Merkel Cell Carcinomas arising in autoimmune disease affected patients treated with biologic drugs, including anti-TNF.

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Statement of translational relevance

In this investigation, three new Merkel cell carcinomas, which arose in patients with rheumatologic diseases treated with biologic drugs were characterized. A possible cause-effect relationship between pharmacological immunosuppressive treatment and MCC onset is plausible. Indeed, the association between MCC and MCPyV infection is well established. It has been reported that biologic drug treatment allows latent viruses to bereactivated. Active viruses, in turn, may exert their tumorigenic potential through viral oncoproteins, such as MCPyV LT/ST. Clinicians/Oncologists should take the possible reactivation of latent viruses with oncogenic potential, such as MCPyV, into consideration. Specific attention should be given to patients treated with biologic drugs that impair immunologic activities. MCPyV reactivation can be detected by determining the presence and titer of IgG antibodies against its antigens. In the case of virus reactivation, treatment with biologic drugs should be withdrawn, thus reducing the risk of MCC onset.
ABSTRACT

Purpose:
The purpose of this investigation was to characterize Merkel cell carcinomas (MCC) arisen in patients affected by auto-immune diseases and treated with biologic drugs.

Experimental Design:
Serum samples from MCC patients were analyzed for the presence and titer of antibodies against antigens of the oncogenic Merkel cell polyomavirus (MCPyV). IgG antibodies against the viral oncoproteins Large T (LT) and small t (ST) antigens and the viral capsid protein 1 were analyzed by indirect E.I.S.As. Viral antigens were recombinant LT/ST and virus-like particles (VLPs), respectively. MCPyV DNA sequences were studied using PCR methods in MCC tissues and in peripheral blood mononuclear cells (PBMCs). Immunohistochemical analyses were carried out in MCC tissues to reveal MCPyV LT oncoprotein.

Results:
MCPyV DNA sequences identified in MCC tissues showed 100% homology with the European MKL-1 strain. PBMCs from patients tested MCPyV-negative. Viral DNA loads in the three MCC tissues were in the 0.1-30 copy/cell range. IgG antibodies against LT/ST were detected in patients 1 and 3, whereas patient 2 did not react to the MCPyV LT/ST antigen. Sera from the three MCC patients contained IgG antibodies against MCPyV VP1. MCC tissues tested MCPyV LT-antigen positive in HIC assays, with strong LT expression with diffuse nuclear localization. Normal tissues tested MCPyV LT-negative when employed as control.

Conclusions:
We investigated 3 new MCCs in patients affected by rheumatologic diseases treated with biologic drugs, incuding TNF. A possible cause-effect relationship between pharmacological
immunosuppressive treatment and MCC onset is suggested. Indeed, MCC is associated with MCPyV LT oncoprotein activity.

Introduction

Merkel cell carcinoma (MCC) is a rare but aggressive neuroendocrine tumor (1). Annual incidence is estimated at about 2.4-3/million/year in Europe (2) and the USA (3), respectively, with increased frequency over the last 20 years.

MCC arises more frequently in elderly people, white subjects of both genders, predominantly in sun-exposed areas, with the average onset age at presentation of 69 years old (4). Its high mortality rate may be partially explained by the advanced onset age, which results in a decrease in immune function, closely related to aging (5). Iatrogenic immunosuppression has been demonstrated to be a risk factor for MCC development: immunosuppressed individuals, such as those affected by AIDS, oncologic or hematological diseases and organ transplant recipients (OTRs), represent approximately 10% of MCC affected patients (4). These MCC immunosuppressed patients have poorer prognoses (6). The role of immunosuppressive therapy in MCC pathogenesis is also suggested by reports of partial spontaneous regression of metastatic MCC after treatment discontinuation (7).

DNA sequences of Merkel cell polyomavirus (MCPyV) were identified in MCC (8). Then, MCPyV sequences were detected in MCC in up to 80% of cases, whereas Ig antibodies against MCPyV were revealed in approximately 80% of healthy subjects and MCC affected patients. These results indicate that MCPyV, which is potentially oncogenic, is a ubiquitous human virus (9).

