Polyomavirus-negative Merkel cell carcinoma: a more aggressive subtype based on analysis of 282 cases using multi-modal tumor virus detection

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Abstract

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Previous studies have reached conflicting conclusions regarding the proportion of Merkel cell carcinomas (MCCs) that contain the Merkel cell polyomavirus (MCPyV) and the clinical significance of tumor viral status. To address these controversies, we detected MCPyV large T antigen using immunohistochemistry with two distinct antibodies and viral DNA using quantitative PCR. Tumors were called virus-positive if two or more of these three assays indicated presence of MCPyV. 53 of 282 (19%) of MCC tumors in this cohort were virus-negative using this multimodal system. Immunohistochemistry with the CM2B4 antibody had the best overall performance (sensitivity=0.882, specificity=0.943) compared to the multimodal classification. Multivariate analysis including age, sex and immunosuppression showed that, relative to MCC patients with virus-positive

tumors, virus-negative MCC patients had significantly increased risk of disease progression (HR=1.77 [95% CI: 1.20 – 2.62]) and death from MCC (HR=1.85 [95% CI: 1.19 – 2.89]). 29 and 41 percent of these respective associations was due to a greater likelihood of virus-negative cases to present at a more advanced clinical stage, even though virus-negative tumors tended to be smaller at presentation. We confirm that a subset of MCCs lack biologically significant levels of MCPyV, and that such tumors represent a more aggressive subtype warranting closer clinical follow-up.

INTRODUCTION

Merkel cell carcinoma (MCC) is a rare and aggressive neuroendocrine skin cancer with a disease-associated mortality of greater than 40%.[1] The incidence of this malignancy has quadrupled over the last 20 years, likely due to the increasing prevalence of risk factors including advanced age, cumulative UV exposure and systemic immune suppression.[2,3] The discovery that HIV-positive patients have greater than tenfold risk of developing MCC suggested the immune system plays a critical role in the development of this cancer, which in turn led to the search for a potential infectious etiology.[4] In 2008, the Moore-Chang group found that a majority of MCCs were associated with the novel, highly prevalent Merkel cell polyomavirus (MCPyV).[5] Following the discovery of MCPyV, viral large and small T antigens have been shown to be constitutively expressed in MCC, and capable of driving oncogenesis through a variety of mechanisms, including binding and inactivation of the retinoblastoma (Rb) tumor suppressor.[6-8]

The initial report identified MCPyV in 8 of 10 (80%) MCCs.[5] Subsequent studies using PCR and/or immunohistochemistry targeting MCPyV large T antigen have at times provided disparate estimates of viral positivity in MCC, ranging from as low as 24% in some series to 100% in another.[9,10] With wide variation in methods and populations, the remaining 10 of 12 major studies to address this question to date have estimated anywhere from 46% to 89% MCPyV positivity, with the aggregate estimate of all studies being 76% (453 of 595 unique MCCs).[5,9-19] At this time, there is currently no accepted ‘gold

standard' method for determining MCC viral status, neither is there consensus on which single assay might be most appropriate for routine clinical application.

Beyond the question of the prevalence of MCPyV in MCC, there are also conflicting reports as to whether tumor viral status has prognostic significance. One study of 114 MCC patients found that virus-negative patients had significantly worse overall survival in comparison to their virus-positive counterparts in a multivariate model (13.0% vs 45.0% 5-year survival).[14] A later study among 60 MCC patients found virus-negative patients had significantly worse overall and recurrence-free survival in a univariate model, but no such association when tumor stage and nodal involvement were taken into account.[17] The largest published cohort to date to address this question, however, found no significant survival difference on the basis of viral status among 127 MCC patients.[16]

Because of the lack of a standard clinical test for MCPyV, as well as the unclear prognostic significance of tumor viral status, MCCs are not currently routinely analyzed for the presence of MCPyV. To identify the prevalence of MCPyV among 282 tumors and determine the best clinical test for viral detection, we used optimized quantitative PCR targeting MCPyV DNA and immunohistochemistry targeting the MCPyV Large T antigen as the virus' mechanism of promoting transformation. We rationalized that in order for the MCPyV to drive oncogenesis within MCC, both viral DNA and active expression of Large T antigen must be present. After viral status determination was made,

we assessed the impact of tumor viral status on clinical outcomes including progression-free survival, MCC-specific survival, and overall survival.

MATERIALS AND METHODS

Patient Selection and Study Approval

1,136 patients with pathologically-verified MCC diagnosed between 1980 and 2015 were referred from around the United States and Canada to the Fred Hutchinson Cancer Research Center and enrolled in an ongoing, IRB-approved (FHCRC-6585) repository of clinical data and specimens based in Seattle between 2004 and 2015. At the time of enrollment, patient data including age, sex, presence of systemic immunosuppression (e.g., chronic lymphocytic leukemia, non-Hodgkin's lymphoma, HIV, solid organ transplantation), tumor characteristics, clinical stage at diagnosis, and therapies received were ascertained. Depending on where patients chose to receive their care, follow-up data were obtained at appointments at the Seattle Cancer Care Alliance or by telephone at intervals varying from 3-12 months on an ongoing basis. Patients were censored at the last follow-up date if they could no longer be reached after 12 months or chose to no longer participate.

For the purposes of this study, all patients who had MCC pathology specimens available for qPCR and IHC analysis, totaling 282 of 1,136 enrolled patients, were included. Additionally, 16 non-MCC skin cancers (12 basal cell carcinomas, 2 squamous cell carcinomas, and 2 melanomas) were obtained from several of these patients to use as controls for qPCR and IHC analysis. The last possible date of follow-up for patients in this cohort was November 2015. One patient did not contribute any follow-up time, and was subsequently excluded from analysis. 169 of 281 total patients were known to have died between the

time of enrollment and November 2015. 86 of the remaining 112 (76.8%) had their last follow-up greater than 12 months prior to November 2015 and were therefore considered lost to follow-up.

Immunohistochemistry

Immunohistochemistry staining was performed at Fred Hutchinson Cancer Research Center Experimental Histopathology Laboratory. FFPE tumor blocks were sectioned and stained with hematoxylin and eosin. Immunohistochemistry was performed using 4μm thick tissue sections in large batches of samples to minimize staining variation between runs. Mouse monoclonal antibodies Ab3[10] (a generous gift from Dr. James DeCaprio, Dana-Farber Cancer Institute) and CM2B4[20] (Santa Cruz Biotechnology, SC-136172) were applied at the following final concentrations: 2.4 ug/mL and 2.0 - 4.0 ug/mL, respectively, after signal optimization for each batch. Of note, due to technical difficulty with CM2B4, non-MCC tumor samples were not successfully stained with that assay. Slides were stained using a DAKO Autostainer platform. Images were acquired using a Leica DFC290 Microscope and accompanying ImageScope Viewer software. Slides were scored on the Allred scoring system, which combines intensity of staining and proportion of cells stained into a single score of 0-8.[21] Samples were scored by either one or two independent pathologists. In cases when two pathologists reviewed the samples, the mean Allred score was used. An Allred score of ≤ 2 (<1% of cells with weak staining) was considered negative for presence of the viral antigen.

