Shared Oncogenic Pathways Implicated in Both Virus-Positive and UV-Induced Merkel Cell Carcinomas

JIDOpen

María del Carmen González-Vela[1,2,17], Soraya Curiel-Olmo[2,17], Sophia Derdak[3,14], Sergi Beltran[3,14], Miguel Santibañez[4], Nerea Martínez[2], Alfredo Castillo-Trujillo[5], Martha Gut[3,14], Roxana Sánchez-Pacheco[1], Carmen Almaraz[2], Laura Cereceda[2], Beatriz Llombart[6], Antonio Agraz-Doblas[7,16], José Revert-Arce[2], José Antonio López Guerrero[8], Manuela Mollejo[9], Pablo Isidro Marrón[10], Pablo Ortiz-Romero[11], Lynnette Fernandez-Cuesta[12,15], Ignacio Varela[7], Ivo Gut[3,14], Lorenzo Cerroni[13], Miguel Ángel Piris[1,2,18] and José Pedro Vaqué[2,7,18]

Merkel cell carcinoma (MCC) is a highly malignant neuroendocrine tumor of the skin whose molecular pathogenesis is not completely understood, despite the role that Merkel cell polyomavirus can play in 55–90% of cases. To study potential mechanisms driving this disease in clinically characterized cases, we searched for somatic mutations using whole-exome sequencing, and extrapolated our findings to study functional biomarkers reporting on the activity of the mutated pathways. Confirming previous results, Merkel cell polyomavirus-negative tumors had higher mutational loads with UV signatures and more frequent mutations in *TP53* and *RB* compared with their Merkel cell polyomavirus-positive counterparts. Despite important genetic differences, the two Merkel cell carcinoma etiologies both exhibited nuclear accumulation of oncogenic transcription factors such as NFAT or nuclear factor of activated T cells (NFAT), P-CREB, and P-STAT3, indicating commonly deregulated pathogenic mechanisms with the potential to serve as targets for therapy. A multivariable analysis identified phosphorylated CRE-binding protein as an independent survival factor with respect to clinical variables and Merkel cell polyomavirus status in our cohort of Merkel cell carcinoma patients.

Journal of Investigative Dermatology (2016) ■, ■–■; doi:10.1016/j.jid.2016.08.015

INTRODUCTION

Merkel cell carcinoma (MCC) is a highly malignant neuroendocrine tumor of the skin, with an increasing number of diagnosed cases and deaths attributable to the tumor. MCC has a relatively low incidence, but the mortality rate of diagnosed cases is 33%, which exceeds that of melanoma in the skin (reviewed in Hughes et al., 2014). MCC primarily affects older and immunosuppressed patients who, at the time of diagnosis, frequently have advanced clinical stage disease. This dramatically affects the 5-year survival rate, which is 64% in patients with localized tumors, 39% in those with loco-regional metastasis, and 18% in those with distant metastasis (Sarnaik et al., 2010).

The origin of MCC remains uncertain. The role of Merkel and/or pluripotent epidermal cells is currently under discussion (McCardle et al., 2010; Ratner et al., 1993). Recent evidence suggests that clonal integration of Merkel cell polyomavirus (MCPyV) is one of the main etiological mechanisms by which MCC develops. It has been found in 55–90% of the cases analyzed (Becker et al., 2009; Bhatia et al., 2010; Duncavage et al., 2009; Feng et al., 2008; Garneski et al., 2009). MCPyV expresses large, small, and

[1]*Pathology Department, Hospital Universitario Marqués de Valdecilla, Santander, Spain;* [2]*Cancer Genomics Laboratory, Instituto de Investigación Marqués de Valdecilla, IDIVAL, Santander, Spain;* [3]*CNAG-CRG, Centre for Genomic Regulation (CRG), Barcelona Institute of Science and Technology (BIST), Baldiri i Reixac 4, 08028, Barcelona, Spain;* [4]*Universidad de Cantabria-IDIVAL, Santander, Spain;* [5]*Oncology Department, Hospital Universitario Marqués de Valdecilla, Santander, Spain;* [6]*Department of Dermatology, Instituto Valenciano de Oncología, Valencia, Spain;* [7]*IBBTEC-UC-CSIC-SODERCAN Instituto de Biomedicina y Biotecnología de Cantabria, Santander, Spain;* [8]*Biobanco -Fundación Instituto Valenciano de Oncología, Valencia, Spain;* [9]*Hospital Universitario Virgen de la Salud, Toledo, Spain;* [10]*Biobanco-Hospital Universitario Central de Asturias, Oviedo, Spain;* [11]*Dermatology Service, Instituto I+12, Hospital Universitario 12 de Octubre, Madrid, Spain;* [12]*International Agency for Research on Cancer (IARC-WHO), Lyon, France;* [13]*Department of Dermatology Medical University of Graz, Austria;* [14]*Universitat Pompeu Fabra (UPF), Barcelona, Spain;* [15]*Department of Translational Genomics, Center of Integrated Oncology Cologne–Bonn, Medical Faculty, University of Cologne, 50931 Cologne, Germany; and* [16]*Josep Carreras Leukemia Research Institute and School of Medicine, University of Barcelona, Barcelona, Spain*

[17]*These authors contributed equally to this work.*

[18]*These authors shared senior authorship.*

Correspondence: José Pedro Vaqué, Instituto de Biomedicina y Biotecnología de Cantabria, PCTCAN, C/Albert Einstein 22, 39011 Santander, Spain. E-mail: vaquej@unican.es

Abbreviations: IHC, immunohistochemistry; KEGG, Kyoto Encyclopedia of Genes and Genomes; MCC, Merkel cell carcinoma; MCPyV, Merkel cell polyomavirus; NFAT, nuclear factor of activated T cells; P-CREB, phosphorylated CRE-binding protein; P-STAT, phosphorylated signal transducer and activator of transcription; RB, retinoblastoma; RTK, receptor with tyrosine kinase activity; SSM, somatic single-base mutation

Received 1 February 2016; revised 3 August 2016; accepted 4 August 2016; accepted manuscript published online 1 September 2016; corrected proof published online XXX

57-kDa T antigens that have the potential to inhibit retinoblastoma (RB) activity (by large T antigen) and promote MCC tumorigenesis, although the mechanisms responsible are poorly understood (Stakaityte et al., 2014). Along with MCPyV, there is increasing evidence that sun exposure may also be a major independent etiological factor. MCC most commonly develops in areas of the skin exposed to the sun, such as the head and neck, as in other malignant skin tumors such as melanoma (Davies et al., 2002; Lunder and Stern, 1998; Rockville Merkel Cell Carcinoma Group, 2009). In this regard, recent work by independent laboratories has shown important genetic differences between $MCPyV^+$ and $MCPyV^-$ MCC tumors, the latter harboring higher mutational burdens with UV signatures (Goh et al., 2015; Harms et al., 2015; Wong et al., 2015).

From a molecular perspective, the main mechanisms of transformation promoting MCCs are still partially unknown. Mutations in *TP53* have been observed in 14–33% of MCC patients. They are considered a rare event in MCCs that are mostly confined to $MCPyV^-$ cases (Harms et al., 2015; Lassacher et al., 2008). Moreover, mutations affecting *PIK3CA* and deregulated PI3K-mTOR activity have been observed in patient samples and cell lines (Hafner et al., 2012; Nardi et al., 2012), and alterations in NOTCH and RAS/mitogen-activated protein kinase signaling pathways (Harms et al., 2015) have been detected in MCCs. However, these advances are not yet used to diagnose or treat the disease. Current treatment options mainly rely on surgical excision combined with loco-regional adjuvant radiation (Harring et al., 2011).