The introduction of biologic disease modifying anti-rheumatic drugs (bDMARDs) in treating some rheumatic diseases, such as rheumatoid arthritis and spondyloarthritis, has completely changed the management and outcome of these disorders. Their efficacy has been widely demonstrated in several randomized clinical trials and observational registries. These
drugs are able to induce significant clinical improvement, often remission, and may prevent functional disability, as well as slowing or halting radiographic damage progression (10,11). Their safety profile has globally been demonstrated as satisfactory with a favorable risk/benefit ratio. However, more information about long term toxicity and harmfulness is necessary to assist clinicians in decision making and follow up. Some reviews have reported on this topic (10,12) comparing the adverse events of tumor necrosis factor (TNF) blockers, interleukin (IL)-1 antagonist, IL-6 antagonist, anti-CD28 and anti-B cell monoclonal antibodies in the treatment of different rheumatic diseases. Overall, the difference between cancer rates was not statistically significant between patients treated with biologics and the control group not exposed to biologics, with conflicting results for non-melanoma skin cancer (NMSC). In UV exposed patients, a significantly increased risk of NMSC was reported by Wolfe and colleagues (13): (RR 1.5, 95% CI 1.2-1.8), in the ARTIS registry (14), in particular in the first year of treatment (RR 2.1, 95% CI 0.8-5.1) and in recent systematic reviews and meta-analysis (12,15). Conversely, a non-significant increased risk was found in the CORRONA (http://www.rheumatology.org/; 1.83, 95% CI 0.85-3.93) and in the BSRBR registries (http://www.rheumatology.org.uk/; HR 1.7, 95% CI 0.9-3.4). Whereas, a study based on the BIOBADASER registry (16) failed to demonstrate an increased NMSC rate in the population treated with TNF inhibitor (TNFi). Previous skin cancer history, ethnic background and solar damage are some of the possible factors that could justify the difference in these results. Furthermore, a different risk profile for malignancies according to the specific autoimmune disease and the biologic drug employed in the treatment should be considered. The most common skin neoplasms related to anti-TNF therapy are basal and squamous cell carcinomas, while cutaneous lymphomas are rarely reported. However, some studies have described cases of patients with rheumatic disease treated with biologic drugs who have developed Merkel cell carcinoma (MCC) (16-18).
Materials and Methods

MCC cases

Three MCC cases, 2 in patients affected by rheumatoid arthritis and 1 in a patient with ankylosing spondylitis, all exposed to biologic drugs, mainly anti-TNF, were studied in detail for clinical features and characterization of MCPyV sequences, antibodies and viral proteins in their peripheral blood mononuclear cell (PBMC) and MCC specimens. Details of cases are reported in the Supplementary data. Written informed consent was obtained from patients at the time of Hospital admission. The study was approved by the Ethic Committee, Ferrara.

MCC and PBMC DNA extraction

Total DNA was extracted from Merkel Cell Carcinoma (MCC), formalin fixed paraffin-embedded (FFPE) specimens, belonging to patients (n=3), using QIAmp DNA FFPE Tissue extraction Kit (Qiagen, Milan, Italy), as described (19). Total DNA was extracted from PBMCs belonging to patients (n=3) using QIAmp DNA Blood Mini Kit (Qiagen, Milan, Italy), as described (20). After DNA extraction, each sample was quantified by the spectrophotometric reading (NanoDrop 2000, Thermo Scientific, Milan, Italy). DNA was stored at -80°C until the time of the analysis. DNA was evaluated for its polymerase chain reaction (PCR) suitability by amplyfing the β-globin gene sequences (21).

