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SHORT COMMUNICATION

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Distinct retinoic gene signatures discriminate Merkel cell polyomavirus-positive from -negative Merkel cell carcinoma cells

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Abstract

Limited molecular knowledge of Merkel cell polyomavirus (MCPyV)-positive and -negative Merkel cell carcinoma (MCC) subsets (MCCP/MCCN) has prevented so far the identification of the MCC origin cell type and, therefore, the development of effective therapies. The retinoic gene signature was investigated in various MCCP, MCCN, and control fibroblast/epithelial cell lines to elucidate the heterogeneous nature of MCC. Hierarchical clustering and principal component analysis indicated that MCCP and MCCN cells were clusterizable from each other and control cells, according to their retinoic gene signature. MCCP versus MCCN differentially expressed genes ($n = 43$) were identified. Protein–protein interaction network indicated SOX2, ISL1, PAX6, FGF8, ASCL1, OLIG2, SHH, and GLI1 as upregulated hub genes and JAG1 and MYC as downregulated hub genes in MCCP compared to MCCN. Numerous MCCP-associated hub genes were DNA-binding/-transcription factors involved in neurological and Merkel cell development and stemness. Enrichment analyses indicated that MCCP versus MCCN differentially expressed genes predominantly encode for to DNA-binding/-transcription factors involved in development, stemness, invasiveness, and cancer. Our findings suggest the neuroendocrine origin of MCCP, by which neuronal precursor cells could undergo an MCPyV-driven transformation. These overarching results might open the way to novel retinoid-based MCC therapies.

KEYWORDS

Merkel cell carcinoma, Merkel cell polyomavirus, retinoic gene, retinoic signaling, retinoid, SOX2

1 | INTRODUCTION

Merkel cell carcinoma (MCC) is rare yet aggressive cutaneous neoplasm with a prevalence of 0.1–2.5 cases/100 000/year worldwide. One-third of patients die of the disease.[1] About 80% of MCCs are related to Merkel cell polyomavirus (MCPyV), that is, MCPyV-positive (MCCP). The remaining cases are virus-negative (MCCN). MCPyV DNA integration into the host genome and the viral oncoprotein large T/small T (LT/sT) expression, are key MCCP events.[2] High UV-induced mutational burden is an MCCN hallmark, with RB1 and p53 mutation and MYCL amplification occurring frequently.[1]

MCCN is more aggressive than MCCP, in terms of increased development, relapse, and death risks. Despite progresses in the field, both MCC subsets appear to be morphologically similar, and information on distinctive diagnostic/prognostic markers is poor.[3] MCC management includes either platinum-/etoposide-based regimens or surgery followed by adjuvant radio-/chemotherapies. However, their effectiveness is unsatisfactory due to the propensity of MCC to metastasize and develop resistance.[3] Identifying distinctive molecular characteristics for the two MCC subsets could improve diagnostic and therapeutic options.

The existence of two MCC aetiologies reflects transformative events possibly occurring in two distinct origin cell types. Given the presence of neuroendocrine markers in MCC, Merkel cells were traditionally considered to be the source of MCC. However, this hypothesis was later doubted since Merkel cells are postmitotic cells and do not undergo cell division, while tumor cells are highly proliferative.[4] Recent evidence points to a multilinear origin, given the expression of neuroendocrine, epithelial/fibroblast, and B-lymphoid markers.[5] Identifying the MCC origin cell is prevented by the limited molecular information on the two aetiologies.[6]

Several retinoic pathway-associated genes are dysregulated in MCC.[5,7] Retinoid responses are mediated by different classes of nuclear receptors, whose signaling activation modulates genes that control morphogenesis, development, and homeostasis.[5,7] Retinoic signaling is dysregulated in carcinoma and components of this pathway are reliable antitumor targets, even for managing cutaneous neoplasms.[8] The impairment of development regulators such as SOX2 and the Hedgehog gene family member SHH has been documented in MCC.[5,7] In particular, SOX2, regulates the embryonic neural crest stem cell maintenance of the Merkel cell lineage, while being reported as expressed in MCC.[9] Similarly, SHH controls stem cell development and neurogenesis. The expression of this gene has been reported in MCC and in association with MCPyV infection.[10]

