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ORIGINAL ARTICLE

Nicotinic Acetylcholine Receptor Expression in Merkel Cell Carcinoma Is Associated With Clinical and Histopathologic Parameters

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ABSTRACT

Background: Merkel cell carcinoma (MCC) is a rare, aggressive cutaneous malignancy with neuroendocrine differentiation. Several molecular pathways have been implicated in MCC development and multiple cell-of-origin candidates have been proposed, including neural crest cells, which express acetylcholine receptors (AChRs). The role of nicotinic acetylcholine receptors (nAChRs) in MCC has not been explored. In this study, we investigated if MCC expresses nAChRs and if nAChR expression correlates with patient characteristics.

Methods: The study included 71 MCC cases diagnosed with sufficient tissue available to perform immunohistochemical analysis. The median follow-up was 29.8 months (range, 2.7–234.1). We performed immunohistochemistry using antibodies against the α3, α5, and α7nAChR subunits.

Results: Our results show that the majority of MCC cases expressed α3, α5, and α7-nAChR subunits. Of the 71 cases, 59 (83%) expressed α3-nAChR, 71 (100%) expressed α5-nAChR, and 63 (88%) expressed α7-nAChR. Location of immunoreactivity differed between cases and included cytoplasmic only and nuclear/peri-nuclear, with variation in the intensity of staining. There were significant correlations between the intensity or location of immunoreactivity and clinical and histopathologic parameters.

Conclusions: These findings seem to support that MCC displays the features of neural crest cells, and suggest the potential for nAChR-targeted therapy.

1 | Introduction

Merkel cell carcinoma (MCC) is a rare, aggressive primary cutaneous malignancy with neuroendocrine differentiation. The exact cause of MCC is unknown, but several potential etiologies have been proposed, including exposure to ultraviolet radiation (UVR) and integration of Merkel cell polyomavirus (MCPyV) [1–5]. MCC was originally thought to arise from innate Merkel cells, which are involved in mechanoreception and also exhibit neuroendocrine differentiation [6]. However, further investigation has raised controversy regarding the cell of origin. Increasingly sophisticated methods have been employed to explore genotypic and phenotypic characteristics of MCC and resolve this confusion regarding the cell of origin, suggesting some cell-of-origin candidates including epidermal, follicular epithelial, lymphoid, fibroblast, mesenchymal stem, and neural crest cells, the latter of which are known to express nicotinic acetylcholine receptors (nAChRs) [3, 7–10].

Among other roles, nAChRs play an essential role in the migration of neural-crest-derived cells during embryonic development. α9-nAChR and α5-nAChRs have been proposed to play a role in the development and progression of certain cutaneous malignancies, such as melanoma, and have been proposed as potential therapeutic targets [7, 8]. However, to our knowledge, the role of nAChRs in MCC has not yet been fully explored. In the present study, we attempted to determine if MCC expresses nAChRs and, if so, whether nAChR expression correlates with patient outcomes. The study was based on a large cohort from a single institution.

2 | Materials and Methods

2.1 | Patients and Samples

With approval from our institutional review board (IRB number: LAB02- 719), we reviewed cases of primary MCC diagnosed and treated at our institution from 2000 through 2021 with available follow-up data. Hematoxylin-eosin–stained slides and formalin-fixed, paraffin-embedded tissue blocks were evaluated to confirm the presence of tumor and available tissue, and 71 MCC cases were included in the final cohort.

2.2 | Immunohistochemistry

To stratify the cases by MCPyV status, immunohistochemical (IHC) studies were performed on unstained slides, using previously validated IHC methods at our CLIA-certified clinical laboratory with antibody against the MCPyV T-antigen (sc-136172, 1:100; Santa Cruz Biotechnology). After appropriate optimization and validation of IHC studies in our research laboratory, unstained slides (5-μm-thick sections cut fresh from the selected FFPE tissue blocks) were evaluated for the expression of nAChRs using the following antibodies and corresponding methods. IHC analysis of anti-nAChR α-3/*CHRNA3* polyclonal (PAB-116458, Invitrogen; dilution1:300), anti-nAChR α-5/*CHRNA5* antibody D-11 (Santa Cruz Biotechnology, dilution1:50), and anti-nAChR α-7/*CHRNA7* antibody (ab216485, dilution1:300) (Abcam) in a paraffin-embedded human tissue as per manufacturer's instruction was performed using on Dako Omnis detection reagents. The section was pre-treated using heat-mediated antigen retrieval with Tris-EDTA buffer (pH 8.0–8.4) for 20 min. The tissues were blocked in 5% BSA for 30 min at room temperature, washed with ddH2O and PBS, and then probed with the primary antibody for 30 min at room temperature. The detection was performed using an HRP conjugated compact polymer system. Diaminobenzidine was used as the chromogen. Tissues were counterstained with hematoxylin and mounted with DPX. Following IHC processing, the IHC slides were reviewed by two board-certified pathologists using light microscopy to assess patterns of immunoreactivity. Comparing against appropriate controls, it was observed that there was variability in the location of nAChR expression and the intensity of immunoreactivity. A classification system was devised in which the location of protein expression was stratified into cytoplasmic only and peri-nuclear, and a range from 0 to 3+ (3+ being diffuse and strong expression and 0 being no expression) regardless of location of staining pattern, to stratify the intensity of immunoreactivity. Each IHC slide was then scored based on this classification system.

2.3 | Statistical Analysis

Demographic, histopathologic, and outcome measures were obtained from patient records and summarized for all patients in the study and by subgroups of interest. Categorical variables were summarized by frequencies and percentages and assessed using Fisher exact test or its generalization. Continuous variables were summarized by medians and ranges and assessed using either Wilcoxon rank-sum test or Kruskal–Wallis test. Overall survival (OS) and disease-specific survival (DSS) times were computed from the date of sample collection to the date of last follow-up. For OS, patients alive at the last follow-up date were censored. For DSS, patients alive at the last follow-up date as well as those who died of causes other than MCC were censored. The Kaplan–Meier method was used to estimate OS and DSS, and the log-rank test was used to assess differences between groups. In addition, univariate Cox proportional hazards regression models were used to assess the association between survival and variables of interest. Statistical analyses were performed using SAS 9.4 for Windows (SAS Institute Inc. Cary, NC). All statistical tests used a significance level of 5%. No adjustments for multiple testing were made.