In this study, we examined a cohort of clinically characterized MCC patients using a combination of whole-exome sequencing approaches and a selection of biomarkers, chosen on the basis of the mechanistic interpretation of the genomic data. We characterized two main MCC etiologies in our cohort: $MCPyV^+$ tumors, which had few mutations and a high survival rate, and $MCPyV^-$ tumors, which had more genomic alterations and a worse clinical outcome. We detected a number of signaling mechanisms with the potential to participate in the development of MCC and studied these using specific endpoint immunohistochemical biomarkers to examine their activity. We found that $MCPyV^+$ and $MCPyV^-$ tumors can share specific disease mechanisms, as implied by the detection of positive nuclear factor of activated T cells (NFAT), phosphorylated CRE-binding protein (P-CREB), and phosphorylated signal transducer and activator of transcription-3 (P-STAT) immunostaining in MCC samples. In addition, $MCPyV^-$ tumors almost exclusively developed alternative mechanisms of disease, as indicated by C-MYC and LEF1 expression in our sample series with respect to their $MCPyV^+$ counterparts. Finally, we found a clinical correlation between P-CREB nuclear expression and a worse prognosis in MCC patients that was independent of sex, age, clinical stage, and MCPyV status.

RESULTS

Inverse correlation between MCPyV and UV mutational signature in MCC

We initially characterized the genomic variants in a series of 15 patients with MCC (henceforth referred to as the *discovery cohort;* see the clinical data in Table 1, and see Supplementary Table S1 online). The mean age of patients was 75.5 years, and 53% of the study population were men. All samples corresponded to primary tumors located in sun-exposed areas in eight of the patients. We generated two paired-end 101-base pair whole-exome sequencing libraries and sequenced paired tumoral and nontumoral genomic DNA for each patient. This allowed us to uniquely map an average of approximately 86 million reads per sample, which were analyzed for the presence of somatic mutations as previously described (Martinez et al., 2014; Vaque et al., 2015). Under these conditions, the mean coverage of the target sequence was ×105 (×53–170), with an average of 92% (range = 71.25–97.07%) of the targeted bases having at least ×15 coverage (see Supplementary Table S2 online for a detailed description of the genomic analysis). Finally, our somatic mutation analyses detected 16,198 somatic single-base mutations (SSMs) in tumor samples (range = 29–3,644 in the discovery cohort of samples), of which 4,913 (range = 5–1,275) are responsible for amino-acid changes (Table 1, and see Supplementary Tables S3 and S4 online).

MCPyV status was determined by immunohistochemistry (IHC) for MCPyV T-antigen protein expression in all patients (except patients 8 and 13), followed by a confirmatory PCR analysis in a subset of cases that showed no discrepancies (see Supplementary Materials online). From a genomic perspective, we detected two clearly different subsets of patients. On one hand, those MCC lesions that tested positive for the Merkel polyomavirus ($MCPyV^+$) had a low number of somatic mutations, with an average mutational index of 0.75 amino acid-changing SSMs/megabase. On the other hand, lesions that had negative test results for this virus ($MCPyV^-$) showed higher mutational indexes, averaging 19.81 amino acid-changing SSMs/megabase, which is comparable to values observed in cutaneous melanomas (Berger et al., 2012). We found that all $MCPyV^-$ lesions, except that from patient 13, harbored a UV signature defined by the detection of more than 60% C>T transitions at dipyrimidine sites or more than 5% CC>TT mutations of the total number of SSMs in each case (Table 1 and Figure 1a) (Brash, 2015; The Cancer Genome Atlas Network, 2015). In contrast, this UV signature was not detected in $MCPyV^+$ patients. Thus, we can conclude that the presence of MCPyV in our discovery cohort was inversely correlated with mutational indexes and the presence of UV signatures.

Common and divergent mechanisms of disease in $MCPyV^+$ and $MCPyV^-$ MCCs

Turning our attention to those somatic mutations that cause amino acid changes, we detected a number of recurrently mutated genes in our discovery cohort, including *FAT4, TP53, RYR2, RPTOR, APC,* and *RB1*, among others, that were found almost exclusively in $MCPyV^-$ MCC lesions. We performed a validation panel over 279 positions, which rendered 98% of validated SSMs (see Supplementary Table S5 online). A list of the most recurrently detected genes with mutations is provided in Supplementary Table S6 online. To gain a deeper insight into the functional relevance of the mutations found in this study, we performed an

Table 1. Clinical and genomic characteristics of patients in the discovery cohort

Patient	Sex	Age in years	Lesion Location	Stage	MCPyV[a]	Sample Type[b]	C>T Dipyrimidine[c]	% C>T Dipyrimidine	CC>TT	% CC>TT	Other Substitutions[d]	% Other Substitutions	Total SSMs	Total SSMs AA Change[e]	MI[f]
1	M	83	Leg	IIIA	Negative	FF	1,875	83.71	6	0.27	365	16.03	2,240	722	24.07
2	M	94	Ear	IV	Negative	FFPE	1,562	79.17	16	0.81	411	20.02	1,973	622	20.73
3	M	74	Nose	IA	Negative	FFPE	296	70.14	3	0.71	126	29.15	422	132	4.40
4	M	81	Face	IIA	Negative	FF	1,875	75.88	12	0.49	596	23.63	2,471	666	22.20
5	F	78	Face	IV	Negative	FF	1,995	75.45	16	0.61	649	23.94	2,644	680	22.67
6	F	92	Leg	IV	Negative	FF	1,306	72.72	8	0.45	490	26.84	1,796	588	19.60
7	F	75	Face	IIA	Negative	FFPE	2,562	70.31	11	0.30	1,082	29.39	3,644	1,275	42.50
8	F	80	Leg	IIIA	Positive	FF	11	37.93	0	0.00	18	62.07	29	7	0.23
9	M	72	Arm	IIIA	Positive	FFPE	143	33.57	0	0.00	283	66.43	426	106	3.53
10	F	88	Thigh	IIA	Positive	FF	12	21.05	0	0.00	45	78.95	57	13	0.43
11	M	86	Leg	IIIB	Positive	FF	17	26.98	0	0.00	46	73.02	63	9	0.30
12	F	80	Face	IV	Positive	FF	9	36.00	0	0.00	16	64.00	25	8	0.27
13	M	69	Lip	IV	Negative	FFPE	71	21.91	0	0.00	253	78.09	324	71	2.37
14	F	80	Face	IIA	Positive	FF	6	16.22	0	0.00	31	83.78	37	5	0.17
15	F	81	Leg	IIB	Positive	FF	8	17.02	0	0.00	39	82.98	47	9	0.30

Note. Clinical data of 15 MCC cases, including sex, age at diagnosis, location of the lesion, and stage. The genomic data from each patient are also represented.

Abbreviations: AA, amino acid; C, cytosine; F, female; FF, freshly frozen; FFPE, formaldehyde-fixed paraffin-embedded; M, male; MCPyV, Merkel cell polyomavirus; MI, mutational index; SSM, somatic single-base mutation; T, thymine.

[a]Detection of MCPyV.