Limited but promising information on retinoic-associated genes in MCC prompted us to explore the heterogeneous nature of MCC by investigating the retinoic gene signature in MCCP and MCCN cells. MCCP versus MCCN differentially expressed genes (DEGs) and the crucial pathways and hub genes possibly implicated in each specific MCC subset were evaluated.

2 | RESULTS

2.1 | Viral DNA detection

MCPyV DNA has been detected in human skin.[11] To exclude the presence of MCPyV genomic sequences in control cells, all cells were evaluated for MCPyV DNA. HDFa, HPF, HaCat, NCTC, MCC13, and MCC26 were MCPyV-negative while PeTa, WaGa, MKL-1, and MS-1 were MCPyV-positive with a mean (±standard deviation of mean) viral load of 2.3 copy/cell (±0.11 copy/cell), 2.51 copy/cell (±0.17 copy/cell), 1.2 copy/cell (±0.31 copy/cell), 0.9 copy/cell (±0.31 copy/cell), respectively (Figure 1A).

2.2 | Clustered heatmap

The highest hierarchical stratification according to the retinoic gene expression data matrix was in relation to MCPyV DNA, as virus DNA-positive cells were separated from the remaining negative cells (Figure 1). A second level of cell stratification enabled three main groups to be identified. Group 1 included PeTa, WaGa, MKL-1, and MS-1 MCCP cells; Peta and WaGa, with the highest amount of MCPyV DNA, were grouped in a specific branch of group 1. Group 2 contained MCC13 and MCC26 MCCN cells. Group 3 included control cells, in which fibroblasts were categorized in a unique branch separated from epithelial cells (Figure 1A). Principal component analysis (PCA) was performed to examine the intrinsic distribution of analyzed cell lines according to retinoic signature. Two-dimensional PCA plots demonstrated a strong separation of MCCP group 1 from MCCN group 2 and control epithelial/fibroblast group 3 with a variance of 17.8% and 31.3%, in PC1 and PC2, respectively (Figure 1B).

2.3 | Retinoic expression, protein–protein interaction (PPI) analysis, and hub genes identification

A total of 43/84 (51.2%) retinoic-associated DEGs were identified between MCCP and MCCN cells, of which 24 were upregulated and 19 downregulated (Figure 2A). The PPI network was constructed with 43 DEGs to identify possible gene interactions (Figure 2B). A total of 41 nodes (genes) and 146 edges (interactions) were identified with a PPI enrichment of $p < 1.0 \times 10^{-16}$. The average node degree, clustering coefficient, and expected number of edges were 6.79, 0.643, and 22, respectively. The overlapping top 10 genes with three different algorithms were deemed as hub genes, namely SOX2, ISL1, PAX6, FGF8, ASCL1, OLIG2, SHH, and GLI1, as upregulated genes, and JAG1, MYC, as downregulated genes, in MCCP compared to MCCN.

2.4 | Enrichment analyses

We next conducted Gene ontology (GO) for biological processes, molecular functions, cellular components, and Kyoto Encyclopedia of