3 | Results

Demographic and histopathologic parameters as well as clinical outcome data for the 71 MCC cases included in the study are summarized in Table 1. Sixty-five percent of patients were male and 35% females. The median age was 71.1 (range, 31.6–91.2) years. Most patients received surgery plus other therapies (e.g., radiotherapy and chemotherapy). MCPyV was positive in 68% of the cases and 66% had metastasis. Thirty-eight (66%) patients died and the median follow-up for all cases was 29.8 (range, 2.7–234.1) months.

We performed immunohistochemistry to evaluate the expression of α3-nAChR, α5-nAChR, and α7-nAChR in MCC. Among the 71 cases, 59 (83%) expressed α3-nAChR, 71 (100%) expressed

TABLE 1 | Cohort clinical and histopathological parameters.

Measure	Level	All ($N=71$)
Gender, *n* (%)	Male	46 (65)
	Female	25 (35)
Age at collection date (years)	Median	71.1
	Range	(31.6–91.2)
Race, *n* (%)	White	57 (83)
	Other	12 (17)
	Unknown	2
Tobacco, *n* (%)	No	38 (58)
	Quit	22 (34)
	Yes	5 (8)
	Unknown	6
Alcohol, *n* (%)	No	27 (43)
	Quit	10 (16)
	Yes	26 (41)
	Unknown	8
History of other cancer, *n* (%)	No	31 (44)
	Yes	39 (56)
	Unknown	1
Primary site, *n* (%)	Head & Neck	24 (34)
	Upper extremity	25 (35)
	Trunk	6 (8)
	Lower extremity	16 (23)
Tumor size (mm)	Number of patients	65
	Median	16.0
	Range	(2.3–93.0)
Tumor thickness (mm)	Number of patients	64
	Median	9.8
	Range	(1.2–60.0)
Lymphovascular invasion, *n* (%)	Absent	19 (29)
	Present	46 (71)
	Unknown	6
Perineural invasion, *n* (%)	Absent	52 (87)
	Present	8 (13)
	Unknown	11
Invasion beyond skin, *n* (%)	No	47 (73)
	Yes	17 (27)
	Unknown	7
Invasion structures, *n* (%)	No	55 (86)
	Yes	9 (14)
	Unknown	7
Mitotic figures (/mm^2)	Number of patients	65
	Median	28.0
	Range	(7.0–138.0)
Growth pattern, *n* (%)	Infiltrative	34 (52)
	Nodular	19 (29)
	Both	12 (18)
	Unknown	6
Treatment, *n* (%)	Surgery alone	13 (19)
	Surgery + Other	56 (81)
	Unknown	2
MCC Polyomavirus, *n* (%)	Negative	23 (32)
	Positive	48 (68)
Metastasis, *n* (%)	No	24 (34)
(At the time of this study)	Yes (nodal)	35 (49)
	Yes (distant)	12 (17)
Metastasis, *n* (%)	No	51 (72)
(At the time of the initial staging)	Yes (nodal)	12 (17)
	Yes (distant)	8 (11)
Vital status, *n* (%)	Alive	20 (34)
	Died	38 (66)
	Lost to follow-up	13 (18)
Follow-up (months)	Median	29.8
	Range	(2.7–234.1)

α5-nAChR, and 63 (88%) expressed α7-nAChR. The location of immunoreactivity differed between cases and included cytoplasmic only and nuclear/peri-nuclear staining patterns (Figure 1), with variation in the intensity of staining (Figure 2).

There were significant correlations between the location or intensity of immunoreactivity and MCPyV status, growth pattern and invasion beyond skin (Tables S1–S6). MCPyV+cases were