DNA extraction and quantification

DNA was extracted from FFPE samples that were originally obtained at the time of diagnosis. Extractions were performed on three to four 4μm tumor tissue curls from paraffin-embedded tumor using the QIAamp DNA-FFPE Tissue Kit (QIAGEN, Valencia, CA) following the manufacturer's instructions. Cell lines that had previously been established to be virus-positive (MKL-1) and virus-negative (UISO)[8], as well as peripheral blood mononuclear cells were used as controls and extracted in parallel with the tumor tissue. DNA was also extracted from the 16 aforementioned non-MCC skin tumors. DNA concentration and quality control for impurities was determined with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

qPCR

Quantitative PCR was performed at the University of Washington Molecular Virology Laboratory.

Sequences of MCPyV were aligned using Sequencher program (Gene Codes Corporation). Multiple real-time Taqman primer/probe sets were then selected from the conserved regions of different genes (Large T, Small T and VP 2) using Primer Express program (LifeTechnology). After comparing the robustness of amplification of the newly selected primer sets and previously published primer sets (LT2, LT3, SET 6, SET 7, SET 9)[10], LT4 and LT3 primers were selected for sensitivity testing on 157 unique tumor samples. The LT4 primer set displayed a

higher rate of amplification of target viral DNA across the tumor set and was thus selected for use in this study. Sequences for the LT4 primer set are: Forward primer: TTCCTCTGGGTATGGGTCCTT; TaqMan probe: Fam-TCAGCGTCCCAGGCT-MGB; Reverse primer: GGTCCTCTGGACTGGGAGTCT.

Primers that target the thyroid peroxidase (TPO) gene were also multiplexed into each PCR reaction. TPO is located on chromosome 2, which is known to have a stable genome copy number in Merkel cell carcinoma, and was thus used as a genomic control for each sample as described by Van Gele et al.[22] MCPyV copy number was calculated relative to the number of copies of TPO; therefore the ratio of LT4/TPO should represent the number of viral copies of DNA per cell. A ratio of LT4 to TPO that was less than 0.01 (i.e., 1 copy of viral DNA per 100 cells) was considered negative for MCPyV DNA.

Taqman Exogenous Internal Positive Control Reagents (Exo IPC) were spiked into all the PCR reactions to monitor PCR inhibition.[23] Negative results were accepted only if the Exo IPC was detected within the acceptable range. Each 30 ul PCR reaction contained 10 ul of DNA, 830 nM of each primer, 100 nM of probe, EXO internal control, 0.03 units of uracil-n-glycosylase (UNG, an enzyme that eliminates carryover PCR products)[24], 15 ul of QuantiTect Multiplex PCR master mix (QIAGEN, Valencia, CA). The thermocycling conditions were as follows: one cycle of 50^{o}C for 2 minutes then 95^{o}C for 15 minutes, followed by 45 cycles of 94^{o}C for 1 minute and 60^{o}C for 1 minute.

Viral Status Determination

Tumor viral status was determined by the consensus of the three tests (DNA qPCR, IHC using Ab3, CM2B4 IHC). For example, if a sample was negative for presence of the virus by qPCR and CM2B4, the sample was deemed virus-negative, even if the third test (Ab3) was positive. We refer to this classification method as the "multi-modal approach." Individual test performance characteristics (e.g., sensitivity and specificity) were estimated using standard 2x2 contingency tables in comparison to the multi-modal approach described above.

As a means of validation, we cross-referenced this classification with available data from a serologic assay that detects circulating antibodies against the MCPyV T antigens.[25] Blood samples considered in this way were all drawn at the time of diagnosis. As has previously been described, 13 of 29 (45%) of those with detectable MCPyV DNA and 11 of 22 (50%) of those with detectable MCPyV Large T antigen had detectable circulating T antigen antibodies. In contrast, 0 of 8 patients lacking detectable MCPyV DNA and 0 of 7 patients lacking detectable MCPyV Large T antigen in their tumors had detectable circulating T antigen antibodies. Thus, circulating T antigen antibodies would not be expected to be present in patients who do not harbor MCPyV in their tumors, and have been found to be present in approximately half of patients whose tumors were virus-positive.

Statistical Analysis

Statistical analyses were performed with STATA Systems software version 13.1 (StataCorp, College Station, TX). Chi-squared tests were used to compare categorical variables. Student's t-tests were used to compare continuous variables. All hypothesis tests were two-tailed and P values less than 0.05 were considered statistically significant.

Progression-free survival was defined as the duration from the date of diagnosis to the date of first progression (new disease, or increase in existing disease) following initial treatment. MCC-specific survival was defined as the duration from the date of diagnosis to the date of death due to MCC. Overall survival was defined as the duration from the date of diagnosis to the date of death due to any cause. Standard Kaplan-Meier survival curves were generated and Cox-proportional hazard models were fit for each clinical outcome. With respect to the latter, we fit three different models: first, a univariate model using MCPyV tumor viral status alone; second, a multivariate model including characteristics known to be associated with survival in MCC (e.g., sex, age, and immunosuppression) but excluding stage; and third, a multivariate model including stage. We considered the second model to best estimate the independent relationship between MCPyV tumor viral status and each outcome as it includes the effect of tumor stage as a potential causal mechanism between viral status and outcome. We fit the third model in order to quantify the effect of stage as a mediator of progression and survival, rather than as a confounder.

RESULTS

Multi-Modal Approach for MCPyV Viral Status Determination

All 282 MCC tumors were analyzed by qPCR for MCPyV DNA with the LT4 primer set normalized against a control gene, thyroid peroxidase (TPO), previously shown to have stable copy number in MCC (**Figure 1**). All samples were also stained with both CM2B4 and Ab3 antibodies that target overlapping portions of MCPyV large T antigen. Using the combination of qPCR, CM2B4, and Ab3 data and requiring a given tumor to have at least two modalities yield positive results to be considered MCPyV-positive, we determined 229 of 282 tumors (81.2%) to be MCPyV-positive and 53 of 282 tumors (18.8%) to be MCPyV-negative (**Table 1**). All three tests agreed on tumor viral status (positive or negative) in 167 (59.2%) of the 282 samples, while the remaining tumors were determined to be positive on two of three assays (**Figure 2**).

