilibrium state", *Nature,* vol. 450, no. 7171, pp. 903-907.

Kohler, S. & Kerl, H. 2006, "Merkel cell carcinoma" in *Pathology and Genetics of Skin Tumours. World Health Organization Classification of Tumours*, eds. P. LeBoit, G. Burg, D. Weedon & A. Sarasin, 6th edn, IARC Press, Lyon, France, pp. 272-273.

Koljonen, V., Kukko, H., Tukiainen, E., Bohling, T., Sankila, R., Joensuu, H. & Pukkala, E. 2010, "Second cancers following the diagnosis of Merkel cell carcinoma: a nationwide cohort study", *Cancer Epidemiology,* vol. 34, no. 1, pp. 62-65.

Koljonen, V., Kukko, H., Tukiainen, E., Bohling, T., Sankila, R., Pukkala, E., Sihto, H., Joensuu, H., Kyllonen, L. & Makisalo, H. 2009, "Incidence of Merkel cell carcinoma in renal transplant recipients", *Nephrology, Dialysis, Transplantation,* vol. 24, no. 10, pp. 3231-3235.

Korhonen, P., Malila, N., Pukkala, E., Teppo, L., Albanes, D. & Virtamo, J. 2002, "The Finnish Cancer Registry as follow-up source of a large trial cohort--accuracy and delay", *Acta Oncologica,* vol. 41, no. 4, pp. 381-388.

Krasagakis, K., Kruger-Krasagakis, S., Eberle, J., Tsatsakis, A., Tosca, A.D. & Stathopoulos, E.N. 2009, "Co-expression of KIT receptor and its ligand stem cell factor in Merkel cell carcinoma", *Dermatology,* vol. 218, no. 1, pp. 37-43.

Krasteva, M., Aubin, F., Laventurier, S., Kehren, J., Assossou, O., Kanitakis, J., Kaiserlian, D. & Nicolas, J.F. 2002, "MHC class II-KO mice are resistant to the immunosuppressive effects of UV light", *European Journal of Dermatology : EJD,* vol. 12, no. 1, pp. 10-19.

Kubo, H., Matsushita, S., Fukushige, T., Kanzaki, T. & Kanekura, T. 2007, "Spontaneous regression of recurrent and metastatic Merkel cell carcinoma", *The Journal of Dermatology,* vol. 34, no. 11, pp. 773-777.

Kukko, H., Bohling, T., Koljonen, V., Tukiainen, E., Haglund, C., Pokhrel, A., Sankila, R. & Pukkala, E. 2012, "Merkel cell carcinoma - a population-based epidemiological study in Finland with a clinical series of 181 cases", *European Journal of Cancer,* vol. 48, no. 5, pp. 737-742.

Kukko, H.M., Koljonen, V.S., Tukiainen, E.J., Haglund, C.H. & Bohling, T.O. 2010, "Vascular invasion is an early event in pathogenesis of Merkel cell carcinoma", *Modern Pathology,* vol. 23, no. 8, pp. 1151-1156.

Kumar, A., Chen, T., Pakkanen, S., Kantele, A., Söderlund-Venermo, M., Hedaman, K. & Franssila R. 2011, "T-helper cell-mediated proliferation and cytokine responses against recombinant Merkel cell polyomavirus-like particles", *PLoS One*, vol. 6, no. 10, pp. e25751.

Kumar, B., Cordell, K.G., Lee, J.S., Worden, F.P., Prince, M.E., Tran, H.H., Wolf, G.T., Urba, S.G., Chepeha, D.B., Teknos, T.N., Eisbruch, A., Tsien, C.I., Taylor, J.M., D'Silva, N.J., Yang, K., Kurnit, D.M., Bauer, J.A., Bradford, C.R. & Carey, T.E. 2008, "EGFR, p16, HPV Titer, Bcl-xL and p53, sex, and smoking as indicators of response to therapy and survival in oropharyngeal cancer", *Journal of Clinical Oncology,* vol. 26, no. 19, pp. 3128-3137.

Kwun, H.J., Guastafierro, A., Shuda, M., Meinke, G., Bohm, A., Moore, P.S. & Chang, Y. 2009, "The minimum replication origin of merkel cell polyomavirus has a unique large T-antigen loading architecture and requires small T-antigen expression for optimal replication", *Journal of Virology,* vol. 83, no. 23, pp. 12118-12128.

Lanoy, E., Costagliola, D. & Engels, E.A. 2010, "Skin cancers associated with HIV infection and solid-organ transplantation among elderly adults", *International Journal of Cancer,* vol. 126, no. 7, pp. 1724-1731.

Lanoy, E., Dores, G.M., Madeleine, M.M., Toro, J.R., Fraumeni, J.F.,Jr & Engels, E.A. 2009, "Epidemiology of nonkeratinocytic skin cancers among persons with AIDS in the United States", *AIDS,* vol. 23, no. 3, pp. 385-393.

Lapuk, A., Marr, H., Jakkula, L., Pedro, H., Bhattacharya, S., Purdom, E., Hu, Z., Simpson, K., Pachter, L., Durinck, S., Wang, N., Parvin, B., Fontenay, G., Speed, T., Garbe, J., Stampfer, M., Bayandorian, H., Dorton, S., Clark, T.A., Schweitzer, A., Wyrobek, A., Feiler, H., Spellman, P., Conboy, J. & Gray, J.W. 2010, "Exon-level microarray analyses identify alternative splicing programs in breast cancer", *Molecular Cancer Research,* vol. 8, no. 7, pp. 961-974.