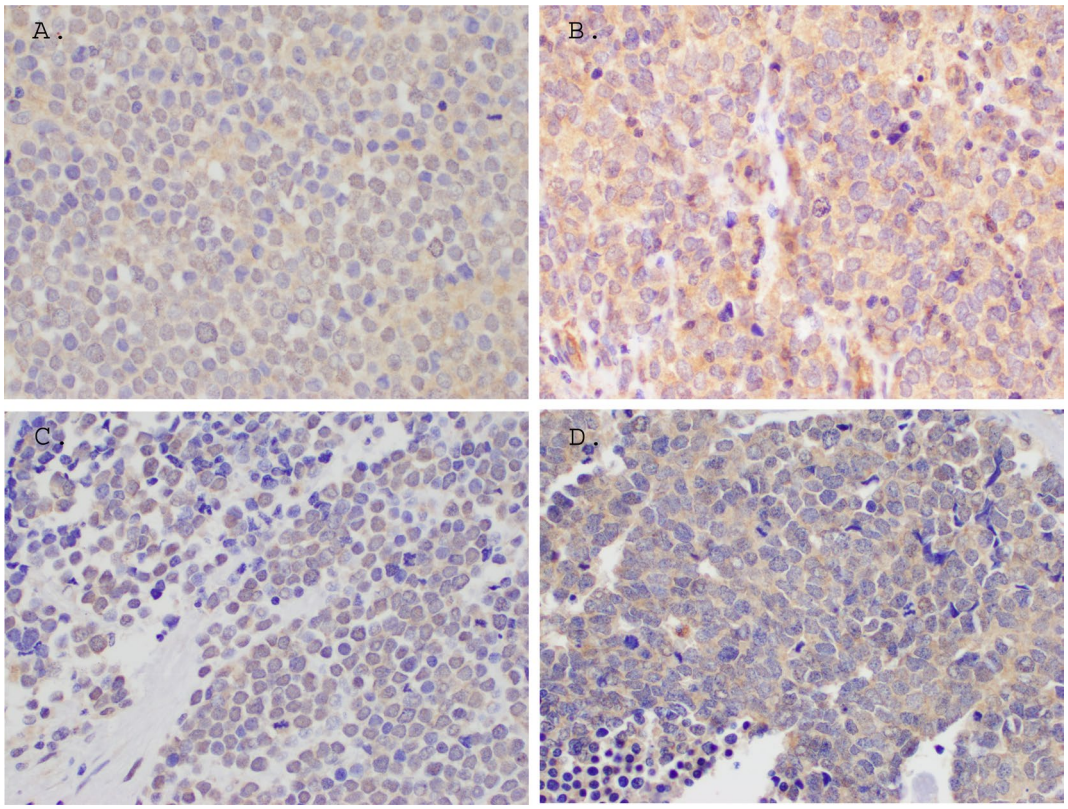

FIGURE 1 | (A–D) Representative images of expression of nAChR in MCC based on location of staining in tumor cells. (A and B) Expression of α7-nAChR in MCC: Nuclear/perinuclear and cytoplasmic pattern (A; 400×, α7-nAChR) versus predominantly cytoplasmic pattern (B; 400×, α7-nAChR). (C and D) Expression of α3-nAChR: Nuclear and cytoplasmic pattern (C; 400×, α3-nAChR) versus predominantly cytoplasmic expression (D; 400×, α3-nAChR).

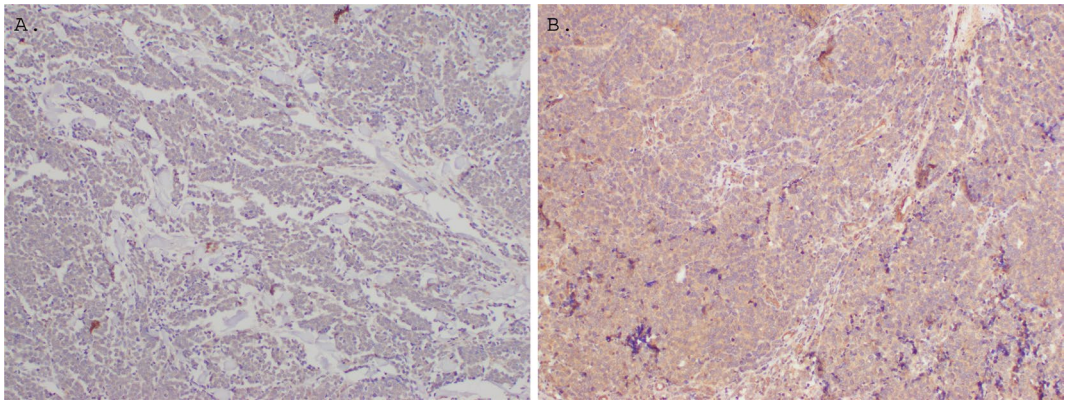

FIGURE 2 | (A and B) Representative images of expression of nAChR in MCC based on staining intensity: Low (A; 100, α7-nAChR) and high (B; 100×, α7-nAChR).

more likely to show α3-nAChR (91% MCPyV+ vs. 9% MCPyV-) and α5-nAChR (75% MCPyV+ vs. 25% MCPyV–) expression in a nuclear and cytoplasmic pattern compared to MCPyV– cases (Tables S1 and S3). MCPyV positivity was significantly associated with higher intensity of α5-nAChR staining; 75% of MCPyV+ cases showed high intensity α5-nAChR expression (Table S4). Expression of α7-nAChR expression in both the nucleus and cytoplasm was associated with an infiltrative tumor growth pattern (Table S5).

The median OS for all cases was 55.2 months with 1-, 3-, and 5-year rates of 88%, 56%, and 47%, respectively. Significant associations with OS were observed for MCPyV status, pattern of α3-nAChR staining and α7-nAChR staining intensity (Table S7). MCPyV+ cases showed improved OS compared to MCPyV– cases (median: 134.1 months vs. 26.2 months, $p < 0.001$; hazard ratio [95% CI] 0.28 [0.14, 0.55], $p < 0.001$) (Figure 3A). The pattern of α3-nAChR was significantly associated with OS, cases with both, cytoplasmic and peri-nuclear staining showed better OS compared with cases showing only cytoplasmic staining (median not reached vs. 46.5 months, $p = 0.048$; 0.39 [0.15, 1.03], $p = 0.058$) (Figure 3B,C). The median DSS for all cases was not reached, with 1-, 3-, and 5-year rates of 91%, 67%, and 63%, respectively. In addition, cases with lower intensity of α7-nAChR staining had longer OS