58 of the 229 patients classified as having MCPyV-positive tumors had serologic data. 37 of 58 (63.8%) of these patients had detectable T antigen antibodies at the time of diagnosis. In addition, 17 of the 53 patients classified as having MCPyV-negative tumors had serologic data available. None of these 17 patients had detectable T antigen antibodies at the time of diagnosis. Thus, results from the oncoprotein antibody assay further support the viral status determinations made with the multi-modal approach.

Detection of MCPyV DNA by qPCR

199 of 282 (70.6%) MCC tumors produced an LT4:TPO ratio ≥ 0.01 (range: 0.01 – 504.5) (**Table 1**), and the remaining 83 (29.4%) samples had LT4:TPO ratios between 0 – 0.009. Based on this classification, qPCR was 82.5% sensitive and 81.1% specific for identifying intratumoral MCPyV in comparison to the multi-modal approach described above.

3 of 16 (18.8%) non-MCC skin tumors (basal cell carcinoma, squamous cell carcinoma, melanoma) LT4:TPO ratios 0.0277 – 1.858, and using immunohistochemistry all of these three tumors had Allred scores ≤2 for the anti-Large T antigen antibody, Ab3.

Detection of MCPyV Large T Antigen by Immunohistochemistry

Using the CM2B4 antibody, 205 of 282 (72.7%) had an Allred score >2 (**Table 1**). The remaining 77 (27.3%) samples had Allred scores ≤2. Based on this classification, CM2B4 was 88.2% sensitive and 94.3% specific for identifying intratumoral MCPyV in comparison to the multi-modal approach.

Using the Ab3 monoclonal antibody, 254 of 282 (90.1%) MCCs had an average Allred score >2 (**Table 1**). The remaining 28 (9.9%) samples had Allred scores ≤2. Based on this classification, Ab3 was 98.3% sensitive and 45.3% specific for identifying intratumoral MCPyV in comparison to the multi-modal approach.

3 of 16 (18.8%) of control tissues (non-MCC skin cancers) had an average Allred score >2 for Ab3 (data not shown). These samples had PCR LT4:TPO ratios <0.01.

MCC Cohort and Survival Analysis

The 282 of 1,136 patients included in this study had nearly identical baseline characteristics to those not included (**Table 2**). Of the 281 patients with available clinical data (**Figure 3**), a total of 1,211 person-years of follow-up time were contributed. Loss to follow-up was high but similar between groups (72 of 96 (75.0%) virus-positive patients; 14 of 16 (87.5%) virus-negative patients). The median age at diagnosis was 71 for both the virus-negative and virus-positive groups (**Table 3**). The proportion of male and white patients, as well as the presence of systemic immunosuppression was similar between groups. Virus-negative tumors tended to be smaller than virus-positive tumors at presentation (median 1.1 cm vs 1.9 cm). Despite this trend, patients with MCPyV-negative tumors were more likely to present with advanced (nodal or distant metastatic) disease than patients with virus-positive tumors (66.7% vs 48.3%). Patients with virus-negative tumors were less likely to undergo surgical excision (74.4% vs 93.0%). This is likely due to the increased tendency to present with Stage III or IV disease, however administration of radiation and chemotherapy were similar between the groups.

88 of 202 (43.6%) virus-positive and 30 of 45 (66.7%) virus-negative MCC patients with stage I-III disease at presentation experienced progression over the assessed time interval (p=0.0052) (**Figure 4A**). The median time to progression for this cohort was 5.1 years (not reached among the virus-positive patients, and 1.2 years among the virus-negative patients). In univariate analysis, virus-

negative patients had a 1.80-fold higher risk of progression (95% CI: 1.22 – 2.64) (**Table 4**). Sex and immune status, but not age or stage at presentation were significantly associated with progression. In multivariate analysis including age, sex, and immune status, virus-negative patients were 1.77 times more likely to progress (95% CI: 1.20 – 2.62). Taking into account disease stage at presentation reduced the increased risk by 29%; however, a positive association remained (HR=1.55 [95% CI: 0.96 – 2.49]).

60 of 228 (26.3%) virus-positive and 24 of 53 (45.3%) virus-negative MCC patients died from MCC over the assessed time interval (p=0.015) (**Figure 4B**). Median time to death from MCC was not reached in the virus-positive patients, and was 3.7 years among the virus-negative patients. In univariate analysis, virus-negative patients had a 1.79-fold higher risk of death from MCC (95% CI: 1.14 – 2.85) (**Table 4**). Sex, immune status, and stage at presentation, but not age, were significantly associated with MCC-specific survival. In multivariate analysis including age, sex, and immune status, virus-negative patients were 1.85 times more likely to die from MCC (95% CI: 1.19 – 2.89). Taking into account disease stage at presentation reduced the increased risk by 41% ; however, a positive association remained (HR=1.50 [95% CI: 0.88 – 2.58]).

132 of 228 (57.9%) virus-positive and 37 of 53 (69.8%) virus-negative MCC patients died over the assessed time interval (p=0.14) (**Figure 4C**). Median overall survival time was 3.7 years in this cohort (4.6 years for virus-positive and 3.3 years for virus-negative patients). Age, sex, immune status, and stage at presentation were all statistically significantly associated with overall survival

(**Table 4**). Tumor viral status was not statistically significantly associated with overall survival in either univariate or multivariate analyses.

Due to its favorable sensitivity and specificity in comparison to the multi-modal approach, we also chose to conduct our survival analysis using CM2B4 Allred score alone to classify tumor viral status with the idea of exploring this antibody as a potential clinical assay. The results of this secondary analysis were almost identical to those described above (**Table 5**).