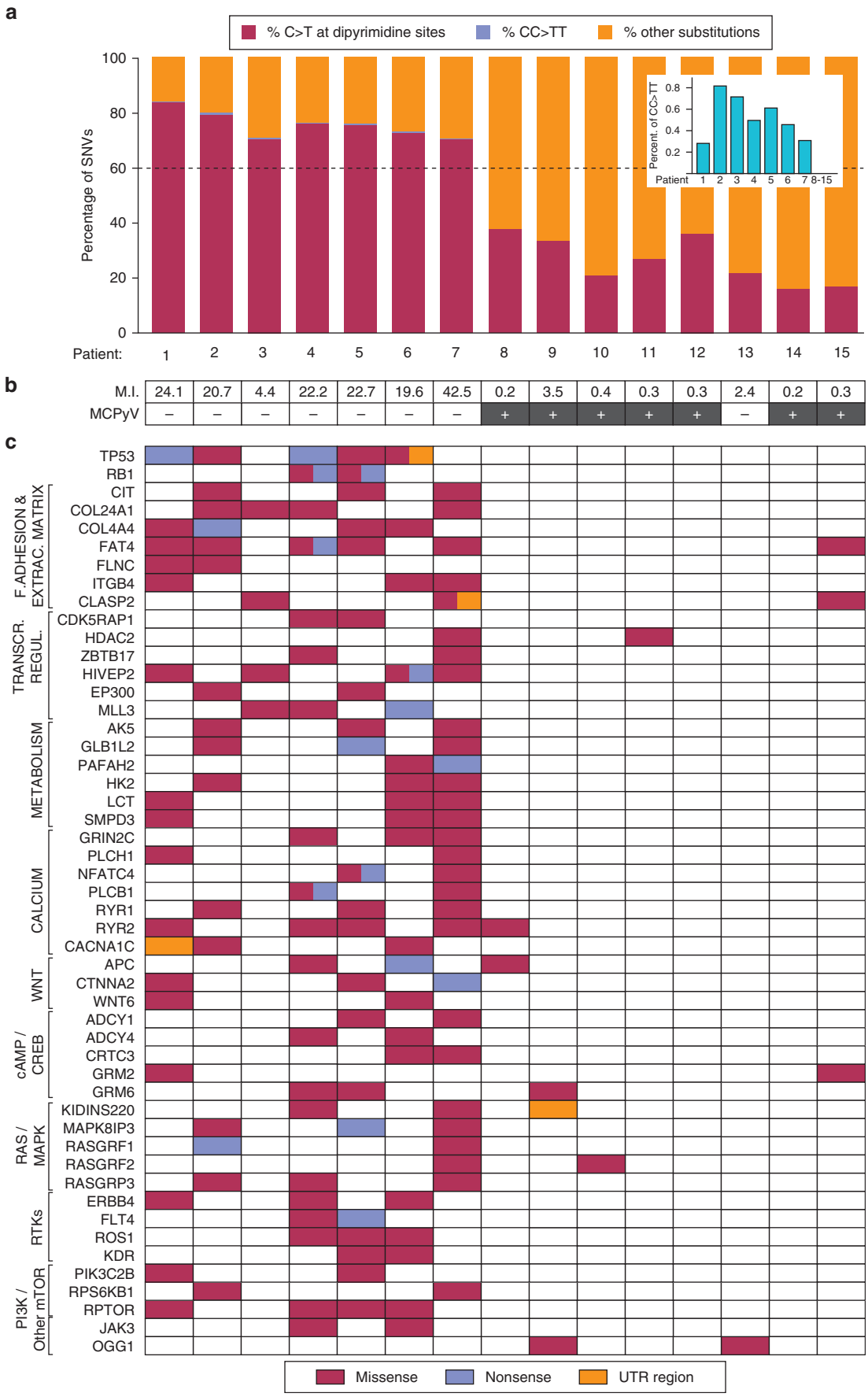
[b]Types of samples from which genomic DNA was extracted.

[c]Number of C>T transitions found at dipyrimidine sites.

[d]Number of other single somatic single-base mutations.

[e]Single somatic nucleotide variants that provoke amino acid changes.

[f]Calculated as the total number of single somatic nucleotide variants that cause an amino acid change per megabase.


a
% C>T at dipyrimidine sites
% CC>TT
% other substitutions
Percentage of SNVs
Percent. of CC>TT
Patient
Patient: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
b
M.I. 24.1 20.7 4.4 22.2 22.7 19.6 42.5 0.2 3.5 0.4 0.3 0.3 2.4 0.2 0.3
MCPyV – – – – – – – + + + + + – + +
c
F.ADHESION & EXTRAC. MATRIX
TRANSCR. REGUL.
METABOLISM
CALCIUM
WNT
cAMP / CREB
RAS / MAPK
RTKs
PI3K / Other mTOR
TP53
RB1
CIT
COL24A1
COL4A4
FAT4
FLNC
ITGB4
CLASP2
CDK5RAP1
HDAC2
ZBTB17
HIVEP2
EP300
MLL3
AK5
GLB1L2
PAFAH2
HK2
LCT
SMPD3
GRIN2C
PLCH1
NFATC4
PLCB1
RYR1
RYR2
CACNA1C
APC
CTNNA2
WNT6
ADCY1
ADCY4
CRTC3
GRM2
GRM6
KIDINS220
MAPK8IP3
RASGRF1
RASGRF2
RASGRP3
ERBB4
FLT4
ROS1
KDR
PIK3C2B
RPS6KB1
RPTOR
JAK3
OGG1
Missense
Nonsense
UTR region

unbiased OncodriveFM analysis (described in the Supplementary Materials and in Gonzalez-Perez and Lopez-Bigas, 2012) using all the SSMs detected in this work (see Supplementary Table S3). This enabled us to detect potential driver genes like *TP53*, *CDK5RAP1*, *FAT4*, *ADAM8*, *GLB1L2*, *OGG1*, *HIVEP2*, and *RB1* with a significant *P*-value (see Supplementary Table S7 online). Moreover, using this approach we were also able to detect a number of significant gene modules from the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway that included TP53, small cell lung carcinoma, non-small cell lung cancer, melanoma, and basal cell carcinoma, to name but a few (see Supplementary Table S8 online). We searched for the main biological and biochemical mechanisms/signaling pathways associated with each significant gene found in our analysis alongside those included in the specific KEGG gene modules detected with OncodriveFM (see Supplementary Tables S7 and S8). To simplify the presentation of our findings, we grouped these into functional categories. These included specific subsets of KEGG gene sets (see Supplementary Table S8) with redundant biological activities (points i–vi as follow) or with shared mechanisms (points vii–ix as follow): (i) focal adhesion and extracellular matrix: including actin cytoskeleton, extracellular matrix, axon guidance, cell adhesion proteins, focal adhesion, and tight junction KEGG gene sets; (ii) metabolism: valine isoleucine degradation, ether lipid metabolism and propanoate metabolism; (iii) transcriptional regulation: transcriptional misregulation in cancer; (iv) RAS/mitogen-activated protein kinase: mitogen-activated protein kinase signaling; (v) PI3K/mTOR: PI3K-AKT and mTOR; (vi) WNT; (vii) receptors with tyrosine kinase activity (RTKs): with many RTKs and associated downstream signaling included in multiple KEGG gene sets such as, for example, neurotrophin signaling, pathways in cancer, non-small cell lung cancer or, ErbB signaling, to name but a few; (viii) cAMP/CRE-binding protein: included in alcoholism and amphetamine addiction; (ix) calcium: vascular smooth muscle contraction or glutaminergic signaling, and (x) other. According to our data, these might act as disease mechanisms in MCC (Figure 1c, and see Supplementary Table S8).

