Molecular aberrations in Merkel cell carcinoma with distinction of polyomavirus positive and negative tumors

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ABSTRACT

Merkel cell carcinoma (MCC) is a rare aggressive skin cancer categorized into two subgroups according to presence of Merkel cell polyomavirus (MCV). Around 20% of the tumors are polyomavirus negative and associate with worse prognosis than MCV-positive tumors. Current treatment modalities for advanced MCC are scarce, and new targeted therapies are warranted. Therefore, we need to further examine the molecular pathology of MCC with respect to the polyomavirus status of the tumor to seek potential therapeutic targets. We hypothesized that there could be significant differences in microRNA, gene mutation and RNA expression patterns of MCV-positive and MCV-negative tumors. In this study, we utilized formalin-fixed paraffin-embedded primary MCC samples to investigate molecular aberrations of MCV-negative and MCV-positive MCC. Microarrays and quantitative reverse transcription PCR were used to determine microRNA expression, while next-generation sequencing was applied for analyzing mutational and RNA expression patterns of MCC tumors. Tumor protein expression was examined by immunohistochemistry. Further, clinical details of MCC patients were statistically correlated to molecular alterations.

Regarding microRNA patterns, we uncovered different expressions of four miRNAs in MCV-negative tumors compared to MCV-positive tumors. The most notable of these miRNAs was miRNA-34a, a known tumor suppressor, which was underexpressed in MCV-negative tumors and therefore might contribute to the pathogenesis of that MCC subgroup. In our sequencing projects, we observed generally higher mutational frequency in MCV-negative tumors.

Notably, we recorded unprecedented *EGFR* mutations in 22% of the MCC tumors studied. In addition, there was overexpression of *ALK* and *EZH2* at RNA level in MCC tumors. We then proceeded to investigate the expression of ALK, EGFR and EZH2 at protein level by immunohistochemistry and found frequent expression of ALK and EZH2. Interestingly, ALK expression by immunohistochemistry correlated strongly with MCV positivity of the tumor. EGFR expression was detected in 7/31 MCV-negative tumors, while none of the MCV-positive tumors expressed EGFR. There was no significant correlation between the alterations and clinical parameters of the patients. We concluded that the relationship of ALK and MCV needs further investigation, while EZH2 could be a potential therapeutic target in MCC since it is frequently expressed, regardless of the MCV status of the tumor. In addition, the subgroup of EGFR positive MCC-negative tumors might benefit from EGFR inhibitor treatment. Our results provide evidence that there are distinct molecular aberrations in MCV-negative and MCV-positive MCC.

LIST OF ORIGINAL PUBLICATIONS

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

I Veija T, Sahi H, Koljonen V, Böhling T, Knuutila S, Mosakhani N.: miRNA-34a underexpressed in Merkel cell polyomavirus-negative Merkel cell carcinoma. *Virchows Archiv.* 2015 Mar;466(3):289-95.

II Veija T, Sarhadi VK, Koljonen V, Böhling T, Knuutila S.: Hotspot mutations in polyomavirus positive and negative Merkel cell carcinomas. *Cancer Genetics.* 2016 Jan-Feb;209(1-2):30-5.

III Veija T, Koljonen V, Böhling T, Kero M, Knuutila S, Sarhadi VK.: Aberrant expression of ALK and EZH2 in Merkel cell carcinoma. *BMC Cancer*. 2017 Mar 31;17(1):236.

IV Veija T, Kero M, Koljonen V, Böhling T.: ALK and EGFR expression by immunohistochemistry are associated with Merkel cell polyomavirus status in Merkel cell carcinoma. *Histopathology*. 2019 Jan epub ahead of print.

ABBREVIATIONS

AJCC – American Joint Committee on Cancer
ALCL – Anaplastic large-cell lymphoma
ALK – Anaplastic lymphoma kinase
BCC – Basal cell carcinoma
BRAF – Serine/Threonine-protein kinase B-Raf
CK20 – Cytokeratin-20
CLL – Chronic lymphocytic leukemia
CML – Chronic myelogenous leukemia
CTLA4 – Cytotoxic T-lymphocyte-associated protein 4
DNA – Deoxyribonucleic acid
EGFR – Epidermal growth factor receptor
EZH2 – Enhancer of zeste homolog 2
FFPE – Formalin-fixed paraffin-embedde
FISH – Fluorescence in situ hybridization
LT – Large T antigen
MCC – Merkel cell carcinoma
MCV – Merkel cell polyomavirus
miRNA – MicroRNA
mRNA – Messenger RNA
NGS – Next-generation sequencing
NSCLC – Non-small cell lung carcinoma
PCR – Polymerase chain reaction
PD-L1 – Programmed death-ligand 1
PD-1 – Programmed cell-death 1 receptor
RB1 – Retinoblastoma gene
RNA – Ribonucleic acid
SCC – Squamous cell carcinoma
SLNB – Sentinel lymph node biopsy
ST – Small T antigen
TMA – Tissue micro array
TTF-1 – Thyroid transcription factor 1

1 INTRODUCTION

Merkel cell carcinoma (MCC) is a rare and aggressive skin cancer of neuroendocrine origin. It most often presents in the head and neck region of aged individuals; however it can also manifest at younger age, particularly alongside an immunocompromising condition (Agelli 2010, Sahi 2010). UV radiation is the main extrinsic risk factor for MCC, and thus the incidence varies according to geographic location, being highest in locations with sustained sun-exposure (Agelli 2010, Youlden 2014).

In 2008, a previously unknown polyomavirus, named Merkel cell polyomavirus (MCV), was discovered from MCC tumor tissue (Feng 2008), and since then, MCC has been categorized into MCV-positive and MCV-negative subgroups. Around 80% of the MCC tumors are MCV-positive, and the so-called T antigens encoded by MCV seem to mediate the oncogenic properties of the virus (Houben 2010, Shuda 2008, Shuda 2015, Verhaegen 2015). Even though MCV-negative tumors represent a minority of MCC cases (20%), they tend to be more aggressive than MCV-positive tumors (Moshiri 2017). Notably, the pathogenesis of MCV-negative MCC is weakly established. Previous mutational studies have reported various infrequent mutations predominantly in MCV-negative tumors, but no key driver mutations have been found (Erstad 2014). Besides the mutational profiles, there is a lack of knowledge regarding the expression of cancer genes at RNA level in MCC. Certain molecules that could alter the expression of oncogenes or tumor suppressors are MicroRNAs. Previously, only one study addressed the MicroRNA profiles of MCC tumors (Xie 2014), and therefore further investigation is warranted.

Management of MCC is based on surgical excision of the primary tumor which can be a curative treatment. However, for advanced MCC the treatment options are scarce (Lebbe 2015). Traditional chemotherapeutics are often ineffective, and even though new immunotherapies seem effective, some MCC tumors do not respond to them (Kaufman 2016, Nghiem 2017). Therefore, there is a demand for new targeted treatments taking into account the MCV status and other individual molecular characteristics of the tumor. For these treatments to emerge, we need to further investigate the genetic and molecular profiles of MCC tumors with respect to the tumor MCV status, ultimately to seek for potential therapeutic targets.

2 REVIEW OF THE LITERATURE

2.1 Skin cancer

Skin cancer is the most frequent type of cancer in humans. There are over 5 million cases per year accounting for as much as 40% of all cancer cases worldwide (GBD 2015 Disease and Injury Incidence and Prevalence Collaborators 2016). In Finland, there were 3824 cases of skin cancer in 2016 according to the Finnish Cancer Registry. The age-standardized mortality was 4.7/100 000 which constitutes for 265 deaths caused by skin cancer during the year 2016 in Finland (Finnish Cancer Registry, https://tilastot.syoparekisteri.fi/syovat) The three main skin cancer types are melanoma, basal cell carcinoma and squamous cell skin carcinoma.

Basal cell carcinoma is the most common of all skin cancers, and together with squamous cell carcinoma they belong to the group of non-melanoma skin cancers that also includes rare cancer types such as Merkel cell carcinoma (Dubas 2013). Figure 1 shows the basic division of skin cancer subgroups, and Figure 2 presents the proportions of different skin cancer types diagnosed in Finland. In addition to the cancers presented in Figure 1, many other cancers can locate on the skin, although not considered as skin cancer, and some of these are mentioned in the Table 1.

Ultraviolet (UV) radiation is the primary cause of skin cancer, augmenting the risk of all main skin cancer types. It is estimated that over 90% of skin cancers result from UV radiation, and exposure to sunlight is the main source of UV radiation. Sunbathing and artificial tanning beds are serious worldwide health risks (Gallagher 2010). Other risk factors include age, Caucasian skin, smoking, ionizing radiation, immunosuppression and viral infections (Apalla 2017, Saladi 2005, Cakir 2012). The appearances of skin cancers are diverse, ranging from discolorations, or ulcers on the skin, to enlargement or other changes in an existing mole.

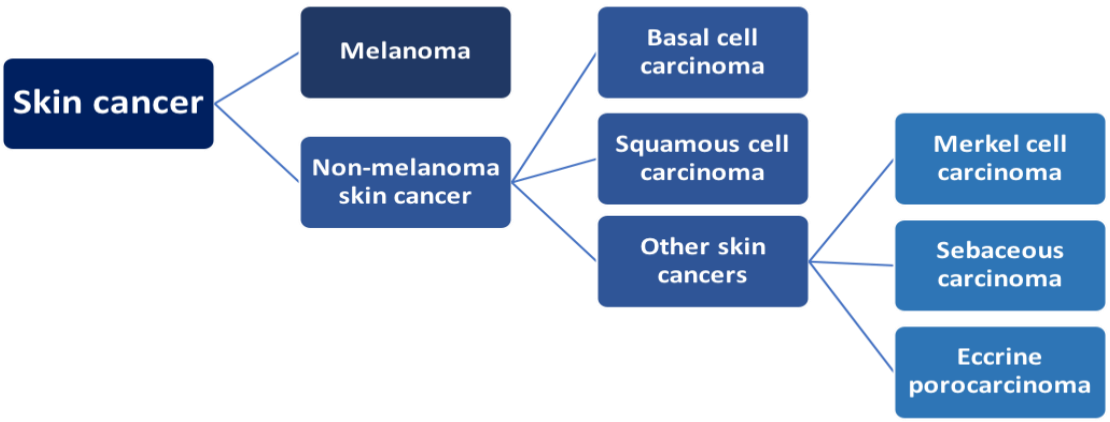

Figure 1. The basic classification of skin cancer subgroups.

2.1.1 Non-melanoma skin cancer

Non-melanoma skin cancers are comprised of a vast number of different tumor types, and only the most frequent types are addressed in this dissertation. Basal cell carcinoma (BCC) is the most frequently diagnosed malignancy, particularly in the Caucasian population (Lomas 2012). Incidence of BCC varies between countries, as it is strongly affiliated with the geographic location of the country (Verkouteren 2017). The incidence has risen around 5 % annually in Europe and 2 % annually in the USA. The average incidence in the UK is 76/100 000 (Lomas 2012, de Vries 2012). In Finland, the age-standardized incidence is 162/100 000 for men and 147/100 000 for women (Finnish Cancer Registry, 2016). The prognosis of BCC is typically excellent. It has remarkably low metastasizing rates and generally does not affect the survival of patients (Lo 1991).

However, it is associated with increased risk of other skin cancers; squamous cell carcinoma by 3-fold and melanoma by 2-fold (Flohil 2013). An even more evident concern is that primary BCC patients have a 17-fold risk of developing another BCC compared to the general population (Flohil 2013).

DNA damage caused by UV radiation is the most renowned cause of BCC. BCC often presents as a shiny red nodule with visible small dilated vessels called teleangiectasia. It can be ulcerated and thus resemble a chronic wound for example. There are three main forms of BCC according to the histologically determined growth pattern of the tumor: superficial, infiltrative and nodular BCC. The nodular form tends to arise in the head and neck region, whereas the superficial form commonly lies on the trunk (Verkouteren 2017). BCC is divided into low-risk and high-risk tumors based on the location of the tumor and histological growth pattern. Superficial and nodular BCC belong to the low-risk group, while the infiltrative type is considered as a high-risk tumor. In addition, all the recurrent BCCs as well as BCCs occurring on immunosuppressed individuals or on a prior radiation site are considered as high-risk tumors (Work Group 2018). Surgical removal of the tumor with 3-5 mm margins is normally sufficient treatment for BCC and is the recommended treatment for all high-risk tumors. Sometimes for low-risk BCC, cryotherapy or topical agents such as 5-Fluorouracil or Imiquimod are utilized as the primary treatment (Work Group 2018).