DISCUSSION

With 282 unique MCC tumors, to the best of our knowledge this study represents the largest to date to address the prevalence and prognostic significance of MCPyV in MCC. We assessed the presence of MCPyV with qPCR using a novel primer set (LT4) with superior performance to previously reported primers, as well as with immunohistochemistry using both CM2B4 and Ab3 antibodies, which each target the MCPyV large T antigen. We required tumors to demonstrate detectable MCPyV by at least two of these three modalities in order to be classified as MCPyV-positive. This approach meant that the presence of viral DNA alone, in the absence of detectable oncoprotein expression in tumor cells, was insufficient to classify a tumor as virus-positive. This requirement is relevant because MCPyV is prevalent in normal skin, and a possible signal from adjacent stromal MCPyV should not be interpreted as causal for tumorigenesis in the absence of viral protein expression in the tumor. Using this multi-modal approach, we estimate that approximately one fifth of MCCs are not driven by the virus. This proportion is highly consistent with the aggregate result from prior studies (76% MCPyV positivity in MCC tumors, as discussed above) although we note that individual rates of viral positivity in MCC cohorts have varied widely between 24-100%.[5,9-19]

In our cohort, immunohistochemistry with CM2B4 had the most favorable sensitivity and specificity profile among the three assays, and survival analysis using CM2B4 alone to determine tumor viral status yielded nearly identical

results to those integrating PCR and Ab3. This suggests CM2B4 is well suited as a clinical assay for detection of MCPyV in MCC.

Patients with virus-negative tumors were more likely to recur after treatment and to die from MCC, even in multivariate models including prognostic factors known to have significant impact on MCC survival. Because advanced stage at presentation is likely to be a causal mechanism between tumor viral status and poorer outcome in MCC, we included this variable in multivariate analyses in an attempt to understand its role as a mediator. When stage at presentation was added to the analysis, the associations with viral status were somewhat weaker and no longer statistically significant, suggesting advanced stage associated with virus-negative cases partially but not entirely accounts for the poorer clinical outcomes seen in the cohort. The lack of statistical significance in overall survival is likely due to the many competing causes of death in this population with median age at diagnosis of 71 years. Taken together, these analyses support the conclusion that virus-negative tumors may be more clinically aggressive even though they tended to be smaller in diameter at presentation.

Plausible biological mechanisms to explain the increased aggressiveness of MCPyV-negative tumors have been previously described. MCPyV-negative tumors have been shown to have a higher number of chromosomal aberrations[26,27], a greater nucleotide mutation burden[27,28], and a higher number of mutations in known oncogenic pathways including PI3K/pAKT, P53, and RB1.[17,27] Virally-driven MCCs may also be more immunogenic due to their

constitutive expression of oncoproteins that may serve as targets for cytotoxic tumor-infiltrating lymphocytes.[27]

The observed stage-independent increased risk of progression and MCC-specific death in virus-negative cases, although not statistically significant, is more difficult to account for. One possible explanation is virus-negative MCC may be more likely to arise in patients who have more medical comorbidities or are immunocompromised in such a way as was not captured in our data, thus leading to increased risk of likelihood of progression and subsequent death. Another possibility is that our classification of stage was inaccurate, and that virus-negative patients thought to have localized disease initially may have in fact had subclinical metastasis earlier in their disease course, thus leading to an apparent “stage-independent” risk of death.

This study had important limitations. During DNA qPCR analysis, tissue samples ranged from 50 - 100% tumor cellularity. Therefore, in some cases, the amount of MCPyV DNA in a sample might actually be higher than that reported by the LT4/TPO ratio after taking into consideration the copies of TPO extracted from surrounding healthy tissue. In order to compensate for the possible dilution of tumor cell DNA by stroma, we opted to use a low viral copy number/cell threshold of 0.01 (equivalent to 1 DNA viral copy per 100 cells) and to integrate these data with immunohistochemistry results. Also, our technique did not specifically demonstrate viral integration into the host genome, however previous studies have demonstrated that MCPyV is nearly universally integrated rather than episomal in MCC.[6] Regarding the clinical significance of tumor viral status,

this retrospective cohort study suffers from possible biases. Specifically, while we included known confounders of survival in MCC (e.g., age, sex, immunosuppression), it is possible that there is confounding from other characteristics not captured by our database (e.g., smoking, medical comorbidities, etc). Loss to follow-up was also high, and while rates were similar between MCPyV-negative and MCPyV-positive patients, there is potential for there to have been differential outcomes not accounted for. Lastly, despite its relatively large size, this study may have been underpowered to detect significant differences in clinical outcomes when including clinical stage in survival models.

The observed higher risk of recurrence, progression, and death from MCC in patients with virus-negative tumors suggest it may be clinically indicated to determine tumor viral status at the time of diagnosis as the results may affect prognosis as well as optimal initial management. Specifically, with the knowledge that virus-negative tumors may be more aggressive, clinicians may consider larger initial surgical margins, larger radiotherapy fields, and the use of regional nodal therapy even in the absence of documented nodal metastasis. Closer clinical follow-up and more frequent radiologic surveillance may be justified for patients with virus-negative MCC tumors because of their higher risk of recurrence and the fact that serologic monitoring[25] is not feasible for this patient population. Randomized clinical trials will need to be conducted to determine whether determining tumor viral status should be incorporated into clinical management of MCC.

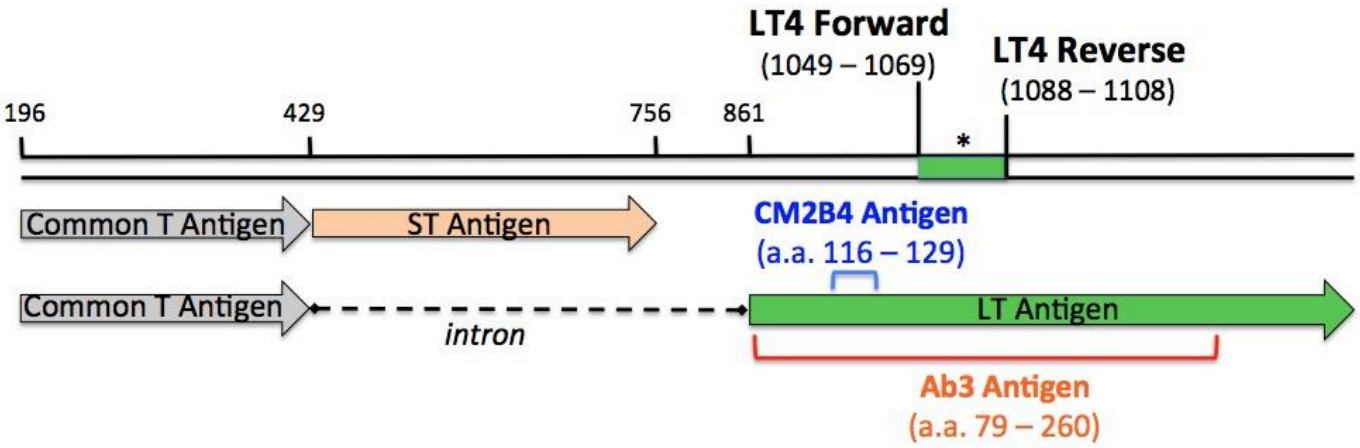


Figure 1. Detection of MCPyV DNA and oncoprotein in MCC tumors. Schematic diagram of the MCPyV small and large T antigens. Numbers correspond to the nucleotide position in the MCPyV genome. The LT4 primer set targets a 60 base pair segment of the persistently expressed portion of the truncated large T antigen. The asterisk indicates the TaqMan probe location (see Methods for details). The colored brackets in the lower portion of the figure show amino acid positions of antigens used to generate the large T antibodies: CM2B4 (blue) and Ab3 (red).

