Fluorescence in situ hybridization and qPCR to detect Merkel cell polyomavirus physical status and load in Merkel cell carcinomas

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The Merkel cell polyomavirus (MCPyV) is detected in 80% of Merkel cell carcinomas (MCC). Clonal integration and tumor-specific mutations in the large T antigen are strong arguments that MCPyV is a human tumor virus. However, the relationship between viral presence and cancer induction remains discussed controversially. Since almost all studies on virus prevalence are based on PCR techniques, we performed MCPyV fluorescence in situ hybridization (FISH) on MCC to gain information about the quality of the viral presence on the single cell level. MCPyV-FISH was performed on tissue microarrays containing 62 formalin-fixed and paraffin-embedded tissue samples including all tumor grades of 42 patients. The hybridization patterns were correlated to the qPCR data determined on corresponding whole tissue sections. Indeed, MCPyV-FISH and qPCR data were highly correlated, i.e. 83% for FISH-positive and 93% for FISH-negative cores. Accordingly, the mean of the qPCR values of all MCPyV-positive cores differed significantly from the mean of the negative cores (p = 0.0076). Importantly, two hybridization patterns were definable in the MCPyV-FISH: a punctate pattern (85%) indicating viral integration, which correlated with a moderate viral abundance and a combination of the punctate with a diffuse pattern (15%), suggesting a possible coexistence of integrated and episomal virus which was associated with very high viral load and VP1 expression. Thus, MCPyV-FISH adds important information on the single cell level within the histomorphological context and could therefore be an important tool to further elucidate MCPyV related carcinogenesis.

Introduction

Merkel cell carcinoma (MCC) is a highly malignant nonmelanoma skin cancer (NMSC) of elderly or immunosuppressed patients. The recent identification of the Merkel cell polyomavirus (MCPyV) in MCC significantly contributed to the understanding of the etiopathogenesis of MCC. MCPyV DNA has been detected consistently in approximately 80% of MCC. The finding of both, clonal viral integration of MCPyV in MCC DNA as well as tumor specific truncating oncogenic mutations in the large T antigen (LT) adds it to the list of human tumor viruses. Interestingly, an association between the viral presence in MCC and metastatic progression was reported and MCPyV-positive MCC showed a favorable prognosis in comparison with tumors without MCPyV. Whether patients with high antibody titers against the VP1 gene product of MCPyV are likely to be associated with a better progression-free survival remains discussed controversially.

Previous analyses on, e.g. high-risk human papillomavirus (hr HPV) 16 and SV40, have demonstrated that viral integration is an important event in tumor virus-associated carcinogenesis. HPV viruses share similar features with polyomaviruses as the small genome size, the double strand circular DNA structure, the use of the host replication apparatus and the LxCxE motive for binding retinoblastoma protein. Importantly, the development of HPV-FISH enabled the discrimination between the integration and the episomal existence of the virus, which can be of clinical relevance if the integration status is associated with tumor progression.
What's new?
It's not clear how the Merkel cell polyomavirus contributes to cancer formation, although it's found in the majority of Merkel cell carcinomas. As work with HPV has shown, the virus needs to situate its DNA into the cell's chromosome in order to get cancer started. Tagging viral DNA with a fluorescent probe illumines whether the virus has integrated itself into the chromosome or not. In this paper, the authors show that FISH can show the location of MCPyV in the cell, as it does HPV. These results suggest this technique could be useful for investigating the cancer-causing activities of MCPyV.

Material and Methods
Tumor material
The tissue microarrays (TMAs) were assembled with 62 formalin-fixed and paraffin-embedded (FFPE) cores from 42 European MCC patients: 25 men (age range: 44–96 years, mean: 74.2 years), 16 women (age range: 60–89 years, mean: 74.4 years); from one core, no data were available (Table 1, Patient 5). All respective tumors had been excised for therapeutic reasons. MCC had been defined by histology and immunohistochemistry for CK20, CD56 and TTF1. Written, informed consent had been obtained from all patients to use tumor material not needed for histopathological diagnosis for further scientific workup; the study was performed according to the guidelines of the local ethics committee (ethical review committee Medical Faculty University of Würzburg; sequential study number 135/06) and the declaration of Helsinki.

In addition to the previously published controls,28 we also used the MCPyV-positive MKL-1 cell line, which previously has been shown by Southern hybridization to harbor integrated MCPyV.6 The cells were embedded in paraffin and cut in 3-μm tissue sections.