We next studied the expression of specific MCC biomarkers, such as MCPyV, p63, RB, and TP53, in conjunction with specific transcription factors such as activated surrogate IHC markers corresponding to the signaling pathways described earlier. For this purpose, we considered 48 clinically characterized MCC patients (henceforth referred to as the *validation cohort*), of whom 15 had been included in the genomic study (see the clinical characteristics in Supplementary Table S1). Along with the aforementioned biomarkers, we analyzed the nuclear expression of β-catenin and LEF-1 (WNT pathway), P-CREB (cAMP/CRE-binding protein), NFAT (calcium), C-MYC, and P-STAT (RTKs) in our validation cohort of samples (Table 2). Our results showed that $MCPyV^+$ lesions had a homogeneous expression landscape, with a uniform staining pattern for specific markers, such as β-catenin (positive in the membrane) and RB (positive) and C-MYC and LEF-1 (negative), and heterogeneous staining for TP53, p63, P-CREB, and NFAT. P-STAT was detected in only four of the 26 $MCPyV^+$ patients. On the other hand, $MCPyV^-$ lesions showed heterogeneous biomarker expression. In total, 50% of the patients had a loss of RB expression, whereas for C-MYC and LEF-1, expression was detected almost exclusively in $MCPyV^-$ patients but was limited to about half of these patients (Table 2). A representation of IHC markers for a representative $MCPyV^+$ and $MCPyV^-$ patient is shown in Figure 2. Despite the different genomic characteristics observed between $MCPyV^+$ and $MCPyV^-$ lesions, it is possible that the two MCC etiologies share common disease mechanisms (TP53, RB, NFAT, P-STAT, and P-CREB), with $MCPyV^-$ lesions developing additional molecular features.

Biological relevance derived from the characterization of MCC tumors

To explore the clinical implications of the molecular mechanisms (biomarkers) identified in this work, we studied their association with the clinical outcome of the patients in our validation cohort. The clinical data for each patient are summarized in Supplementary Table S1. We analyzed the death events caused by MCC in different sets of patient tumors characterized with respect to the positive or negative expression of each biomarker depicted as follows: (i) biomarkers already studied by other groups in different cohorts of patients, that is, MCPyV, TP53, RB, and p63 and (ii) biomarkers expressed in both $MCPyV^+$ and $MCPyV^-$ tumors: NFAT, P-CREB, and P-STAT. We could not include biomarkers in this study that were mainly expressed in $MCPyV^-$ tumors (C-MYC and LEF1) alone because of the limited cohort size. As expected, and in accordance with previous observations by other laboratories (Carson et al., 1998; Nardi et al., 2012; Sihto et al., 2011; Waltari et al., 2011), MCC patients with tumors that were negative for the expression of MCPyV and RB tended to have a poorer clinical outcome, whereas, at least in our hands, TP53, p63, and NFAT did not show this tendency (see Supplementary Figure S1 online).

Those cancers that tested positive for the expression of P-CREB or P-STAT showed significantly shorter survival than their negative counterparts ($P = 0.011$ and $P = 0.024$, respectively), with a crude hazard ratio of 3.89 for P-CREB and 3.37 for P-STAT (Figure 3). We decided to perform

Figure 1. Inverse correlation between MCPyV expression and UV mutational signature in MCC. Tumor samples from the discovery cohort are presented from left to right. (**a**) Mutation spectra for each sample, showing the percentage of C>T transitions in dipyrimidine sites (red), the percentage of CC>TT mutations (purple) and other substitutions (orange); (**b**) mutational index (MI) calculated as the number of amino-acid-changing mutations per megabase and MCPyV presence (black boxes) or absence (white boxes). (**c**) Mutated genes (written vertically on the left side and grouped into gene-sets) are represented for each sample (colored box). Mutation type is highlighted in a colored box: missense (red), nonsense (purple) and UTR region (orange). C, cytosine; CREB, CRE-binding protein; EXTRAC., extracellular; F., focal; MAPK, mitogen-activated protein kinase; MCPyV, Merkel cell polyomavirus; MI, mutational index; PI3K, phosphatidylinositol 3 kinase; REGUL., regulation; RTK, receptor with tyrosine kinase activity; SNV, single nucleotide variation; SSM, somatic single-base mutation; T, thymine; TRANSCR., transcription; UTR, untranslated region.

Table 2. Immunohistochemical detection of specific MCC biomarkers in patients characterized in the validation cohort