Table 1: Individual performance of the three assays (qPCR, CM2B4 and Ab3) in classifying intratumoral MCPyV in Merkel Cell Carcinoma in comparison to the multi-modal approach.

	Virus-Positive*	Virus-Negative*	Total	Assay Sensitivity	Assay Specificity
qPCR-pos	189	10	199		
qPCR-neg	40	43	83		
Total	229	53	282	0.825	0.811
CM2B4-pos	202	3	205		
CM2B4-neg	27	50	77		
Total	229	53	282	0.882	0.943
Ab3-pos	225	29	254		
Ab3-neg	4	24	28	0.983	0.453
Total	229	53	282		

*tumor viral status as determined by the 'multi-modal' approach (at least two of the three assay results in agreement)

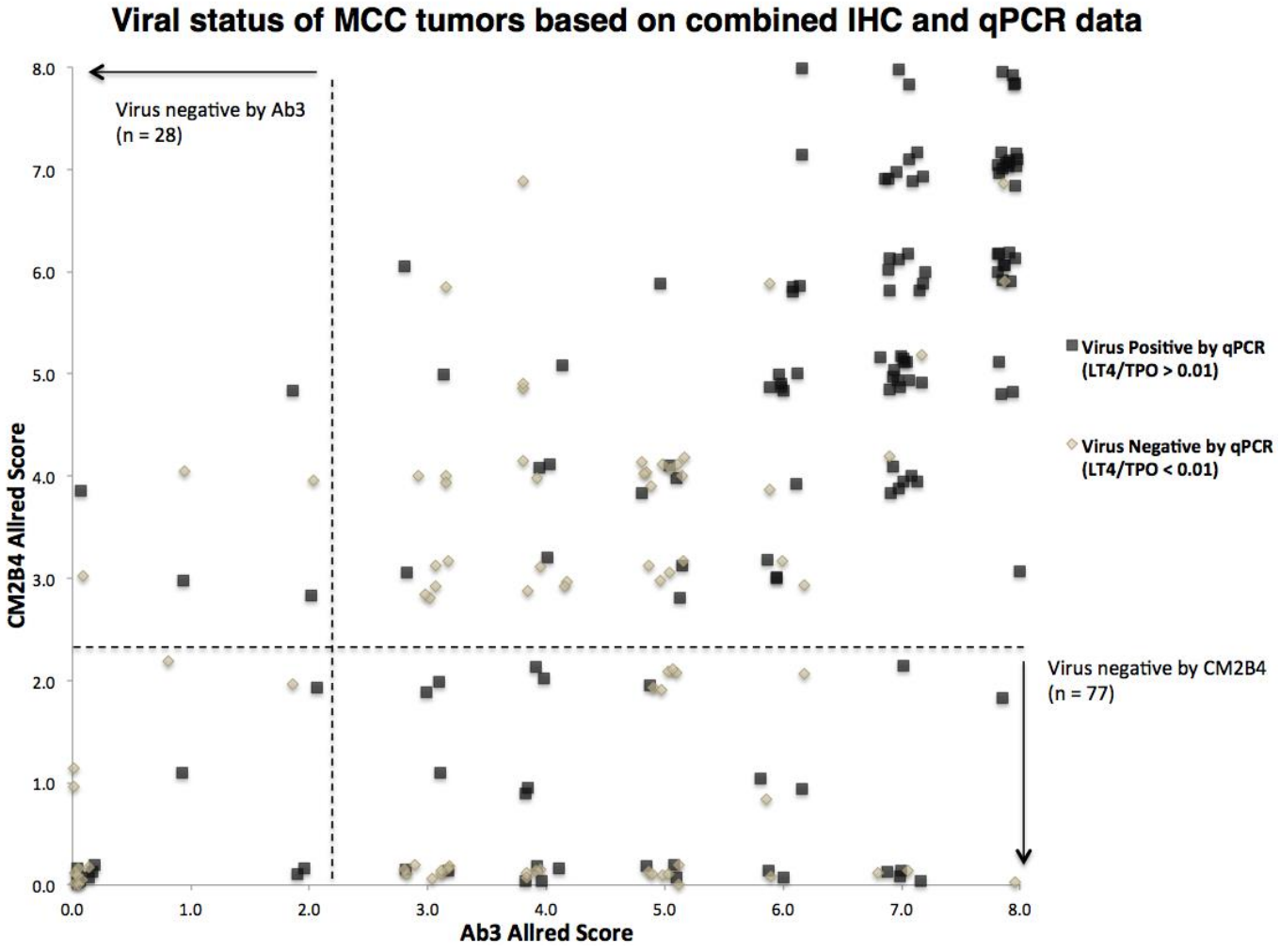


Figure 2. Graphical representation of each MCC tumor analyzed with CM2B4, Ab3 and qPCR. An Allred score of ≤2 was considered negative for presence of MCPyV large T antigen. For qPCR analysis, LT4/TPO < 0.01 (corresponding to 1 copy of viral DNA/100 cells) was considered negative for biologically relevant MCPyV.

Table 2. Baseline characteristics of 1,136 MCC patients, by inclusion in this study, Fred Hutchinson Cancer Research Center, 2004 – 2015.