Probe
MCPyV DNA was isolated from a MCC and cloned into a StrataClone PCR Cloning Vector (pSC-A-amp/kan; Stratagene, Santa Clara, CA). The plasmid DNA was purified with the plasmid DNA Purification Kit NucleoBond® PC 2000 (Macherey-Nagel, Dueren, Germany) and sequenced using T7 and T3 primers. The HPyV-6 and -7 probes were generated from the 24272 plasmid: pHPyV6-607a and 24278 plasmid: pHPyV7-13a, respectively (Addgene, Cambridge, MA). The 5,104 bp MCPyV DNA contained the VP2, VP1 and LT with a 4-bp deletion within the LT introducing a stop codon prior to the helicase domain. Labeling was performed by standard nick translation with Biotin-Nick Translation Mix (Roche, Mannheim, Germany) containing biotin (Bio)-16-dUTPs. The final concentration of the labeled DNA was 2 ng/μl in 50% formamide, 20% dextran sulfate, 2× SSC pH 7.0, 50× excess carrier DNA from salmon sperm (Sigma Chemical, St Louis, MO) and 50× tRNA from S. cerevisiae (Sigma Chemical).

Detection of MCPyV by FISH MCPyV
FISH was optimized and performed according to Hopman et al.29 on 3-μm thick TMA or MKL-1 cell block sections