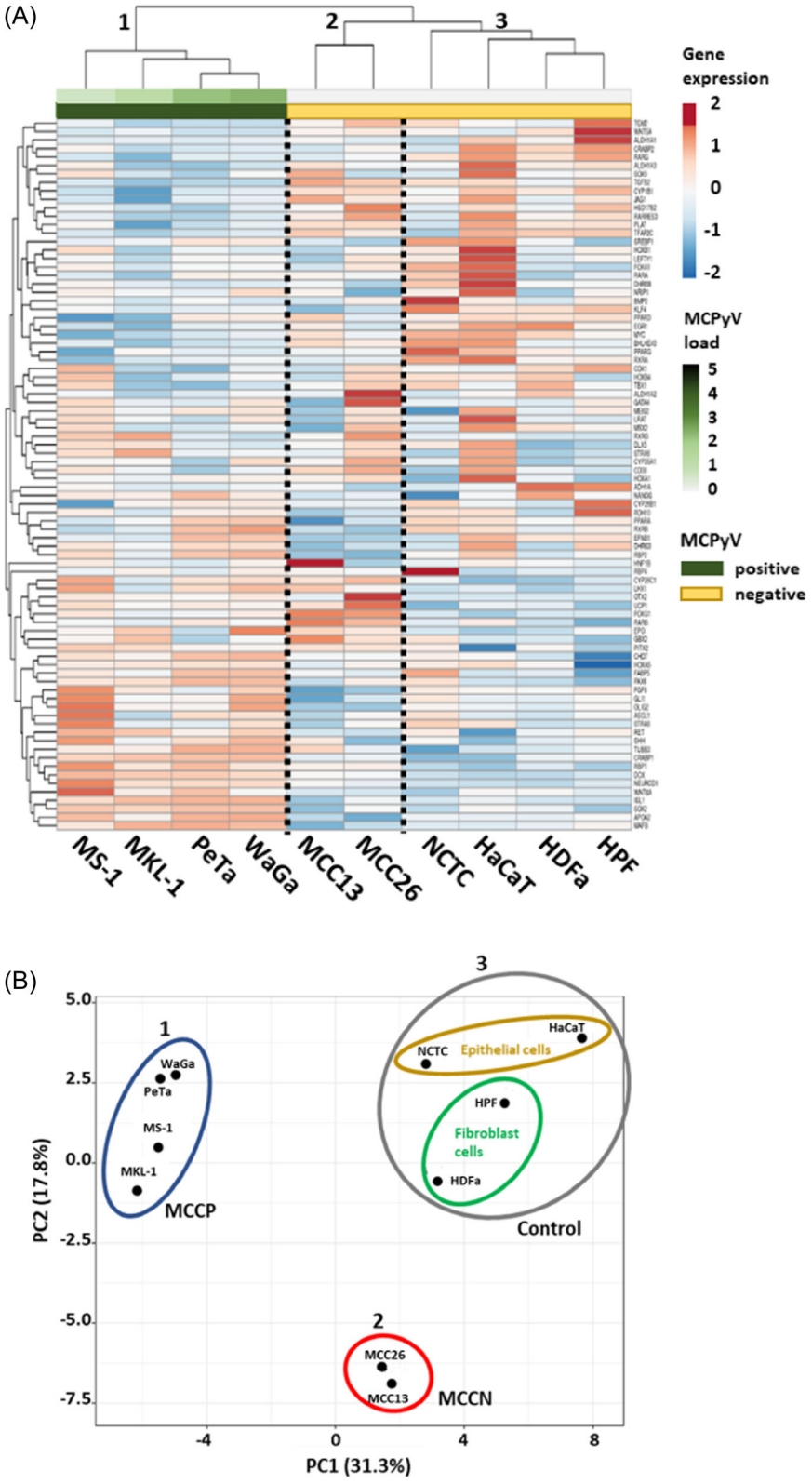

FIGURE 1 Clustered heatmap and principal component analysis (PCA) of 84 retinoic-associated genes across Merkel cell poliomavirus (MCPyV)-positive Merkel cell carcinoma (MCCP) cells and MCPyV-negative MCC (MCCN) cells, as well as epithelial and fibroblast control cells. (A) MCCP cells were MS-1, MKL-1, PeTa, and WaGa. MCCN cells were MCC13 and MCC26. Control cells were fibroblasts HDFa and HPF and ephitelial cells NCTC and HaCat. Cells are depicted on the *x*-axis along with their MCPyV status and MCPyV DNA load, as viral DNA copies/cell. MCPyV-positive and -negative cells are depicted in the dark green and yellow, respectively. A green-to-white color gradient depicts the amount of MCPyV DNA in MCCP cells. Cells groups 1, 2, and 3 are numbered on the sample dendrogram. Genes symbols are depicted on the *y*-axis, on the right. Heatmap data matrix of the retinoic gene signature visualizes the values in cells using a red-to-blue color gradient which gives an overview from the highest to the lowest expression values in the matrix. (B) PCA analysis of 84 retinoic-associated genes across MCCP, MCCN, and control cells. The two-dimensional PCA plot comprises *x* and *y* axes which depicts PC1 and PC2 corresponding to 31.3% and 17.8% of total variance, respectively. PCA demonstrated a significant separation of MCCP, MCCN, and control cells in three distinct clusters, that is, cluster 1, 2, and 3. PCA plot and heatmap were performed using Clustvis software.

Genes (KEGG) enrichment analyses on 43 DEGs between MCCP and MCCN cells. The complete list of enriched pathways is shown in Figure 2 and Supporting Information: Table 1. Enrichment analysis indicated that regionalization and ear, embryonic organ, and mesenchyme development were the most enriched biological processes. Molecular function exhibited retinoid, isoprenoid, E-box, and retinal binding and DNA-binding as the most enriched functions. Cellular component mainly included transcription regulator complex, collagen-containing extracellular matrix, and RNA polymerase II transcription regulator complex. KEGG analysis illustrated that DEGs