Larramendy, M.L., Koljonen, V., Böhling, T., Tukiainen, E. & Knuutila, S. 2004, "Recurrent DNA copy number changes revealed by comparative genomic hybridization in primary Merkel cell carcinomas" *Modern Pathology*, vol. 17, no. 5, pp. 561-567.

Lassacher, A., Heitzer, E., Kerl, H. & Wolf, P. 2008, "p14ARF hypermethylation is common but INK4a-ARF locus or p53 mutations are rare in Merkel cell carcinoma", *Journal of Investigative Dermatology*, vol. 128, no. 7, pp. 1788-1796.

Laude, H.C., Jonchere, B., Maubec, E., Carlotti, A., Marinho, E., Couturaud, B., Peter, M., Sastre-Garau, X., Avril, M.F., Dupin, N. & Rozenberg, F. 2010, "Distinct merkel cell polyomavirus molecular features in tumour and non tumour specimens from patients with merkel cell carcinoma", *PLoS Pathogens,* vol. 6, no. 8, pp. e1001076.

Lee, S., Paulson, K.G., Murchison, E.P., Afanasiev, O.K., Alkan, C., Leonard, J.H., Byrd, D.R., Hannon, G.J. & Nghiem, P. 2011, "Identification and validation of a novel mature microRNA encoded by the Merkel cell polyomavirus in human Merkel cell carcinomas", *Journal of Clinical Virology*, vol. 52, no. 3, pp. 272-275.

Lemos, B.D., Storer, B.E., Iyer, J.G., Phillips, J.L., Bichakjian, C.K., Fang, L.C., Johnson, T.M., Liegeois-Kwon, N.J., Otley, C.C., Paulson, K.G., Ross, M.I., Yu, S.S., Zeitouni, N.C., Byrd, D.R., Sondak, V.K., Gershenwald, J.E., Sober, A.J. & Nghiem, P. 2010, "Pathologic nodal evaluation improves prognostic accuracy in Merkel cell carcinoma: analysis of 5823 cases as the basis of the first consensus staging system", *Journal of the American Academy of Dermatology,* vol. 63, no. 5, pp. 751-761.

Lewis, C.E. & Pollard, J.W. 2006, "Distinct role of macrophages in different tumor microenvironments", *Cancer Research,* vol. 66, no. 2, pp. 605-612.

Liu, X., Hein, J., Richardson, S.C., Basse, P.H., Toptan, T., Moore, P.S., Gjoerup, O.V. & Chang, Y. 2011, "Merkel cell polyomavirus large T antigen disrupts lysosome clustering by translocating human Vam6p from the cytoplasm to the nucleus", *The Journal of Biological Chemistry,* vol. 286, no. 19, pp. 17079-17090.

Llombart, B., Monteagudo, C., Lopez-Guerrero, J.A., Carda, C., Jorda, E., Sanmartin, O., Almenar, S., Molina, I., Martin, J.M. & Llombart-Bosch, A. 2005, "Clinicopathological and immunohistochemical analysis of 20 cases of Merkel cell carcinoma in search of prognostic markers", *Histopathology,* vol. 46, no. 6, pp. 622-634.

Loyo, M., Guerrero-Preston, R., Brait, M., Hoque, M.O., Chuang, A., Kim, M.S., Sharma, R., Liegeois, N.J., Koch, W.M., Califano, J.A., Westra, W.H. & Sidransky, D. 2010, "Quantitative detection of Merkel cell virus in human tissues and possible mode of transmission", *International Journal of Cancer,* vol. 126, no. 12, pp. 2991-2996.

Mahmoud, S.M., Lee, A.H., Paish, E.C., Macmillan, R.D., Ellis, I.O. & Green, A.R. 2012, "The prognostic significance of B lymphocytes in invasive carcinoma of the breast", *Breast Cancer Research and Treatment,* vol. 132, no. 2, pp. 545-553.

Mahmoud, S.M., Paish, E.C., Powe, D.G., Macmillan, R.D., Grainge, M.J., Lee, A.H., Ellis, I.O. & Green, A.R. 2011, "Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer", *Journal of Clinical Oncology,* vol. 29, no. 15, pp. 1949-1955.

Manjili, M.H. 2011, "Revisiting cancer immunoediting by understanding cancer immune complexity", *The Journal of Pathology,* vol. 224, no. 1, pp. 5-9.

Mantovani, A., Sozzani, S., Locati, M., Allavena, P. & Sica, A. 2002, "Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes", *Trends in Immunology,* vol. 23, no. 11, pp. 549-555.

Maricich, S.M., Wellnitz, S.A., Nelson, A.M., Lesniak, D.R., Gerling, G.J., Lumpkin, E.A. & Zoghbi, H.Y. 2009, "Merkel cells are essential for light-touch responses", *Science,* vol. 324, no. 5934, pp. 1580-1582.

Martel-Jantin, C., Filippone, C., Cassar, O., Peter, M., Tomasic, G., Vielh, P., Briere, J., Petrella, T., Aubriot-Lorton, M.H., Mortier, L., Jouvion, G., Sastre-Garau, X., Robert, C. & Gessain, A. 2012, "Genetic variability and integration of Merkel cell polyomavirus in Merkel cell carcinoma", *Virology,* vol. 426, no. 2, pp. 134-142.