	Patients Included n=282		Patients Not Included n=854	
CHARACTERISTIC				
	Median	**SD**	**Median**	**SD**
Median age	71	12.4	69	12.1
	n	**%**	**n**	**%**
Sex				
Female	105	37.2	300	36.5
Male	177	62.8	522	63.5
Race				
White	272	97.1	612	95.6
Other	8	2.9	28	4.4
Immunosuppression				
Absent	262	92.9	681	88.3
Present	20	7.1	90	11.7
TUMOR CHARACTERISTICS				
	Median	**SD**	**Median**	**SD**
Primary Tumor Size (cm)	1.7	1.7	1.5	1.5
	n	**%**	**n**	**%**
Site of Primary Tumor				
Head & Neck	96	34.0	285	34.9
Trunk	16	5.7	58	7.1
Buttock/Genitalia	20	7.1	40	4.9
Extremity	110	39.0	311	38.0
Unknown Primary	40	14.2	123	15.1
Stage at Diagnosis				
Local	107	48.2	375	51.7
I	75	33.8	274	37.8
II	32	14.4	101	13.9
Advanced	115	51.8	351	48.3
III	81	36.5	293	40.3
IV	34	15.3	58	8.0
TREATMENT				
Surgical Excision				
Yes	216	90.0	562	89.5
No	24	10.0	66	10.5
Radiation				
Yes	167	63.7	523	73.8
No	95	36.3	186	26.2
Chemotherapy				
Yes	40	14.4	116	16.6
No	237	85.6	584	83.4

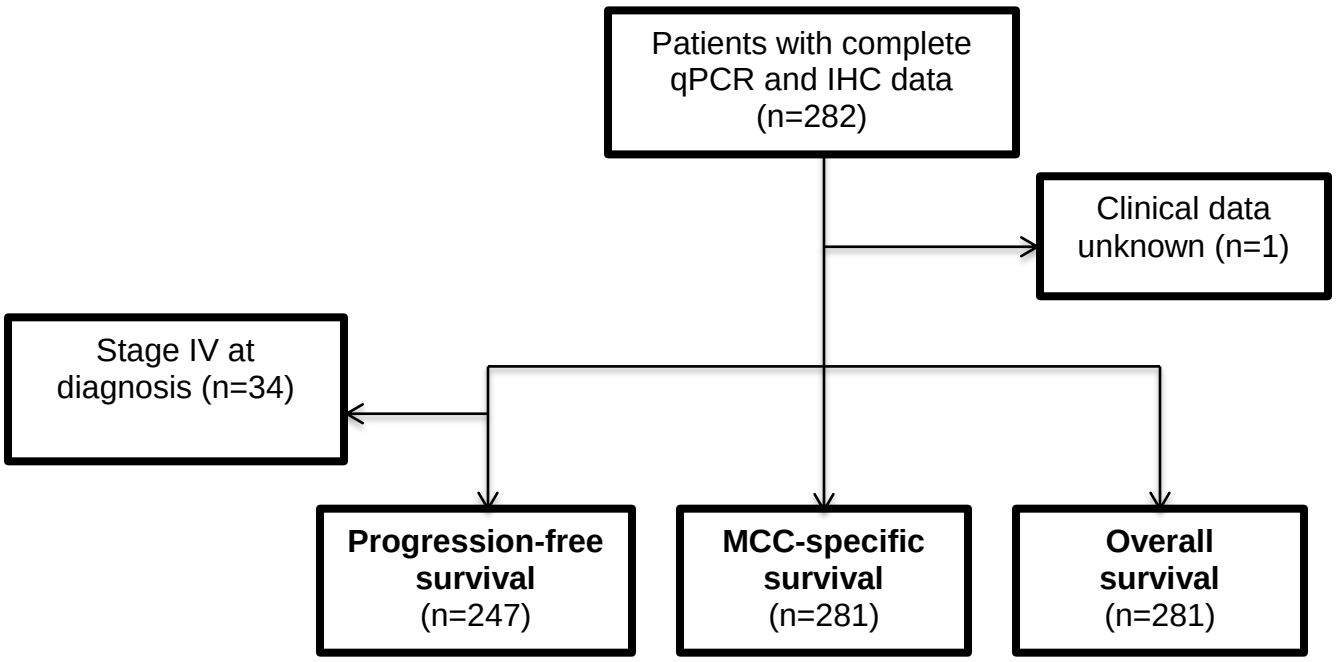
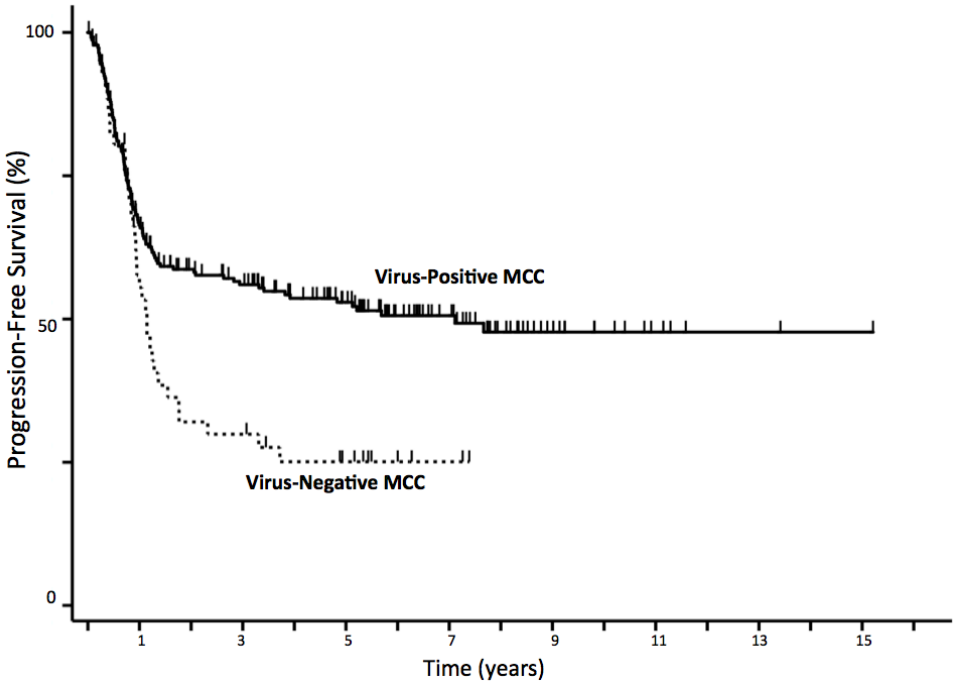


Figure 3. Flowchart of survival analysis inclusion criteria. All patients with clinical follow-up data were included in survival analysis. For progression-free survival, patients who presented with metastatic disease were excluded from analysis.