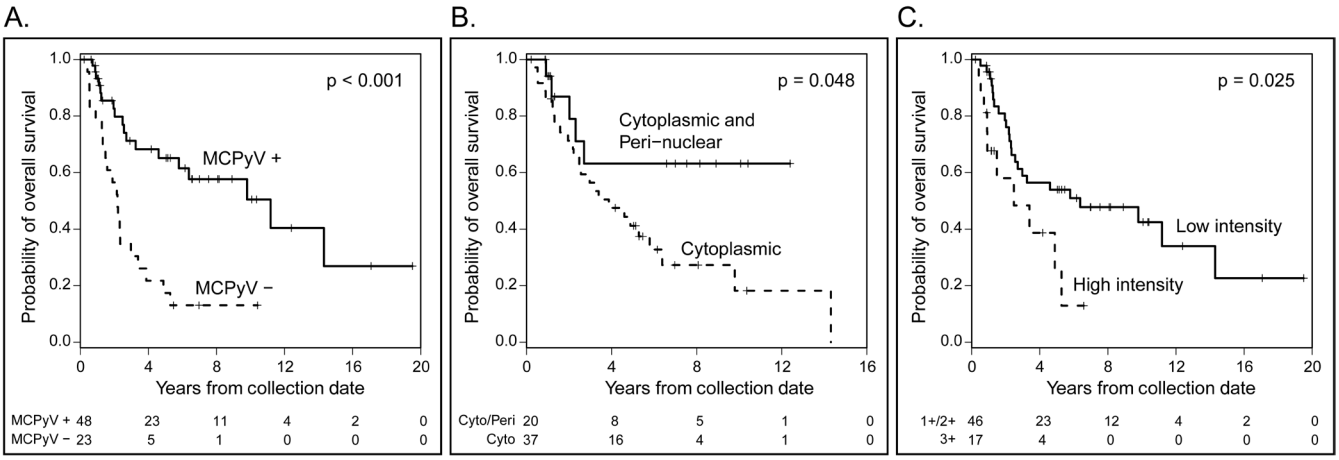

FIGURE 3 | (A) OS by MCPyV status. Patients with MCPyV+e tumors have better OS than patients with MCPyV– tumors. (B) OS by pattern of α3-nAChR expression; cytoplasmic only expression is associated with worse OS. (C) OS by intensity of α7-nAChR expression; cases with high intensity of α7-nAChR expression have worse OS.

compared with those with high intensity of staining (76.5 months vs. 29.8 months, $p = 0.025$; 0.43 [0.20, 0.92],$p = 0.030$). MCPyV status was the only measure significantly associated with DSS, where cases with neMCPyV+ experienced better survival compared with MCPyV– cases (median not reached vs. 26.6 months, $p < 0.001$; 0.21 [0.08, 0.52], $p < 0.001$). In multivariable analysis, MCPyV status was the only variable associated with OS (Table 2).

TABLE 2 | Summary of overall survival—multivariable assessments.

		Overall survival	
Measure	**Level**	**Hazard ratio (95% CI)**	***p***
MCC polyomavirus	Negative	*ref*	
	Positive	0.45 (0.21, 1.00)	**0.049**
Location of α3-nAChR	Cytoplasmic	*ref*	
	Nuclear + both	0.49 (0.19, 1.26)	0.14
Intensity of α7-nAChR	Low	*ref*	
	High	1.66 (0.71, 3.85)	0.24

Abbreviations: CI, confidence interval; ref, reference group. Bold values indicate a *p* value of less than 0.05.

4 | Discussion

The incidence of MCC is increasing globally, with higher rates reported in regions with fair-skinned populations and high levels of UVR exposure. In the United States and Europe, the incidence rate is approximately 0.44 cases per 100,000 person-years [3, 8, 9, 11, 12]. However, in Australia, the incidence rate is significantly higher, reported to be 1.6 cases per 100 000 person-years [13]. Unfortunately, no relief is in the forecast, as it is predicted that as climate change worsens, cases of skin cancer will gradually increase due to increased UVR exposure [13]. As seen in our cohort, MCC is more commonly diagnosed in males, with a male-to-female ratio of approximately 2:1, and is typically diagnosed in individuals over the age of 50 years, with a median age at diagnosis of 75 years [12–15].

The etiology of MCC is a topic of ongoing research. However, several potential etiologies have been identified, including MCPyV, UVR exposure, and chronic immunosuppression, particularly in solid organ transplant recipients [2, 12, 16]. MCPyV has been detected in up to 80% of MCC tumors in the United States, and its presence is associated with a better prognosis, with a five-year OS rate of approximately 50% [2, 12, 15]. The nAChRs are pentameric ligand-gated ion channels that mediate the transmission of nerve impulses at the neuromuscular junction [17]. nAChRs are also expressed in non-neuronal tissues, including various types of cancer cells (e.g., lung, breast, pancreas, prostate, and colon cancer) and immune cells [18]. In cancer cells, they have been shown to promote cell proliferation by activating the MAPK and PI3K/Akt pathways, leading to cell cycle progression and inhibition of apoptosis [19, 20]. nAChRs have also been shown to promote angiogenesis in tumors through activation of endothelial cells, promoting the release of pro-angiogenic factors such as vascular endothelial growth factor [21]. Importantly, immune cells such as macrophages and dendritic cells express nAChRs, including the α7-nAChR, which inhibits the production of inflammatory cytokines via the well-known cholinergic anti-inflammatory pathway [22, 23]. These immune cells have also been found in MCC.

Although associations between nAChRs and other malignancies have been established, little work has been done on the role of nAChRs in MCC, possibly owing to the rarity of the disease. A pilot study in 15 patients with MCC demonstrated expression of muscarinic AChRs in most of the tumors analyzed [24]. The current study, to our knowledge, is among the first to evaluate the expression of nAChRs in MCC and their possible clinical implications.

In this study, there was variation in our findings between the three antigens evaluated (α3, α5, and α7-nAChR), but for most of the examined antigens, either location or intensity of staining

correlated with MCPyV status, suggesting an association between nAChR expression and MCPyV. Furthermore, expression of the α7-nAChR antigen in MCC was associated with a histopathologic parameter (pattern of growth) and an environmental exposure (alcohol use). In a previous study, an association was found between the α5 subunit of the nAChR and some behavioral effects of ethanol [25]. It has also been demonstrated that α7-nAChRs may be important mediator of the motor-impairing effects of moderate ethanol consumption [26]. Given these associations in addition to our findings, there is likely a complex interplay between nAChRs, the microenvironment, and MCC growth that merits further investigation.

An interesting finding of our study was the correlation between OS and the intensity of the α5 and α7-nAChR antibodies as well as the cytoplasmic location of staining of the α3-nAChR antibody (Table S3). The finding that the vast majority of MCC cases expressed nAChRs, and intensity of expression of nAChRs correlated with OS, strongly suggest the potential for targeting nAChRs for the treatment of this aggressive malignancy. Additional studies are needed to fully understand this possibility.

One of the limitations of our results is the possible interobserver variability in interpreting the pattern and intensity of nAC-hR expression. Therefore, we are currently undertaking molecular analyses on a subset of our cohort to more accurately evaluate the link between expression of nAChRs and MCC.