Medina-Franco, H., Urist, M.M., Fiveash, J., Heslin, M.J., Bland, K.I. & Beenken, S.W. 2001, "Multimodality treatment of Merkel cell carcinoma: case series and literature review of 1024 cases", *Annals of Surgical Oncology,* vol. 8, no. 3, pp. 204-208.

Mehanna, H., Beech, T., Nicholson, T., El-Hariry, I., McConkey, C., Paleri, V. & Roberts, S. 2012, "Prevalence of human papillomavirus in oropharyngeal and nonoropharyngeal head and neck cancer-systematic review and meta-analysis of trends by time and region", *Head & Neck,* published online ahead of print January 20, 2012; doi:10.1002/hed.22015.

Mills, L.A., Durrani, A.J. & Watson, J.D. 2006, "Merkel cell carcinoma in South East Scotland, 1993-2003", *The Surgeon,* vol. 4, no. 3, pp. 133-138.

Mogha, A., Fautrel, A., Mouchet, N., Guo, N., Corre, S., Adamski, H., Watier, E., Misery, L. & Galibert, M.D. 2010, "Merkel cell polyomavirus small T antigen mRNA level is increased following in vivo UV-radiation", *PloS One,* vol. 5, no. 7, pp. e11423.

Mortier, L., Mirabel, X., Fournier, C., Piette, F. & Lartigau, E. 2003, "Radiotherapy alone for primary Merkel cell carcinoma", *Archives of Dermatology,* vol. 139, no. 12, pp. 1587-1590.

Mott, R.T., Smoller, B.R. & Morgan, M.B. 2004, "Merkel cell carcinoma: a clinicopathologic study with prognostic implications", *Journal of Cutaneous Pathology,* vol. 31, no. 3, pp. 217-223.

Movahedi, K., Laoui, D., Gysemans, C., Baeten, M., Stange, G., Van den Bossche, J., Mack, M., Pipeleers, D., In't Veld, P., De Baetselier, P. & Van Ginderachter, J.A. 2010, "Different tumor microenvironments contain functionally distinct subsets of macrophages derived from Ly6C(high) monocytes", *Cancer Research,* vol. 70, no. 14, pp. 5728-5739.

Murakami, Y., Chen, L.F., Sanechika, N., Kohzaki, H. & Ito, Y. 2007, "Transcription factor Runx1 recruits the polyomavirus replication origin to replication factories", *Journal of Cellular Biochemistry,* vol. 100, no. 5, pp. 1313-1323.

Nardi, V., Song, Y., Santamaria-Barria, J.A., Cosper, A.K., Lam, Q., Faber, A.C., Boland, G.M., Yeap, B.Y., Bergethon, K., Scialabba, V.L., Tsao, H., Settleman, J., Ryan, D.P., Borger, D.R., Bhan, A.K., Hoang, M.P., Iafrate, A.J., Cusack, J.C., Engelman, J.A. & Dias-Santagata, D. 2012, "Activation of PI3K signaling in Merkel cell carcinoma", *Clinical Cancer Research,* vol. 18, no. 5, pp. 1227-1236.

Okkenhaug, K., Bilancio, A., Farjot, G., Priddle, H., Sancho, S., Peskett, E., Pearce, W., Meek, S.E., Salpekar, A., Waterfield, M.D., Smith, A.J. & Vanhaesebroeck, B. 2002, "Impaired B and T cell antigen receptor signaling in p110delta PI 3-kinase mutant mice", *Science,* vol. 297, no. 5583, pp. 1031-1034.

Onizuka, S., Tawara, I., Shimizu, J., Sakaguchi, S., Fujita, T. & Nakayama, E. 1999, "Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody", *Cancer Research,* vol. 59, no. 13, pp. 3128-3133.

Padgett, B.L., Walker, D.L., ZuRhein, G.M., Eckroade, R.J. & Dessel, B.H. 1971, "Cultivation of papova-like virus from human brain with progressive multifocal leucoencephalopathy", *Lancet,* vol. 1, no. 7712, pp. 1257-1260.

Pages, F., Berger, A., Camus, M., Sanchez-Cabo, F., Costes, A., Molidor, R., Mlecnik, B., Kirilovsky, A., Nilsson, M., Damotte, D., Meatchi, T., Bruneval, P., Cugnenc, P.H., Trajanoski, Z., Fridman, W.H. & Galon, J. 2005, "Effector memory T cells, early metastasis, and survival in colorectal cancer", *The New England Journal of Medicine,* vol. 353, no. 25, pp. 2654-2666.

Paik, J.Y., Hall, G., Clarkson, A., Lee, L., Toon, C., Colebatch, A., Chou, A. & Gill, A.J. 2011, "Immunohistochemistry for Merkel cell polyomavirus is highly specific but not sensitive for the diagnosis of Merkel cell carcinoma in the Australian population", *Human Pathology,* vol. 42, no. 10, pp. 1385-1390.

Pape, E., Rezvoy, N., Penel, N., Salleron, J., Martinot, V., Guerreschi, P., Dziwniel, V., Darras, S., Mirabel, X. & Mortier, L. 2011, "Radiotherapy alone for Merkel cell carcinoma: a comparative and retrospective study of 25 patients", *Journal of the American Academy of Dermatology,* vol. 65, no. 5, pp. 983-990.