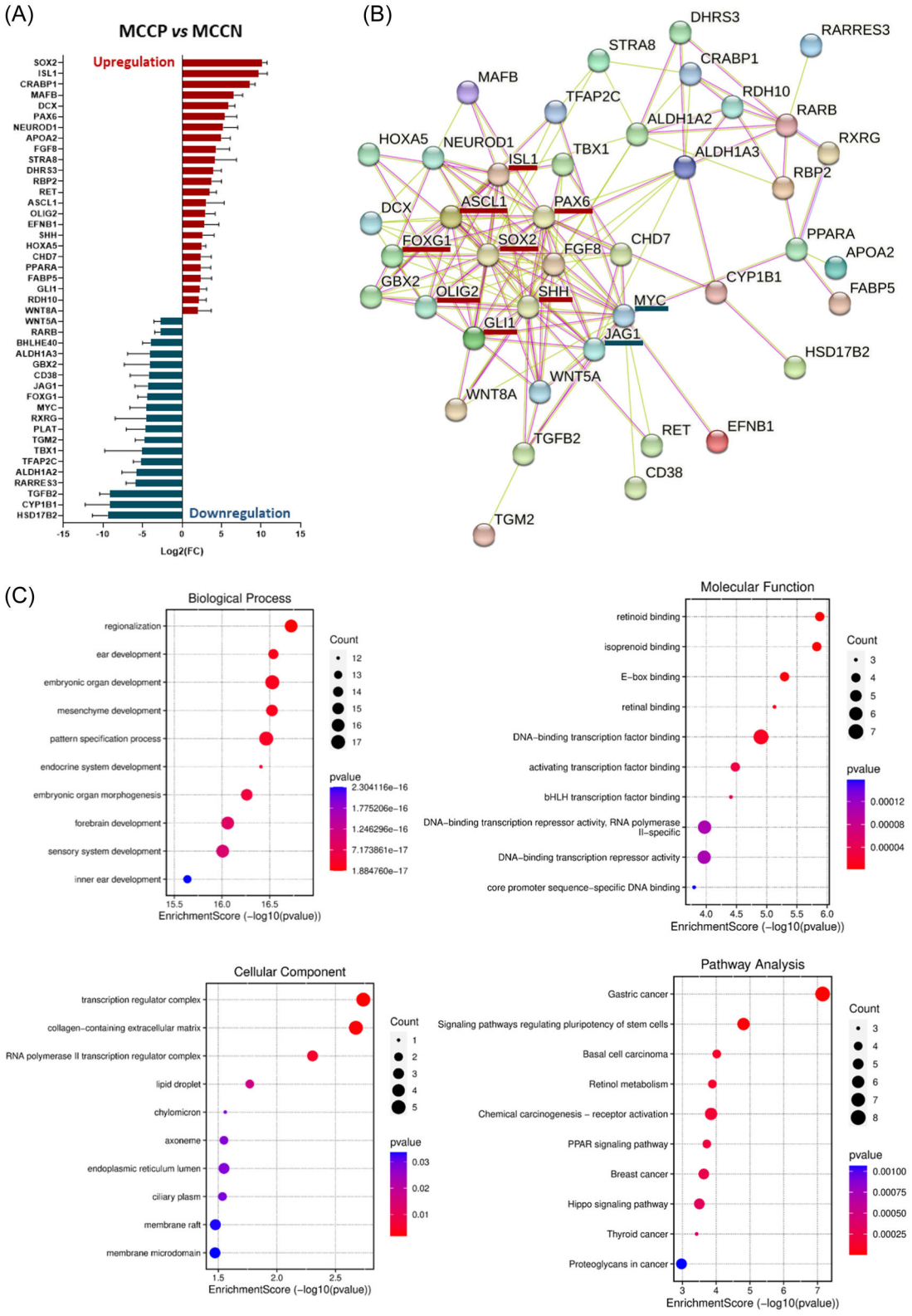

FIGURE 2 (See caption on next page).

were predominantly enriched in gastric cancer, stem cell pluripotency, basal cell carcinoma, retinol metabolism, and chemical carcinogenesis (Figure 2C). The majority of hub genes resulted to be highly distributed across all identified GO/KEGG pathways (Supporting Information: Table 1).

3 | DISCUSSION

Herein, the retinoic gene signature was investigated in MCCP and MCCN cells to deepen the heterogeneous nature of MCC. Given the identification of MCPyV DNA in the skin,[11] we first excluded the presence possible MCPyV-related gene expression bias in control cells. Control cells were MCPyV DNA-negative while, as expected,[12] MCCP and MCCN cells were MCPyV DNA-positive and -negative, respectively.

Heatmap analysis of retinoic signature indicated that cells were hierarchically divisible according to MCPyV positivity. Moreover, MCCP and MCCN resulted highly distinguishable from each other and control cells. Cell clusterization was confirmed by PCA, which provides the cell separation degree based on retinoic gene expression variance. Distinct MCCP and MCCN clusters, which were remarkably distant from each other and from the third cluster including control cells, were observed. These novel data indicate that the retinoic signature is highly divergent in MCCP and MCCN. Consistently, MCCP cells were hierarchically stratified according to MCPyV load, as high MCPyV load cells were categorized in their own branch. MCPyV might perturb the retinoic gene profile in MCCP. Understanding the underlying mechanisms is an open question.