5 | Conclusion

Our findings show support an association between nAChRs and MCC and add to the body of knowledge regarding molecular pathways involved in MCC. Targeted therapies and immunotherapies have shown promising results in the treatment of MCC, and our findings raise the possibility that targeting nAChRs might improve patient outcomes. Further research is needed to fully elucidate these molecular mechanisms and to develop effective treatment strategies.

Author Contributions

Christopher R. Cunningham wrote the paper, interpreted the IHC slides, helped collect the clinical and histopathologic data as well as take photographs. Yiannis P. Dimopoulos helped collect the clinical and histopathologic data. Denái R. Milton analyzed the data and interpreted the results, helped create the tables and helped edit the paper. Ian M. García-Quiñones helped perform the molecular analyses and edit the paper. Manuel Delgado-Vélez helped perform the molecular analyses and edit the paper. José A. Lasalde-Dominicci helped perform the molecular analyses and edit the paper. Woo Cheal Cho helped collect the clinical and histopathologic data and edit the paper. Victor G. Prieto helped edit the paper. Leomar Y. Ballester designed and performed the IHC validation/testing/interpreting and edit the paper. Phyu P. Aung designed the study, interpreted the IHC slides, helped write the paper, and took the photomicrographs.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supplemental table 1. Patient and Clinical Characteristics – by Location of nAChR α3

		Location of nAChR α3		
Measure	**Level**	**Cytoplasmic (N=37)**	**Nuclear and cytoplasmic (N=22)**	**p-value**
Gender, n (%)	Male	27 (73)	10 (45)	0.052
	Female	10 (27)	12 (55)	
Age at collection date (years)	Median	73.0	73.5	0.83
	Range	(31.6 - 90.7)	(44.0 - 86.8)	
Race, n (%)	White	28 (78)	19 (86)	0.51
	Other	8 (22)	3 (14)	
	Missing	1	0	
Tobacco, n (%)	No	20 (56)	13 (72)	0.36
	Quit	13 (36)	5 (28)	
	Yes	3 (8)	0	
	Missing	1	4	
Alcohol, n (%)	No	16 (46)	7 (41)	0.92
	Quit	5 (14)	2 (12)	
	Yes	14 (40)	8 (47)	
	Missing	2	5	
History of other cancer, n (%)	No	14 (38)	11 (50)	0.42
	Yes	23 (62)	11 (50)	
	Missing	0	0	
Primary site, n (%)	Head & Neck	12 (32)	7 (32)	1.00
	Upper Extremity	14 (38)	9 (41)	
	Trunk	3 (8)	1 (5)	
	Lower Extremity	8 (22)	5 (23)	
Tumor size (mm)	Number of patients	35	19	0.82
	Median	16.0	16.0	
	Range	(2.3 - 93.0)	(8.0 - 43.0)	
Tumor thickness (mm)	Number of patients	34	19	0.25
	Median	9.2	11.0	
	Range	(1.5 - 60.0)	(1.2 - 22.0)	
LVI, n (%)	Absent	10 (29)	4 (21)	0.75
	Present	25 (71)	15 (79)	
	Missing	2	3	
PNI, n (%)	Absent	29 (91)	15 (88)	1.00
	Present	3 (9)	2 (12)	

PNI	Missing	5	5	
Invasion beyond skin, n (%)	No	27 (79)	13 (68)	0.51
	Yes	7 (21)	6 (32)	
	Missing	3	3	
Invasion structures, n (%)	No	32 (94)	15 (79)	0.17
	Yes	2 (6)	4 (21)	
	Missing	3	3	
Mitotic figures (/mm^2)	Number of patients	35	19	0.78
	Median	27.0	28.0	
	Range	(8.0 - 138.0)	(7.0 - 117.0)	
Growth pattern, n (%)	Infiltrative	16 (46)	12 (63)	0.52
	Nodular	11 (31)	4 (21)	
	Both	8 (23)	3 (16)	
	Missing	2	3	
Treatment, n (%)	Surgery alone	7 (19)	4 (18)	1.00
	Surgery+other	29 (81)	18 (82)	
	Missing	1	0	
MCC polyomavirus, n (%)	Negative	16 (43)	2 (9)	**0.008**
	Positive	21 (57)	20 (91)	
Metastasis, n (%)	No	10 (27)	10 (45)	0.17
	Yes	27 (73)	12 (55)	
Vital status, n (%)	Alive	11 (30)	8 (36)	**< 0.001**
	Died	25 (68)	6 (27)	
	Lost to follow-up	1 (3)	8 (36)	
Follow-up (months)	Median	39.0	26.0	0.89
	Range	(2.7 - 171.7)	(8.6 - 204.9)	

Supplemental table 2. Patient and Clinical Characteristics – by Intensity of nAChR α3

		Intensity of nAChR α3		
Measure	**Level**	**Low (N=45)**	**High (N=14)**	**p-value**
Gender, n (%)	Male	29 (64)	8 (57)	0.75
	Female	16 (36)	6 (43)	
Age at collection date (years)	Median	72.8	76.0	0.84
	Range	(52.0 - 90.7)	(31.6 - 89.7)	
Race, n (%)	White	37 (82)	10 (77)	0.70
	Other	8 (18)	3 (23)	
	Missing	0	1	
Tobacco, n (%)	No	24 (59)	9 (69)	0.78
	Quit	14 (34)	4 (31)	
	Yes	3 (7)	0	
	Missing	4	1	
Alcohol, n (%)	No	17 (44)	6 (46)	0.07
	Quit	3 (8)	4 (31)	
	Yes	19 (49)	3 (23)	
	Missing	6	1	
History of other cancer, n (%)	No	19 (42)	6 (43)	1.00
	Yes	26 (58)	8 (57)	
Primary site, n (%)	Head & Neck	13 (29)	6 (43)	0.54
	Upper Extremity	19 (42)	4 (29)	
	Trunk	4 (9)	0	
	Lower Extremity	9 (20)	4 (29)	
Tumor size (mm)	Number of patients	41	13	0.30
	Median	15.0	24.0	
	Range	(5.9 - 93.0)	(2.3 - 50.0)	
Tumor thickness (mm)	Number of patients	40	13	0.79
	Median	9.8	10.1	
	Range	(1.2 - 60.0)	(1.5 - 22.0)	
LVI, n (%)	Absent	12 (29)	2 (15)	0.47
	Present	29 (71)	11 (85)	
	Missing	4	1	
PNI, n (%)	Absent	33 (87)	11 (100)	0.57
	Present	5 (13)	0	
	Missing	7	3	
Invasion beyond skin, n (%)	No	30 (73)	10 (83)	0.71