MCPyV DNA detection and sequence analysis

In order to verify the presence of Merkel Cell Polyomavirus (MCPyV) DNA sequences, DNA from MCC and PBMC samples were analyzed by qualitative PCR with
MCPyLT1709.F and MCPyLT1846.R primer set, which generate an amplicon of 138 bp (20). The forward and reverse primers are located at nucleotide position 1709-1846, based on the GenBank sequence EU375803. The pUC57MC1 recombinant plasmid carrying DNA of the MCC 350 strain was used as a positive control (8). This recombinant plasmid was a kind gift from Dr. Allander, Karolinska Institutet, Stockholm, Sweden. Amplified MCPyV PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Milan, Italy) according to the manufacturer’s instructions. MCPyV genotype was identified by direct sequence analysis. PCR amplicons were sequenced with automated ABI Prism 3730xl Genetic Analyser (Applied Biosystems, Monza, Italy). The resulting MCPyV DNA sequences were BLAST vs. MCPyV DNA belonging to different viral strains present in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Specifically, MCPyV DNA sequences were aligned against the reference sequences of the North America (MCC350, EU375803.1; MCC339, EU375804.1), Japan (TKS, FJ 464337), Sweden, France and Italy MCPyV isolates (MKL-1, FJ173815) (22,23).

MCPyV DNA load quantification

Specific quantitative real-time PCR assays, using TaqMan chemistry, were performed using the CFX96 Touch™ Real-Time PCR Detection System (Biorad, Segrate, Milan, Italy) for MCPyV DNA load quantification. Recombinant plasmid pMCPyVLT.1, which contains 258 bp of the large T antigen (24) coding sequence (FJ472933), was used as positive control (25). Standard calibration curves were generated using 10-fold dilutions, from 10¹ to 10⁷ copies, of pMCPyVLT.1 (20). Cellular RNase P gene was used to determine the human cell equivalents of each sample under analysis (26). Samples and controls were analyzed in
duplicate. The sensitivity of the method was 10 viral copies. Viral DNA loads are reported as MCPyV DNA copy/cell equivalent.

**Immunohistochemical analysis**

Immunohistochemical (IHC) analysis was performed on the three MCC FFPE samples. IHC staining was performed by using the Multimeric Detection Kit (Universal DAB Detection Kit Ultraview, Roche Tissue Diagnostics [CH]), on a BenchMark XT immunostainer (Roche T. D.) (21). FFPE slices, 4 µm thick, were stained with mouse monoclonal antibody against the MCPyV LT-antigen (CM2B4) (Santa Cruz Biotechnology, Santa Cruz, CA) (dilution, 1:50). Staining intensity and its distribution were assessed by the pathologists of our working group. Staining was graded as negative (no staining) and as weak, moderate, or strong intensity.

**Detection of serum IgG antibodies against MCPyV VP1 protein and LT/ST antigens**

Serum antibodies against MCPyV viral protein 1 (VP 1), large T and small t antigens (LT/ST) (27), were detected by enzyme-linked immunosorbent assay (ELISA) using MCPyV virus-like particles (VLPs) and the recombinant LT/ST. VLPs and LT/ST were generated and purified as previously described (27,28). Detection of VP1 and LT/ST antibodies by indirect ELISA was performed as previously described (27,28).
Results

MCPyV genome analysis

MCC (n=3) and PBMC (n=3) samples of patients affected by autoimmune diseases, were analyzed by qualitative PCR for the presence of MCPyV DNA, targeting MCPyV LT gene coding sequences. In a previous study, we detected MCPyV DNA sequences in 22% of PBMCs from healthy subjects (20), whereas other groups found MCPyV sequences in PBMCs from MCC patients (32). Herein, MCPyV DNA was detected in all 3 MCC samples, while none of the 3 PBMCs analyzed showed MCPyV DNA sequences (Table 1).

The viral DNA load from the three MCPyV-positive MCC samples was evaluated by specific real-time quantitative PCR (qPCR) analysis (Table 2). In the MCC sample from patient 1, the mean MCPyV DNA load was 29.89 copy/cell (range: 18.27 copy/cell to 42.68 copy/cell). In the MCC sample from patient 2, the mean MCPyV DNA load was 0.1 copy/cell (range: 0.05 copy/cell to 0.15 copy/cell). In the MCC sample from patient 3, the mean MCPyV DNA load was 1.5 copy/cell (range: 0.7 copy/cell to 2.37 copy/cell), (Table 2).