The second most frequent non-melanoma skin cancer is squamous cell carcinoma (SCC). Similarly to BCC, the incidence of SCC is bound to the latitudinal location of the country, as UV radiation is the main risk factor. In the UK, the average incidence rate is around 23/100 000 (Lomas 2012). In

Finland, the age-standardized incidence in 2016 was 40.2/100 000 for men and 23.4/ 100 000 for women. In total, there were 1719 confirmed cases of SCC in 2016 (Finnish Cancer Registry). Prognosis of local SCC is fairly good since the 5-year survival rate is around 90%. However, SCC is prone to metastasize, and the 5-year survival rate lowers to 42% in advanced disease (Que 2018). In addition to UV radiation, risk factors for SCC include age, male sex, immunosuppression and the human papilloma virus (HPV) infection (Didona 2018). The average age of SCC onset is around 65-years, and it is three times more common in men than women. Solid organ transplantation and associated immunosuppressive treatment can increase the risk of SCC as much as 250 times compared to the general population (Que 2018).

The clinical appearance of SCC is highly variable. It often arises from a pre-malignant lesion called actinic keratosis or solar keratosis (Fernandez-Figueras 2015). Actinic keratoses are scaly white or reddish macules typically located on sun-exposed skin. Once SCC appears it can form an ulcer or necrosis on its center. Sometimes it grows on an existing wound or scar tissue. SCC that only grows in the epidermis and not in the deeper layers of the skin, in situ carcinoma, is also known as Morbus Bowen (Apalla 2017, Ratushny 2012). Like BCC, the treatment of choice for SCC is surgical removal of the tumor, either by traditional excision or applying Mohs micrographic surgery. Radiation treatment and chemotherapeutics can be utilized for advanced SCC (Apalla 2017).

About 1% of non-melanoma skin cancers rise from the appendages or adnexa of the skin, including sebaceous glands, sweat glands and sensory organs. Examples of such entities are sebaceous carcinoma, eccrine porocarcinoma and Merkel cell carcinoma (Dubas 2013).

2.1.2 Melanoma

Cutaneous melanoma evolves from melanocytes that are cells located in the basal layer of the epidermis. As in non-melanoma skin cancers, the incidence of melanoma is bound to geographics and ethnicity (Apalla 2017, Erdei 2010). Melanoma is the second most common form of skin cancer. In Finland, there were 1947 confirmed cases in 2016. The age-standardized incidence being 38.4/100 000 for men and 33.8/100 000 for women (2016) The respective mortality rates are 6.1/100 000 and 2.1/100 000 (Finnish Cancer Registry). Melanoma is seemingly more aggressive and lethal compared to non-melanoma skin cancers. According to data from the Finnish Cancer Registry, from the 265 skin cancer deaths in Finland in 2016, 80% were caused by melanoma and only 20% by non-melanoma skin cancers.

UV radiation exposure is the fundamental risk factor for melanoma. Particularly intense, occasional exposure to fair skinned individuals at a young age is harmful and increases the risk for melanoma dramatically (Psaty 2010, Garibyan 2010). In addition to UV radiation, fair skin, positive family history and immunosuppression associated with HIV infection or organ transplant are risk augmenting factors. Beyond that, previous non-

melanoma skin cancer multiplies the risk for melanoma by 4-fold (Psaty 2010, Leachman 2009). Even though quite few melanomas develop on an existing mole, the presence of numerous moles clearly increases the risk for melanoma (Holly 1987, Tucker 1997).

The classical warning signs or characteristics of melanoma can be remembered with ABCDE mnemonic which stands for: Asymmetry, Border irregularity, Color variation, Diameter more than 6 mm, Enlarging or Evolving (Friedman 1985, Friedman 1991). Standard histological evaluation of melanoma includes the definition of Breslow thickness, that is the tumor's maximal thickness in millimeters, and the Clark level, which depicts the tumor's invasion depth according to different skin structures. Clark levels range from Level 1, or in situ melanoma, to level 5, or melanoma infiltrating the subcutaneous fat (Kauffmann 2014). However, the Clark level currently has no significance in clinical practice, whereas the Breslow thickness is essential for staging melanoma. The most important prognostic factors in addition to the Breslow thickness are the presence of ulceration and the number of mitotic cells in the tumor, all of which predict poorer prognosis (Gershenwald 2017).

Wide surgical excision is the main treatment for local invasive melanoma and is often curative. Breslow thickness is considered when determining sufficient healthy tissue margins. For tumors with thickness over 4 mm, a margin of at least 2 cm is recommended (Ethun 2016). Melanoma spreads to the lymph nodes vividly, and the employment of Sentinel Lymph Node Biopsy (SLNB) is decided based on the Breslow thickness, and SLNB is

recommended for at least all melanomas between 1-4 mm in thickness (Wong 2018).

In addition to surgery, treatment options for advanced melanoma include systemic targeted therapies with BRAF, CTLA4 and PD-1 inhibitors (Coit 2016).

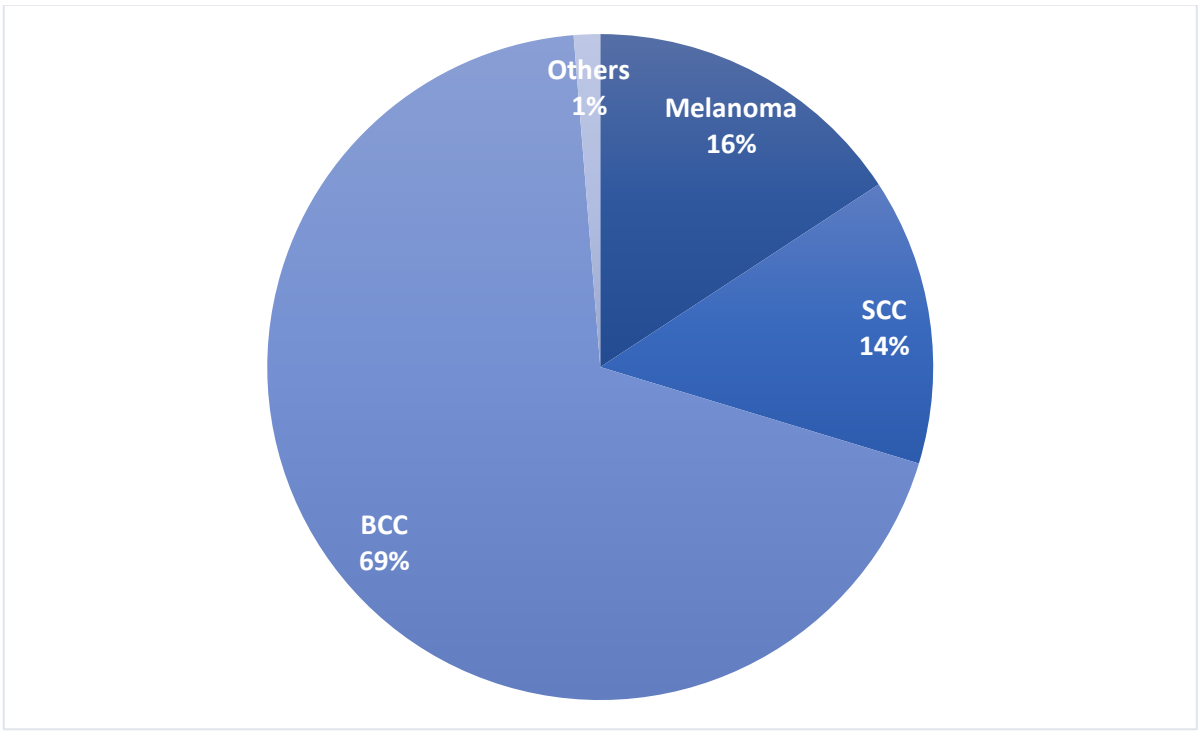

Figure 2. The relative proportions of skin cancer diagnoses in Finland according to Finnish Cancer Registry data in 2016.

Table 1. Many other forms of cancer besides melanoma and non-melanoma skin cancers can appear on the skin. Some of those are presented in this table.

Other cancers that can locate on the skin
Dermatofibrosarcoma Protuberans
Kaposi's Sarcoma
Paget's Disease
Leiomyosarcoma
Angiosarcoma
Cutaneous T-cell lymphoma

2.2 Merkel cell carcinoma

Merkel cell carcinoma (MCC) was first introduced in 1972 as trabecular carcinoma of the skin (Toker 1972). Since then, many names have been coupled to this malignancy such as primary small-cell carcinoma of the skin, neuroendocrine carcinoma of the skin and Merkel cell carcinoma (Hitchcock 1988). MCC was historically thought to arise from Merkel cells, mechanoreceptors located on the basal layer of epidermis (Figure 6). However, more recent evidence hints, for example, that epidermal stem cells might in fact be the cell of origin (Tilling 2012). Even though the cell of origin in MCC has been widely disputed and remains an open debate, Merkel cell carcinoma has become the most applied term.

2.2.1 Epidemiology

UV radiation is the most important exogenous predisposing factor for MCC (Lunder 1998, Popp 2002, Wong 2015). Thus, over 50% of the tumors occur in the sun-exposed head and neck region (Agelli 2010, Kukko 2012, Albores-Saavedra 2010). MCC primarily manifests in the elderly population, the mean age of the patients being around 75 years (Agelli 2010, Kukko 2012). Additionally, it infrequently appears in the younger population with immunocompromising conditions including, an HIV-infection or an organ transplant recipient associated with immunosuppressive treatment (Sahi 2010, Koljonen 2009b). The male-to-female ratio is somewhat controversial, as some population-based studies have reported male predominance (Hodgson 2005, Allen 2005) and others either found no difference (Lyhne 2011) or claimed a minor female predominance (Kukko 2012). MCC patients

have a higher risk for developing another cancer and vice versa. The elevated risk for Chronic lymphocytic leukemia and BCC is especially documented in Finnish MCC patients (Koljonen 2009a, Koljonen 2010).

MCC is a relatively rare tumor. The incidence rate is highest in Australia, being 1.6/100 000 (age-standardized to USA standard population), probably due to strong, sustained sun exposure (Youlden 2014). In the USA, the incidence rate has been growing substantially and was 0.79/100 000 in 2011 (not age-standardized) (Fitzgerald 2015). In Finland, there were 295 cases of Merkel cell carcinoma reported to the Finnish Cancer Registry from 1989 to 2008. Accordingly, the age-standardized (World standard population) incidence was 0.11/100 000 for men and 0.12/100 000 for women (Kukko 2012).

MCC is an exceedingly aggressive form of cancer. Around one-quarter of the cases have local lymph node involvement, while distant metastasis is found in 7-12% of MCC cases (Lebbe 2015, Harms 2016, Lemos 2010). Disease-specific overall mortality is estimated at 33-46%, (Lemos 2010, Becker 2010) which surpasses the mortality rates of melanoma (Hodgson 2005, Grabowski 2008).

2.2.2 Histology

Microscopic evaluation of hematoxylin and eosin stained MCC samples commonly illustrate spherical, small blue cells with hyperchromatic nuclei (Figure 3). The ratio of cytoplasm to nucleus is diminished (Bichakjian 2007). MCC cells embody neuroendocrine characteristics such as neurosecretory

granules seen in electron microscopy and expression of neuron-specific enolase (Hitchcock 1988). There are three histologic subtypes of MCC: intermediate, small-cell and trabecular subtype (Ratner 1993, Jaeger 2012). Intermediate is the most common type (Figure 3), while trabecular is the rarest. The small-cell type resembles small-cell lung carcinoma; it is poorly differentiated and advances rapidly, as does the intermediate type (Jaeger 2012).