More than 50% of retinoic genes were DEGs between MCCP and MCCN cells, thus highlighting profound differences between the two MCC subsets. Interactions across DEGs were determined by PPI network construction and the 10 topmost hub genes based on the interaction values degree were identified. Most of the 10 hub genes including SHH and GLI1 Hedgehog genes and SOX2, as well as PAX6, ISL1, ASCL1, and JAG1, encode for transcription factors regulating neurological development and Merkel cell lineage/differentiation. In particular, SOX2 controls (i) embryonic neural crest stem cell maintenance and (ii) cancer stem cell self-renewal/proliferation.[9] while SHH and GLI1 are known to be two stem cell development and neurogenesis regulators.[10] JAG1 is a ligand of the Merkel cell development-regulator Notch,[13] while PAX6 and ISL1 are known to be expressed in neuroendocrine neoplasms.[14] In agreement with our findings, previous studies indicated that SOX2, ISL1, and the Hedgehog genes are expressed in MCCP tissues/cells.[9] Concerning JAG1, we confirm previous data indicating that the gene is under microRNA negative regulation in MCCP.[3] Since the PAX6 and ASCL1 role in MCC is unclear, they could be considered as novel MCCP-associated genes. Our data improves the current MCC origin cell theories, confirming the presence of similarities between MCC and Merkel cells.[5]