In order to check PCR amplicons specificity as obtained in the three MCC samples from patients, the amplified products were subjected to DNA sequencing (Fig. 1). DNA sequence data were compared to the reference sequence, shown in Fig. 1, A, of the North American (MCC339 strain, gene bank EU375804.1; MCC350 strain, gene bank EU375803.1), European (Sweden, France and Italy) (MKL-1 strain, gene bank FJ173815) and Japanese MCPyV isolates (TKS strain, gene bank FJ464337). DNA analysis indicated that MCPyV LT coding sequences, were identified as belonging to the MCPyV genome in all three MCC samples. Specifically, DNA analysis indicated that MCPyV LT sequences were 100% homologous to the European strain MCPyV MKL-1 in all three MCC samples (fig. 1, B), as shown before (16).
MCPyV LT-antigen analysis by IHC staining

In order to evaluate MCPyV LT-antigen protein expression, IHC staining on MCC tissue samples from patients (n=3) was carried out. MCPyV LT-antigen staining was present in all tissue sections. Specifically, MCPyV LT-antigen was strongly expressed with diffuse nuclear localization (Fig.2, panels A-C) in MCC tissue samples from patients. In the corresponding normal tissues, MCPyV LT-antigen expression was not detected (Fig. 2, panel D).

MCPyV antibodies detection by indirect ELISA

Indirect ELISA was carried out in order to determine the presence of serum antibodies against MCPyV in MCC patients. Sera from the three patients, who were found to be MCPyV-positive, were analyzed for the presence of IgG antibodies against MCPyV VP1 and LT/ST antigens. Anti-MCPyV VP1 antibodies were detected in all patients, whereas anti-MCPyV LT/ST antibodies were detected in patients 1 and 3. Serum from patient 2 failed to react with the MCPyV LT/ST antigen. Serum samples were diluted from 1:100 to 1:2,048,000 (anti-MCPyV VP1) and from 1:100 to 1:17,714,700 (anti-MCPyV LT/ST) in order to determine antibody titers. Patient 1 presents anti-MCPyV VP1 and LT/ST end point titers of 1:512,000 (optical density, OD: 0.218) and 1:900 (OD: 0.207), respectively; the patient 2 has an anti-MCPyV VP1 end point titer of 1:800 (OD: 0.201); patient 3 presents anti-MCPyV VP1 and LT/ST end point titers of 1:128,000 (OD: 0.22) and 1:300 (OD: 0.246), respectively.
Discussion

In this report, we characterized 3 new MCC cases in patients affected by rheumatologic diseases, all of whom had been exposed to biologic drugs, where a possible cause-effect relationship between the pharmacological immunosuppressive treatment and cancer arisen could be hypothesized. It has been established that, MCC onset/progression is linked to MCPyV and viral LT gene expression promotes tumor onset/progression (8,29).

In this study, MCPyV LT DNA sequences were detected in all three MCC samples. Our results are in agreement with previous works where approximately 80% of MCCs are associated with MCPyV (8,29), suggesting that this virus is likely to be the etiology agent of MCC (8,29). We detected a viral DNA copy/cell ratio within a 0.06-1.2 range, which is sufficient to contribute to MCC (30). Molecular analysis of MCPyV LT DNA sequences from the three cases showed high homology with previously published MCPyV sequences obtained from MCC specimens. Specifically, we identified the MKL-1 strain, suggesting that this strain is the main MPCyV isolate in Europe (20,31). Strong MCPyV LT antigen (LTA) expression in immunohistochemistry, with diffuse nuclear localization, was evaluated in the three MCC tissues. These findings support the thesis that MCPyV has an essential role in MCC-genesis (30). It is interesting to note that the corresponding PBMCs from the same patients tested negative for MCPyV DNA. In this context, it should be recalled that MCPyV DNA in PBMCs, when revealed, is present at low copy number in patients affected by MCC (32), as well as in healthy subjects (20).