So far, the determination of the MCPyV prevalence in MCC is based on PCR or qPCR techniques.33 Such assays, however, cannot distinguish between clonal integration or episomal MCPyV presence on the single cell level. In the initial description of MCPyV, Feng et al. demonstrated the monoclonal integration of MCPyV in six of ten MCC patients by Southern blotting. In two additional patients, the results of Southern hybridization suggested episomal presence or concatenated integration.1 Southern hybridization confirmed clonal integration of MCPyV in several MCC cell lines in subsequent studies,6,24,25 often suggesting concatenated MCPyV presence although technically southern blotting cannot distinguish between episomal and concatenated MCPyV presence. In another study, Sastre-Garau et al. reported the viral integration of MCPyV into MCC using a detection of integrated papillomavirus sequences (DIPS-PCR)-based approach.26 The viral integration was shown on fresh frozen tissue sections of one patient by FISH, revealing punctate signals. This was associated with 6.3 copies per cell. From the ten investigated patients, one case was associated with a high, 62.2 per cell, viral copy number. By DIPS-PCR, the MCPyV DNA was found integrated with truncated LT at the 4q13.1 locus. Further PCR analyses using inverse and adjacent primers revealed also the full-length MCPyV DNA (5,387 bp). Sequencing this product showed no mutations leading to truncated proteins in none of the viral genes; thus, the authors interpreted it as episomal virus presence. Interestingly, one of them contained in addition a viral junction region in the LT. Moreover, Laude et al.10 identified by DIPS-PCR downstream the truncated proteins in none of the viral genes; thus, the authors interpreted it as episomal virus presence. In another study, Sastre-Garau et al. reported the viral integration of MCPyV into MCC using a detection of integrated papillomavirus sequences (DIPS-PCR)-based approach.26 The viral integration was shown on fresh frozen tissue sections of one patient by FISH, revealing punctate signals. This was associated with 6.3 copies per cell. From the ten investigated patients, one case was associated with a high, 62.2 per cell, viral copy number. By DIPS-PCR, the MCPyV DNA was found integrated with truncated LT at the 4q13.1 locus. Further PCR analyses using inverse and adjacent primers revealed also the full-length MCPyV DNA (5,387 bp). Sequencing this product showed no mutations leading to truncated proteins in none of the viral genes; thus, the authors interpreted it as episomal virus presence. Interestingly, one of them contained in addition a viral junction region in the LT. Moreover, Laude et al.10 identified by DIPS-PCR downstream the truncated proteins in none of the viral genes; thus, the authors interpreted it as episomal virus presence.
| TMA No | Patient No | Age | Gender | Tissue type | rel. MCPyV presence | Tissue type | rel. MCPyV presence | Tissue type | rel. MCPyV presence |
|--------|------------|-----|--------|-------------|---------------------|-------------|---------------------|-------------|---------------------|
| 1      | 1          | 76  | m      | P           | 3.5                 | -           | -                   | -           | -                   |
| 2      | 1          |     |        | LN-M        | 1.16                | -           | -                   | -           | -                   |
| 3      | 2          | 75  | f      | LN-M        | 0.0009              | -           | -                   | -           | -                   |
| 4      | 3          | 80  | f      | relapse     | 9.2                 | -           | -                   | -           | -                   |
| 5      | 4          | 75  | m      | LN-M        | 0.04                | -           | -                   | -           | -                   |
| 6      | 5          |     |        | no data     | 0.01                | -           | -                   | -           | -                   |
| 7      | 6          | 63  | f      | P           | 0.65                | -           | -                   | -           | -                   |
| 8      | 7          | 53  | m      | P           | 0.004               | -           | -                   | -           | -                   |
| 9      | 8          | 76  | m      | relapse     | 0.24                | -           | -                   | -           | -                   |
| 10     | 8          |     |        | P           | 0.33                | -           | -                   | -           | -                   |
| 11     | 8          |     |        | LN-M        | 0.41                | -           | -                   | -           | -                   |
| 12     | 9          | 60  | f      | P           | 0.28                | -           | -                   | -           | -                   |
| 13     | 10         | 55  | m      | LN-M        | 0.34                | -           | -                   | -           | -                   |
| 14     | 10         |     |        | -           | -                   | -           | -                   | -           | P 0.26              |
| 15     | 11         | 71  | f      | P           | 0.39                | -           | -                   | -           | -                   |
| 16     | 12         | 75  | m      | LN-M        | 1.2                 | -           | -                   | -           | -                   |
| 17     | 12         |     |        | M           | 0.72                | -           | -                   | -           | -                   |
| 18     | 13         | 80  | f      | P           | 1.3                 | -           | -                   | -           | -                   |
| 19     | 14         | 61  | f      | M           | 5.7                 | -           | -                   | -           | -                   |
| 20     | 14         |     |        | relapse     | 11.2                | -           | -                   | -           | -                   |
| 21     | 15         | 69  | m      | P           | 3.9                 | -           | -                   | -           | -                   |
| 22     | 16         | 96  | m      | relapse     | 0.03                | -           | -                   | -           | -                   |
| 23     | 17         | 84  | m      | re-Excision | 0.5                 | -           | -                   | -           | -                   |
| 24     | 17         |     |        | -           | -                   | -           | -                   | -           | M 0.5               |
| 25     | 18         | 72  | f      | P           | 0.7                 | -           | -                   | -           | -                   |
| 26     | 18         |     |        | M           | 0.2                 | -           | -                   | -           | -                   |
| 27     | 19         | 67  | m      | M           | 0.8                 | -           | -                   | -           | -                   |
| 28     | 20         | 74  | f      | M           | 1.15                | -           | -                   | -           | -                   |
| 29     | 20         |     |        | M           | 1.09                | -           | -                   | -           | -                   |
| 30     | 20         |     |        | M           | 0.82                | -           | -                   | -           | -                   |
| 31     | 20         |     |        | M           | 4.9                 | -           | -                   | -           | -                   |
| 32     | 21         | 83  | f      | re-excursion | 0.3                 | -           | -                   | -           | -                   |
| 33     | 22         | 77  | m      | P           | 1.1                 | -           | -                   | -           | -                   |
| 34     | 23         | 84  | m      | P           | 1.4                 | -           | -                   | -           | -                   |
| 35     | 24         | 44  | m      | M           | 1.001               | -           | -                   | -           | -                   |
| 36     | 25         | 83  | m      | M           | 1.37                | -           | -                   | -           | -                   |
| 37     | 25         |     |        | -           | -                   | -           | -                   | -           | M 0.008             |
| 38     | 26         | 74  | m      | P           | 0.31                | -           | -                   | -           | -                   |
| 39     | 26         |     |        | M           | 0.35                | -           | -                   | -           | -                   |
| 40     | 27         | 55  | m      | M           | 25.6                | -           | -                   | -           | -                   |
| 41     | 27         |     |        | M           | 2.0                 | -           | -                   | -           | -                   |
| 42     | 28         | 64  | m      | M           | 6.3                 | -           | -                   | -           | -                   |
| 43     | 28         |     |        | -           | -                   | M           | 52                  | -           | -                   |
with modifications: sections were deparaffinized, pretreated with 0.2 M HCl for 20 min at room temperature and washed once in dH2O and twice in 2× SSC. The preparations were then treated with 1 M NaSCN for 30 min at 80°C, washed in dH2O and 2× SSC, digested with 1 mg/ml pepsin (2,500–3,500 U/mg protein from porcine stomach mucosa; Sigma Chemical) in 0.14 M NaCl solution, pH 2. For DNase I or RNase A treatment, the tissue sections were treated with 0.2 g/100 ml pepsin (Sigma) in 0.01 M HCl at 37°C for 10 min. Slides were then incubated 30 min at 37°C with 5.7 U DNase I (QIagen) in RDD buffer (QIagen) or 250 μg/ml RNase A (QIagen) in 2× SSC, respectively, and cooled on ice. The slides were washed twice in 2× SSC and postfixed in 4% formaldehyde in PBS for 10 min at room temperature. After washing in 2× SSC and rinsing in dH2O, the sections were dehydrated in an ethanol series starting with 70% ethanol in dH2O. The biotin-labeled MCPyV probe was added under the coverslip at a concentration of 2 ng/ml. Hybridization mixture and target DNA were denatured simultaneously for 5 min at 80°C prior to hybridization overnight at 37°C in a humid chamber. After hybridization the preparations were washed stringently in 2× SSC, pH 7.0 at 70°C for 2 min. The biotin (Bio) labeled probe was detected with sequential incubations of fluorescein isothiocyanate (FITC) biotinylated avidin (AvFITC, 1:500; Vector, Brunswig Chemie, Amsterdam, The Netherlands), biotin conjugated goat anti-avidin (BioGaA, 1:100; Vector) and FITC-conjugated avidin 1:500, all for 30 min at 37°C and diluted in 4× SSC/0.05% Tween-20 (Janssen Chimica). Finally, the slides were washed in 4× SSC/0.05% Tween-20 (Janssen Chimica) and PBS, dehydrated in an ascending ethanol series and mounted in Vectashield (Vector) containing 0.2 mg/ml 40,6-diamidino-2-phenyl indole (DAPI; Sigma Chemical). MCPyV-FISH slides were evaluated using a fluorescence microscope (DM 5000 B fluorescence microscope, Leica, Wetzlar, Germany) equipped with DAPI and FITC filters. Images were recorded with the Leica DC 300 Fx camera (Leica) mounted on top of the fluorescence microscope. Nuclear FISH signals were evaluated by four investigators (AH, AzH, EJS, DR) according to the criteria from Hafkamp et al.19: We defined the “punctate pattern” (Group “1”) with maximal five discrete signals per nucleus showing comparable fluorescence intensity and size indicating viral integration. As the cell density in our cores was very high and therefore nuclei overlapping, we decided to allow one to two signals more compared to Hafkamp et al. to avoid misinterpreting when signals shine through overlaying nuclei. In addition to this, we observed nuclei with a higher and very high number of tiny signals, in part overlapping. Because of the latter, we named it “diffuse pattern,” which in parallel to Hafkamp et al. is indicating the presence of