	Yes	11 (27)	2 (17)	
	Missing	4	2	
Invasion structures, n (%)	No	36 (88)	11 (92)	1.00
	Yes	5 (12)	1 (8)	
	Missing	4	2	
Mitotic figures (/mm^2)	Number of patients	41	13	0.74
	Median	27.0	28.0	
	Range	(7.0 - 138.0)	(16.0 - 77.0)	
Growth pattern, n (%)	Infiltrative	20 (49)	8 (62)	0.49
	Nodular	11 (27)	4 (31)	
	Both	10 (24)	1 (8)	
	Missing	4	1	
Treatment, n (%)	Surgery alone	9 (20)	2 (14)	1.00
	Surgery+other	35 (80)	12 (86)	
	Missing	1	0	
MCC polyomavirus, n (%)	Negative	13 (29)	5 (36)	0.74
	Positive	32 (71)	9 (64)	
Metastasis, n (%)	No	18 (40)	2 (14)	0.11
	Yes	27 (60)	12 (86)	
Vital status, n (%)	Alive	13 (29)	6 (43)	0.49
	Died	24 (53)	7 (50)	
	Lost to follow-up	8 (18)	1 (7)	
Follow-up (months)	Median	32.4	35.7	0.99
	Range	(2.7 - 204.9)	(10.5 - 120.8)	

Supplemental table 3. Patient and Clinical Characteristics – by Location of nAChR α5

		Location of nAChR α5		
Measure	**Level**	**Cytoplasmic (N=18)**	**Nuclear and cytoplasmic (N=53)**	**p-value**
Gender, n (%)	Male	9 (50)	37 (70)	0.16
	Female	9 (50)	16 (30)	
Age at collection date (years)	Median	67.7	71.4	0.96
	Range	(44.8 - 90.7)	(31.6 - 91.2)	
Race, n (%)	White	15 (88)	42 (81)	0.72
	Other	2 (12)	10 (19)	
	Missing	1	1	
Tobacco, n (%)	No	12 (71)	26 (54)	0.25
	Quit	3 (18)	19 (40)	
	Yes	2 (12)	3 (6)	
	Missing	1	5	
Alcohol, n (%)	No	9 (53)	18 (39)	0.45
	Quit	1 (6)	9 (20)	
	Yes	7 (41)	19 (41)	
	Missing	1	7	
History of other cancer, n (%)	No	9 (50)	22 (42)	0.59
	Yes	9 (50)	30 (58)	
	Missing	0	1	
Primary site, n (%)	Head & Neck	5 (28)	19 (36)	0.57
	Upper Extremity	5 (28)	20 (38)	
	Trunk	2 (11)	4 (8)	
	Lower Extremity	6 (33)	10 (19)	
Tumor size (mm)	Number of patients	15	50	0.43
	Median	15.0	16.0	
	Range	(2.5 - 65.0)	(2.3 - 93.0)	
Tumor thickness (mm)	Number of patients	15	49	0.50
	Median	9.0	10.0	
	Range	(1.2 - 60.0)	(1.5 - 22.0)	
LVI, n (%)	Absent	6 (40)	13 (26)	0.34
	Present	9 (60)	37 (74)	
	Missing	3	3	
PNI, n (%)	Absent	13 (87)	39 (87)	1.00
	Present	2 (13)	6 (13)	

PNI	Missing	3	8	
Invasion beyond skin, n (%)	No	11 (73)	36 (73)	1.00
	Yes	4 (27)	13 (27)	
	Missing	3	4	
Invasion structures, n (%)	No	13 (87)	42 (86)	1.00
	Yes	2 (13)	7 (14)	
	Missing	3	4	
Mitotic figures (/mm^2)	Number of patients	15	50	0.57
	Median	35.0	26.0	
	Range	(11.0 - 72.0)	(7.0 - 138.0)	
Growth pattern, n (%)	Infiltrative	8 (53)	26 (52)	1.00
	Nodular	4 (27)	15 (30)	
	Both	3 (20)	9 (18)	
	Missing	3	3	
Treatment, n (%)	Surgery alone	4 (22)	9 (18)	0.73
	Surgery+other	14 (78)	42 (82)	
	Missing	0	2	
MCC polyomavirus, n (%)	Negative	10 (56)	13 (25)	**0.021**
	Positive	8 (44)	40 (75)	
Metastasis, n (%)	No	7 (39)	17 (32)	0.77
	Yes	11 (61)	36 (68)	
Vital status, n (%)	Alive	4 (22)	16 (30)	0.82
	Died	11 (61)	27 (51)	
	Lost to follow-up	3 (17)	10 (19)	
Follow-up (months)	Median	59.5	27.7	0.15
	Range	(6.2 - 171.7)	(2.7 - 234.1)	

Supplemental table 4. Patient and Clinical Characteristics – by Intensity of nAChR α5