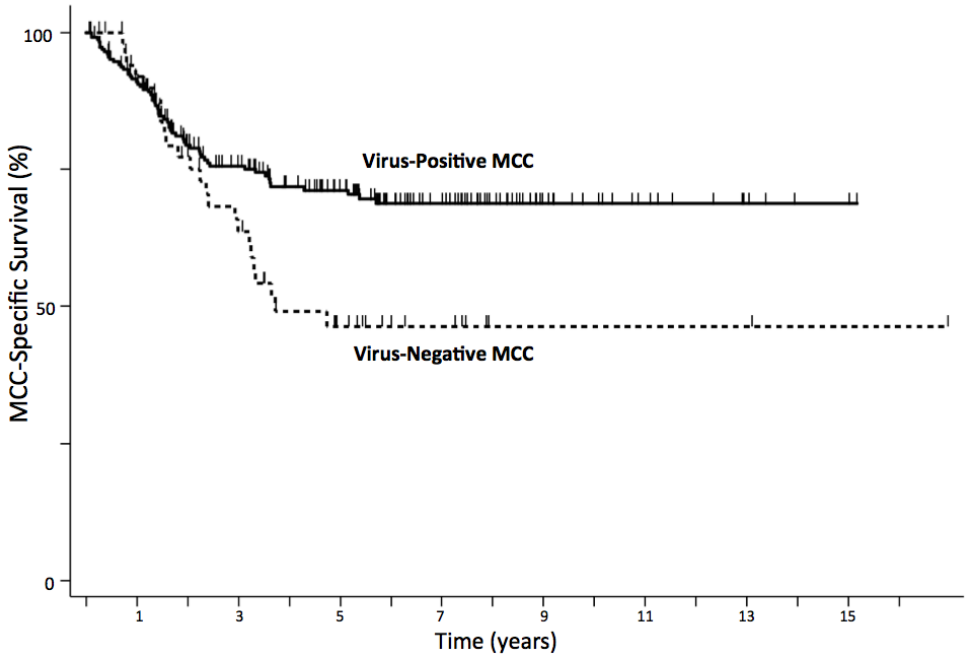
Table 3. Baseline characteristics of 281 MCC patients, by tumor MCPyV status, Fred Hutchinson Cancer Research Center, 2004 – 2012.

	Virus-Negative n=53		Virus-Positive n=229	
CHARACTERISTIC				
	Median	**SD**	**Median**	**SD**
Median age	71	11.2	71	12.6
	n	**%**	**n**	**%**
Sex				
Female	18	34.0	87	38.0
Male	35	66.0	142	62.0
Race				
White	51	98.1	221	96.9
Other	1	1.9	7	3.1
Immunosuppression				
Absent	51	96.2	211	92.1
Present	2	3.8	18	7.9
TUMOR CHARACTERISTICS				
	Median	**SD**	**Median**	**SD**
Primary Tumor Size (cm)	1.1	1.7	1.9	1.7
	n	**%**	**n**	**%**
Site of Primary Tumor				
Head & Neck	22	41.5	74	32.3
Trunk	6	11.3	10	4.4
Buttock/Genitalia	2	3.7	18	7.9
Extremity	10	18.8	100	43.7
Unknown Primary	13	24.5	27	11.8
Stage at Diagnosis				
Local	14	33.3	93	51.7
I	10	23.8	65	36.1
II	4	9.5	28	15.6
Advanced	28	66.7	87	48.3
III	20	47.6	61	33.9
IV	8	19.1	26	14.4
TREATMENT				
Surgical Excision				
Yes	29	74.4	187	93.0
No	10	25.6	14	7.0
Radiation				
Yes	29	61.7	138	64.2
No	18	38.3	77	35.8
Chemotherapy				
Yes	10	19.2	30	13.3
No	42	80.8	195	86.7

A


100
50
0
Progression-Free Survival (%)
Virus-Positive MCC
Virus-Negative MCC
1
3
5
7
9
11
13
15
Time (years)

B


100
50
0
MCC-Specific Survival (%)
Virus-Positive MCC
Virus-Negative MCC
1
3
5
7
9
11
13
15
Time (years)

C

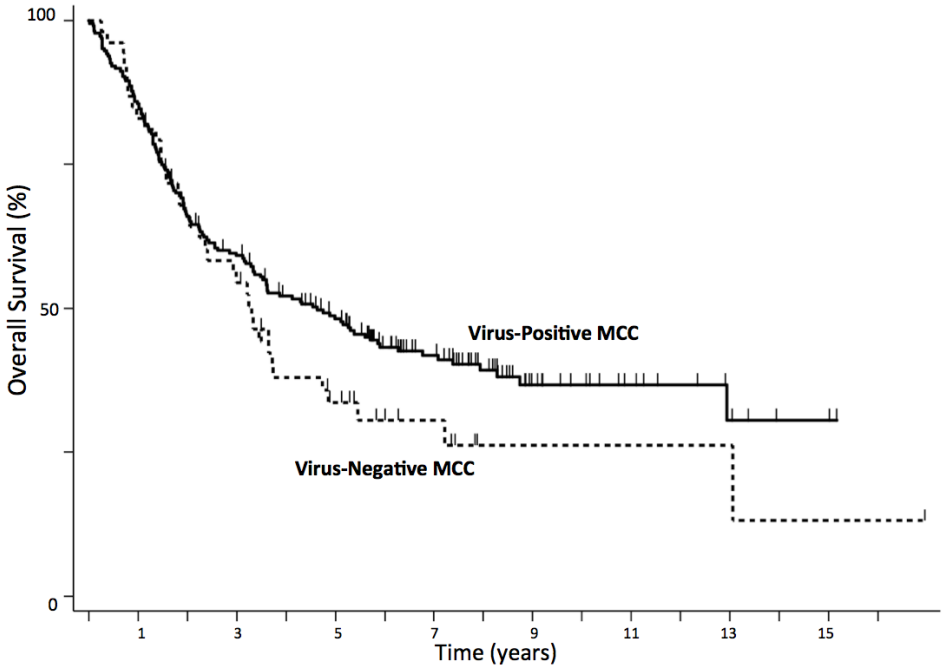


Figure 4. Kaplan-Meier curves of survival among MCC patients whose tumors were MCPyV-positive or -negative. **A**) Patients with virus-negative tumors had significantly decreased progression-free survival (p = 0.0052). **B**) Patients with virus-negative tumors had significantly decreased MCC-specific survival (p = 0.015). **C**) Patients with virus-negative tumors had a non-significant trend for poorer overall survival (p= 0.14).

Table 4. Univariate and multivariate analysis of progression-free survival, MCC-specific survival, and overall survival on the basis of MCPyV tumor viral status.