We investigated the presence of serum antibodies against MCPyV in samples of the same three MCC patients. Anti-MCPyV VP1 antibodies were detected in all three patients, whereas anti-MCPyV LT/ST antibodies were detected in patients 1 and 3. It could be speculated that the absence of LT/ST antibodies in patient 2 is due to the small tumor size.
Previous serologic studies have shown that anti-MCPyV antibodies are present in higher proportions in MCC patients compared to controls (33,34). Moreover, MCC patients with high MCPyV antibody titers may present better progression-free survival (35).

Anti-MCPyV antibodies were detected in up to 80% of adults and from 20% to 43% of 1 to 5-years old children. These data suggest that MCPyV infects a large proportion of the population, where it induces a physiological immune response (28,33,34).

The three MCC patients were pharmacologically treated with immunosuppressive biological drugs due to being affected by rheumatologic diseases. In general, about 10% of MCC cases are detected in immunosuppressed individuals (36), whereas it is well known that patients with impaired immune functions are more at risk of developing virus-related diseases (37). For instance, immunosuppression induced by HIV/AIDS in HIV-positive individuals, increases the risk of developing MCC compared to HIV-negative individuals (38). It is well established that the immune system counteracts MCPyV infection and associated MCC development. Indeed, MCC regression occurs when immune suppression therapy is suspended/withdrawn (39).

It is also known that pharmacological therapies using immunosuppressive agents to prevent organ rejection result in immunodeficiency inducing skin cancer (40), non-melanoma skin cancer (41) and MCC (42).

Reactivation of other human polyomaviruses (HPyVs) by pharmacological immunosuppressants has been observed in cases of progressive multifocal leukoencephalopathy (PML) (43), due to polyomavirus JC (JCPyV) reactivation during treatment with natalizumab and rituximab for multiple sclerosis (44) and in acute interstitial nephritis and hemorrhagic cystitis development in stem cell transplant recipients caused by BK polyomavirus (BKPyV) (45). All these viruses are acquired during childhood (46) and the development of an immunocompromised state can result in active replication of viruses
and disease occurrence. According to the current evidence, screening for polyomavirus infections should be considered prior to initiating treatment with biologics.

Pharmacological immunosuppressive therapies also represent an important additional risk factor for MCC development (16-18) when biologic drugs are employed in managing rheumatologic disorders. Other immunosuppressive drugs used in the treatment of acquired factor VIII inhibitor (47), B-cell chronic lymphocytic leukemia (48), relapsing follicular and chronic lymphocytic lymphomas (49,50), have been found to be associated with MCC.

To the best of our knowledge, in the current literature there are only anecdotal reports of MCC occurrence in patients treated with biologic drugs, for a total number of 7 patients: 4 patients treated with Rituximab, respectively, for acquired factor VIII inhibitor (47), B-cell chronic lymphocytic leukemia (48), relapsing follicular lymphoma (49) and chronic lymphocytic lymphoma (50). The remaining 3 cases are of patients affected by rheumatic diseases, one with long-standing rheumatoid arthritis treated with adalimumab (16), one with etanercept (18) and the last one affected by psoriatic arthritis treated with etanercept (17).

In this study, patient 1 represents the first case of MCC that appeared in a patient treated with abatacept after 5 months of therapy. It is noteworthy that, in this patient there was previous exposure to two anti-TNFα drugs (etanercept 10 months and adalimumab 8 months) and to non biologic-DMARDs; therefore the possible consequences of antecedent exposure to other immunosuppressive drugs cannot be ruled out. In patient 2, MCC was diagnosed after 5 years of infliximab therapy; it rapidly grew after the last infusion and then quickly stopped after the withdrawal of the biological drug. Patient 3 developed MCC after 42 months of etanercept therapy.

In our case, the lag time between initial exposure to a biologic (anti-TNF in all case) and MCC occurrence was rather wide, ranging from 23 to 60 months, compared to the 18-24 months reported by others (16-18). Advanced age in patients affected by MCC has been
reported as a risk factor for the onset/progression of this tumor (4). Indeed, in our study, patients 1 and 3 were 70 years old and patient 2 was 56 years old. Previously, the highest incidence of MCC had been observed in people above 65 years of age (3). However, in previous reports of patients affected by rheumatic diseases in whom an MCC did occur after exposition to anti-TNF, only 1 out of 3, was over 70 (16-18). Nevertheless, advanced age combined with impaired immune function in the elderly seems to be an important risk factor for MCPyV-related MCC (39).