		Intensity of nAChR α5		
Measure	**Level**	**Low (N=15)**	**High (N=56)**	**p-value**
Gender, n (%)	Male	9 (60)	37 (66)	0.76
	Female	6 (40)	19 (34)	
Age at collection date (years)	Median	69.2	71.2	0.84
	Range	(55.4 - 88.1)	(31.6 - 91.2)	
Race, n (%)	White	14 (93)	43 (80)	0.44
	Other	1 (7)	11 (20)	
	Missing	0	2	
Tobacco, n (%)	No	9 (64)	29 (57)	0.89
	Quit	4 (29)	18 (35)	
	Yes	1 (7)	4 (8)	
	Missing	1	5	
Alcohol, n (%)	No	8 (57)	19 (39)	0.47
	Quit	1 (7)	9 (18)	
	Yes	5 (36)	21 (43)	
	Missing	1	7	
History of other cancer, n (%)	No	6 (40)	25 (45)	0.78
	Yes	9 (60)	30 (55)	
	Missing	0	1	
Primary site, n (%)	Head & Neck	8 (53)	16 (29)	0.32
	Upper Extremity	3 (20)	22 (39)	
	Trunk	1 (7)	5 (9)	
	Lower Extremity	3 (20)	13 (23)	
Tumor size (mm)	Number of patients	13	52	0.37
	Median	11.0	16.0	
	Range	(2.3 - 65.0)	(4.0 - 93.0)	
Tumor thickness (mm)	Number of patients	13	51	0.62
	Median	8.2	9.9	
	Range	(1.2 - 60.0)	(1.5 - 22.5)	
LVI, n (%)	Absent	5 (38)	14 (27)	0.50
	Present	8 (62)	38 (73)	
	Missing	2	6	
PNI, n (%)	Absent	11 (92)	41 (85)	1.00
	Present	1 (8)	7 (15)	

PNI	Missing	3	10	
Invasion beyond skin, n (%)	No	10 (83)	37 (71)	0.49
	Yes	2 (17)	15 (29)	
	Missing	3	6	
Invasion structures, n (%)	No	10 (83)	45 (87)	0.67
	Yes	2 (17)	7 (13)	
	Missing	3	6	
Mitotic figures (/mm^2)	Number of patients	13	52	0.47
	Median	25.0	28.5	
	Range	(13.0 - 72.0)	(7.0 - 138.0)	
Growth pattern, n (%)	Infiltrative	7 (54)	27 (52)	0.85
	Nodular	3 (23)	16 (31)	
	Both	3 (23)	9 (17)	
	Missing	2	6	
Treatment, n (%)	Surgery alone	2 (13)	11 (20)	0.72
	Surgery+other	13 (87)	43 (80)	
	Missing	0	2	
MCC polyomavirus, n (%)	Negative	9 (60)	14 (25)	**0.015**
	Positive	6 (40)	42 (75)	
Metastasis, n (%)	No	6 (40)	18 (32)	0.56
	Yes	9 (60)	38 (68)	
Vital status, n (%)	Alive	2 (13)	18 (32)	0.26
	Died	11 (73)	27 (48)	
	Lost to follow-up	2 (13)	11 (20)	
Follow-up (months)	Median	27.7	31.6	0.85
	Range	(6.2 - 124.9)	(2.7 - 234.1)	

Supplemental table 5. Patient and Clinical Characteristics – by Location of nAChR α7

		Location of nAChR α7		
Measure	**Level**	**Cytoplasmic (N=40)**	**Nuclear and cytoplasmic (N=23)**	**p-value**
Gender, n (%)	Male	25 (63)	14 (61)	1.00
	Female	15 (38)	9 (39)	
Age at collection date (years)	Median	71.3	72.8	0.39
	Range	(31.6 - 90.7)	(54.8 - 87.3)	
Race, n (%)	White	31 (79)	19 (83)	1.00
	Other	8 (21)	4 (17)	
	Missing	1	0	
Tobacco, n (%)	No	23 (61)	12 (60)	1.00
	Quit	12 (32)	7 (35)	
	Yes	3 (8)	1 (5)	
	Missing	2	3	
Alcohol, n (%)	No	13 (35)	10 (53)	0.48
	Quit	7 (19)	2 (11)	
	Yes	17 (46)	7 (37)	
	Missing	3	4	
History of other cancer, n (%)	No	19 (48)	8 (35)	0.43
	Yes	21 (53)	15 (65)	
Primary site, n (%)	Head & Neck	12 (30)	10 (43)	0.27
	Upper Extremity	14 (35)	10 (43)	
	Trunk	3 (8)	0	
	Lower Extremity	11 (28)	3 (13)	
Tumor size (mm)	Number of patients	38	19	0.41
	Median	16.0	14.0	
	Range	(2.5 - 93.0)	(2.3 - 43.0)	
Tumor thickness (mm)	Number of patients	37	19	0.43
	Median	9.3	9.9	
	Range	(1.5 - 60.0)	(1.2 - 21.0)	
LVI, n (%)	Absent	8 (21)	6 (32)	0.52
	Present	30 (79)	13 (68)	
	Missing	2	4	
PNI, n (%)	Absent	33 (89)	14 (93)	1.00
	Present	4 (11)	1 (7)	
	Missing	3	8	

Invasion beyond skin, n (%)	No	27 (71)	13 (72)	1.00
	Yes	11 (29)	5 (28)	
	Missing	2	5	
Invasion structures, n (%)	No	33 (87)	15 (83)	0.70
	Yes	5 (13)	3 (17)	
	Missing	2	5	
Mitotic figures (/mm^2)	Number of patients	38	19	0.75
	Median	28.0	28.0	
	Range	(7.0 - 96.0)	(7.0 - 138.0)	
Growth pattern, n (%)	Infiltrative	16 (42)	14 (74)	**0.047**
	Nodular	14 (37)	2 (11)	
	Both	8 (21)	3 (16)	
	Missing	2	4	
Treatment, n (%)	Surgery alone	8 (21)	4 (17)	1.00
	Surgery+other	31 (79)	19 (83)	
	Missing	1	0	
MCC polyomavirus, n (%)	Negative	12 (30)	7 (30)	1.00
	Positive	28 (70)	16 (70)	
Metastasis, n (%)	No	12 (30)	9 (39)	0.58
	Yes	28 (70)	14 (61)	
Vital status, n (%)	Alive	15 (38)	5 (22)	0.09
	Died	22 (55)	12 (52)	
	Lost to follow-up	3 (8)	6 (26)	
Follow-up (months)	Median	34.8	27.7	0.69
	Range	(2.7 - 234.1)	(6.2 - 204.9)	