Pastrana, D.V., Tolstov, Y.L., Becker, J.C., Moore, P.S., Chang, Y. & Buck, C.B. 2009, "Quantitation of human seroresponsiveness to Merkel cell polyomavirus", *PLoS Pathogens,* vol. 5, no. 9, pp. e1000578.

Patel, R., Adsay, V. & Andea, A. 2010, "Basal cell carcinoma with progression to metastatic neuroendocrine carcinoma", *Rare Tumors,* vol. 2, no. 1, pp. e8.

Paulson, K.G., Carter, J.J., Johnson, L.G., Cahill, K.W., Iyer, J.G., Schrama, D., Becker, J.C., Madeleine, M.M., Nghiem, P. & Galloway, D.A. 2010, "Antibodies to merkel cell polyomavirus T antigen oncoproteins reflect tumor burden in merkel cell carcinoma patients", *Cancer Research,* vol. 70, no. 21, pp. 8388-8397.

Paulson, K.G., Iyer, J.G., Tegeder, A.R., Thibodeau, R., Schelter, J., Koba, S., Schrama, D., Simonson, W.T., Lemos, B.D., Byrd, D.R., Koelle, D.M., Galloway, D.A., Leonard, J.H., Madeleine, M.M., Argenyi, Z.B., Disis, M.L., Becker, J.C., Cleary, M.A. & Nghiem, P. 2011, "Transcriptome-wide studies of merkel cell carcinoma and validation of intratumoral CD8+ lymphocyte invasion as an independent predictor of survival", *Journal of Clinical Oncology,* vol. 29, no. 12, pp. 1539-1546.

Paulson, K.G., Lemos, B.D., Feng, B., Jaimes, N., Penas, P.F., Bi, X., Maher, E., Cohen, L., Leonard, J.H., Granter, S.R., Chin, L. & Nghiem, P. 2009, "Array-CGH reveals recurrent genomic changes in Merkel cell carcinoma including amplification of L-Myc", *The Journal of Investigative Dermatology,* vol. 129, no. 6, pp. 1547-1555.

Penn, I. & First, M.R. 1999, "Merkel's cell carcinoma in organ recipients: report of 41 cases", *Transplantation,* vol. 68, no. 11, pp. 1717-1721.

Perrone, F., Suardi, S., Pastore, E., Casieri, P., Orsenigo, M., Caramuta, S., Dagrada, G., Losa, M., Licitra, L., Bossi, P., Staurengo, S., Oggionni, M., Locati, L., Cantu, G., Squadrelli, M., Carbone, A., Pierotti, M.A. & Pilotti, S. 2006, "Molecular and cytogenetic subgroups of oropharyngeal squamous cell carcinoma", *Clinical Cancer Research,* vol. 12, no. 22, pp. 6643-6651.

Pfeifer, G.P., You, Y.H. & Besaratinia, A. 2005, "Mutations induced by ultraviolet light", *Mutation Research,* vol. 571, no. 1-2, pp. 19-31.

Plettenberg, A., Pammer, J. & Tschachler, E. 1996, "Merkel cells and Merkel cell carcinoma express the BCL-2 proto-oncogene", *Experimental Dermatology*, vol. 5, no. 2, pp. 102-107.

Popp, S., Waltering, S., Herbst, C., Moll, I. & Boukamp, P. 2002, "UV-B-mutations and chromosomal imbalances indicate common pathways for the development of Merkel and skin squamous cell carcinomas", *International Journal of Cancer*, vol. 99, no. 3, pp. 352-360.

Poulsen, M. 2004, "Merkel-cell carcinoma of the skin", *The Lancet Oncology,* vol. 5, no. 10, pp. 593-599.

Prabhu, S., Smitha, R.S. & Punnya, V.A. 2010, "Merkel cell carcinoma of the alveolar mucosa in a young adult: a rare case report", *The British Journal of Oral & Maxillofacial Surgery,* vol. 48, no. 1, pp. 48-50.

Qin, Z., Richter, G., Schuler, T., Ibe, S., Cao, X. & Blankenstein, T. 1998, "B cells inhibit induction of T cell-dependent tumor immunity", *Nature Medicine,* vol. 4, no. 5, pp. 627-630.

Rampias, T., Sasaki, C., Weinberger, P. & Psyrri, A. 2009, "E6 and e7 gene silencing and transformed phenotype of human papillomavirus 16-positive oropharyngeal cancer cells", *Journal of the National Cancer Institute,* vol. 101, no. 6, pp. 412-423.

Reichgelt, B.A. & Visser, O. 2011, "Epidemiology and survival of Merkel cell carcinoma in the Netherlands. A population-based study of 808 cases in 1993-2007", *European Journal of Cancer,* vol. 47, no. 4, pp. 579-585.

Reuschenbach, M., von Knebel Doeberitz, M. & Wentzensen, N. 2009, "A systematic review of humoral immune responses against tumor antigens", *Cancer Immunology, Immunotherapy,* vol. 58, no. 10, pp. 1535-1544.

Richetta, A.G., Mancini, M., Torroni, A., Lore, B., Iannetti, G., Sardella, B. & Calvieri, S. 2008, "Total spontaneous regression of advanced merkel cell carcinoma after biopsy: review and a new case", *Dermatologic Surgery,* vol. 34, no. 6, pp. 815-822.