Conclusion

In conclusion, immunosuppression due to biologic drug therapies, chiefly anti-TNF, could represent a risk factor for MCC development in rheumatic patients. Our study data, along with previously reported results, may indicate that pharmacological immunosuppressive therapy with biologics could modify the immune response to MCPyV thus undermining the outcome for some who resulted positive for MCPyV, and increasing the probability of MCC development. In as much as biologic DMARDs are potent immunosuppressive agents, their use in managing rheumatologic disorders may enhance susceptibility to MCPyV infections, as well as, reducing host anti-tumoral immune response, thus inducing cancer development. Whether the reintroduction of non-biologic immunosuppressants could be a safe option and whether an alternative biologic with different mechanisms could be considered after an adequate period free from cancer still remains an unanswered question. More data, along with better knowledge of MCC pathogenesis, are needed.
Acknowledgments

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References


Table 1. MCPyV screening in MCCs and PBMC samples by qualitative PCR and real-time quantitative PCR (qPCR) analysis.

<table>
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<tr>
<th>Patient</th>
<th>PCR MCC</th>
<th>PBMCs</th>
<th>qPCR MCC</th>
<th>PBMCs</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
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<td>+</td>
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<tr>
<td>3</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>MCPyV positive</td>
<td>3/3</td>
<td>0/3</td>
<td>3/3</td>
<td>0/3</td>
</tr>
</tbody>
</table>
Table 2. Mean MCPyV DNA load in MCPyV positive MCCs revealed by real-time quantitative PCR (qPCR) analysis. Viral DNA loads are reported as MCPyV DNA copy/cell equivalent.

<table>
<thead>
<tr>
<th>Patient</th>
<th>MCPyV DNA Load (copy/cell)</th>
<th>Range (copy/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$2.989 \times 10^1$</td>
<td>$1.827 \times 10^1$ – $4.268 \times 10^1$</td>
</tr>
<tr>
<td>2</td>
<td>$1.031 \times 10^{-1}$</td>
<td>$5.054 \times 10^{-2}$ – $1.483 \times 10^{-1}$</td>
</tr>
<tr>
<td>3</td>
<td>$0.153 \times 10^1$</td>
<td>$7.0192 \times 10^{-1}$ – $0.237 \times 10^1$</td>
</tr>
<tr>
<td>Mean MCPyV DNA load</td>
<td>$1.05 \times 10^1$</td>
<td>$0.634 \times 10^{-1}$ – $1.566 \times 10^1$</td>
</tr>
</tbody>
</table>
Figures’ legends

Figure 1.

MCPyV sequence analysis. A, alignment of MCPyV LT sequences of 4 MCPyV genotype strains, MCC339 [GenBank, accession no. EU375804.1], MCC350 [GenBank, accession no. EU375803.1], MKL-1 [GenBank, accession no. FJ 464337], [GenBank, accession no. FJ173815], and TKS [GenBank, accession no. FJ 464337] are indicated. Nucleotide substitutions in MCPyV strains are numbered and marked in grey. B, alignment of MCPyV LT sequences identified in MCC sample from patients 1, 2 and 3. Nucleotide substitutions in MCPyV strains are marked in grey. The sequence homology of MCPyV genotypes was compared with the BLAST using flat master-slave with identities of the National Centre for Biotechnology Information (NCBI).

Figure 2.

Immunohistochemical analysis of MCPyV large T-antigen (LT) in MCC and normal tissues. Immunohistochemical analysis with anti-MCPyV large T (LT) monoclonal antibody confirmed the elevated nuclear expression of this viral oncoprotein LT in MCC tissues from patients 1 (A), 2 (B) and 3 (C); the control represented by the normal epidermis (D), from patient 3, does not demonstrate immunoreactivity.
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