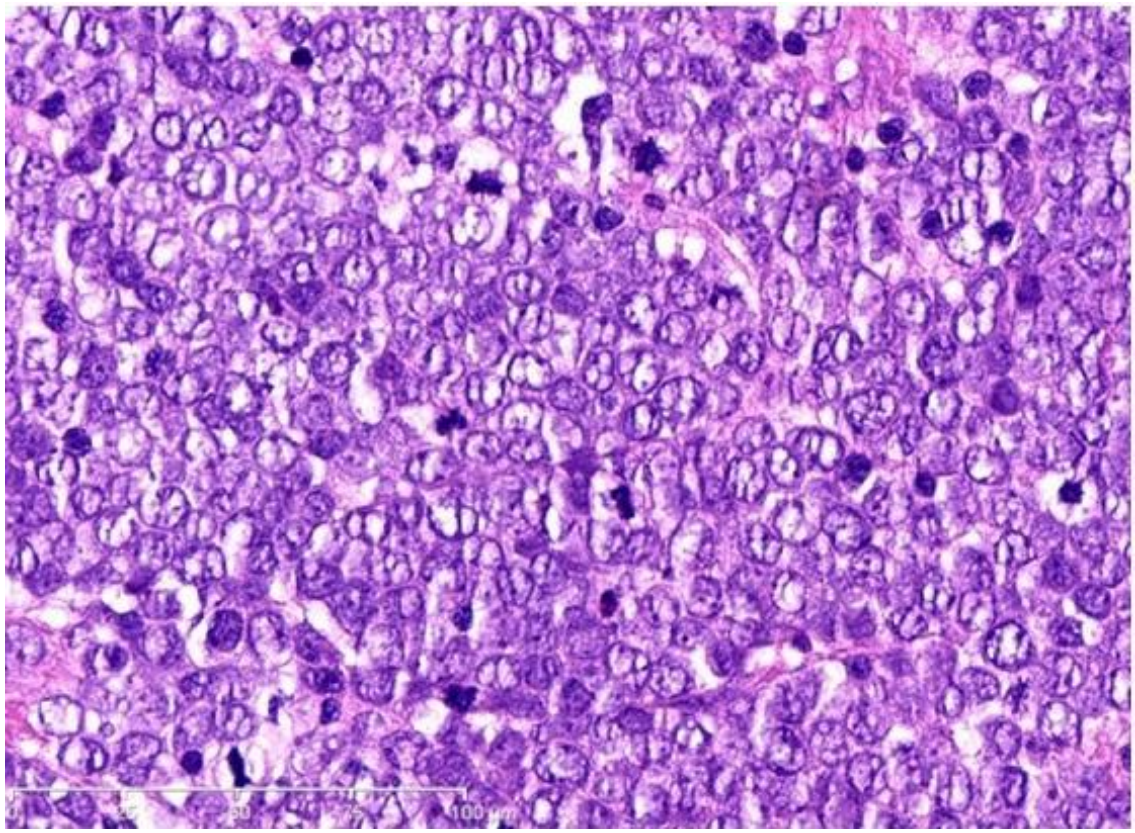

Figure 3. Microscopic view of intermediate type MCC. Hematoxylin & eosin staining. Modified from (Jaeger 2012).

2.2.3 Clinical presentation

MCC typically presents as a nodular mass, the size of which can vary from millimeters to several centimeters. The tumor is located in the dermal layer of the skin and is often covered by intact epidermis. MCC may, however, ulcerate to the skin surface (Heath 2008). Typical macroscopic findings are displayed in Figures 4 and 5. MCC has a strong propensity to metastasize. The invasion of local lymph nodes is notably more common in MCC when compared to other malignant skin tumors (Prewett 2015). The most common sites of distant metastasis are distant lymph nodes, liver and lung (Kouzmina 2017). Beyond being a skin lesion, Merkel cell carcinoma can manifest in unusual and curious ways. Examples of this are presented in Table 2.

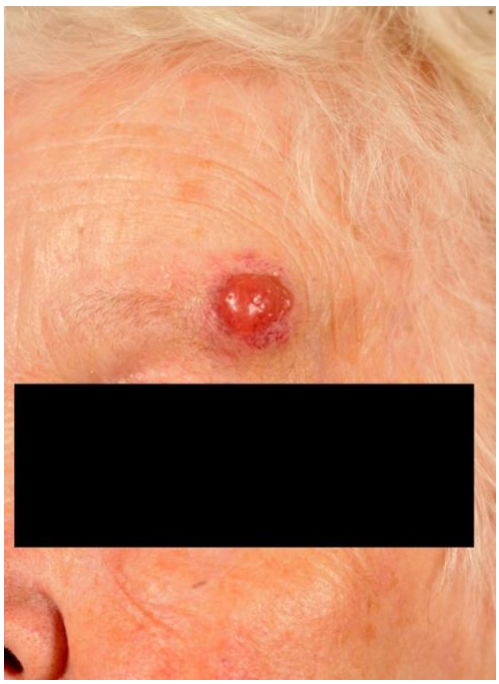

Figure 4. Typical nodular MCC on eyebrow. Courtesy of Dr. Virve Koljonen.

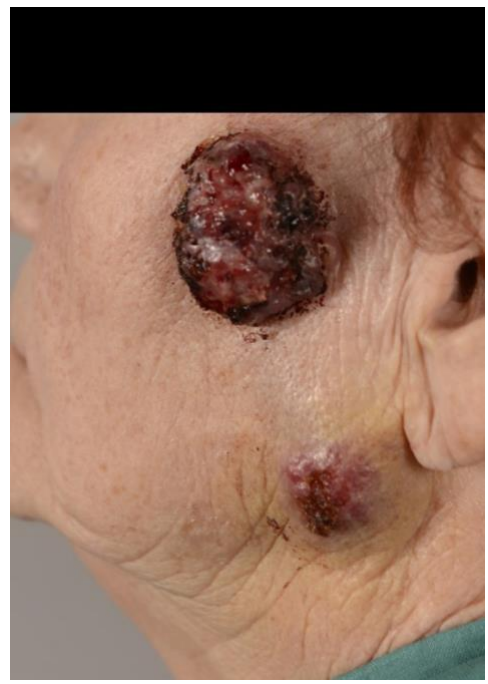

Figure 5. Ulcerated large MCC on the cheek and local metastasis on the neck. Courtesy of Dr. Virve Koljonen

Table 2. This table presents rare and somewhat bizarre cases of MCC found in the literature.

MCC mimicking cellulitis	**49-year-old woman was treated with antibiotics for presumed osteomyelitis of the left fifth digit. Debridement of the digit after failure of antibiotic treatment revealed MCC and patient was further diagnosed with metastatic disease.** (Safa 2018)
Pleural fluid metastasis of MCC	**68-year-old woman was first diagnosed with MCC of the buttocks which was surgically excised. However, the disease relapsed with vast nodal spread. 2-years after the primary excision, metastasis to left axillary lymph nodes and pleural fluid was discovered.** (Rhee 2018)
Intra-thoracic MCC	**64-year-old woman presented with large intra-thoracic mass which was biopsied and established as MCC. She was treated for vulvar MCC 10-years earlier but no local recurrence was present.** (Kong 2017)
Secondary MCC on skin graft donor site	**92-year-old male was diagnosed with MCC on his left cheek which was excised and full thickness skin graft was applied. 4-years later, secondary MCC on the supraclavicular donor site was diagnosed.** (Aloraifi 2017)
Testicular MCC	**66-year-old male presented with left testicular swelling and was diagnosed with MCC of the testis. 3-months later tumor metastasized to right testis. No primary skin lesion was identified.** (Mweempwa 2016)
Anal canal MCC	**42-year old woman was diagnosed with MCC on the anal canal. She died 13 months after diagnosis due to metastatic disease.** (Paterson 2003)
Subcutaneous MCC	**63-year-old woman presented with subcutaneous mass of the left arm which was diagnosed as MCC and completely confined to subcutis and showed no dermal or epidermal involvement in MRI scan or histopathologic examination.** (Huang 2005)
Oral MCC	**28-year old male was diagnosed with oral MCC in alveolar mucosa extending to floor of the mouth and submandibular lymph node metastasis. Hemimandibulectomy and neck dissection was performed followed by radiotherapy.** (Prabhu 2010)

2.2.4 Diagnosis and staging

The diagnosis of MCC begins with surgical excision of the suspected skin lesion with 1-2 mm clinical margins, or if the location or size of the tumor is challenging, a punch biopsy may be used. A pathologist then performs the histological examination of the tumor sample combined with immunohistochemical staining. Cancers that can cause challenges in differential diagnostics include small-cell lung carcinoma, carcinoid tumor, malignant lymphoma, and small-cell melanoma. Positive staining of Cytokeratin-20 (CK20), which is fairly specific for MCC among skin tumors, and negative staining of Thyroid transcription factor 1 (TTF-1) is typically required for MCC diagnosis. The absence of TTF-1 expression differentiates MCC from small-cell lung carcinoma (Dancey 2006).

The first adequate staging system for MCC was produced by the American Joint Committee on Cancer (AJCC) in 2009 (Edge 2010). Since then, an updated staging system was presented in the 8th edition of the AJCC cancer staging manual, replacing the previous system as of January 2018 (Harms 2016). In this system, the size of the primary tumor, invasion to the local lymph nodes and presence of distant metastases are taken into account. Stage is defined as either clinical or pathological depending on whether the evaluation is based on clinical examination, as for example lymph node palpation, or microscopic examination by a pathologist. Table 3 presents the system in detail.

Due to the high tendency to invade and metastasize, pathological examination of the local lymph nodes via the Sentinel lymph node biopsy

(SLNB) is recommended in all cases of MCC (Lebbe 2015). About 30% of MCC cases that clinically present lymph node negative show micrometastasis in SLNB. Thus, SLNB aids considerably in staging MCC precisely and planning adequate treatment (Lebbe 2015, Prewett 2015)

Table 3. The new TNM classification and staging of MCC according to AJCC cancer staging manual. (Harms 2016) Reproduced with permission from Springer nature.

	Clinical stage groups (cTNM)				**Pathological stage groups (pTNM)**		
	T	**N**	**M**		**T**	**N**	**M**
0	T_{is}	N0	M0	0	T_{is}	N0	M0
I	T1	N0	M0	I	T1	N0	M0
IIA	T2–3	N0	M0	IIA	T2–3	N0	M0
IIB	T4	N0	M0	IIB	T4	N0	M0
III	T0–4	N1–3	M0	IIIA	T1–4	N1a(sn) or N1a	M0
					T0	N1b	M0
				IIIB	T1–4	N1b–3	M0
IV	T0–4	Any N	M1	IV	T0–4	Any N	M1

T	**N**		**M**
Tx, primary tumor cann	cNx, regional lymph nodes cannot be clinically asse	pNx, regional lymph nodes cannot be assessed (e.g., previously removed	M0, no distant metastasis M1, distant metastasis

T	N		M
ot be assessed T0, no primary tumor T_{is}, in situ primar y tumor T1, primary tumor ≤2 cm T2, primary tumor >2 cm but ≤5 cm T3, primary tumor >5 cm T4, primary tumor inva des fascia, muscle, cartilage, o r bone	ssed (e.g., previously removed for another reason, body habitus) cN0, no regional lymph node metastasis by clinical or radiologica l evaluation cN1, clinically dete cted regional nodal metastasis cN2, in-transit metast asis without lymph node metasta sis cN3, in-transit metast asis with lymph node metasta sis	for another reason) or not removed for pathological evaluation pN0, no regional lymph node metastasis detected on pathological evaluation pN1a(sn), clinically occult nodal metastasis identified only by sentinel lymph node biopsy pN1a, clinically occult regional lymph node metastasis following lymph node dissection pN1b, clinically or radiologically detect ed regional lymph node metastasis, patholog ically confirmed pN2, in-transit metastasis without lymph node metastasis pN3, in-transit metastasis with lymph node metastasis	M1a, metastasis to distant skin, distant subcutaneous tissue, or distant lymph nodes M1b, lung M1c, all other distant sites

2.2.5 Treatment

MCC treatment is based on wide surgical excision of the primary tumor with clear margins. No extensive studies have been conducted to evaluate sufficiency of the margins, but 1-2cm of healthy tissue is the most customary practice (Lebbe 2015, Prewett 2015). Definitive wide excision (re-excision) is often done after the initial diagnostic excision and pathological confirmation of MCC. Direct closure of the wound is desirable, although skin grafts, local skin flaps or even microvascular flaps are occasionally necessary for reconstruction. When planning the reconstruction, the need for following adjuvant treatment such as local radiation should be taken into account.

Mohs micrographic surgery, developed in the 1930s, is occasionally utilized overseas for primary treatment, and it might be beneficial in reaching clear margins, especially in areas where preservation of tissue is crucial (Tai 2013). In this technique, tissue is removed gradually, and each resection is microscopically examined by a pathologist for tumor cells and indicating a demand for further removal which continues until the removed tissue is free of tumor cells (Mikhail 1999). In a recent review study, Mohs surgery and traditional wide excision appeared equally efficient in treating local MCC (Stage I-II). There was no difference in overall survival or histological presence of residual tumor in surgical margins between the two modalities (Singh 2018). However, In Finland, Mohs surgery has not been established in the treatment of MCC, and wide excision with 1-2 cm margins remains as the treatment of choice (Personal communication with Dr. Koljonen and Dr. Koskivuo).

Radiotherapy has been used as a primary treatment without surgical intervention for local MCC. However, surgery has a superior impact on survival and remains the cornerstone of initial treatment (Wright 2018). Furthermore, adjuvant radiotherapy to the surgical excision site is recommended due to its positive effect on survival in retrospective studies (Lebbe 2015, Fiedler 2018). After initial tumor removal, lymph node dissection or local radiotherapy to the nodal area is rational in cases where SLNB is positive or has not been carried out (Lebbe 2015, Prewett 2015).