Riou-Gotta, M.O., Fournier, E., Danzon, A., Pelletier, F., Levang, J., Mermet, I., Blanc, D., Humbert, P. & Aubin, F. 2009, "Rare skin cancer: a population-based cancer registry descriptive study of 151 consecutive cases diagnosed between 1980 and 2004", *Acta Oncologica,* vol. 48, no. 4, pp. 605-609.

Roberge, C. & Bastin, M. 1988, "Site-directed mutagenesis of the polyomavirus genome: replication-defective large T mutants with increased immortalization potential", *Virology,* vol. 162, no. 1, pp. 144-150.

Robert, C., Thomas, L., Bondarenko, I., O'Day, S., M, D.J.W., Garbe, C., Lebbe, C., Baurain, J.F., Testori, A., Grob, J.J., Davidson, N., Richards, J., Maio, M., Hauschild, A., Miller, W.H.,Jr, Gascon, P., Lotem, M., Harmankaya, K., Ibrahim, R., Francis, S., Chen, T.T., Humphrey, R., Hoos, A. & Wolchok, J.D. 2011, "Ipilimumab plus dacarbazine for previously untreated metastatic melanoma", *The New England Journal of Medicine,* vol. 364, no. 26, pp. 2517-2526.

Sahi, H., Koljonen, V., Kavola, H., Haglund, C., Tukiainen, E., Sihto, H. & Böhling, T. 2012, "Bcl-2 expression indicates better prognosis of Merkel cell carcinoma regardless of the presence of Merkel cell polyomavirus", *Virchows Archiv*, published ahead of print September 14, 2012; doi:10.1007/s00428-0012-1310-3.

Sahi, H., Kukko, H., Böhling, T., Tukiainen, E., Sihto, H., Joensuu, H. & Koljonen, V. 2010, "Unusually young Merkel cell carcinoma patients are Merkel cell polyomavirus positive and frequently immunocompromised", *European Journal of Plastic Surgery*, vol. 33, no. 6, pp. 349-353.

Samlowski, W.E., Moon, J., Tuthill, R.J., Heinrich, M.C., Balzer-Haas, N.S., Merl, S.A., DeConti, R.C., Thompson, J.A., Witter, M.T., Flaherty, L.E. & Sondak, V.K. 2010, " A phase II trial of imatinib mesylate in merkel cell carcinoma (neuroendocrine carcinoma of the skin): A Southwest Oncology Group study (S0331)", *American Journal of Clinical Oncology,* vol. 33, no. 5, pp. 495-9.

Schmitt, M., Wieland, U., Kreuter, A. & Pawlita, M. 2012, "C-terminal deletions of Merkel cell polyomavirus large T-antigen, a highly specific surrogate marker for virally induced malignancy", *International Journal of Cancer*, published ahead of print June 6, 2012; doi:10.1002/ijc.27607.

Schoenborn, J.R. & Wilson, C.B. 2007, "Regulation of interferon-gamma during innate and adaptive immune responses", *Advances in Immunology,* vol. 96, pp. 41-101.

Schowalter, R.M., Pastrana, D.V., Pumphrey, K.A., Moyer, A.L. & Buck, C.B. 2010, "Merkel cell polyomavirus and two previously unknown polyomaviruses are chronically shed from human skin", *Cell Host & Microbe,* vol. 7, no. 6, pp. 509-515.

Schrama, D., Peitsch, W.K., Zapatka, M., Kneitz, H., Houben, R., Eib, S., Haferkamp, S., Moore, P.S., Shuda, M., Thompson, J.F., Trefzer, U., Pfohler, C., Scolyer, R.A. & Becker, J.C. 2011, "Merkel cell polyomavirus status is not associated with clinical course of Merkel cell carcinoma", *The Journal of Investigative Dermatology,* vol. 131, no. 8, pp. 1631-1638.

Schumacher, K., Haensch, W., Roefzaad, C. & Schlag, P.M. 2001, "Prognostic significance of activated CD8(+) T cell infiltrations within esophageal carcinomas", *Cancer Research,* vol. 61, no. 10, pp. 3932-3936.

Scuda, N., Hofmann, J., Calvignac-Spencer, S., Ruprecht, K., Liman, P., Kuhn, J., Hengel, H. & Ehlers, B. 2011, "A novel human polyomavirus closely related to the african green monkey-derived lymphotropic polyomavirus", *Journal of Virology,* vol. 85, no. 9, pp. 4586-4590.

Seo, G.J., Chen, C.J. & Sullivan, C.S. 2009, "Merkel cell polyomavirus encodes a microRNA with the ability to autoregulate viral gene expression", *Virology,* vol. 383, no. 2, pp. 183-187.

Shah, S., Divekar, A.A., Hilchey, S.P., Cho, H.M., Newman, C.L., Shin, S.U., Nechustan, H., Challita-Eid, P.M., Segal, B.M., Yi, K.H. & Rosenblatt, J.D. 2005, "Increased rejection of primary tumors in mice lacking B cells: inhibition of anti-tumor CTL and TH1 cytokine responses by B cells", *International Journal of Cancer,* vol. 117, no. 4, pp. 574-586.

Shah, M.H., Varker, K.A., Collamore, M., Zwiebel, J.A., Coit, D., Kelsen, D. & Chung, K.Y. 2009, "G3139 (Genasense) in patients with advanced Merkel cell carcinoma", *American Journal of Clinical Oncology*, vol. 32, no. 2, pp. 174-179.