Supplemental table 6. Patient and Clinical Characteristics – by Intensity of nAChR α7

		Intensity of nAChR α7		
Measure	**Level**	**Low (N=46)**	**High (N=17)**	**p-value**
Gender, n (%)	Male	30 (65)	9 (53)	0.40
	Female	16 (35)	8 (47)	
Age at collection date (years)	Median	70.1	74.7	0.30
	Range	(31.6 - 90.7)	(47.7 - 89.7)	
Race, n (%)	White	37 (80)	13 (81)	1.00
	Other	9 (20)	3 (19)	
	Missing	0	1	
Tobacco, n (%)	No	24 (56)	11 (73)	0.46
	Quit	16 (37)	3 (20)	
	Yes	3 (7)	1 (7)	
	Missing	3	2	
Alcohol, n (%)	No	16 (38)	7 (50)	0.11
	Quit	5 (12)	4 (29)	
	Yes	21 (50)	3 (21)	
	Missing	4	3	
History of other cancer, n (%)	No	20 (43)	7 (41)	1.00
	Yes	26 (57)	10 (59)	
Primary site, n (%)	Head & Neck	14 (30)	8 (47)	0.56
	Upper Extremity	19 (41)	5 (29)	
	Trunk	3 (7)	0	
	Lower Extremity	10 (22)	4 (24)	
Tumor size (mm)	Number of patients	43	14	0.68
	Median	16.0	19.0	
	Range	(2.5 - 93.0)	(2.3 - 50.0)	
Tumor thickness (mm)	Number of patients	42	14	0.41
	Median	10.1	8.6	
	Range	(1.2 - 60.0)	(1.5 - 22.0)	
LVI, n (%)	Absent	9 (21)	5 (36)	0.30
	Present	34 (79)	9 (64)	
	Missing	3	3	
PNI, n (%)	Absent	36 (88)	11 (100)	0.57
	Present	5 (12)	0	

	Missing	5	6	
Invasion beyond skin, n (%)	No	32 (74)	8 (62)	0.49
	Yes	11 (26)	5 (38)	
	Missing	3	4	
Invasion structures, n (%)	No	38 (88)	10 (77)	0.37
	Yes	5 (12)	3 (23)	
	Missing	3	4	
Mitotic figures (/mm^2)	Number of patients	43	14	0.42
	Median	29.0	26.5	
	Range	(7.0 - 138.0)	(7.0 - 96.0)	
Growth pattern, n (%)	Infiltrative	22 (51)	8 (57)	0.92
	Nodular	12 (28)	4 (29)	
	Both	9 (21)	2 (14)	
	Missing	3	3	
Treatment, n (%)	Surgery alone	8 (17)	4 (25)	0.49
	Surgery+other	38 (83)	12 (75)	
	Missing	0	1	
MCC polyomavirus, n (%)	Negative	12 (26)	7 (41)	0.35
	Positive	34 (74)	10 (59)	
Metastasis, n (%)	No	18 (39)	3 (18)	0.14
	Yes	28 (61)	14 (82)	
Vital status, n (%)	Alive	15 (33)	5 (29)	1.00
	Died	24 (52)	10 (59)	
	Lost to follow-up	7 (15)	2 (12)	
Follow-up (months)	Median	47.1	14.0	**0.003**
	Range	(6.2 - 234.1)	(2.7 - 79.0)	

Supplemental table 7. Summary of Overall Survival – Univariate Assessments

		Overall Survival					
Measure	**Level**	**Total # : # Died**	**Median (95% CI) (in months)**	**Rate (%) YR1 : YR3 : YR5 : LAST**	**p-value**	**Hazard Ratio (95% CI)**	**p-value**
All patients		71 : 38	55.2 (28.1, 117.5)	88 : 56 : 47 : 19			
MCC polyomavirus	Negative	23 : 20	26.2 (15.8, 35.6)	78 : 30 : 17 : 13	**< 0.001**	*ref*	
	Positive	48 : 18	134.1 (55.2, NE)	93 : 71 : 65 : 27		0.28 (0.14, 0.55)	**< 0.001**
Location of nAChR α5	Cytoplasmic	18 : 11	63.3 (23.4, 171.7)	94 : 59 : 53 : 0	0.74	*ref*	
	Nuclear + cytoplasmic	53 : 27	40.7 (27.7, 117.5)	86 : 55 : 44 : 30		1.13 (0.56, 2.29)	0.74
Intensity of nAChR α5	Low	15 : 11	28.1 (22.6, 117.5)	93 : 43 : 29 : 14	0.18	*ref*	
	High	56 : 27	63.3 (30.7, 171.7)	87 : 60 : 52 : 23		0.62 (0.30, 1.26)	0.18
Location of nAChR α3	Cytoplasmic	37 : 25	46.5 (26.6, 69.4)	86 : 56 : 41 : 0	**0.036**	*ref*	
	Nuclear + cytoplasmic	22 : 6	NE (24.2, NE)	90 : 63 : 63 : 63		0.39 (0.16, 0.97)	**0.042**
Intensity of nAChR α3	Low	45 : 24	55.2 (26.6, 171.7)	88 : 56 : 48 : 17	0.97	*ref*	
	High	14 : 7	58.6 (15.8, NE)	86 : 68 : 48 : 39		0.98 (0.42, 2.30)	0.97
Location of nAChR α7	Cytoplasmic	40 : 22	63.3 (26.2, 134.1)	90 : 58 : 52 : 12	0.95	*ref*	
	Nuclear + cytoplasmic	23 : 12	40.7 (24.2, NE)	86 : 52 : 41 : 35		1.02 (0.51, 2.07)	0.95
Intensity of nAChR α7	Low	46 : 24	76.5 (28.1, 171.7)	96 : 59 : 54 : 23	**0.025**	*ref*	
	High	17 : 10	29.8 (10.8, 63.3)	68 : 48 : 26 : 13		2.34 (1.09, 5.01)	**0.030**

Abbreviations: CI = confidence interval; NE = not estimated/not reached; *ref* = reference group.