For advanced MCC, chemotherapy is employed, and response is often desirable. However, the recurrence is frequent. The most utilized pharmaceuticals are carboplatin or cisplatin coupled with etoposide (Nghiem 2017, Cassler 2016). In addition to these traditional chemotherapeutics, In 2016 a human anti–PD-L1 monoclonal antibody called Avelumab became the first treatment approved by the US Food and Drug Administration (FDA) for patients with stage IV MCC (Kaufman 2016, Kaufman 2018). Avelumab targets the transmembrane protein Programmed death-ligand-1 (PD-L1) that forms a complex with programmed cell death-1 receptor (PD-1) and inhibits T cell activity. Avelumab prevents this T cell inhibition by blocking the formation of PD-L1/PD-1 complex and therefore preserves immune response. It has been suggested that cancers can evade the host's immune system via upregulation of PD-L1 (Dong 2002, Sheppard 2004, Thompson 2004). On a one-year follow-up study, Avelumab seems to have durable a response in patients that underwent at least one previous line of chemotherapy for stage IV MCC (Kaufman 2018). In addition to Avelumab, the PD-1 inhibitor Pembrolizumab has shown encouraging and durable response rates in advanced MCC as a first-line treatment and just

recently in early 2019 became approved by the FDA in treatment of metastatic MCC (Nghiem 2016, Nghiem 2019).

2.2.6 Merkel cell polyomavirus

In 2008, Feng et al. discovered the Merkel cell polyomavirus (MCV) from the tumor tissue of MCC patients (Feng 2008). The virus was clonally integrated into MCC genome, thus suggesting infectious etiology to MCC. Further studies have demonstrated that MCV is present in approximately 80% of MCC tumors (Feng 2008, Sihto 2009, Becker 2009, Garneski 2009, Kassem 2008, Mangana 2010).

Polyomaviruses are small, double-stranded DNA viruses that are very common and most of the time do not cause disease in humans. As of 2016, 13 polyomaviruses with human hosts have been identified (Polyomaviridae Study Group of the International Committee on Taxonomy of Viruses 2016, Kean 2009). MCV is the first and yet only polyomavirus known to cause cancer, although five other polyomaviruses are associated with human disease (Table 4). Like all polyomaviruses, the MCV genome encodes so-called T antigens. MCV infected MCC cells harbor truncating mutations in viral Large T antigen (LT) genomic sequence, which makes the virus incapable of replicating, and thus viruses cannot propagate outside of the infected cell. A functioning viral replication process would also facilitate cell death in tumor cells. These facts indicate that MCV infection occurs before clonal expansion of MCC tumor cells, and truncating LT mutations are required to sustain tumor growth (Shuda 2008). Furthermore, T antigens are necessary

for MCV infected MCC to maintain growth, because inhibiting T antigens trigger death of MCV-positive MCC cell lines (Houben 2010).

It has been suggested that with improved detection methods, MCV can be found in all MCC tumors (Rodig 2012). In fact, MCV DNA can occur in low quantities on normal or malignant cells and is in that case considered an occupant virus without a role in oncogenesis (Shuda 2009, Loyo 2010, Foulongne 2010). MCV tumors that harbor oncogenic, integrated MCV genome express LT, which can be demonstrated with mouse monoclonal antibody CM2B4. Thus, the presence of a sufficient copy number of MCV DNA is required for the MCC tumor to be determined MCV-positive (Sihto 2009, Higaki-Mori 2012, Bhatia 2010a, Bhatia 2010b).

Table 4. In addition to MCV, five other human polyomaviruses are known to have a disease correlate.

Polyomavirus	**Associated disease**
Trichodysplasia spinulosa polyomavirus	Trichodysplasia spinulosa (DeCaprio 2013)
BK polyomavirus	Polyomavirus associated nephropathy (DeCaprio 2013)
JC polyomavirus	Proggressive multifocal leukoencephalopathy (DeCaprio 2013)
Human polyomavirus 6	Pruritic and dyskeratotic dermatosis (Nguyen 2017)
Human polyomavirus 7	Epithelial hyperplasia (Nguyen 2017)

2.2.7 MCV-positive MCC

The majority of MCCs (80%) embodies MCV infection and is consequently called MCV-positive MCC. MCV-positive MCC is associated with a better outcome than MCV-negative MCC (Moshiri 2017, Sihto 2009, Sihto 2011, Andres 2009). These tumors tend to be located on a limb more often than MCV-negative, and express tumor suppressor RB1 (Sihto 2009, Sihto 2011).

Pathogenesis of MCV-positive MCC has been under tremendous research since the discovery of MCV. A major oncogenic factor is inactivation of tumor suppressor RB1 by the LT antigen (Borchert 2014). It has been demonstrated in vitro and in xenograft models that inactivation of RB1 by sequestration is necessary for MCV-positive MCC to maintain growth (Houben 2012).
In addition to the LT antigen, MCV-positive MCC express Small T antigen (ST). Oncogenic properties of the ST antigen are established in murine models, and are believed to initiate malignant transformation while the LT antigen sustains it (Shuda 2015, Verhaegen 2015). Recent study reported that the ST antigen binds to MYCL proto-oncogene and unites it to the histone acetyltransferase and chromatin remodeling complex, named as EP400 complex (Cheng 2017). The ST-MYCL-EP400 complex binds to specific gene promoters, activates their expression and promotes oncogenesis. MYCL and EP400 are necessary for MCV-positive MCC cell lines to maintain viability (Cheng 2017).

MCV-positive MCC tumors are associated with a high number of tumor infiltrating lymphocytes (TILs) which refer to better survival (Sihto 2012). PD-L1 expression in MCC seems to be bound to MCV-positive tumors, and PD-

L1 positivity associates with a high number of TILs, suggesting that immune response promotes PD-L1 expression in the presence of MCV (Lipson 2013).

2.2.8 MCV-negative MCC

MCV-negative tumors comprise nearly 20% of MCC cases and they are more aggressive, having a greater risk to metastasize and cause death (Moshiri 2017). The pathogenesis of MCV-negative MCC is more uncharted than is MCV-positive MCC. Studies have shown that MCV-negative tumors entail a remarkably higher mutational burden, and UV light associated mutations are especially frequent (Wong 2015, Goh 2016, Harms 2015, Cimino 2014).

Loss of RB1 expression is characteristic for MCV-negative MCC, but the underlying mechanism is not completely understood (Bhatia 2010a, Sihto 2011). However, deletions of *RB1* locus are observed more frequently in MCV-negative MCC (Paulson 2009, Sahi 2014, Van Gele 1998, Larramendy 2004). In addition to RB1 deletions, *TP53* mutations are seen more frequently in MCV-negative MCC tumors (Sihto 2011). *TP53* is a gene encoding for a well-established tumor suppressor protein p53. It is estimated that over 50% of human cancers harbor *TP53* mutations (Hollstein 1991).

In MCV-negative MCC there are fewer TILs than in MCV-positive tumors, but when present the number of TILs associates with a better outcome, also in MCV-negative tumors, and correlates to PD-L1 positivity (Sihto 2012). In the

tumor series of Lipson et al. all of the MCV-negative tumors were PD-L1 negative (Lipson 2013).

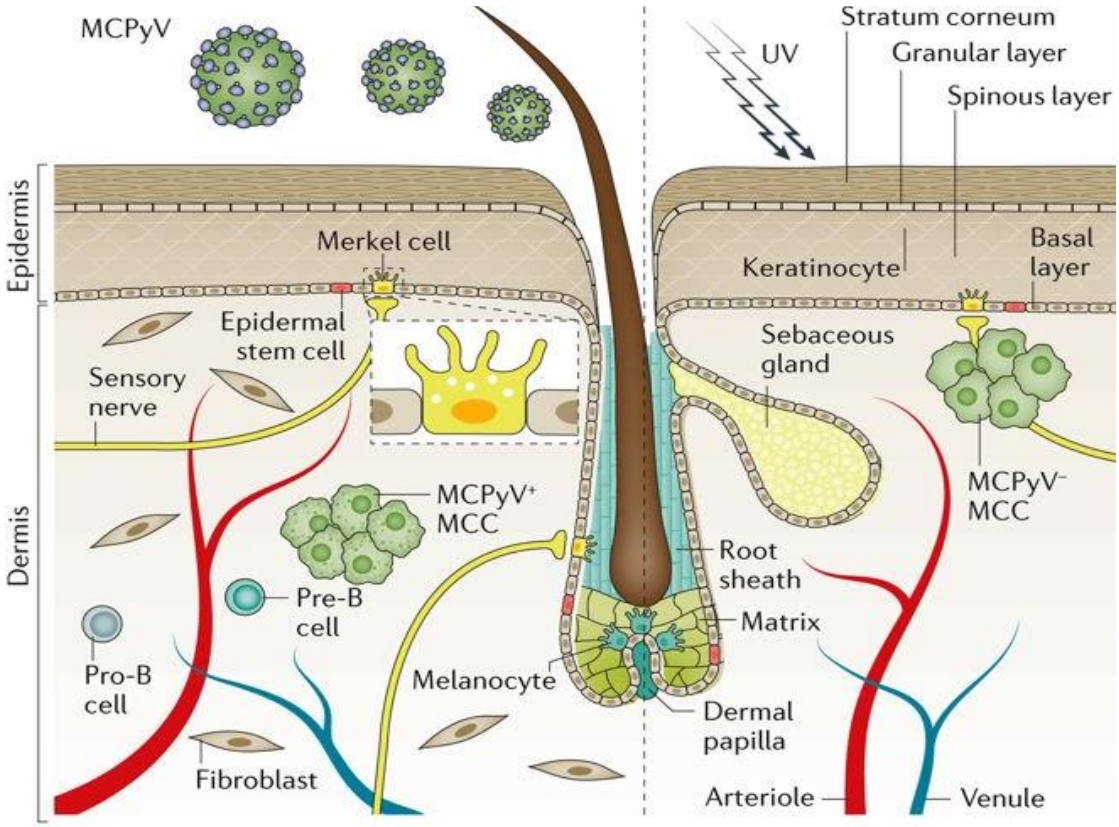

Figure 6. Figure presents the simplified normal skin structures and main risk factors for MCC; Merkel cell polyomavirus (MCPyV) on the left and UV radiation on the right. MCC tumor is growing in the dermis. Potential cells of origin are present; Merkel cell, epidermal stem cell and pre-B cell. (Becker 2017) Reproduced and modified with permission from Springer Nature.

2.3 Cancer genetics

2.3.1 Gene mutations and cancer

Mutations are persistent alterations of the nucleotide sequence of the DNA. Mutations arise from DNA damage caused by environmental factors such as radiation and irritant chemicals, or from defects in the DNA replication process. Mutations can be categorized into minor changes comprising one or a few nucleotides and larger alterations that affect large portions of nucleotide sequence.

The smallest mutational change in the DNA is a point mutation, which means that a single nucleotide has been deleted, substituted with another nucleotide or a new nucleotide has been inserted in the genomic sequence (Yi 2018). During the translation process, the nucleotide sequence is decoded by the codons, triplets of nucleotides that code for one amino acid, to form an amino acid chain, which after further modification forms a protein. The alteration of single nucleotide in the codon can transform the amino acid which that codon encodes for and thus leads to the formation of a defective protein. This type of point mutation is called a missense mutation. Another type of point mutation is silent mutation, which means that the change in the nucleotide sequence does not alter the amino acid that is decoded, and therefore has no effect on the created protein. Furthermore, a point mutation can convert a normal codon into a stop codon that terminates the translation of an amino acid chain. This type of truncating point mutation is called a nonsense mutation.

Tumor suppressor genes protect the cells from uncontrolled cell proliferation and cancer formation. Tumor suppressors often avert cell cycle progression or promote apoptosis in response to DNA damage. Mutation in a tumor suppressor gene can cause oncogenic transformation of the cells if the mutation distorts the translation of normal functioning protein. One of the key tumor suppressor genes in the human genome is the *RB1* gene. The tumor suppressing function of RB1 mainly results from its inhibitory effect on the transcription of genes required in the S-phase of the cell cycle. This effect is mediated by inactivation of the transcription factor E2F (Du 2006). Dysfunction of *RB1* is present in many cancers, although it is named due to its relationship with retinoblastoma, cancer of the retina (Murphree 1984).

A proto-oncogene is a gene that if mutated, can transform to an oncogene which has properties that promote malignant cell growth. Mutation of a proto-oncogene can cause constant abnormal expression of the gene and high activity of the coded protein that promotes cell growth and thus contributes to oncogenesis (Todd 1999). Typical examples of such proto-oncogenes are genes belonging to the RAS subfamily. Proteins they encode, for example KRAS, function as mediators in various signal transduction cascades and mutations that cause high expression or constant activation of these proteins support cancer formation (Bos 1989).

2.3.2 Chromosomal aberrations and cancer

Alterations in the chromosomal structure can result in abnormal expression of multiple genes. Translocation means that parts of different chromosomes are rearranged to form hybrid chromosomes. Translocation can be balanced where there is no loss of genetic information; the exchange of genetic information is equal between the affected chromosomes. Alternatively, a translocation can be unbalanced with either loss of genes or gain of extra genes. Sometimes, translocation results in the formation of a fusion gene with oncogenic properties. The best-known fusion gene is the *BRC-ABL1,* described first in chronic myelogenous leukemia (CML) and known as the Philadelphia chromosome. It consists of balanced and reciprocal exchange of chromosomal parts between chromosomes 9 and 22, which leads to coding of fusion protein that is a constantly active tyrosine kinase, facilitating continuous cell proliferation (Melo 1996). In addition to translocations, chromosome abnormalities include deletions, duplications and inversions. Deletion means that a part of a chromosome is lost, whereas in duplication a part is multiplied. When a chromosome portion has first detached and then reattached inversely, the alteration is called an inversion. All these structural abnormalities are a result of unsuccessful cell division.