| TMA No | Patient No | Age | Gender | Tissue type | rel. MCPyV presence | Tissue type | rel. MCPyV presence | Tissue type | rel. MCPyV presence |
|--------|------------|-----|--------|-------------|---------------------|-------------|---------------------|-------------|---------------------|
| 44     | 28         | -   | -      | -           | -                   | M           | 52                  | -           | -                   |
| 45     | 28         | 83  | f      | P           | 10.4                | -           | -                   | -           | -                   |
| 46     | 30         | 67  | f      | -           | -                   | P           | 4485                | -           | -                   |
| 48     | 31         | 77  | m      | -           | -                   | M           | 494                 | -           | -                   |
| 49     | 32         | 76  | f      | -           | -                   | P           | 75                  | -           | -                   |
| 50     | 33         | 91  | m      | -           | -                   | re-excision | 341                | -           | -                   |
| 51     | 34         | 85  | m      | -           | -                   | -           | -                   | -           | re-excision 0.004   |
| 52     | 35         | 82  | m      | -           | -                   | -           | -                   | -           | P 0.13              |
| 53     | 36         | 69  | m      | -           | -                   | -           | -                   | -           | LN-M 0.03           |
| 54     | 36         | -   | -      | -           | -                   | -           | -                   | -           | P 0.2               |
| 55     | 37         | 74  | f      | -           | -                   | -           | -                   | -           | P 0.04              |
| 56     | 38         | 82  | f      | -           | -                   | -           | -                   | -           | P 0.14              |
| 57     | 39         | 89  | m      | -           | -                   | -           | -                   | -           | P nd                |
| 58     | 39         | -   | -      | -           | -                   | -           | -                   | -           | LN-M nd             |
| 59     | 39         | -   | -      | -           | -                   | -           | -                   | -           | LN-M nd             |
| 60     | 40         | 75  | m      | -           | -                   | -           | -                   | -           | re-excision nd      |
| 61     | 41         | 77  | m      | -           | -                   | -           | -                   | -           | re-excision nd      |
| 62     | 42         | 89  | f      | -           | -                   | -           | -                   | -           | P nd                |

The data were sorted according to the FISH evaluation groups and matched with qPCR data. Abbreviations: P; primary tumor; M; metastasis; LN-M; lymph node metastasis; nd: not detected.
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concentrations. After antigen retrieval (Dako, #S6199) at 95°C

alyzed with TCS SP2 confocal fluorescence microscope (Leica).

with vectashield with DAPI (Vector Laboratories) and ana-

ological comparison of the qPCR data and FISH analysis.

MCPyV detection by qPCR

Genomic DNA was isolated from whole FFPE tissue sections

(whole tissue section) using a DNA Isolation Kit (Qiagen, Hilden,

Germany). Real time PCR for MCPyV detection was performed in

triplicates with a primer pair specific for the LT region as previ-
nously described. Genomic DNA of a MCC cell line that harbours

at least two concatemerized copies of the MCPyV genome served

as calibrator for the relative quantification calculated by the ΔΔCt

method normalized to the repetitive DNA elements LINE1. The

used LINE-1 primer and probe sequences:

forward: 5′- CGC TTT TCA GAC CGG CTT AA G-3′,

reverse: 5′- AGA TTC CGT GGG CGT AGG A-3′,

probe: 5′- CGC ACC ACG AGA CTA TAT CCC ACA CCT G- 3′.