Patient	β-Catenin	p63	NF-ATC1	P-CREB	P-STAT-3	TP53	RB	MYC	LEF1-L	MCPyV
9	Membrane	**Positive**	**Positive**	**Positive**	Negative	Negative	Positive	Negative	Negative	Positive
10	Membrane	Negative	**Positive**	**Positive**	**Positive**	Negative	**Loss**	Negative	Negative	Positive
11	Membrane	Negative	**Positive**	Negative	Negative	**Positive**	Positive	Negative	Negative	Positive
12	Membrane	**Positive**	**Positive**	**Positive**	Negative	Negative	Positive	Negative	Negative	Positive
14	Membrane	**Positive**	**Positive**	Negative	Negative	Negative	Positive	Negative	Negative	Positive
15	Membrane	**Positive**	Negative	Negative	Negative	**Positive**	Positive	**Positive**	Negative	Positive
16	Membrane	Negative	Negative	Negative	Negative	Negative	Positive	Negative	Negative	Positive
17	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Negative	Negative	Positive
18	Membrane	Negative	Negative	Negative	Negative	Negative	**Loss**	Negative	Negative	Positive
19	Membrane	**Positive**	Negative	Negative	**Positive**	Negative	Positive	Negative	Negative	Positive
20	Membrane	**Positive**	**Positive**	Negative	Negative	**Positive**	Positive	Negative	Negative	Positive
21	Membrane	**Positive**	Negative	**Positive**	Negative	**Positive**	Positive	Negative	Negative	Positive
22	Membrane	Negative	Negative	Negative	Negative	Negative	Positive	Negative	Negative	Positive
23	Membrane	Negative	Negative	**Positive**	Negative	Negative	Positive	Negative	Negative	Positive
24	Membrane	**Positive**	**Positive**	Negative	**Positive**	Negative	Positive	Negative	Negative	Positive
25	Membrane	Negative	Negative	Negative	Negative	Negative	Positive	Negative	Negative	Positive
26	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Negative	Negative	Positive
27	Membrane	Negative	Negative	Negative	Negative	Negative	Positive	Negative	Negative	Positive
8	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	Positive
28	Membrane	N/D	Negative	**Positive**	Negative	**Positive**	Positive	Negative	Negative	Positive
29	Membrane	Negative	Negative	Negative	Negative	**Positive**	Positive	Negative	Negative	Positive
30	Membrane	Negative	**Positive**	Negative	Negative	Negative	Positive	Negative	Negative	Positive
31	Membrane	**Positive**	**Positive**	Negative	Negative	Negative	Positive	Negative	Negative	Positive
32	Membrane	**Positive**	**Positive**	**Positive**	Negative	**Positive**	Positive	Negative	**Positive**	Positive
33	Membrane	**Positive**	Negative	**Positive**	Negative	**Positive**	Positive	Negative	Negative	Positive
34	Membrane	**Positive**	**Positive**	**Positive**	Negative	**Positive**	Positive	Negative	Negative	Positive
35	Membrane	**Positive**	**Positive**	Negative	**Positive**	**Positive**	Positive	Negative	Negative	Positive
1	Negative	**Positive**	Negative	**Positive**	**Positive**	Negative	Positive	**Positive**	**Positive**	Negative
2	Negative	Negative	Negative	Negative	Negative	**Positive**	**Loss**	Negative	**Positive**	Negative
3	Membrane	**Positive**	Negative	**Positive**	**Positive**	**Positive**	Positive	**Positive**	Negative	Negative
4	Negative	**Positive**	Negative	**Positive**	Negative	**Positive**	**Loss**	Negative	Negative	Negative
5	Membrane	**Positive**	**Positive**	Negative	Negative	**Positive**	**Loss**	**Positive**	Negative	Negative
6	Membrane	**Positive**	Negative	Negative	**Positive**	**Positive**	Positive	**Positive**	Negative	Negative
7	Negative	**Positive**	Negative	**Positive**	Negative	**Positive**	Positive	Negative	Negative	Negative
13	N/D	N/D	Negative	N/D	N/D	N/D	N/D	N/D	N/D	Negative
36	Membrane	**Positive**	**Positive**	Negative	Negative	**Positive**	**Loss**	Negative	Negative	Negative
37	Membrane	**Positive**	Negative	**Positive**	**Positive**	**Positive**	**Loss**	**Positive**	**Positive**	Negative
38	Membrane	**Positive**	Negative	**Positive**	**Positive**	**Positive**	**Loss**	Negative	Negative	Negative
39	Membrane	**Positive**	**Positive**	**Positive**	Negative	**Positive**	**Loss**	Negative	Negative	Negative
40	Negative	**Positive**	Negative	**Positive**	**Positive**	Negative	**Loss**	Negative	Negative	Negative
41	Membrane	Negative	Negative	Negative	**Positive**	Negative	Positive	**Positive**	Negative	Negative
42	Membrane	**Positive**	Negative	**Positive**	Negative	Negative	Positive	Negative	**Positive**	Negative
43	Membrane	Negative	Negative	Negative	Negative	**Positive**	**Loss**	**Positive**	Negative	Negative
44	Negative	Negative	**Positive**	Negative	**Positive**	**Positive**	**Loss**	Negative	Negative	Negative
45	Membrane	Negative	Negative	Negative	Negative	Negative	**Loss**	Negative	Negative	Negative
46	Membrane	**Positive**	**Positive**	**Positive**	**Positive**	Negative	Positive	**Positive**	Negative	Negative
47	Negative	Negative	**Positive**	**Positive**	**Positive**	Negative	Positive	Negative	**Positive**	Negative
48	Membrane	**Positive**	**Positive**	**Positive**	**Positive**	Negative	Positive	**Positive**	**Positive**	Negative

Note. Assessment of the expression of specific markers by immunohistochemistry. All these markers are related to pathways detected by an unbiased approach as being especially altered in tumor samples. Boldface type indicate potentially pathogenic markers.

Abbreviations: P-CREB, phosphorylated CRE-binding protein; P-STAT, phosphorylated signal transducer and activator of transcription; RB, retinoblastoma; MCPyV, Merkel cell polyomavirus.

multivariable analyses of P-CREB and P-STAT including clinical data (age, sex, and stage) with MCPyV status and detected stage as a confounding variable (not shown). After including all of these variables in the same multivariable model, only P-CREB–positive status remained as an independent predictor of mortality: adjusted HR for P-CREB = 5.56; 95% confidence interval = 1.22–25.33 (Figure 3).

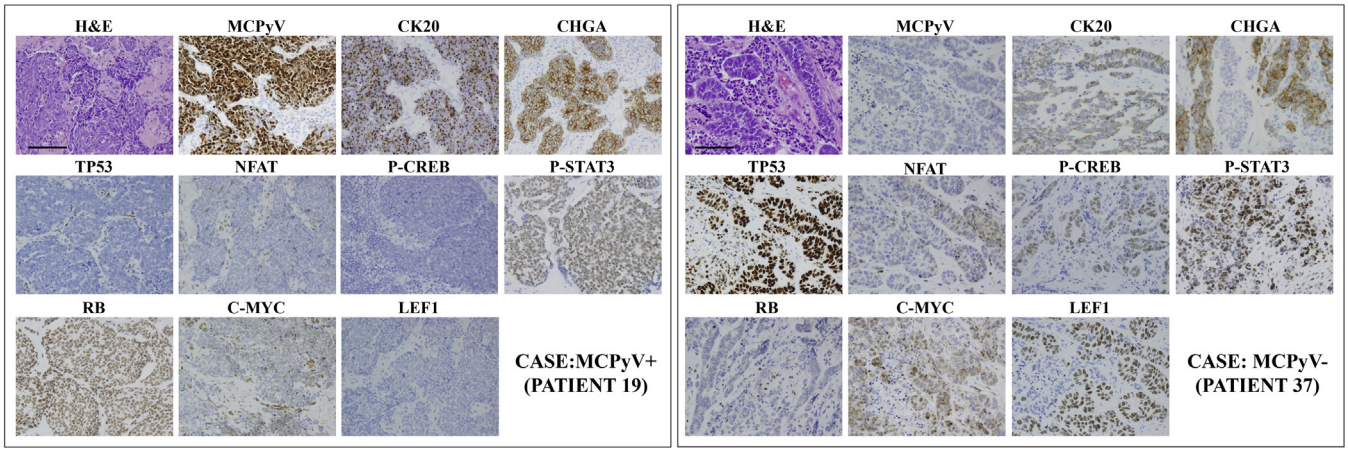


Figure 2. Pathway characterization of MCPyV$^+$ and MCPyV$^-$ MCC tumors. Immunohistochemical analyses of two representative cases of MCPyV$^+$ (left, patient 19) and MCPyV$^-$ (right, patient 37). The immunohistochemical analyses show staining for the indicated marker in each case. In patient 37 staining for NFAT is negative in the nucleus of the tumoral cell, but there are some positive lymphocytes. Staining for P-CREB shows nuclear positivity in tumor cells. Scale bar = 100 μm. H&E, hematoxylin and eosin; MCC, Merkel cell carcinoma; MCPyV, Merkel cell polyomavirus; NFAT, nuclear factor of activated T cells; P-CREB, phosphorylated CRE-binding protein; RB, retinoblastoma.

DISCUSSION

MCC is a type of cancer with increasing incidence and a high mortality rate. Despite recent advances in our understanding of the main biological mechanisms involved in its clinical evolution, we still know little about the molecular biology of this disease, as is reflected by the current lack of any specific therapy (Becker, 2010; Hughes et al., 2014).

In this study, we used a combination of genomic techniques and clinical data to mechanistically and biologically characterize MCC tumors. These methods included deep-sequencing approaches (whole-exome sequencing) in samples from 15 patients (the discovery cohort) and a selection of immunohistochemical markers in 48 samples from MCC patients (the validation cohort).