2.3.3 Cancer genes

2.3.3.1 Anaplastic lymphoma kinase

Studies on anaplastic large-cell lymphoma (ALCL) indicated that a chromosomal translocation is frequently present in ALCL samples. It was discovered that the translocation led to the formation of a fusion gene that consisted of a nucleophosmin (*NPM*) gene and a tyrosine kinase gene, later named the anaplastic lymphoma kinase gene (*ALK*). The resulting fusion protein is the oncogenic driver in 60% of the ALCL cases (Morris 1994). Another known fusion related to *ALK* is the EML4-ALK fusion gene that accounts for 3-5% of non-small-cell lung cancers (NSCLC) and also occurs in other cancers such as colorectal adenocarcinoma and breast carcinoma (Soda 2007, Lin 2009). The fusion of *ALK* with another gene leads to overexpression of ALK that promotes cancer formation. In addition to chromosomal translocation, ALK can be overexpressed, in result of mutation of the *ALK* gene, which is apparent in some thyroid cancers (Murugan 2011). An ALK inhibitor, crizotinib, has received FDA approval for treatment of NSCLC, and there are multiple inhibitors currently undergoing clinical trials.

2.3.3.2 Epidermal growth factor receptor

The epidermal growth factor receptor (EGFR) is a cell membrane receptor for extracellular protein ligands that belong to the epidermal growth factor family. These ligands include the epidermal growth factor (EGF) and the transforming growth factor α (TGFα). Binding of ligand to EGFR initiates its

intrinsic intracellular tyrosine kinase activity which triggers downstream signaling cascades that ultimately lead to DNA synthesis and cell proliferation (Oda 2005, Yarden 1987). Mutations in the *EGFR* gene that induces overexpression or overactivity of EGFR have been reported in many types of cancer, for example NSCLC, prostate carcinoma and anal squamous cell carcinoma (Peraldo-Neia 2011, Walker 2009, Murray 2006). Many EGFR inhibitors are currently in clinical use as cancer treatment (Liang 2014). In NSCLC, occurrence of *EGFR* mutations as well as expression of EGFR predicts responsiveness to EGFR inhibitor treatment (Brandao 2012, Han 2005, Huang 2004).

2.3.3.3 Enhancer of zeste homolog 2

Enhancer of zeste homolog 2 (EZH2) is an enzyme that catalyzes DNA methylation and thus induces heterochromatin formation and repression of gene function (Vire 2006). Many types of cancer harbor overexpression of EZH2. Its oncogenic effect originates from inactivation of important tumor suppressor genes (Zingg 2015, Kim 2016). In renal cell carcinoma, overexpression of EZH2 is suggested to predict poor prognosis (Lee 2012). Inhibitors of EZH2 are currently in development, and their use in cancer treatment is an active area of research (Kim 2016).

2.3.4 MicroRNA and cancer

MicroRNAs (miRNA) are small non-coding RNA molecules that function as post-transcriptional regulators of gene expression. This group of RNA molecules were acknowledged in the early 2000s and is an emerging field in medical research. Abnormal miRNA expression can consequently modify expression of important oncogenes or tumor suppressors to promote malignant growth. miRNAs regulate gene expression by binding to messenger RNA (mRNA) and prohibit its translation to a functional protein. The human genome encodes hundreds of miRNAs, all of which have multiple target genes. Further, one gene can be regulated by many miRNAs (Williams 2008). The first human cancer that was associated with aberrant miRNA expression is chronic lymphocytic leukemia (CLL). Since then, miRNA profiling of various cancer types has expressed that abnormalities in miRNA expression are frequent in cancer. miRNAs are expected to be applicable particularly well in cancer screening and as prognostic factors (Reddy 2015).

3 AIMS OF THE STUDY

The aim of this study was to examine molecular alterations in Merkel cell carcinoma tumor samples utilizing methods of modern cancer research with special interest on distinction of polyomavirus negative and positive MCC. A further aim was to assess the correlation of molecular aberrations and clinical characteristics of MCC patients with emphasis put on disease progression.

Specified aims were to:

1) Explore and compare the MicroRNA profiles of MCV-negative and MCV-positive MCC tumors. (**STUDY I**)

2) Analyze the mutational patterns in MCV-negative and MCV-positive MCC tumors by targeted Next-generation sequencing. (**STUDY II**)

3) Quantify mRNA expression of 50 cancer-related genes in MCC by targeted NGS. (**STUDY III**)

4) Determine expression of ALK, EGFR and EZH2 and their association to MCV status in MCC tumors and evaluate clinical significance of the proteins. (**STUDY IV**)

4 MATERIALS AND METHODS

4.1 Merkel cell carcinoma samples and clinical data

The Ethics Committee of Helsinki University Hospital approved the study. The Ministry of Health and Social Affairs granted permission to collect patient data and the National Authority for Medicolegal Affairs to collect and analyze tissue samples.

Formalin-fixed, paraffin-embedded (FFPE) MCC tumor samples were collected from our nationally gathered pool of around 270 MCC samples (years 1979 – 2013) in the pathology archives. MCC diagnoses were confirmed by morphology compatible with MCC in microscopy and by immunohistochemistry positive for CK-20 (DakoCytomation, Glostrup, Denmark) and negative for TTF-1 (Novocastra reagents, Leica Biosystems, Wetzlar, Germany). MCV-detection was done previously by quantitative PCR and proportioned to reference the gene protein tyrosine phosphatase gamma receptor gene (*PTPRG*). The sample was considered positive if the MCV DNA copy number per reference gene was greater than 0.1. The process is described in detail elsewhere (Sihto 2009). Tissue micro arrays (TMA) were constructed from FFPE tumor samples. 0.6-mm tissue area of each tumor sample was inserted into an empty well on the tissue array block (**IV**).

Clinical details were extracted from clinical files and hospital records. Gathered details included patient age, sex, primary tumor location, tumor

size, information about development of metastasis, date of diagnosis and date of death from MCC.

4.2 DNA and RNA extraction

DNA was extracted from FFPE samples according to the manufacturer's instructions with a QIAamp DNA Mini Kit (250) (QIAGEN, Hilden, Germany). DNA concentration was measured with a Qubit® 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) (**II**).

Extraction of total RNA, including miRNA was carried out utilizing a miRNeasy mini Kit (QIAGEN) according to the manufacturer's instructions. RNA was quantified by either The NanoDrop-1000 Spectrophotometer (Thermo Fisher Scientific) (**I**) or Qubit® 2.0 fluorometer (Thermo Fisher Scientific) (**III**). Quality of RNA was measured by either Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) (**I**) or 2200 TapeStation System in combination with RNA ScreenTape assay (Agilent Technologies) (**III**).

4.3 Microarrays and miRNA target prediction (Study I)

Microarrays were constructed with the miRNA Complete Labeling and Hybridization kit protocol version 2.4 (Agilent Technologies). Hybridization of RNA samples was performed on Agilent's miRNA Microarray System V16 (1205 human and 144 human viral miRNAs, Sanger miRBase 16.0). The microarrays were washed with the manufacturer's washing buffers. Arrays

were scanned with Agilent's Feature Extraction (v.11.0.1.1.), and default parameters were applied to extract the data.

The following databases were employed to search for predicted mRNA targets of the miRNAs: TargetScan (http://www.targetscan.org), miRanda (http:// www.microrna.org/microrna/home.do), mirTarget2 (http:// mirdb.org/miRDB), Tarbase (http://diana.cslab.ece.ntua.gr/ tarbase), miRBase target prediction database (http://www. mirbase.org/), and PICTAR (pictar.mdc-berlin.de). To exclude false positives, only those mRNA targets that were predicted by at least four of the six programs were accounted for. Predicted mRNA targets were screened by Chipster v1.4.7 (http://chipster.csc.fi/) for significant enrichment of overrepresented pathways in the ConsensusPathDB (CPDB) by the hypergeometric test ($p < 0.05$).

4.4 Quantitative Real-Time PCR (Study I)

To validate the microarray results. Reverse transcription of the RNA was executed with a miScript II RT Kit (50) (QIAGEN) according to the manufacturer's instructions. Quantitative Real-Time PCR (qRT-PCR) was conducted with a miScript SYBR Green PCR Kit (QIAGEN) and a LightCycler 480 (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturers' protocols.

Amplification primers for miRNAs (miR-34a, miR- 30a, miR-181d, miR-142-3p, and miR-1539) and U6 were purchased from QIAGEN. Data was

normalized according to U6 expression. Duplicate RNA samples with a negative control (no template of cDNA) were included in every PCR run. Potential nonspecific amplification was assessed with melting curve analysis.

The ΔΔCt method was used for relative quantification of miRNA expression. The relative quantity (RQ) for each miRNA, compared with the quantity of U6, was calculated. Mean and median RQs for MCV-positive and MCV-negative tumors were calculated. Expression data of miRNAs was presented also as fold change (fold change = log 2 RQ).

4.5 Next-generation sequencing (Studies II and III)

Mutation and RNA expression projects (**II** and **III**) were performed by amplicon-based next-generation sequencing (NGS) using Ion Torrent technology (Thermo Fisher Scientific). The Ion AmpliSeq™ Cancer Hotspot Panel v2 (Thermo Fisher Scientific) and the Ion AmpliSeq™ RNA Cancer Panel (Thermo Fisher Scientific) were utilized to amplify target amplicons in 50 cancer-related genes and mRNA.

The DNA libraries were constructed from 10ng of DNA with the Ion AmpliSeq™ Library Kit 2.0 (Thermo Fisher Scientific). The RNA libraries were constructed from 20ng of RNA with the Ion AmpliSeq™ RNA Library Kit (Thermo Fisher Scientific). All the libraries were barcoded with the Ion Xpress™ Barcode Adapter Kit (Thermo Fisher Scientific) and the library concentrations were measured using the Qubit 2.0 Fluorometer.

Templates for sequencing were prepared using either the Ion PGM™ Template OT2 200 Kit (**II**) or the Ion PGM Hi-Q™ OT2 Kit (**III**) and Ion OneTouch™ 2 System (Thermo Fisher Scientific). Ion Sphere™ particles were enriched with Ion OneTouch ES (Thermo Fisher Scientific) and loaded onto Ion 316™ Chip (II) or to Ion 318™ Chip (**III**). Sequencing was performed on the Ion Torrent PGM™ System with the Ion PGM™ Sequencing 200 Kit v2 (**II**) and the Ion PGM Hi-Q™ Sequencing Kit (**III**).

To analyze the mutation data (**II**), the Ion Torrent Suite™ Software 4.0.2 and the Torrent Variant Caller Plugin 4.0 were applied for signal processing, base calling, sequence alignment, variant analysis, and hotspot mutation analysis. The aligned reads were visually inspected with the integrative Genomics Viewer (IGV v2.2, Broad Institute, Cambridge, MA, USA). In silico evaluation of the effect of nucleotide variation on the protein function was executed with SIFT and PROVEAN.
The average sequencing mean depth for all tumor samples was 1542X, with an average of 95% uniformity across targets. To rule out possible technical errors, the quality score threshold for variant calling was set at Q30.

For the RNA expression data (**III**), the Coverage Analysis plugin was used to create amplicon counts. The amplicon count data created was imported into a Chipster (http://chipster.csc.fi/index.shtml) for further expression analysis. Expression differences between tumor and normal skin tissue and MCV-positive and MCV-negative tumors were analyzed with DESeq2. Differently expressed genes were determined from adjusted p-values and log2 fold changes. To control for false positives, the p-values were corrected for multiple testing and the adjusted p-value or FDR (false discovery rate)

calculated with Benjamini-Hochberg correction. An average of 344,458 reads (after quality check) were obtained for each sample with an average of 95% on target.

4.6 Fluorescence in situ hybridization (Study III)

2 µm thick FFPE tumor sections fixed on microscopic slides were deparaffinized, pretreated with protease and hybridized with Vysis LSI ALK Dual Color Break Apart FISH probes according to the manufacturer's guidelines (Abbott Molecular Inc., Des Plaines, IL, USA). Slides were interpreted under a fluorescence microscope. The presence of at least one green and orange signal pair split apart by ≥2 signal diameters (pair-signal type fusion), or a single orange without corresponding green signal (single-signal type fusion) was required to determine a cell to be ALK gene rearrangement positive. The cells were defined as ALK rearrangement negative if they had fused or if they had a close orange and green signal.