Correlation of FISH and qPCR data

The qPCR data were matched with the according cores in each

group. Inconclusive cores were retested by MCPyV-FISH on

whole tissue section to verify the FISH analysis. The concord-
ance was determined by a cutoff for the qPCR values (see

below) as follows: in Group “0” all cores with a qPCR value ≤

threshold, in all other groups qPCR values > threshold. Of

this, the percentage of correlation between FISH analysis and

qPCR data was calculated from the total group number.

VP-1 staining by immunohistochemistry

The staining for VP1/Vp2 was performed as described previ-

ously.30 In brief, tissue sections of MCC cases were deparafla-
nized in xylene followed by rehydration in declining ethanol

concentrations. After antigen retrieval (Dako, #S6199) at 95°C

for 30 min, slides were rinsed in water, transferred to PBS and

protein was blocked (Dako, #X0909) for 10 min. After 2h incu-

bation at 37°C incubation with polyclonal rabbit anti-MCV

(VP1/Vp2) serum (1:400 diluted; kind gift of C. Buck), slides

were washed and incubated with goat anti-rabbit FITC

(DAKO) for 45 min. After rinsing in PBS, slides were mounted

with vectashield with DAPI (Vector Laboratories) and anal-

alyzed with TCS SP2 confocal fluorescence microscope (Leica).

Statistical analysis

All statistical tests were performed with GraphPad Prism 5

(GraphPad Software, La Jolla, CA, U.S.A.). Outliers were

tested and defined according to the Grubb’s test. To distin-

guish the copy number of negative and positive FISH results,

a cut-off was set at the highest relative MCPyV presence in

the negative group.

The unpaired t-test was used to determine statistical sig-

nificant differences of the mean values from two groups cal-
culated from the log of the qPCR data. p Values of <0.05

were considered to be significant.

The log of the qPCR data of the different FISH evaluation

groups were compared (p < 0.0001) by ANOVA (Kruskal–

Wallis test) as the data were approximately corresponding to

Gaussian distribution tested by D’Agostino and Pearson nor-

mality test.

Results

MCPyV-FISH on MKL-1 cell line, MCC and negative controls

To demonstrate the feasibility of our MCPyV-FISH, we first

performed it on FFPE-treated MCPyV-positive MKL-1 cells.

These cells have previously been shown to harbor integrated

MCPyV.6 MCPyV-FISH of MKL-1 cells and a MCC case,

which served as a positive control28 revealed single punctate

fluorescent signals (Figs. 1b and 1h) in the nuclei (Figs. 1a

and 1g) indicating genomic integration of MCPyV. By DNase

I treatment, the MCPyV signals disappeared (Figs. 1c, 1i

and 1j) whereas RNase A did not lead to a significant decrease

in signal intensity (Figs. 1d, 1k and 1l) demonstrating that

the observed FISH signals are mostly based on interaction of

FISH probe and MCPyV DNA. In contrast, in diverse

MCPyV-negative tissues as tested by PCR, e.g. colon carci-
nomas, no specific MCPyV-FISH signals could be detected

(Figs. 1e and 1f; data not shown). Additionally, omission of

the probe did also not generate any FISH signal on the inves-

tigated MCC tissue (data not shown). To exclude possible

cross-reactions with other polyomaviruses, we hybridized a

HPyV 6 probe on the MKL-1-positive control which showed

no signal (data not shown).

MCPyV-FISH on MCC TMAs

Next, MCPyV detection by FISH was carried out on MCC

TMAs containing a total of 62 different tumor samples of 42

MCC patients. Of these 62 samples, 47 cores (76%) were pos-

itive for MCPyV by FISH and 15 (24%) cores were negative

(Group “0”) (Figs. 2a and 2b; Supporting Information Fig.

S1, upper rows A–F). Since the signal pattern varied among

the different cores, these cores were further subdivided (table

in Fig. 4) into 40 MCC cores (85%) with punctate signal pat-

tern (Group “1”); Figs. 2c and 2d; Supporting Information Fig.