Shi, Y., Liu, H., Gai, D., Ma, J. & Chen, X.S. 2009, "A computational analysis of ATP binding of SV40 large tumor antigen helicase motor", *PLoS Computational Biology,* vol. 5, no. 9, pp. e1000514.

Shuda, M., Arora, R., Kwun, H.J., Feng, H., Sarid, R., Fernandez-Figueras, M.T., Tolstov, Y., Gjoerup, O., Mansukhani, M.M., Swerdlow, S.H., Chaudhary, P.M., Kirkwood, J.M., Nalesnik, M.A., Kant, J.A., Weiss, L.M., Moore, P.S. & Chang, Y. 2009, "Human Merkel cell polyomavirus infection I. MCV T antigen expression in Merkel cell carcinoma, lymphoid tissues and lymphoid tumors", *International Journal of Cancer,* vol. 125, no. 6, pp. 1243-1249.

Shuda, M., Feng, H., Kwun, H.J., Rosen, S.T., Gjoerup, O., Moore, P.S. & Chang, Y. 2008, "T antigen mutations are a human tumor-specific signature for Merkel cell polyomavirus", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 105, no. 42, pp. 16272-16277.

Shuda, M., Kwun, H.J., Feng, H., Chang, Y. & Moore, P.S. 2011, "Human Merkel cell polyomavirus small T antigen is an oncoprotein targeting the 4E-BP1 translation regulator", *The Journal of Clinical Investigation,* vol. 121, no. 9, pp. 3623-3634.

Siebrasse, E.A., Reyes, A., Lim, E.S., Zhao, G., Mkakosya, R.S., Manary, M.J., Gordon, J.I. & Wang, D. 2012, "Identification of MW polyomavirus, a novel polyomavirus in human stool", *Journal of Virology*, vol. 86, no. 19, pp. 10321-10326.

Sihto, H., Tynninen, O., Bützow, R., Saarialho-Kere, U. & Joensuu, H. 2007, "Endothelial cell KIT expression in human tumors", *Journal of Pathology*, vol. 211, no. 4, pp. 481-488.

Skene, P.J., Illingworth, R.S., Webb, S., Kerr, A.R., James, K.D., Turner, D.J., Andrews, R. & Bird, A.P. 2010, "Neuronal MeCP2 is expressed at near histone-octamer levels and globally alters the chromatin state", *Molecular Cell,* vol. 37, no. 4, pp. 457-468.

Skoczylas, C., Henglein, B. & Rundell, K. 2005, "PP2A-dependent transactivation of the cyclin A promoter by SV40 ST is mediated by a cell cycle-regulated E2F site", *Virology,* vol. 332, no. 2, pp. 596-601.

Sorbye, S.W., Kilvaer, T., Valkov, A., Donnem, T., Smeland, E., Al-Shibli, K., Bremnes, R.M. & Busund, L.T. 2011, "Prognostic impact of lymphocytes in soft tissue sarcomas", *PloS One,* vol. 6, no. 1, pp. e14611.

Srinivasan, A., McClellan, A.J., Vartikar, J., Marks, I., Cantalupo, P., Li, Y., Whyte, P., Rundell, K., Brodsky, J.L. & Pipas, J.M. 1997, "The amino-terminal transforming region of simian virus 40 large T and small t antigens functions as a J domain", *Molecular and Cellular Biology,* vol. 17, no. 8, pp. 4761-4773.

Su, L.D., Fullen, D.R., Lowe, L., Uherova, P., Schnitzer, B. & Valdez, R. 2002, "CD117 (KIT receptor) expression in Merkel cell carcinoma", *The American Journal of Dermatopathology,* vol. 24, no. 4, pp. 289-293.

Sullivan, C.S., Grundhoff, A.T., Tevethia, S., Pipas, J.M. & Ganem, D. 2005, "SV40-encoded microRNAs regulate viral gene expression and reduce susceptibility to cytotoxic T cells", *Nature,* vol. 435, no. 7042, pp. 682-686.

Sullivan, C.S. & Pipas, J.M. 2002, "T antigens of simian virus 40: molecular chaperones for viral replication and tumorigenesis", *Microbiology and Molecular Biology Reviews,* vol. 66, no. 2, pp. 179-202.

Sutmuller, R.P., van Duivenvoorde, L.M., van Elsas, A., Schumacher, T.N., Wildenberg, M.E., Allison, J.P., Toes, R.E., Offringa, R. & Melief, C.J. 2001, "Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses", *The Journal of Experimental Medicine,* vol. 194, no. 6, pp. 823-832.

Swick, B.L., Ravdel, L., Fitzpatrick, J.E. & Robinson, W.A. 2008, "Platelet-derived growth factor receptor alpha mutational status and immunohistochemical expression in Merkel cell carcinoma: implications for treatment with imatinib mesylate", *Journal of Cutaneous Pathology,* vol. 35, no. 2, pp. 197-202.

Swick, B.L., Ravdel, L., Fitzpatrick, J.E. & Robinson, W.A. 2007, "Merkel cell carcinoma: evaluation of KIT (CD117) expression and failure to demonstrate activating mutations in the C-KIT proto-oncogene - implications for treatment with imatinib mesylate", *Journal of Cutaneous Pathology,* vol. 34, no. 4, pp. 324-329.