4.7 Immunohistochemistry

For immunohistochemistry, three- or five-micron sections were cut from the FFPE samples and processed further. In study **II** and **III**, staining for EGFR (clone 31G7 Invitrogen, Waltham, MA, USA) and ALK (clone 5A4, NovocastraTM, Leica Biosystems, Wetzlar, Germany and clone D5F3 Ventana/Roche, Tucson, AZ, USA) was performed on the BenchMark XT (Ventana/Roche). Detection of EGFR was with the *ultra* VIEW Universal DAB Detection Kit (Ventana/Roche) and ALK detection with OptiView (760–700, Roche) amplification kit (Roche/Ventana, 760–099).

In study **IV**, the ALK (clone D5F3, Roche 790-4794) and EGFR (clone 5B7, Roche 790-4347) stainings were performed with the Ventana Benchmark Ultra instrument (Roche), while the EZH2 staining (clone 11/EZH2, BD Transduction 612666, USA) was performed in the LabVision immunostainer (Labvision, CA, USA). Detection of ALK and EGFR was with OptiView, while EZH2 was detected with the polymer-based detection system (Envision, K5007, Agilent, USA). All of the IHC slides were finally stained with hematoxylin (Mayer, S3099, Dako, Glostrup, Denmark) and then assessed with a microscope by two researchers (**II**, **III** and **IV**).

4.8 Statistical analysis

Statistical analysis of the microarray data was accomplished via GeneSpring GX Analysis Software v11.0.2 (Agilent). The data was preprocessed by log 2 transformation, and all arrays were normalized by the 75th percentile method. We excluded miRNAs that were absent in any of the samples or controls from further analysis. miRNAs not expressed in at least 100 % of one group of samples were excluded. The significance of differential expression between every two groups of samples was estimated by t test for those miRNAs with at least a 2.0-fold reduced or increased mean expression level between the two groups. Expression of mirRNAs was considered to differ significantly when the adjusted p value (q value) was <0.2 (Benjamini correction for multiple testing) (**I**). To determine statistically significant differences in miRNA expression between the defined MCC subgroups, the students' t test and Mann- Whitney U test were applied. P values of less than 0.05 were considered statistically significant (**I**).

The relationship between mutation count and MCV status was evaluated with the Mann–Whitney U-test (**II**). The correlation of tumor location, size as well as patient's age with the MCV status was examined with Fisher's test (**II**). Statistical analysis to correlate protein expression to MCV status and clinical data was with the Chi-Squared and Fisher's exact test (**IV**). To analyze the relationship between protein expression and MCC-specific survival, the Kaplan-Meier method and the log rank test were utilized. MCC-specific survival was calculated from the date of diagnosis to the date of death from MCC (**IV**).

5 RESULTS

5.1 Patients

Patients involved in studies I, II and III were selected from a cohort of 32 patients (16 MCV-positive and 16 MCV-negative), 17 patients were included in all three studies I, II and III. This cohort of 32 patients is presented in Table 5. From these 32 patients, 9 (28%) were males while 23 (72%) were females. 11 tumors (34%) were located in the head and neck region. The mean age of the patients was 79 years.

Study IV included 112 MCC tumor samples in tissue micro arrays (TMA). From the 112 corresponding patients, 30 (27 %) were males and 82 (73 %) females. Over half of the tumors, 60 (54%) were located in the head and neck region. 31 tumors were MCV-negative (28%) and 81 MCV-positive (72%). The mean age of the patients was 78 years. Development of metastasis was reported in 28 out of 112 patients (25%), and the MCC-specific mortality rate was 20%. The clinical follow-up for these patients ended 12th of June 2013.

5.2 MicroRNA profiles (Study I)

Microarray was utilized for 15 MCV-positive and 13 MCV-negative MCC tumors and revealed five differently expressed miRNAs between the two cohorts. miR-34a, miR-30a, miR- 1539, and miR-142-3p were significantly

underexpressed, and miR-181d was overexpressed in MCV-negative tumors compared to MCV-positives.

To validate the array results, we performed qRT-PCR on 14 MCV-positive and 12 MCV-negative tumors, and confirmed underexpression of miR-34a, miR-30a, miR-142-3p, and miR-1539 in MCV-negative tumors, although only the distinction of miR-34a was statistically significant. (p-value 0.0043, Mann-Whitney test) (Figure 7) Regarding miR-181d, qRT-PCR and microarray provided conflicting results and therefore we did not reach conclusions about expression of miR-181d. In this study, miRNA expression did not correlate to the disease stage or survival.

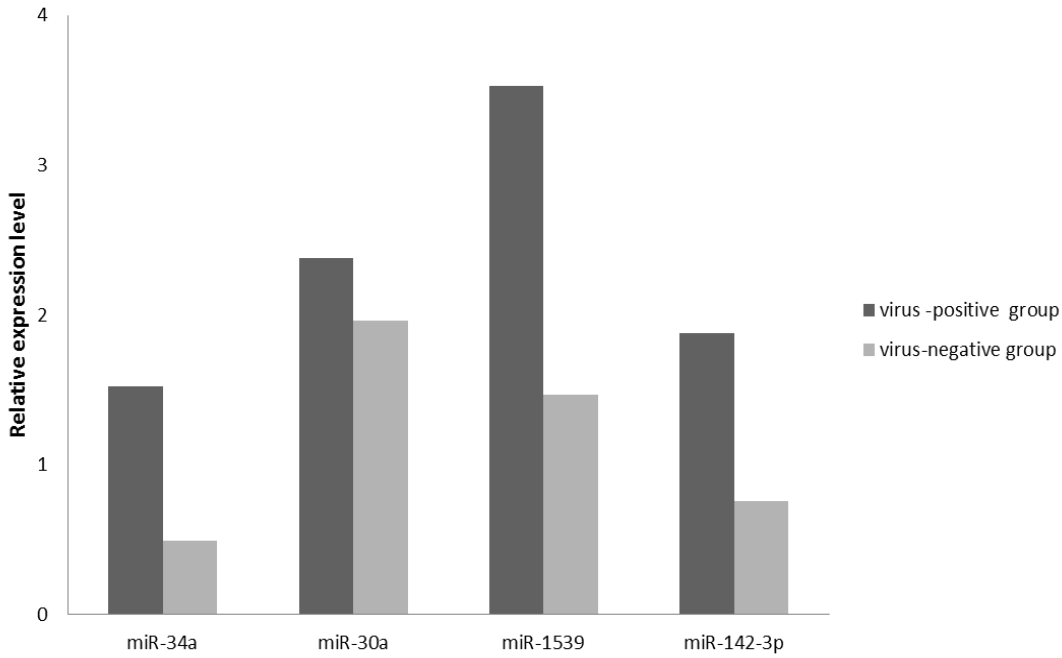

Figure 7. The relative expression of four miRNAs in MCV-positive and MCV-negative tumors by qPCR. (Veija 2015) Reproduced with permission from Springer Nature.

5.3 Gene mutations and RNA expression (Studies II and III)

Mutational analysis was done with 15 MCV-negative and 12 MCV-positive tumors (**II**). 13 (48%) tumors harbored hotspot mutations in one or more of 21 cancer genes that were included in the panel of 50 genes. MCV-negative tumors had a higher mutational burden (Not statistically significant, p-value = 0.24) and had mutations in 20 different genes, while MCV-positive tumors had them in only 5 genes. Mostly mutated genes were *TP53* (4 MCV-negative and 3 MCV-positive), *EGFR* (4 MCV-negative and 2 MCV-positive), *KIT* (4 MCV-negative and 1 MCV-positive), and *PIK3CA* (4 MCV-negative and 1 MCV-positive). The frequency of hotspot mutations in MCV-negative and MCV-positive tumors are shown in Figure 8. In addition, we found infrequent novel RB1 mutations only in MCV-negative tumors.

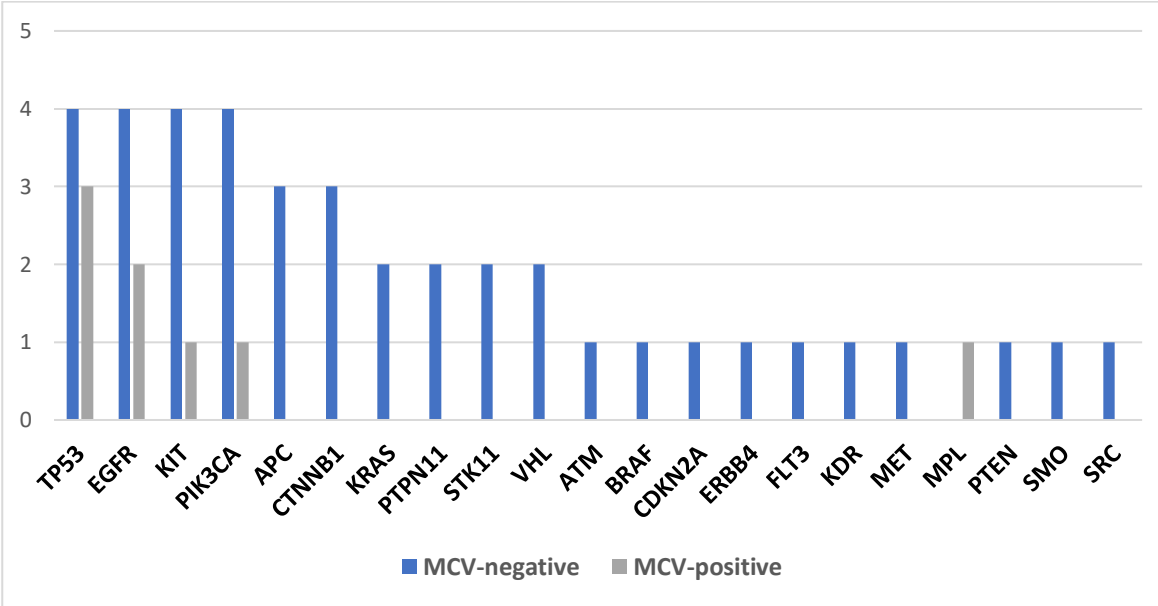

Figure 8. Frequency of hotspot mutations in MCV-negative and MCV-positive tumors. Y-axis = number of tumors.

RNA expression analysis was executed for 13 MCV-positive and 13 MCV-negative MCC tumors. (**III**) Significant overexpression of *ALK, CDKN2A, EZH2* and *ERBB4* and underexpression of *EGFR, ERBB2, PDGFRA* and *FGFR1* was evident in MCC tumors compared to normal skin regardless of the MCV-status. (p-value <0.005 and log2 fold change of at least 2) *ALK* was the most highly expressed gene, while *EGFR* expression was the lowest. (fold change 7,6 for *ALK* and -4.8 for *EGFR*) In addition, six genes *MET*, *NOTCH1*, *FGFR3*, *SMO*, *JAK3* and *NPM1* were differently expressed in MCV-positive and MCV-negative tumors.

Expression of *ALK* mRNA was seen in all 26 MCC cases, and expression was evidently high in 22 tumors. There was no difference in expression between MCV-positive and MCV-negative tumors. Fluorescence in situ hybridization (FISH) was successfully carried for 21 tumors, but no *ALK* fusion was recorded. However, in five tumors there were indications of possible gain or polyploidy involving *ALK.*

Table 5. This table presents the cohort of 32 MCC patients that were included in studies I, II and III. MicroRNA column states if the tumor was included (yes/no) in the study. In columns ALK NGS, IHC and FISH, na (not applicable) = not included in the study, + = positive expression/gain or polyploidy in FISH analysis, - = negative expression/no findings in FISH. (+) means weak positive expression in ALK IHC.