S1, middle rows G–L) and seven cores (15%) with a punctate

combined with a “diffuse pattern” (Group “2”; Figs. 2e

and 2f; Supporting Information Fig. S1, lower rows M–R). To

further elaborate the latter cores, we analyzed corresponding

whole tissue sections, which confirmed the coexistence of a

punctate and diffuse pattern in one and the same tumor

(Supporting Information Fig. S1, M–P). We tested this pattern

with DNase I and RNase A treatment on the whole tissue

section of Patient 30 (Figs. 3a–3f). These stainings

confirmed the previous observation that RNase A treatment
did not lead to an obvious decrease in signals per nucleus

(Figs. 3e and 3f), whereas applying DNase I erased the FISH

signals (Figs. 3c and 3d).
Figure 1. Specific punctate FISH signal in MKL-1 cells and MCPyV-positive MCC. The MCPyV signal is shown as green fluorescent punctuated dot; the nuclear localization was verified by DAPI staining in blue. Scale bar 10 μm. (a and b) Positive control on MCPyV-positive MKL-1 cells demonstrating a punctate pattern with distinct fluorescence signals dots located in the nuclei (arrows). (c) DNase I treatment erased the signals completely. (d) RNase A diminished the fluorescence intensity of the signal dots slightly (arrows). The number of punctate nuclei stays approximately the same. (e and f) Negative control on normal colon tissue without signal. (g and h) MCPyV-FISH on a MCPyV-positive MCC tissue section reveals the same punctate pattern as on the MKL-1-positive control. (i and j) In MKL-1 cells, DNase I erased the signal dots. (k and l) The signals were slightly decreased after RNase A treatment (see arrows).
The FISH results revealed 30 patients (71.5%) positive including 24 patients (57% \( n = 42 \)) with a punctate pattern and nine patients (21.4%) negative. The remaining three patients (3/42; 7.1%) had discordant results in multiple lesions as one sample displayed a punctate pattern while the other was FISH negative. In total, the TMA included 14 patients with multiple lesions (Table 1). These were derived either from different tumor lesions such as primary tumor, local recurrences or metastases (\( n = 9 \) patients) as well as from different metastases (\( n = 5 \) patients). The FISH results of examples from different lesions are shown in Supporting Information Fig. S1. Among these patients, 10/14 (71.4%) displayed concordant results two being FISH negative and six demonstrating an equal punctate pattern in all samples from the same patient (Supporting Information Fig. S1II–L). Four patients revealed discordant results: three patients as mentioned above with a negative and a punctate TMA core and, in one patient, we observed a punctate and a mixed pattern in different metastases.

MCPyV qPCR

In six of 62 cases, no MCPyV sequences were detectable by qPCR (qPCR negative). In the remaining 56 tissue sections from which the analyzed cores were obtained, the relative presence level of MCPyV ranged from 0.0009 to 4,485 (interquartile range IQR 0.22–4.4; Fig. 4b). When samples were stratified upon MCPyV-FISH result in negative and positive, the difference in the relative MCPyV presence detected by qPCR reached statistical significance (\( p = 0.0076; \) Fig. 4a). Moreover, all FISH evaluation groups differed significantly according to their respective qPCR values tested by ANOVA (\( p < 0.0001 \)) (Fig. 4a). Group “1” with the punctate pattern showed qPCR values from 0.0009 to 25.6 (IQR 0.305–1.7; Fig. 4b) whereas the mixed group was associated with the highest values of relative presence from 29.9 up to 4,485 (IQR 52–1,947; Fig. 4b). Notably, the relative viral abundance detected by qPCR was higher in these areas in which more diffuse hybridization patterns were detected in the whole tissue section and \textit{vice versa}. For example the case with the highest qPCR value displayed intense diffuse hybridization signals combined with a strong integrated signal pattern over almost the complete tissue section. Accordingly, cases with lower viral load revealed less diffuse stained areas.

The clonal aspect of MCPyV integration in the patients with multiple lesions in the punctate group (\( n = 8 \)) was reflected by similar relative MCPyV presence in five patients (Table 1): Patients 8 (2/3 cores), 12, 17, 20 (3/4 cores) and 26.

**Correlation of the MCPyV-FISH with MCPyV qPCR**

The threshold was set at the highest qPCR value in the negative FISH group at 0.26, after the Grubb’s test determined the qPCR value 0.5 for sample 24 as outlier. Based on this cutoff, 83% of the MCPyV-FISH-positive cores correlated with the qPCR data and 93% of the negative cores in Group “0” (Fig. 4b, table). On patient basis, copy numbers of 30 cores from 23 patients (76.7%) correlated in the positive group and nine cores from nine (100%) patients in the negative FISH group (Table 1). From three patients (nos. 10; 17; 25) with each, a FISH-positive and a FISH-negative core, the cores of two patients (nos. 10 and 25) correlated with their according copy number (Table 1).
Figure 3. The mixed pattern is associated with VP1 staining. MCPyV-FISH on samples of Patient 30 performed without (a and b), with DNase I (c and d) or RNase A (e and f) treatment revealed that the diffuse (b, circle) MCPyV signals disappear after DNase I (d) treatment. After RNase treatment (f), the signals did not change in number per nucleus or fluorescence intensity confirming the specificity of the MCPyV-FISH probe to DNA hybridization (see circles exemplarily). (a), (c) and (e) The according nuclei were stained with DAPI (blue). (g–i) The same case was stained with VP1. (g) DAPI shows the nuclei. (h) VP1 expression in green. (i) Overlay. (j–l) VP1 staining of Case 29 with a punctate FISH-pattern. (j) DAPI staining, (k) VP1 staining green. (l) The overlay did not reveal any signal for VP1 expression. 10-μm scale bar.
No significant correlations were found of MCPyV status, either by FISH or by qPCR data, with clinical data as age, gender, tumor grade or survival (data not shown).