First, we found different genetic patterns of disease mechanisms in our cohort of patients with MCPyV$^+$ and MCPyV$^-$ MCC tumors. Consistent with previous observations, our cohort of patients with MCPyV$^-$ tumors

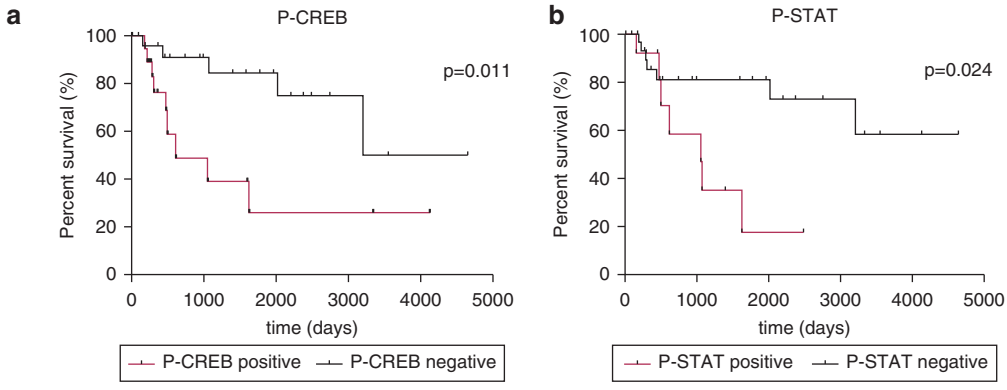


Figure 3. Association of survival of MCC patients with specific characterization. Kaplan-Meier curves showing percentage of survival from patients with positive (red) or negative (black) expression of the indicated biomarkers and their respective *P*-values: (**a**) P-CREB ($P = 0.011$) and (**b**) P-STAT ($P = 0.024$). (**c**) Crude hazard ratio and hazard ratio adjusted for P-STAT3, P-CREB, MCPyV status, sex, age, and stage, with 95% confidence intervals. CI, confidence interval; HRa, adjusted hazard ratio; HRc, crude hazard ratio; MCC, Merkel cell carcinoma; P-CREB, phosphorylated CRE-binding protein; P-STAT, phosphorylated signal transducer and activator of transcription.

c Hazard Ratios for P-CREB and STAT markers, in relation to mortality

	Vital status Death N=15	Survival N=33	HRc[a]	(95%	CI)	HRa[b]	(95%	CI)
P-CREB								
Negative	5	20	1	--		1	--	
Positive	9	12	3.89	1.28	11.87	5.56	1.22	25.33
P-STAT								
Negative	7	24	1	--		1	--	
Positive	7	8	3.37	1.1	10.3	1.65	0.22	12.35

a HRc = Crude Hazard Ratio.
b HRa=Hazard Ratio adjusted for P-CREB, P-STAT, MCPyV status, sex, age and stage.

accumulated most of the detected somatic mutations, giving an average mutational index of 20 amino acid-changing mutations/megabase and a UV signature comparable to that of malignant melanoma (C>T at dipyrimidine sites above 60% of the total SSMs) (The Cancer Genome Atlas Network, 2015; Goh et al., 2015; Harms et al., 2015; Wong et al., 2015). By contrast, MCPyV$^+$ tumors had lower mutational indexes (average mutational index = 0.75 amino acid-changing mutations/megabase) that are similar to those observed for small B-cell lymphomas (Vaque et al., 2014). To provide our mutational data with a functional interpretation we used an unbiased approach (OncodriveFM), which enabled us to identify specific genes and pathways that were significantly altered in our MCC samples, presumably with the potential to participate as disease mechanisms in MCC. We explored this possibility by focusing on the expression of a group of transcription factors as endpoint surrogates for the activity of the genes and signaling pathways identified in this study. These included β-catenin and LEF-1 (WNT pathway [Duchartre et al., 2016]), NFAT (calcium, reviewed in Mognol et al., 2016), P-CREB (cAMP/CRE-binding protein signaling [Rodriguez and Setaluri, 2014]), P-STAT (RTKs signaling, reviewed in Yu et al., 2014) and C-MYC (RAS/mitogen-activated protein kinase, PI3K, and RTKs signaling pathways [Kress et al., 2015]), along with MCPyV, TP53, p63, and RB (previously identified MCC biomarkers [Zager et al., 2011]) (Figures 1 and 4).

Our data enabled a number of MCC mutations (with a previous identification number in the Catalogue of Somatic Mutations in Cancer identification, as shown in Supplementary Table S3) to be associated with specific biomarker expression in MCC samples from the validation cohort (Table 2 and Figure 1). For example, in patient 5 we detected two potentially inactivating *TP53* mutations provoking amino-acid changes, R280K (COSM129830) and H47Y (COSM129851), and a truncating *RB1* mutation (W195*, COSM214151) with positive (TP53) and negative (RB) protein expression. These can be due to a direct effect on a specific gene, but we cannot rule out potential indirect effects of specific gene mutations on the activity of its associated signaling pathway (see Figure 4). In this regard and to serve as an example, *HIVEP2* (also known as Myc intron binding protein 1, MIBP1) is a significantly mutated gene in our analysis, detected in patients 1, 3, 7 (missense mutations), and 6 (a truncating mutation). In our work, IHC data showed positive results for C-MYC in patients 1, 3, and 6, suggesting a potential correlation between MIBP1 mutations (presumably inactivating) and C-MYC expression. Furthermore, it has been shown that MIBP1 can inhibit transcription of *C-MYC* (Iwashita et al., 2012), and the expression of both genes has been shown to be inversely correlated in human cells (Zajac-Kaye et al., 2000). Also, *ERBB4* mutations in patients 1 (two missense mutations) and 6 (R711C, COSM160827) could contribute to activating c-MYC and STAT (see also Figure 4). On the other hand, we detected a large number of mutations in adenylate cyclases and G-protein coupled receptors that could participate in cAMP/CRE-binding protein activation, for example ADCY10-P241H (COSM899133) and GRM3-E538K (COSM229505), which were found in patient 1 (P-CREB−positive). In our study, perhaps with the exception of TP53, we did not detect hotspot mutations with functional validation to predict activation or repression of a specific signaling pathway. It is possible that pursuing the study of inactivating mutations in *MIBP1* or presumably activating mutations such as ERBB4-R711C could improve our ability to understand important MCC oncogenic mechanisms. Nevertheless, it is conceivable that by analyzing the expression of a specific subset of biomarkers in MCC samples, we could detect deregulated mechanisms of disease, as might be expected given the mutational data. From a mechanistic perspective, and despite substantial differences in the number of mutations, the MCPyV$^+$ and MCPyV$^-$ tumors examined here unexpectedly shared several deregulated signaling mechanisms, as indicated by the detection of specific