Patient	age	sex	Primary tumor location	Metastasis	MCV	MicroRNA	ALK NGS	ALK IHC D5F3	ALK FISH	Genes with hotspot mutations
1	90	female	Right temple	Right neck	neg	yes	na	na	na	na
2	68	male	unknown	Scalp	pos	yes	+	+	-	na
3	80	female	Posterior thigh	Inguinal and axillary lymph nodes	pos	yes	+	+	+	none
4	59	male	Thorax	none	pos	yes	+	+	-	none
5	72	female	unknown	none	pos	yes	+	+	-	na
6	67	male	left cheek	none	neg	yes	na	na	na	*APC, EGFR, KIT, TP53*
7	81	female	unknown	none	pos	yes	+	+	-	na
8	83	female	Right arm	none	neg	yes	+	+	+	none
9	85	female	Left temple	none	neg	yes	-	-	-	*ATM, BRAF, CTNNB1, EGFR, ERBB4, KRAS, PIK3CA, PTEN, PTPN11, STK11, TP53, VHL*
10	90	female	Forehead	none	pos	yes	+	+	-	none
11	71	male	Right buttock	none	pos	yes	+	+	-	*EGFR*
12	95	female	Left cheek	none	pos	yes	+	+	-	none
13	87	female	Left shoulder	Axillary lymph nodes	pos	yes	na	na	na	none
14	77	female	Right cheek	Mediastinum, pleura and brain	neg	yes	+	+	-	*KIT*
15	79	female	Right breast	none	neg	yes	+	+	-	*APC, CDKN2A, EGFR, FLT3, KIT, PTPN11, SRC*
16	72	female	Calf	Inguinal lymph nodes	neg	yes	+	+	-	*CTNNB1, EGFR, KDR, KIT, KRAS, TP53*
17	57	female	Right cheek	none	pos	yes	na	na	na	na
18	78	male	Neck	Anal canal, pancreas	neg	yes	+	+	-	none
19	79	male	Left forearm	none	pos	yes	+	+	-	none
20	81	female	Left upper back	none	neg	yes	+	+	+	none

21	84	female	Right shoulder	none	pos	yes	+	+	-	none
22	82	male	Neck	none	neg	yes	na	na	na	none
23	85	male	Left arm	Axillary lymph nodes	pos	yes	+	-	-	*EGFR, TP53*
24	84	female	Back	none	neg	yes	-	-	-	none
25	87	female	cheek	none	pos	yes	na	na	na	TP53
26	60	female	Left foot	Heart, lung	neg	yes	+	+	-	none
27	80	male	Right breast	none	neg	no	+	(+)	-	*PIK3CA*
28	83	female	Front of left ear	none	neg	no	+	(+)	-	*PIK3CA*
29	69	female	Flank	none	pos	no	+	+	+	*KIT, MPL, PIK3CA, TP53*
30	68	female	Upper abdomen	none	neg	no	+	+	-	*APC, CTNNB1, MET, PIK3CA, STK11, TP53, VHL*
31	79	female	Right buttock	none	pos	no	+	-	-	none
32	100	female	Right cheek	none	neg	no	+	+	+	*SMO*

5.4 Immunohistochemistry (Studies II, III and IV)

5.4.1 ALK immunohistochemistry

In study **III**, ALK immunohistochemistry was positive in 22 of 24 MCC tumors (91,7%) with antibody clone D5F3 and in 4 of 26 tumors (15.4%) with clone 5A4. Staining was clearly more intense with antibody D5F3, and it detected ALK positivity more frequently than the clone 5A4 (Figure 9).

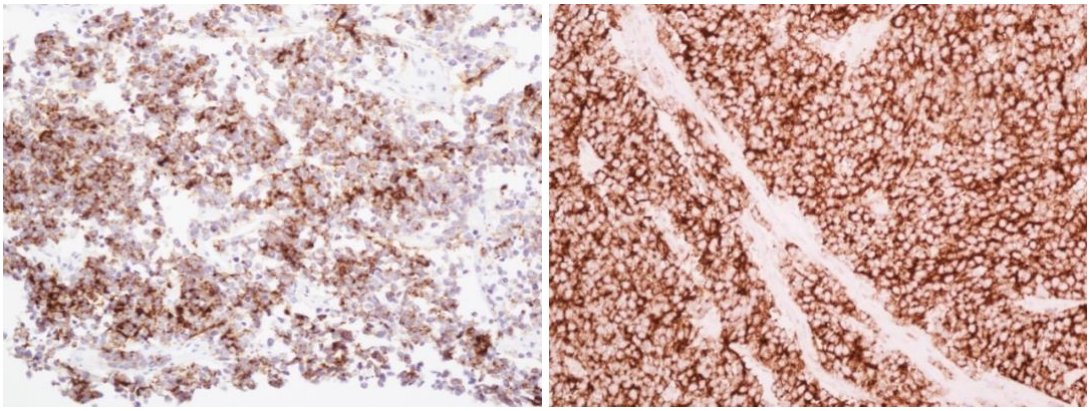

Figure 9. The figure illustrates the difference between ALK positive staining when using antibody clone 54A (Left) and clone D5F3 (Right) (**III**).

In study **IV**, 110 tumor samples in TMA slides were stained for ALK with antibody clone D5F3. During microscopic assessment of the stainings, we noticed that individual samples stained with different intensity. Therefore, we interpreted the expression as either strong positive, weak positive or negative. (Figure 10) Consequently, there were 56 strong positive, 16 weak positive and 38 negative tumors (51%, 15% and 34% respectively). In further analysis, ALK expression did not correlate with patient sex, tumor location or development of metastasis or MCC-specific death. However, we recorded that ALK expression associated with MCV positivity. 78% of the MCV-positive tumors were ALK positive, while only 32% of the MCC-negative tumors expressed ALK. This association was pronounced with tumors that expressed strong positivity for ALK, since 51 of the 56 (91%) strong positive tumors were MCV positive (Figure 12). This association of ALK positivity to MCV positivity was statistically significant ($p < 0.05$).

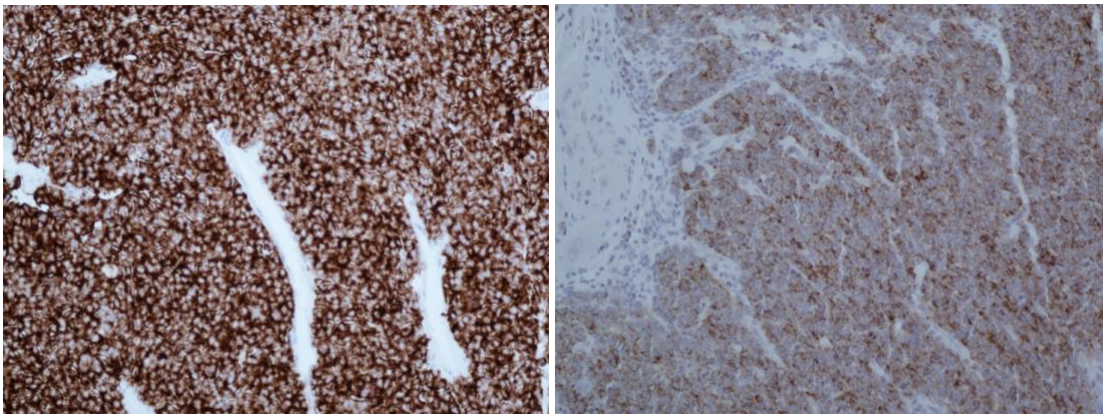

Figure 10. Strong ALK staining on the left and weak staining on the right(**IV**).

5.4.2 EGFR immunohistochemistry

In study **II**, EGFR IHC staining was negative in all 27 MCC cases studied. In study **IV**, we recorded an unprecedented expression of EGFR in 7 of the 111 MCC tumors. There was absolute association between EGFR positivity and MCV negativity since, all of those 7 tumors were MCV-negative. (Figure 11) EGFR expression did not correlate to patient sex, tumor location, development of metastasis or MCC specific death (**IV**). From those seven EGFR positive tumors, three (42,9%) developed metastasis, while from 104 EGFR negative tumors only 25 (24,0%) developed metastasis, proposing that disease course of EGFR positive MCC might be more aggressive than in EGFR negative disease. However, no statistical significance was observed regarding this matter.

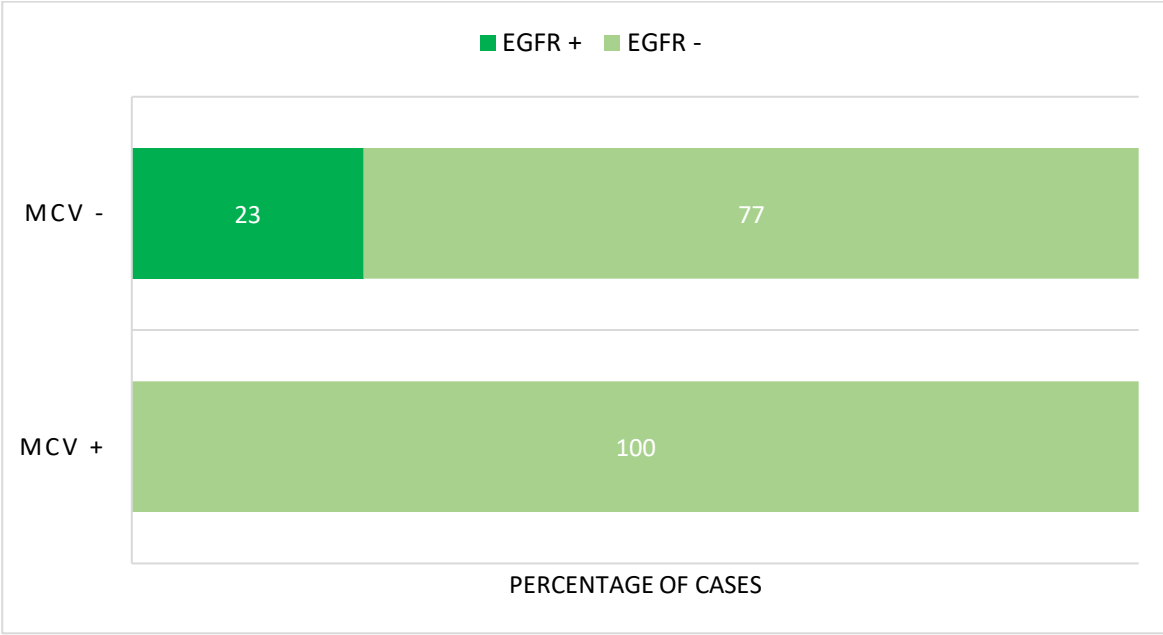

Figure 11. The fractions of EGFR positive and negative cases according to MCV-status (**IV**).

5.4.3 EZH2 immunohistochemistry

In study **IV,** we observed frequent expression of EZH2 by immunohistochemistry. Similarly to ALK stainings, we interpreted the staining as either strong positive, weak positive or negative. From 111 tumors, 84 (76 %) showed strong positivity, while 18 (16%) were weak positive, and only 9 (8%) were negative. EZH2 expression was not associated with MCV-status of the tumors, development of metastasis or MCC-specific survival (Figure 12).

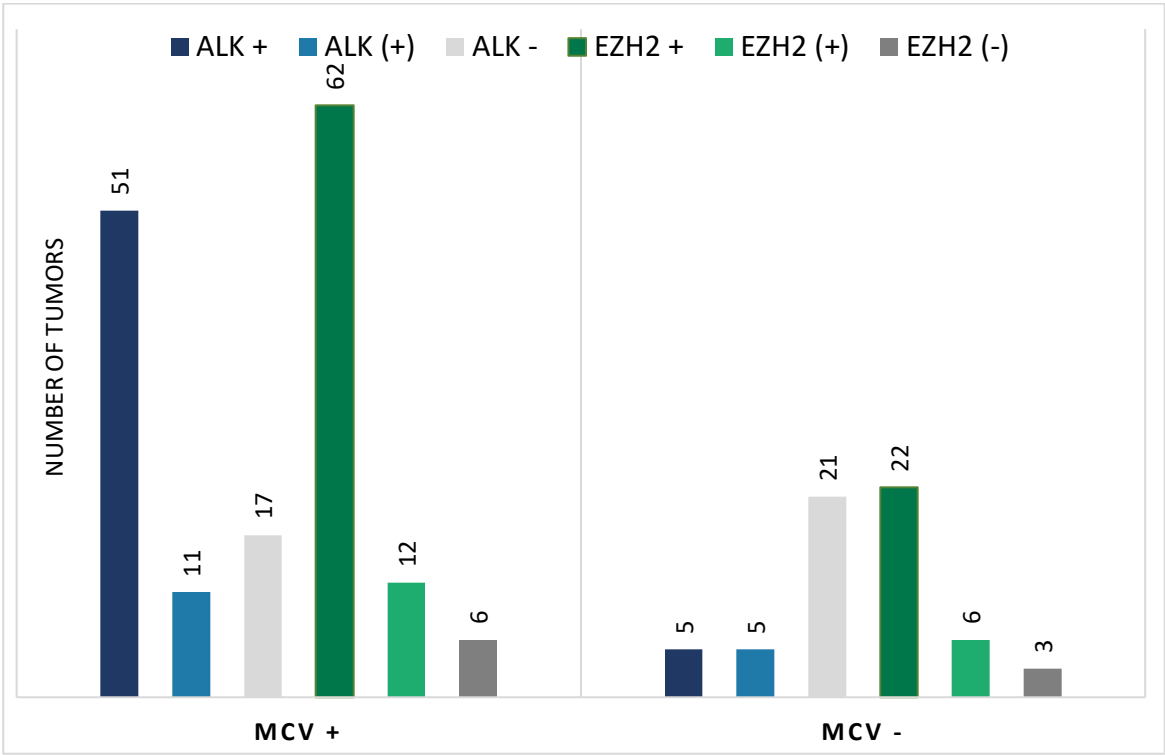

Figure 12. Number of MCV-positive and MCV-negative cases according to ALK and EZH2 staining (**IV**).