**Immunofluorescence staining VP1**

The VP1 staining on a case with punctate pattern did not lead to a signal (Figs. 3j–3l). In contrast, in Patient 30 with a mixed pattern and a viral load of 4,485 copies per genome VP1 was expressed which is shown by the positive staining in Figures 3g–3i.

**Discussion**

In the current study, we visualized MCPyV-DNA by FISH on FFPE MCC specimens and correlated these results to the qPCR MCPyV-DNA detection of the corresponding tissues. The MKL-1 cell line, which contains integrated MCPyV-DNA, served as positive control for the MCPyV-FISH. For the evaluation of the MCPyV-FISH, we applied the criteria which were established by Hafkamp et al. for HPV 16. Indeed, the punctate pattern of the specific MCPyV-FISH in MKL-1 cells were in high agreement with those described for HPV 16-FISH signal pattern for integrated viral DNA. This enabled us to confirm MCPyV-integration in MKL-1 cells morphologically by FISH analysis.

In addition, we assessed MCPyV-specificity by DNase I treatment. No specific MCPyV-hybridization signals were detected after DNase I treatment. In contrast, RNase A treatment altered the MCPyV-specific FISH signals only marginally, which possibly indicates that a minimal portion of the FISH signal is due to the hybridization of the MCPyV-probe to MCPyV-RNA.

Analysis of the 62 TMA cores by MCPyV-specific FISH revealed concordant positivity in 72% of the patients, which is in accordance with the frequency of MCPyV detection in MCC by PCR as reported in the literature; e.g. by Kassem et al. (77%), Busam et al. (75%), Waltari et al. (77%) and Schrama et al. (86%). In addition to the information on the mere presence of MCPyV within the tumor tissue, MCPyV-FISH also provided data about the quality of the MCPyV presence. In 57% of all MCCs, we identified a punctate pattern with equal intensity in different tumor types from primary tumor and local recurrences to lymph node metastases and distant metastases of the same patient. Moreover, the clonal aspect of the integration reported by Feng et al. was underlined by the detection of the punctate pattern with equal intensity in different tumor types from primary tumor and local recurrences to lymph node metastases and distant metastases of the same patient (Supporting Figure 4).
Information Fig. S11–L). Similarly, the finding of a punctate pattern in two patients from which only metastases were on the TMA was as well in line with clonal integration.

Of interest, the punctate MCPyV-FISH hybridization pattern was found to be associated with moderate qPCR values. This has also recently been demonstrated by Sastre-Garau et al. on cryoconserved tumor tissue of one MCC patient revealing distinct signals by FISH. 26

The qPCR is highly sensitive as extremely low values starting from 0.0009 relative MCPyV presence were detected, and, in only six MCC cases, MCPyV-DNA was present below this detection limit. In few cases, however, positive qPCR values did not correlate with the FISH evaluation. This might be explained by the fact that FISH was performed on TMA cores whereas the qPCR represented the relative MCPyV presence from whole tissue sections. Therefore, the high concordance of 83% for all MCPyV-FISH-positive cores and 93% for the negative cores is remarkable. Indeed, in comparison to similar studies investigating papillomaviruses (HPV)21,33 the concordance in the here presented study was remarkable high; thus, the TMA screening represented a very good approximation of the situation in the whole tissue section. For example Ho et al. reported 75.2% concordance between positive HPV-FISH and HPV-E6 type-specific PCR results analyzing cervical swabs; vice versa, there was an 82% concordance in HPV E6 type-specific qPCR for HPV-negative FISH cases.21

Besides a few discordant results between FISH and qPCR, in our series, there were also three patients with different FISH signal patterns within different localizations, e.g. a negative primary tumor and a punctate pattern in the metastasis. Similar observations have previously been reported for HPV-FISH studies on cervical cancer.34–36 Possible reasons for the observed heterogeneity of tumor lesions of an individual patient could be due to tissue quality e.g. age of the tissue, fixation techniques etc. In addition, since some MCPyV-positive tissues contained negative parts observed on whole tissue section as well, a casual cutout of a negative tissue part is a conceivable risk for artifacts. For the qPCR data, some variation in relative MCPyV presence was also observable between different samples of the same patients, e.g. for Patients 1, 14 and 27. However, since qPCR was performed on whole sections, different tumor cell percentages on the respective sections can contribute to these differences.