Progression-Free Survival		
Risk Factor	**Hazard Ratio**	**95% Confidence Interval**
Univariate Model		
Tumor Viral Status		
MCPyV Positive	Reference	Reference
MCPyV Negative	1.80	1.22 – 2.64
Multivariate Model excluding Stage		
Tumor Viral Status		
MCPyV Positive	Reference	Reference
MCPyV Negative	1.77	1.20 – 2.62
Age	1.01	0.99 – 1.02
Sex		
Female	Reference	Reference
Male	1.73	1.14 – 2.62
Immunosuppression		
Absent	Reference	Reference
Present	2.14	1.15 – 4.01
Multivariate Model including Stage		
Tumor Viral Status		
MCPyV Positive	Reference	Reference
MCPyV Negative	1.55	0.96 – 2.49
Age	1.02	1.00 – 1.03
Sex		
Female	Reference	Reference
Male	1.76	1.07 – 2.91
Immunosuppression		
Absent	Reference	Reference
Present	2.28	1.12 – 4.63
Stage		
I	Reference	Reference
II	1.61	0.91 – 2.86
III	1.42	0.87 – 2.32
IV	Omitted	Omitted

MCC-Specific Survival		
Risk Factor	**Hazard Ratio**	**95% Confidence Interval**
Univariate Model		
Tumor Viral Status		
MCPyV Positive	Reference	Reference
MCPyV Negative	1.79	1.14 – 2.80
Multivariate Model excluding Stage		
Tumor Viral Status		
MCPyV Positive	Reference	Reference
MCPyV Negative	1.85	1.19 – 2.89

Age	1.00	0.99 – 1.02
Sex		
Female	Reference	Reference
Male	1.84	1.13 – 2.99
Immunosuppression		
Absent	Reference	Reference
Present	2.64	1.29 – 5.41
Multivariate Model including Stage		
Tumor Viral Status		
MCPyV Positive	Reference	Reference
MCPyV Negative	1.50	0.88 – 2.58
Age	1.00	0.98 – 1.02
Sex		
Female	Reference	Reference
Male	1.93	1.11 – 3.33
Immunosuppression		
Absent	Reference	Reference
Present	3.94	2.14 – 7.26
Stage		
I	Reference	Reference
II	1.79	0.77 – 4.17
III	2.92	1.52 – 5.63
IV	5.29	2.21 – 12.65

Overall Survival		
Risk Factor	**Hazard Ratio**	**95% Confidence Interval**
Univariate Model		
Tumor Viral Status		
MCPyV Positive	Reference	Reference
MCPyV Negative	1.32	0.93 – 1.87
Multivariate Model excluding Stage		
Tumor Viral Status		
MCPyV Positive	Reference	Reference
MCPyV Negative	1.25	0.90 – 1.73
Age	1.05	1.03 – 1.06
Sex		
Female	Reference	Reference
Male	1.52	1.11 – 2.06
Immunosuppression		
Absent	Reference	Reference
Present	2.39	1.29 – 4.44
Multivariate Model including Stage		
Tumor Viral Status		
MCPyV Positive	Reference	Reference
MCPyV Negative	1.30	0.86 – 1.96
Age	1.04	1.02 – 1.06
Sex		
Female	Reference	Reference
Male	1.47	1.01 – 2.14

Immunosuppression		
Absent	Reference	Reference
Present	3.25	1.81 – 5.81
Stage		
I	Reference	Reference
II	1.60	0.91 – 2.81
III	2.03	1.30 – 3.17
IV	3.38	1.91 – 5.97

Table 5. Univariate and multivariate analysis of progression-free survival, MCC-specific survival, and overall survival on the basis of MCPyV tumor viral status as determined by CM2B4 Allred Score.

Progression-Free Survival		
Risk Factor	**Hazard Ratio**	**95% Confidence Interval**
Univariate Model		
CM2B4 Staining		
Allred ≤2	Reference	Reference
Allred >2	1.67	1.16 – 2.40
Multivariate Model excluding Stage		
CM2B4 Staining		
Allred ≤2	Reference	Reference
Allred >2	1.65	1.12 – 2.42
Age	1.01	0.99 – 1.02
Sex		
Female		
Male	1.73	1.14 – 2.60
Immunosuppression		
Absent		
Present	2.13	1.18 – 3.83
Multivariate Model including Stage		
CM2B4 Staining		
Allred ≤2	Reference	Reference
Allred >2	1.38	0.91 – 2.10
Age	1.02	1.00 – 1.03
Sex		
Female	Reference	Reference
Male	1.74	1.06 – 2.89
Immunosuppression		
Absent	Reference	Reference
Present	2.23	1.13 – 4.41
Stage		
I	Reference	Reference
II	1.67	0.95 – 2.93
III	1.47	0.91 – 2.37
IV	Omitted	Omitted

MCC-Specific Survival		
Risk Factor	**Hazard Ratio**	**95% Confidence Interval**
Univariate Model		
CM2B4 Staining		
Allred ≤2	Reference	Reference
Allred >2	1.60	1.04 – 2.45
Multivariate Model excluding Stage		
CM2B4 Staining		
Allred ≤2	Reference	Reference
Allred >2	1.64	1.05 – 2.58

Age	1.01	0.99 – 1.02
Sex		
Female	Reference	Reference
Male	1.83	1.11 – 2.97
Immunosuppression		
Absent	Reference	Reference
Present	2.61	1.33 – 5.14
Multivariate Model including Stage		
CM2B4 Staining		
Allred ≤2	Reference	Reference
Allred >2	1.41	0.85 – 2.32
Age	1.00	0.98 – 1.02
Sex		
Female	Reference	Reference
Male	1.92	1.11 – 3.33
Immunosuppression		
Absent	Reference	Reference
Present	3.80	2.12 – 6.81
Stage		
I	Reference	Reference
II	1.82	0.78 – 4.22
III	2.93	1.51 – 5.66
IV	5.47	2.28 – 13.15

Overall Survival		
Risk Factor	**Hazard Ratio**	**95% Confidence Interval**
Univariate Model		
CM2B4 Staining		
Allred ≤2	Reference	Reference
Allred >2	1.37	0.99 – 1.89
Multivariate Model excluding Stage		
CM2B4 Staining		
Allred ≤2	Reference	Reference
Allred >2	1.32	0.95 – 1.83
Age	1.05	1.03 – 1.06
Sex		
Female	Reference	Reference
Male	1.51	1.09 – 2.09
Immunosuppression		
Absent	Reference	Reference
Present	2.43	1.44 – 4.11
Multivariate Model including Stage		
CM2B4 Staining		
Allred ≤2	Reference	Reference
Allred >2	1.41	0.97 – 2.05
Age	1.04	1.02 – 1.06
Sex		
Female	Reference	Reference
Male	1.48	1.02 – 2.16

Immunosuppression		
Absent	Reference	Reference
Present	3.29	1.88 – 5.76
Stage		
I	Reference	Reference
II	1.63	0.93 – 2.84
III	2.01	1.29 – 3.13
IV	3.46	1.97 – 6.10

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