6 DISCUSSION

6.1 Patients

Our cohort of 32 MCC patients involved in studies **I**, **II** and **III** was selected based on the known MCV status of the tumor and sufficient amount of sample available. This cohort was selected mainly so that there would be equal numbers of MCV-positive and negative cases, and therefore it does not reflect a random cohort of 32 MCC patients.

The group of 112 MCC patients in study **IV** reflects quite well an average MCC cohort, since previous literature shows that tumors are frequently located on the head and neck (over 50%), the mean age of the patients is around 75 years and around 80% tumor MCC positivity is observed (Agelli 2010, Kukko 2012, Kukko 2012, Albores-Saavedra 2010, Sihto 2009). In this cohort, a clear female predominance was seen, however previous literature is ambivalent regarding the typical male-to-female ratio in MCC (Kukko 2012, Hodgson 2005, Allen 2005, Lyhne 2011). In our cohort of 112 patients, the development of metastasis was more frequent (25% vs 7-12%) than what was anticipated (Lebbe 2015, Harms 2016, Lemos 2010). However, the mortality in our cohort was lower than what was estimated in the literature (Lemos 2010, Becker 2010). Lower mortality in our cohort might be due to short follow-up in more recent cases that were diagnosed in the early 2010s (Follow-up ended 2013). Large variation in follow-up time between the cases is also a major limitation of this study and may impair the association of molecular alterations and survival. However, due to low incidence and high

mortality of MCC, performing equalized follow-up, for example 5 years, for all patients in the cohort is challenging.

6.2 MicroRNA profiles (Study I)

In study **I** we compared the miRNA expression of MCC tumors according to tumor MCV status. We recorded differential expression of four miRNAs in MCV-negative and MCV-positive tumors with both Microarray and qPCR analysis. We did not find correlation between miRNA expression and disease stage or survival, and larger studies are required to ascertain whether miRNAs have prognostic value in MCC.

The most intriguing finding in the miRNA study was the underexpression of miR-34a in MCV-negative tumors. In concordance to our results, another group had reported miR-34a underexpression in MCV-negative MCC compared to MCV-positive (Xie 2014). miR-34a is considered a tumor suppressor miRNA that acts alongside tumor suppressor protein p53, activating it and increasing its own production via p53 (He 2007, Yamakuchi 2009, Yamakuchi 2008). Thus, underexpression of miR-34a could promote oncogenesis in MCV-negative MCC. In comparison to our study, Xie et al. also found underexpression of miR-30a in MCV-negative tumors. In addition, they reported overexpression of miR-375, miR-769-5p and underexpression of miR-203 in MCV-positive tumors. Those three miRNAs showed no differential expression in our study. It could be that some miRNAs, like miR-34a are typically dysregulated in MCC, but there is also divergence in the miRNA profiles between tumors.

To the best of our knowledge, only these two studies assessing miRNA profiles of MCV-positive and MCV-negative MCCs have been conducted, and it is thus intriguing to gain more knowledge about miRNAs, their relation to MCV and whether they have use as biomarkers or have other clinical value in MCC cases. Our findings, that are partly in concordance with another study, suggest that miRNA profiles in MCC tumors do differ according to MCV status. However, whether they have a role in pathogenesis or clinical use as biomarkers in MCC remains to be deciphered.

6.3 Gene mutations (Study II)

In study **II** we used targeted NGS for seeking mutations of known cancer genes in MCC tumors. Generally, mutations were more frequent in MCV-negative tumors, and even though we were unable to demonstrate that it is statistically significant, (p-value 0.24) our observation sits well with other mutational studies that were published consecutively in 2015-2016. All of these studies report more numerous mutations in MCV-negative MCC (Wong 2015, Goh 2016, Harms 2015). Notably, mutations in MCV-negative tumors at nucleotide level are commonly Cytosine to Thymine transitions, which is considered as signature nucleotide change caused by UV radiation. This provides further evidence that especially for MCV-negative tumors, the UV is an undisputed etiological factor (Wong 2015, Harms 2015, Alexandrov 2013).

TP53 mutations are previously reported to associate with MCV-negative tumors (Erstad 2014, Sihto 2011). On the contrary, we recorded them also in MCV-positive tumors. The most notable finding was *EGFR* mutations in 6

tumors (22% of 27) which had not been published before in MCC. Unfortunately these mutations were not cofirmed by Sanger sequencing (**III**), however that could be due to low mutant allele frequency of the tumors. Non-cancerous tissue obtained from 2 of these 6 MCC patients was negative for *EGFR* mutations, suggesting that the mutations in the tumors were real and somatic. Unfortunately, we failed to witness that these mutations lead to activation of the *EGFR* gene and EGFR protein expression. A future interest would be to go back to these samples and examine whether the EGFR receptor is active in these tumors that contained *EGFR* mutations. Hotspot mutations of *EGFR* are reported in various malignancies, including NSCLC (Murray 2006), where some of the mutations predict responsiveness to EGFR tyrosine kinase inhibitors (Brandao 2012, Han 2005). In fact, at least one of the mutations that we recorded in MCC is reported responsive to EGFR inhibitor in lung adenocarcinoma (Brandao 2012). Since there are hotspot mutations of *EGFR* also in MCC, it could be a potential therapeutic target in the subset of MCC tumors, provided that the mutations are activating.

In keeping with previous a exome sequencing study, we recorded *RB1* mutations only in MCV-negative tumors (Cimino 2014). In our study, novel mutations were seen in three MCV-negative tumors. These mutations were not recorded in either the dbSNP142 or the COSMIC database. (http://cancer.sanger.ac.uk/cosmic) We also found *PIK3CA* and *KIT* mutations more commonly in MCV-negative tumors. *PIK3CA* mutations in MCV-negative tumors are also reported by another research group (Nardi 2012). They suggested that PIK3 signaling might have a pathologic role in the subset of MCC.

In conclusion, many mutational studies have been conducted for MCC, and no prevalent driver mutations have been found. It seems that some genes might be contributing to the pathogenesis of a subset of MCC tumors, and especially MCV-negative tumors are associated with more potential oncogenic genes. Thus, new molecular targets for treatment might emerge.

6.4 RNA expression (Study III)

In study **III**, we utilized targeted NGS to explore the expression of cancer genes at RNA level in MCC tumors. To our knowledge, this was the first study to address the RNA expression of oncogenes and tumor suppressors in MCC. The expression in the tumors was compared to normal skin and in addition, the MCV-negative and MCV-positive tumors were compared. The most notable overexpressed genes were *ALK* and *EZH2,* while *EGFR* emerged as strongly underexpressed. Six genes - *MET*, *NOTCH1*, *FGFR3*, *SMO*, *JAK3* and *NPM1* - were differently expressed in MCV-positive and MCV-negative tumors, adding more to the landscape of varying molecular characteristics of the two MCC subgroups.

We recorded over 7-fold overexpression of *ALK* in our cohort of 26 MCC tumors. Particularly high expression of *ALK* mRNA was present in 22/26 tumors regardless of tumor MCV status. ALK is known for its involvement in fusion proteins such as NPM-ALK in ALCL (Morris 1994). To investigate potential ALK fusion in MCC, we performed Fluorescence in situ hybridization (FISH) for the tumor samples, but no fusion was recorded.

However, in five tumors there were indications of possible gain or polyploidy involving *ALK.* Since no chromosomal rearrangements or activating *ALK* mutations are present, the mechanism of *ALK* mRNA expression in MCC remains unknown.

We also observed underexpression of EGFR mRNA in MCC tumors compared to normal skin in study **III**. Although the underexpression was almost 5-fold, this observation might be due to strong expression of EGFR in normal skin and not because of nonexistent expression in MCC tumors.

EZH2 mRNA expression in MCC was first reported by our group (**III**). We detected high expression of EZH2 regardless of tumor MCV status. One study has reported activating EZH2 mutation in one MCC case (Harms 2016), but these mutations have been absent in other mutational studies in addition to our study **II** (Goh 2016, Harms 2015). Thus, the overexpression of EZH2 in MCC might be due to epigenetic regulation rather than genetic origin. Whether EZH2 functions as a silencer of tumor suppressors in MCC is undeciphered.

6.5 Protein expression (Studies II, III and IV)

Studies **II** and **III** were essentially sequencing projects, but based on the sequencing results, we also analyzed protein expression by immunohistochemistry with the same tumor samples that were sequenced. In study **IV**, we wanted to further explore the protein expression of the three standout oncoproteins in previous studies, ALK, EGFR and EZH2. Therefore,

we utilized TMAs containing 112 MCC samples to study expression of these proteins on a larger scale (**IV**).

In study **III**, we used two antibodies (D5F3 and 5A4) for detecting ALK expression and saw that D5F3 detected ALK more frequently. Therefore, in study **IV** we only used antibody clone D5F3 for ALK staining. ALK expression by IHC in MCC has been previously studied. They reported frequency of ALK positivity as high as 93.8% on 32 MCC tumors studied (Filtenborg-Barnkob 2013). In study **III**, we recorded expression frequency almost as high as they did with antibody D5F3. However, when we investigated the expression in a much larger tumor cohort in study **IV**, we showed that ALK expression is common in MCC but not as common as previously reported. A notable, novel finding was the correlation of ALK positivity to MCV positivity which has not been published before. In study **III,** we recorded ALK mRNA expression regardless of MCV status. It remains to be discovered why ALK expression at protein level associates with MCV positivity while ALK mRNA seems to be expressed in all MCC tumors. A future interest would be to study the possible interaction of MCV and ALK; whether MCV infection facilitates ALK expression in MCC.

Brunner et al. had previously studied EGFR expression by immunohistochemistry in 32 MCC TMA samples, and the staining was negative in all cases (Brunner 2008). Yet, since we recorded EGFR mutations in a subset of MCC tumors (**II**), we were hopeful to discover EGFR protein expression in MCC. However, in study **II**, EGFR staining was negative in all 27 MCC cases studied. In that study, we used an antibody clone that reacts with the extracellular domain of EGFR. We hypothesized that the reason we could not detect EGFR protein expression in any of the tumor samples was either

because we used an antibody that does not detect truncated forms of the EGFR or because only a few tumor cells contain the *EGFR* mutation. In study **IV**, we used a different type of antibody clone that binds to the intracellular domain of EGFR and detects constitutively active and truncated forms of EGFR (Mascaux 2011). This antibody clone (5B7) has been used in NSCLC samples, and positive staining was found to predict the efficacy of EGFR Tyrosine Kinase inhibitor treatment (Mascaux 2011, Chang 2016). With this antibody, we recorded EGFR expression in 23% (7/31) of the MCV-negative tumors, while none of the MCV-positive tumors expressed EGFR (**IV**). Hence, we propose that EGFR might be a desirable treatment target also in the subset of MCC that express EGFR by immunohistochemistry.

In study **IV,** we observed frequent expression of EZH2 in MCC tumors regardless of MCV-status. Harms et al. had studied EZH2 expression in 29 primary MCCs, and they reported that strong expression of EZH2 associates with disease progression (Harms 2017). They did not observe completely negative cases, while our much larger cohort of primary MCCs harbored 8% of negative tumors. They also showed that EZH2 expression is higher on metastases compared to primary tumors. In contradiction to their study, we only had primary tumors in our cohort, and we did not find correlation between primary tumor EZH2 expression and development of metastasis or MCC-specific death. Therefore, a suggestion can be made that EZH2 is not a prognostic factor but rather could be a potential therapeutic target since the majority of MCC tumors express EZH2.

7 CONCLUSIONS

In study **I,** we showed that MicroRNA profiles of MCV-positive and MCV-negative tumors are partially distinct. However, their potential in clinical use is uncharted. Notably MiRNA-34a is underexpressed in MCV-negative tumors and its deprivation might promote pathogenesis of MCV-negative MCC.

Mutational analysis (Study **II**) of MCC recorded that MCV-negative tumors harbor gene mutations more frequently compared to MCV-positive, however no frequently presenting driver mutations were observed. However, a subset of MCC tumors contain *EGFR* mutations unprecedented in MCC. Further, in our immunohistochemistry study (study **IV**), we observed expression of EGFR in small proportion of MCV-negative tumors, suggesting that these tumors might respond to EGFR inhibitors.

RNA sequencing (Study **III**) introduced that ALK is commonly expressed at RNA level in MCC tumors regardless of tumor MCV status, although *ALK* fusions are absent. Intriguingly we recorded that expression of ALK at protein level by immunohistochemistry associates strongly with tumor MCV positivity (Study **IV**). However, the mechanism of ALK overexpression in MCC remains unknown.

Based on RNA sequencing in study **III** and immunohistochemistry in study **IV,** we concluded that EZH2 is frequently expressed in MCC both at RNA and protein level regardless of MCV status and might be a potential therapeutic target.

In statistical analysis comparing protein expression and clinical parameters of MCC patients; (Study **IV**) Expression of ALK, EGFR and EZH2 does not appear to correlate with development of metastasis or MCC-specific mortality.

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