Tadmor, T., Aviv, A. & Polliack, A. 2011, "Merkel cell carcinoma, chronic lymphocytic leukemia and other lymphoproliferative disorders: an old bond with possible new viral ties", *Annals of Oncology,* vol. 22, no. 2, pp. 250-256.

Tadmor, T., Liphshitz, I., Aviv, A., Landgren, O., Barchana, M. & Polliack, A. 2012, "Increased incidence of chronic lymphocytic leukaemia and lymphomas in patients with Merkel cell carcinoma - a population based study of 335 cases with neuroendocrine skin tumour", *British Journal of Haematology,* vol. 157, no. 4, pp. 457-462.

Talmadge, J.E., Meyers, K.M., Prieur, D.J. & Starkey, J.R. 1980, "Role of natural killer cells in tumor growth and metastasis: C57BL/6 normal and beige mice", *Journal of the National Cancer Institute,* vol. 65, no. 5, pp. 929-935.

Tang, C.K. & Toker, C. 1978, "Trabecular carcinoma of the skin: an ultrastructural study", *Cancer,* vol. 42, no. 5, pp. 2311-2321.

Teppo, L., Pukkala, E. & Lehtonen, M. 1994, "Data quality and quality control of a population-based cancer registry. Experience in Finland", *Acta Oncologica,* vol. 33, no. 4, pp. 365-369.

Terabe, M. & Berzofsky, J.A. 2004, "Immunoregulatory T cells in tumor immunity", *Current Opinion in Immunology,* vol. 16, no. 2, pp. 157-162.

Toda, M., Wang, L., Ogura, S., Torii, M., Kurachi, M., Kakimi, K., Nishikawa, H., Matsushima, K., Shiku, H., Kuribayashi, K. & Kato, T. 2011, "UV irradiation of immunized mice induces type 1 regulatory T cells that suppress tumor antigen specific cytotoxic T lymphocyte responses", *International Journal of Cancer,* vol. 129, no. 5, pp. 1126-1136.

Toker, C. 1972, "Trabecular carcinoma of the skin", *Archives of Dermatology,* vol. 105, no. 1, pp. 107-110.

Tolstov, Y.L., Pastrana, D.V., Feng, H., Becker, J.C., Jenkins, F.J., Moschos, S., Chang, Y., Buck, C.B. & Moore, P.S. 2009, "Human Merkel cell polyomavirus infection II. MCV is a common human infection that can be detected by conformational capsid epitope immunoassays", *International Journal of Cancer,* vol. 125, no. 6, pp. 1250-1256.

Touze, A., Le Bidre, E., Laude, H., Fleury, M.J., Cazal, R., Arnold, F., Carlotti, A., Maubec, E., Aubin, F., Avril, M.F., Rozenberg, F., Tognon, M., Maruani, A., Guyetant, S., Lorette, G. & Coursaget, P. 2011, "High levels of antibodies against merkel cell polyomavirus identify a subset of patients with merkel cell carcinoma with better clinical outcome", *Journal of Clinical Oncology,* vol. 29, no. 12, pp. 1612-1619.

Tucci, M.G., Lucarini, G., Giangiacomi, M., Zizzi, A., Criante, P., Ricotti, G. & Biagini, G. 2006, "Immunohistochemical study of apoptosis markers and involvement of chemokine CXCR4 in skin Merkel cell carcinoma", *Journal of the European Academy of Dermatology and Venereology*, vol. 20, no. 10, pp. 1220-1225.

Turk, T., Orlic, Z.C., Smoljan, I., Nacinovic, A., Bekafigo, I.S., Radic, J. & Zamolo, G. 2009, "Spontaneous regression of Merkel cell carcinoma in a patient with chronic lymphocytic leukemia: a case report", *Journal of Medical Case Reports,* vol. 3, pp. 7270.

Tzeng, H.T., Tsai, H.F., Liao, H.J., Lin, Y.J., Chen, L., Chen, P.J. & Hsu, P.N. 2012, "PD-1 Blockage Reverses Immune Dysfunction and Hepatitis B Viral Persistence in a Mouse Animal Model", *PloS One,* vol. 7, no. 6, pp. e39179.

Urbani, S., Amadei, B., Tola, D., Massari, M., Schivazappa, S., Missale, G. & Ferrari, C. 2006, "PD-1 expression in acute hepatitis C virus (HCV) infection is associated with HCV-specific CD8 exhaustion", *Journal of Virology,* vol. 80, no. 22, pp. 11398-11403.

van der Meijden, E., Janssens, R.W., Lauber, C., Bouwes Bavinck, J.N., Gorbalenya, A.E. & Feltkamp, M.C. 2010, "Discovery of a new human polyomavirus associated with trichodysplasia spinulosa in an immunocompromized patient", *PLoS Pathogens,* vol. 6, no. 7, pp. e1001024.

Van Gele, M., Kaghad, M., Leonard, J.H., Van Roy, N., Naeyaert, J.M., Geerts, M.L., Van Belle, S., Cocquyt, V., Bridge, J., Sciot, R., De Wolf-Peeters, C., De Paepe, A., Caput, D. & Speleman, F. 2000, "Mutation analysis of P75 and TP53 in Merkel cell carcinoma", *British Journal of Cancer,* vol. 82, no. 4, pp. 823-826.