Limited knowledge of the molecular hallmarks of the two MCC etiologies prevents the MCC origin cell, which could provide insights into novel diagnoses and therapies, from being identified. The expression of neuroendocrine, epithelial/fibroblast, and B-lymphoid markers, which suggested a possible multilinear origin for MCC, brought its Merkel cell origin into doubt. Given the high mutational burden of MCCN, its origin cell might be a sun-exposed, skin keratinocyte. MCCP might instead derive from (i) pre-/pro-B cells, as expressing B-cell maturation markers, (ii) dermal fibroblasts, as being MCPyV-infection susceptible, and (iii) neuronal cell precursors.[5] Our novel findings on retinoic signature in MCC might suggest the neuroendocrine origin of MCCP, by which neuronal precursor cells could undergo an MCPyV-driven transformation into Merkel cell-like cancer cells.[5] This hypothesis requires further investigations.

Regarding the remaining hub genes, MYC overexpression is frequent in MCPyV-independent MCC.[3] Despite no information is available on FGF8 and OLIG2 in MCC, they are dysregulated in cancer.[15,16] Both genes can be considered as novel candidate MCCP-related genes.

The identification of hub MCCP/MCCN differentiating genes might allow the development of novel MCC subset-specific anti-tumor strategies. Indeed, SOX2, Hedgehog genes, PAX6, OLIG2, ASCL1, and JAG1, are all reliable targets for cancer therapy.[17,18] These genes can, therefore, be considered novel candidate targets for MCCP treatment, and we thus encourage further studies in this direction.

Enrichment analyses were next performed on DEGs to identify critical discriminating pathways. GO analysis indicated that DEGs predominantly consisted of genes involved in embryonic development, which is known to be dysregulated in cancer.[19] GO analysis of molecular function and cellular component revealed that DEGs were predominantly associated with transcription regulator/DNA binding functions and extracellular matrix. We can theorize that DNA-binding

FIGURE 2 Differentially expressed retinoic genes (DEGs) between pooled Merkel cell poliomavirus (MCPyV)-positive Merkel cell carcinoma (MCCP) cells and MCPyV-negative MCC (MCCN) cells and functional enrichment analyses of DEGs. MCCP cells were MS-1, MKL-1, PeTa, and WaGa. MCCN cells were MCC13 and MCC26. (A) Graphical representation of DEGs in MCCP versus MCCN cells. Red color indicated upregulated ($\log_2$ FC > 2) genes and blue color indicated downregulated genes ($\log_2$ FC < −2). (B) Protein–protein interaction (PPI) network was constructed with STRING database with the entire set of 43 DEGs between MCCP and MCCN cells. Network nodes correspond to genes, while edges represented associations. An edge may be drawn with up to two different colored lines. Purple line: experimental evidence. Yellow line: textmining evidence. Ten hub genes were identified using Cytoscape. Hub upregulated genes between MCCP and MCCN are underlined in red, while hub downregulated genes are underlined in blue. (C) Bubble plots represent the signaling pathways of the entire set of 43 DEGs resulting in the comparison between MCCP and MCCN cells using Gene ontology (GO) and Kyoto Encyclopedia of Genes (KEGG) databases. Enrichment analyses show GO for biological process, molecular functions and cellular component terms, and KEGG pathways.

proteins such as transcription factors might sustain the expression of genes whose activation could favor an invasive phenotype in MCCP. KEGG pathway enrichment indicates that DEGs were enriched mostly for pathways in (i) multiple cancer types, suggesting that MCCP might present more common features to other malignancies than MCCN and (ii) stem cell pluripotency. Hence, dysregulated retinoic gene signature might confer a highly invasive/transformative potential to MCCP. Lastly, all identified pathways were highly enriched by all PPI hub genes, thus underlining the prominent role of these pathways in MCCP.