Interestingly, in seven samples, we observed a punctate hybridization pattern combined with a diffuse hybridization pattern in some areas associated with a high, up to 4,485 copies, viral load. In parallel to this finding, Sastre-Garau et al.26 observed a complete LT sequence in a case associated with 62.2 copies. This was the highest viral load the authors detected out of 10 cases with a range from 0.6 to 10.3 in the other nine cases. The authors interpreted this as episomal presence. This is sustained by Carbone et al. who detected high copy numbers in the range of 500–1,000 per cell linked to the episomal presence of SV-40 in the S-HM cell line.27 In addition to our observation, the diffuse FISH pattern observed in hr HPV-associated carcinomas is well known to be associated with the episomal presence.19–22 We conclude from these reports and our own observations that the diffuse pattern indicates most likely the episomal presence of MCPyV and that we then would see in consequence in the mixed pattern a coexistence of the integrated and episomal form.

Moreover, Harms et al. reported the detection of VP1 mRNA expression in some of MCPyV-positive tumors.38 In the case with the highest viral load (4,485 copies per genome), we also observed a positive VP1 staining. In this respect, it should be mentioned that the VP1 expression does not per se indicate the episomal presence as this could also derive from intact integrated VP1. Nevertheless, our observations suggest that in MCPyV-positive MCC tumor cells a secondary infection by MCPyV can occur. This relationship is also known in SV40 transformed mesothelial cell lines (S-HML). The episomal existence of SV40 virus was demonstrated by Fahrbach et al.39 The recombination might also contribute to the high anti-VP1 antibody titers in MCC patients. In such cases, MCPyV-FISH can be especially helpful to distinguish between a mere infection or an integrated status of MCPyV. In the latter case, a punctate pattern should be observable at least in some of the cells. For example Patient 28 had one metastasis with a punctate pattern and three additional metastases with a mixed pattern. Sequence analysis of the MCPyV LT region demonstrated the same MCC characteristic6 premature stop codon mutation deleting the helicase domain in all four tumors (data not shown) supporting the presence of clonal integrated MCPyV in all metastases. The wild type sequences were not detected what was possibly due to limits by isolating episomal DNA out of FFPE tissues, thus providing not sufficient quality for subsequent sequencing unless the copy number is extremely high as in Case 30. Interestingly, the samples with 1,947 and 52 copies were obtained on the same day, which was already the second surgery. The metastases with 6.3 copies in the punctuated pattern and 29.9 copies in the mixed pattern were collected in the same year at a later time point on the same day. One year later, two other metastases were resected at different time points but were not available for our study. Supposed, tumor progression would be linked to the change of the physical virus status from the episomal to the integrated form as it is known from hr HPV in cervical cancer,39 then this observation would fit to the ongoing metastases growth as the copy number decreased and the pattern became punctuated. Unfortunately, the primary tumor of the respective patient was not available for further analyses. Interestingly, especially in Patient 30, we observed extremely large signal dots among the mixed pattern (Fig. 2f). This case had the highest MCPyV presence detected by qPCR and we were not able to identify a stop codon in the LT region by Sanger sequencing. Thus, pointing to wild type LT and an episomal presence seems reasonable. Recently, Wang et al. presented a
replication system in C33A cells cotransfected with pcDNA4C-MCV Ori and pcDNA4C-MCV LT encoding the full length LT. The replication was verified by BrdU labeling at the LT loci. Under the same experimental condition, the FISH analysis gives an accumulation pattern in the nuclei at Brd4. Thus, possibly indicating that in real situations episomal replicating MCPyV-DNA accumulate in foci and, therefore, very big FISH signals could be observed. This could possibly explain the enormous signals detected in this case.

In summary, two different MCPyV-positive FISH hybridization patterns could be distinguished in MCC. A punctate pattern with single punctate signals of comparable fluorescence intensities indicating MCPyV integration and this mixed with a diffuse pattern, containing a high number of tiny and partially overlapping signals, with much higher fluorescence signals in some tumor areas. The latter suggests the coexistence of both, integrated and episomal presence of the virus in some cases. The punctate pattern was associated with moderate qPCR data whereas the mixed pattern demonstrated high qPCR values for MCPyV presence supporting the additional episomal presence. Indeed, there was a strong correlation between MCPyV-FISH and the relative MCPyV presence detected by qPCR. Thus, while presence of MCPyV can be verified by qPCR, the quality of the presence can be visualized by MCPyV-specific FISH analysis. In this regard, MCPyV qPCR and MCPyV-FISH are important complementary tools to gain maximum biological information of the presence of MCPyV in MCC, but also other tumor entities, and thus to further elucidate MCPyV-related carcinogenesis. By correlation with clinical data, the physical status of MCPyV could provide further knowledge if tumor progression is accompanied by episomal presence to integration, which can be an additional tool in tumor diagnostic, prognosis or treatment.

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