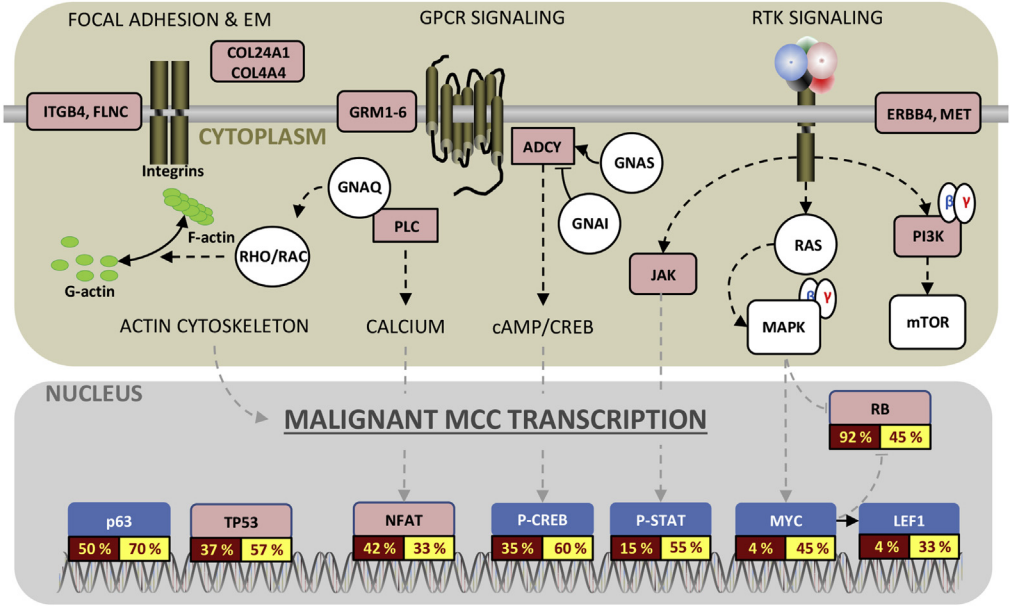


Figure 4. Deregulated mechanisms of disease in MCC. Schematic representation of the main signaling pathways found to be altered in MCC. Pink boxes indicate recurrently mutated genes in our discovery cohort. Percentage of expression for each biomarker in our validation cohort is represented in maroon (MCPyV$^+$) or yellow (MCPyV$^-$) boxes. EM, extracellular matrix; GPCR, G protein-coupled receptor; MCPyV, Merkel cell polyomavirus; NFAT, nuclear factor of activated T cells; P-CREB, phosphorylated CRE-binding protein; MAPK, mitogen-activated protein kinase; P-STAT, phosphorylated signal transducer and activator transcription; PI3K, phosphatidylinositol 3 kinase; RTK, receptor with tyrosine kinase activity.

biomarkers such as P-STAT, P-CREB, and NFAT in samples from our validation cohort of MCC patients. On the other hand, we also found that C-MYC and LEF1 were expressed almost exclusively in $MCPyV^-$ patients. At first glance, this could be due to nothing more than the accumulation of somatic mutations in this type of MCC tumor. However, $MCPyV^+$ patients showed activated P-STAT−, P-CREB−, and NFAT−associated signaling in the absence of specific mutations, resulting in the mechanistic mimicry of $MCPyV^-$ tumors. In our opinion, these findings suggest that $MCPyV^-$ tumors with high mutational loads can acquire specific biological properties through the activation of alternative oncogenic pathways including, but not restricted to, those detected in this work: C-MYC and LEF1. Our results strongly suggest a need for further studies that would determine their contribution to the biology and targeted therapy of this disease.

From a biological perspective, the ability of a range of markers to predict a worse clinical evolution of MCC patients has been examined. Of these, $MCPyV^-$ tumors (Nardi et al., 2012), p63 expression (Asioli et al., 2007), and the percentage of MCC cells expressing Ki67 (Llombart et al., 2005) have been proposed as factors associated with bad prognosis. We found a tendency toward worse survival in patients characterized with specific biomarkers used individually: $MCPyV^-$ and RB^-. Under these circumstances, it is possible that more significant results could be obtained from additional methods that attempt to better determine the status of specific biomarkers such as TP53, or from increasing the cohort size. Two mechanisms shared by $MCPyV^+$ and $MCPyV^-$ MCC tumors were highly significant predictors of survival: P-CREB and P-STAT. A multivariable analysis detected P-CREB as being a strong predictor of mortality independent of sex, age, stage, MCPyV status, and P-STAT. Thus, our findings argue for a potential role for these disease mechanisms in the biological evolution of both types of MCC lesions. Further efforts should be made to explore the biological role of these markers in larger series of samples from clinically characterized MCC patients. Our results also suggest that a better characterization of the molecular mechanisms that can control CREB activation in MCC cancers with different MCPyV status could be useful for developing tools for diagnosis, prognosis, and treatment of this disease.

In summary, we characterized a cohort of MCC patients using an original combination of genomic and IHC approaches applied to this field. Confirming previous data, we found two distinct etiologies with clearly divergent mutational signatures. Despite these differences, the data arising from our work show that $MCPyV^+$ and $MCPyV^-$ MCC tumors can develop similar mechanisms of disease with clinical implications for patient survival and potential to serve as targets for therapy. Moreover, a subset of MCC patients with CRE-binding protein and/or signal transducer and activator of transcription activation developed a more aggressive disease that was associated with worse survival. In fact, within our patient cohort, we found P-CREB to be a strong independent survival factor for this disease. Thus, in the current MCC clinical setting, upon mechanistic characterization of MCC tumors at diagnosis, we may be able to predict poorer outcomes and explore approaches for specific therapy.

MATERIALS AND METHODS

Ethics statement

All human samples used in this study were collected following the Declaration of Helsinki protocols after obtaining written informed consent from each patient and the doctors involved, as required by the Comité Ético de Investigación Clínica, Cantabria (CEIC). We kept the original records under specific restricted conditions to fulfill the current legal requirements. All processes were approved and conducted in adherence with the specific recommendations of the CEIC.

Patient samples

Samples from 48 clinically characterized MCC patients were used (validation cohort; see Supplementary Table S1). From these, 15 patients (discovery cohort; Table 1, and see Supplementary Table S1) with a total of 30 paired (nontumoral and tumoral) formalin-fixed, paraffin-embedded and freshly frozen samples were selected for whole-exome sequencing analysis. Patients were included consecutively.

Additional methods

See Supplementary Materials online. Supplementary data is also included, since it is a single file with additional methods and supplementary information.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by grants from Instituto de Salud-Carlos III (ISCIII); cofinanced by the European Union; (FEDER) (PI12/00357), and a Ramón and Cajal research program (MINECO; RYC-2013-14097) to JPV, Asociación Española Contra el Cáncer and ISCIII grants (RD06/0020/0107, RD012/0036/0060) to MAP, and Coordinated Project of Excellence inter-Institutos de investigación acreditados institutes (ISCIII; PIE15/00081) to MAP. The Ramón and Cajal research program also supports IV. SD was supported by the Torres Quevedo subprogram (MICINN; PTQ-12-05391).

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.08.015.

REFERENCES

Asioli S, Righi A, Volante M, Eusebi V, Bussolati G. p63 expression as a new prognostic marker in Merkel cell carcinoma. Cancer 2007;110:640−7.

Becker JC. Merkel cell carcinoma. Ann Oncol 2010;21(Suppl 7):vii81−5.

Becker JC, Houben R, Ugurel S, Trefzer U, Pfohler C, Schrama D. MC polyomavirus is frequently present in Merkel cell carcinoma of European patients. J Invest Dermatol 2009;129:248−50.

Berger MF, Hodis E, Heffernan TP, Deribe YL, Lawrence MS, Protopopov A, et al. Melanoma genome sequencing reveals frequent PREX2 mutations. Nature 2012;85(7399):502−6.

Bhatia K, Goedert JJ, Modali R, Preiss L, Ayers LW. Merkel cell carcinoma subgroups by Merkel cell polyomavirus DNA relative abundance and oncogene expression. Int J Cancer 2010;126:2240−6.

Brash DE. UV signature mutations. Photochem Photobiol 2015;91:15−26.