Van Gele, M., Leonard, J.H., Van Roy, N., Van Limbergen, H., Van Belle, S., Cocquyt, V., Salwen, H., De Paepe, A. & Speleman, S. 2002 "Combined karyotyping, CGH and M-FISH analysis allows detailed characterization of unidentified chromosomal rearrangements in Merkel cell carcinoma", *International Journal of Cancer*, vol. 101, no. 2, pp. 137-145.

Van Ghelue, M., Khan, M.T., Ehlers, B. & Moens, U. 2012, "Genome analysis of the new human polyomaviruses", *Reviews in Medical Virology,* published ehead of print March 28, 2012; doi:10.1002/rmv.1711.

Van Keymeulen, A., Mascre, G., Youseff, K.K., Harel, I., Michaux, C., De Geest, N., Szpalski, C., Achouri, Y., Bloch, W., Hassan, B.A. & Blanpain, C. 2009, "Epidermal progenitors give rise to Merkel cells during embryonic development and adult homeostasis", *The Journal of Cell Biology,* vol. 187, no. 1, pp. 91-100.

Vesely, M.J., Murray, D.J., Neligan, P.C., Novak, C.B., Gullane, P.J. & Ghazarian, D. 2008, "Complete spontaneous regression in Merkel cell carcinoma", *Journal of Plastic, Reconstructive & Aesthetic Surgery : JPRAS,* vol. 61, no. 2, pp. 165-171.

Vlahova, L., Doerflinger, Y., Houben, R., Becker, J.C., Schrama, D., Weiss, C., Goebeler, M., Helmbold, P., Goerdt, S. & Peitsch, W.K. 2012, "P-cadherin expression in Merkel cell carcinomas is associated with prolonged recurrence-free survival", *British Journal of Dermatology,* vol. 166, no. 5, pp. 1043-1052.

Voog, E., Biron, P., Martin, J.P. & Blay, J.Y. 1999, "Chemotherapy for patients with locally advanced or metastatic Merkel cell carcinoma", *Cancer,* vol. 85, no. 12, pp. 2589-2595.

Vukmanovic-Stejic, M., Rustin, M.H., Nikolich-Zugich, J. & Akbar, A.N. 2011, "Immune responses in the skin in old age", *Current Opinion in Immunology,* vol. 23, no. 4, pp. 525-531.

Walsh, N.M. 2001, "Primary neuroendocrine (Merkel cell) carcinoma of the skin: morphologic diversity and implications thereof", *Human Pathology,* vol. 32, no. 7, pp. 680-689.

Wang, H.X., Li, Q., Sharma, C., Knoblich, K. & Hemler, M.E. 2011, "Tetraspanin protein contributions to cancer", *Biochemical Society Transactions,* vol. 39, no. 2, pp. 547-552.

Werling, A.M., Doerflinger, Y., Brandner, J.M., Fuchs, F., Becker, J.C., Schrama, D., Kurzen, H., Goerdt, S. & Peitsch, W.K. 2011, "Homo- and heterotypic cell-cell contacts in Merkel cells and Merkel cell carcinomas: heterogeneity and indications for cadherin switching", *Histopathology,* vol. 58, no. 2, pp. 286-303.

Woegerbauer, M., Thurnher, D., Houben, R., Pammer, J., Kloimstein, P., Heiduschka, G., Petzelbauer, P. & Erovic, B.M. 2010, "Expression of the tetraspanins CD9, CD37, CD63, and CD151 in Merkel cell carcinoma: strong evidence for a posttranscriptional fine-tuning of CD9 gene expression", *Modern Pathology,* vol. 23, no. 5, pp. 751-762.

Wong, W.F., Kohu, K., Chiba, T., Sato, T. & Satake, M. 2011, "Interplay of transcription factors in T-cell differentiation and function: the role of Runx", *Immunology,* vol. 132, no. 2, pp. 157-164.

Yang, Q., Hornick, J.L., Granter, S.R. & Wang, L.C. 2009, "Merkel cell carcinoma: lack of KIT positivity and implications for the use of imatinib mesylate", *Applied Immunohistochemistry & Molecular Morphology,* vol. 17, no. 4, pp. 276-281.

Yiengpruksawan, A., Coit, D.G., Thaler, H.T., Urmacher, C. & Knapper, W.K. 1991, "Merkel cell carcinoma. Prognosis and management", *Archives of Surgery,* vol. 126, no. 12, pp. 1514-1519.

Zebedin, E., Simma, O., Schuster, C., Putz, E.M., Fajmann, S., Warsch, W., Eckelhart, E., Stoiber, D., Weisz, E., Schmid, J.A., Pickl, W.F., Baumgartner, C., Valent, P., Piekorz, R.P., Freissmuth, M. & Sexl, V. 2008, "Leukemic challenge unmasks a requirement for PI3Kdelta in NK cell-mediated tumor surveillance", *Blood,* vol. 112, no. 12, pp. 4655-4664.

Zhang, L., Conejo-Garcia, J.R., Katsaros, D., Gimotty, P.A., Massobrio, M., Regnani, G., Makrigiannakis, A., Gray, H., Schlienger, K., Liebman, M.N., Rubin, S.C. & Coukos, G. 2003, "Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer", *The New England Journal of Medicine,* vol. 348, no. 3, pp. 203-213.

zur Hausen, H. 2008, "A specific signature of Merkel cell polyomavirus persistence in human cancer cells", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 105, no. 42, pp. 16063-16064.