The limitation of this study is the small number of cell lines investigated, while analyses were performed observationally and at transcriptional level, only.

In conclusion, MCC cells were stratified according to retinoic signature. Ten MCCP-associated hub genes, mostly encoding for Merkel cell-specific transcription factors implicated in neurological development and stemness, were identified. Enrichment analyses indicated that DEGs are predominantly related to DNA-binding/transcription factor functions, development, stemness, and cancer. Likely, a dysregulated retinoic signature might confer a high invasive/transformative potential to MCCP. The results of the present study might open the way to novel retinoid-based MCC therapies.[8]

4 | METHODS

4.1 | Cells and nucleic acids

PeTa, WaGa, MKL-1 and MS-1 MCCP cells, and MCC13 and MCC26 MCCN cells were cultured in RPMI 1640 (EuroClone). Control cells were HDFa and HPF human skin/primary fibroblasts and HaCat and NCTC skin keratinocytes, cultured in Medium 106 and Dulbecco's modified Eagle medium/F-12 (EuroClone), respectively. Nucleic acids were isolated with RNeasy Plus and QIAmp DNA Blood/Tissue Kits (Qiagen), respectively, and stored (−80°C).[20]

4.2 | Viral DNA

MCPyV DNA was quantified by ddPCR using QX200 Droplet Digital (Bio-Rad). Amplification conditions were: 95°C, 5 min, followed by 94°C, 30 s, 48°C, 1 min, for 45 cycles, then 90°C, 5 min, ending at 4°C. Data were analyzed using the QuantaSoft tool. EIF2C1 housekeeping gene was used to determine human cell equivalents. DNA isolation/H_2O controls were included.

4.3 | Retinoic expression profile

cDNA was synthesized using 250 ng of total RNA and an RT^2 First Strand Kit, while the RT^2 Human Retinoic Acid Signaling PCR array (Qiagen) was used to measure the expression of 84 retinoic genes involved in retinoic acid/retinol pathways and in developmental pathways, including adipogenesis, body pattern formation, and cardiovascular, neurological, sex determination, skin, and stem cell development. Plates were run on CFX96 Touch Real-Time PCR (Bio-Rad). Reactions were performed in triplicate.

4.4 | Statistics

Gene expression analysis was performed using the $2^{-\Delta\Delta C_t}$ method. Log2 fold change <−2/>2 and $p < 0.05$ were considered statistically significant. Hierarchical clustering and PCA were performed using ClustVis. GO/KEGG were performed to categorize DEGs. PPI network was constructed using STRING (cut-off > 0.4). Ten PPI hub genes were identified via the cytoHubba plugin in Cytoscape, with maximum neighborhood/edge percolated components and degree algorithms.

AUTHOR CONTRIBUTIONS

Chiara Mazziotta, Christian Felice Cervellera, and Giada Badiale performed the experiments. Ilaria Vitali supported the execution of experiments and performed the statistical analysis. Antoine Touzé provided the cell lines. John Charles Rotondo organized and supervised the work, and corrected the manuscript draft. Chiara Mazziotta and John Charles Rotondo wrote the final version of the manuscript. Fernanda Martini, Mauro Tognon, and Antoine Touzé critically revised the manuscript. John Charles Rotondo, Chiara Mazziotta, Mauro Tognon, Fernanda Martini, and Antoine Touzé contributed to supervision, to funding acquisition, and project administration. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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