The Cancer Genome Atlas Network. Genomic classification of cutaneous melanoma. Cell 2015;161:1681−96.

Carson HJ, Reddy V, Taxy JB. Proliferation markers and prognosis in Merkel cell carcinoma. J Cutan Pathol 1998;25:16−9.

Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, et al. Mutations of the BRAF gene in human cancer. Nature 2002;417(6892):949−54.

Duchartre Y, Kim YM, Kahn M. The Wnt signaling pathway in cancer. Crit Rev Oncol Hematol 2016;99:141−9.

Duncavage EJ, Zehnbauer BA, Pfeifer JD. Prevalence of Merkel cell polyomavirus in Merkel cell carcinoma. Mod Pathol 2009;22:516−21.

Feng H, Shuda M, Chang Y, Moor PS. Clonal integration of a polyomavirus in human Merkel cell carcinoma. Science 2008;319:1096−100.

Garneski KM, Warcola AH, Feng Q, Kiviat NB, Leonard JH, Nghiem P. Merkel cell polyomavirus is more frequently present in North American than Australian Merkel cell carcinoma tumors. J Invest Dermatol 2009;129:246–8.

Goh G, Walradt T, Markarov V, Blom A, Riaz N, Doumani R, et al. Mutational landscape of MCPyV-positive and MCPyV-negative Merkel cell carcinomas with implications for immunotherapy. Oncotarget 2015;7:3403–15.

Gonzalez-Perez A, Lopez-Bigas N. Functional impact bias reveals cancer drivers. Nucleic Acids Res 2012;40:e169.

Hafner C, Houben R, Baeurle A, Ritter C, Schrama D, Landthaler M, et al. Activation of the PI3K/AKT pathway in Merkel cell carcinoma. PLoS One 2012;7:e31255.

Harms PW, Vats P, Verhaegen ME, Robinson DR, Wu YM, Dhanasekaran SM, et al. The distinctive mutational spectra of polyomavirus-negative Merkel cell carcinoma. Cancer Res 2015;75:3720–7.

Harring TR, Nguyen NT, Goss JA, O'Mahony CA. Treatment of liver metastases in patients with neuroendocrine tumors: a comprehensive review. Int J Hepatol 2011:154541.

Hughes MP, Hardee ME, Cornelius LA, Hutchins LF, Becker JC, Gao L. Merkel cell carcinoma: epidemiology, target, and therapy. Curr Dermatol Rep 2014;3:46–53.

Iwashita Y, Fukuchi N, Waki M, Hayashi K, Tahira T. Genome-wide repression of NF-kappaB target genes by transcription factor MIBP1 and its modulation by O-linked beta-N-acetylglucosamine (O-GlcNAc) transferase. J Biol Chem 2012;287:9887–900.

Kress TR, Sabo A, Amati B. MYC: connecting selective transcriptional control to global RNA production. Nat Rev Cancer 2015;15:593–607.

Lassacher A, Heitzer E, Kerl H, Wolf P. p14ARF hypermethylation is common but INK4a-ARF locus or p53 mutations are rare in Merkel cell carcinoma. J Invest Dermatol 2008;128:1788–96.

Lunder EJ, Stern RS. Merkel-cell carcinomas in patients treated with methoxsalen and ultraviolet A radiation. N Engl J Med 1998;339:1247–8.

Llombart B, Monteagudo C, Lopez-Guerrero JA, Carda C, Jorda E, Sanmartin O, et al. Clinicopathological and immunohistochemical analysis of 20 cases of Merkel cell carcinoma in search of prognostic markers. Histopathology 2005;46:622–34.

Martinez N, Almaraz C, Vaque JP, Varela I, Derdak S, Beltran S, et al. Whole-exome sequencing in splenic marginal zone lymphoma reveals mutations in genes involved in marginal zone differentiation. Leukemia 2014;28:1334–40.

McCardle TW, Sondak VK, Zager J, Messina JL. Merkel cell carcinoma: pathologic findings and prognostic factors. Curr Probl Cancer 2010;34:47–64.

Mognol GP, Carneiro FR, Robbs BK, Faget DV, Viola JP. Cell cycle and apoptosis regulation by NFAT transcription factors: new roles for an old player. Cell Death Dis 2016;7:e2199.

Nardi V, Song Y, Santamaria-Barria JA, Cosper AK, Lam Q, Faber AC, et al. Activation of PI3K signaling in Merkel cell carcinoma. Clin Cancer Res 2012;18:1227–36.

Ratner D, Nelson BR, Brown MD, Johnson TM. Merkel cell carcinoma. J Am Acad Dermatol 1993;29(2 Pt 1):143–56.

Rockville Merkel Cell Carcinoma Group. Merkel cell carcinoma: recent progress and current priorities on etiology, pathogenesis, and clinical management. J Clin Oncol 2009;27:4021–6.

Rodriguez CI, Setaluri V. Cyclic AMP (cAMP) signaling in melanocytes and melanoma. Arch Biochem Biophys 2014;563:22–7.

Sarnaik AA, Lien MH, Nghiem P, Bichakjian CK. Clinical recognition, diagnosis, and staging of merkel cell carcinoma, and the role of the multidisciplinary management team. Curr Probl Cancer 2010;34:38–46.

Sihto H, Kukko H, Koljonen V, Sankila R, Bohling T, Joensuu H. Merkel cell polyomavirus infection, large T antigen, retinoblastoma protein and outcome in Merkel cell carcinoma. Clin Cancer Res 2011;17:4806–13.

Stakaityte G, Wood JJ, Knight LM, Abdul-Sada H, Adzahar NS, Nwogu N, et al. Merkel cell polyomavirus: molecular insights into the most recently discovered human tumour virus. Cancers (Basel) 2014;6:1267–97.

Vaque JP, Martinez N, Batlle-Lopez A, Perez C, Montes-Moreno S, Sanchez-Beato M, et al. B-cell lymphoma mutations: improving diagnostics and enabling targeted therapies. Haematologica 2014;99:222–31.

Vaque JP, Martinez N, Varela I, Fernandez F, Mayorga M, Derdak S, et al. Colorectal adenomas contain multiple somatic mutations that do not coincide with synchronous adenocarcinoma specimens. PLoS One 2015;10:e0119946.

Waltari M, Sihto H, Kukko H, Koljonen V, Sankila R, Bohling T, et al. Association of Merkel cell polyomavirus infection with tumor p53, KIT, stem cell factor, PDGFR-alpha and survival in Merkel cell carcinoma. Int J Cancer 2011;129:619–28.

Wong SQ, Waldeck K, Vergara IA, Schroder J, Madore J, Wilmott JS, et al. UV-associated mutations underlie the etiology of MCV-negative Merkel cell carcinomas. Cancer Res 2015;75:5228–34.

Yu H, Lee H, Herrmann A, Buettner R, Jove R. Revisiting STAT3 signalling in cancer: new and unexpected biological functions. Nat Rev Cancer 2014;14:736–46.

Zager JS, Rollison DE, Messina J. Prognostication in Merkel cell carcinoma. Expert Rev Anticancer Ther 2011;11:665–7.

Zajac-Kaye M, Ben-Baruch N, Kastanos E, Kaye FJ, Allegra C. Induction of Myc-intron-binding polypeptides MIBP1 and RFX1 during retinoic acid-mediated differentiation of haemopoietic cells. Biochem J 2000;345:535–41.