Seroreactivity against Merkel cell polyomavirus and other polyomaviruses in chronic lymphocytic leukaemia, the MCC-Spain study

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Merkel cell polyomavirus (MCPyV) has been suspected to cause chronic lymphocytic leukaemia (CLL) but previous data are inconsistent. We measured seroreactivities of nine polyomaviruses (MCPyV, BKPyV, JCPyV, LPyV, KIPyV, WUPyV, HPyV-6, HPyV-7 and TSPyV) in 359 CLL cases and 370 controls using bead-based multiplex serology technology. We additionally tested two herpesviruses (HSV-1 and CMV). Associations between disease and viral seroreactivities were assessed using logistic regression. All human viruses showed high seroprevalences (69–99 %) against structural proteins in controls but significantly lower viral seroprevalences in cases (58–94 %; OR range = 0.21–0.70, P value <0.05), except for MCPyV
Chronic lymphocytic leukaemia (CLL) is one of the most common B-cell malignancies in Europe with an incidence rate around five cases per 100000 (Sant et al., 2010). It shows an average 2:1 male/female ratio and a higher frequency in the elderly, with a median age of 65–72 years at CLL diagnosis (Montserrat & Moreno, 2008). An increased risk of CLL is consistently observed among those with family history of haematological malignancies. Other suspected risk factors include long-term use of pesticides and regular use of hair dyes (Benavente et al., 2005; Cocco et al., 2013; Montserrat & Moreno, 2008; Slager et al., 2014). However, its aetiology remains unknown and infections have been suggested as potential candidates.

Recently, Merkel cell polyomavirus (MCPyV) has been identified as an oncogenic virus responsible for Merkel cell carcinoma, a rare skin tumour. Interestingly, Merkel cell carcinoma incidence has been reported to be higher among CLL patients and vice versa (Koljonen et al., 2009; Teman et al., 2012; Cimino et al., 2013) do not support an aetiological role. However, the presence of a truncated LT-Ag MCPyV sequence in the nucleus, a peculiarity of MCPyV logical role. However, the presence of a truncated LT-Ag detected in tumour tissue or peripheral blood mononuclear cells of CLL subjects (Shuda et al., 2009; Toracchio et al., 2009; Teman et al., 2011; Comar et al., 2012; Imajoh et al., 2012; Cimino et al., 2013) do not support an aetiological role. However, the presence of a truncated LT-Ag MCPyV sequence in the nucleus, a peculiarity of MCPyV in Merkel cell carcinoma cells, has been reported in highly purified CD19+CD5+ CLL cells (Haugg et al., 2011; Pantulu et al., 2010). Serology data are furthermore inconsistent; in a previous case-control study we identified an increased MCPyV seroprevalence in CLL patients (Robles et al., 2012) whereas in a recent paper a decreased risk was observed (Teras et al., 2015), although none of them significant. The latter paper also observed null associations for seven additional polyomaviruses and subsequent risk of CLL, although potential concerns in sample size were raised.

CLL cases are often diagnosed with hypogammaglobulinemia (Dearden, 2008; Hamblin & Hamblin, 2008), a decline in total immunoglobulin G (IgG) levels that could be related to a general poor antibody response. It is, however, unclear whether hypogammaglobulinemia relates to progression of disease or if it is already present at early stages.

Within the context of a large multicentric case-control study in Spain, we explored the potential association between polyomaviruses and CLL at different stages of disease using serology data.

CLL cases were recruited within the MCC-Spain study (www.mccspain.org), an epidemiological population-based multicase-control study (Castaño-Vinyals et al., 2015), in collaboration with the International Cancer Genome Consortium on Chronic Lymphocytic Leukaemia Project (ICGC-CLL; www.cllgenome.es), previously described elsewhere (International Cancer Genome Consortium et al., 2010; Puente et al., 2011).

For the present study, a case-control study frequency-matched by sex, region and age at interview (±5 years) by random selection was performed. Eligible subjects included incident and prevalent CLL cases and population based controls with no prior history of lymphoproliferative disorders, recruited between 2010 and July 2012 from seven centres in Asturias, Barcelona, Cantabria and Granada regions. The final study population included 370 controls and 359 CLL cases (by disease stage: 204 Rai 0, 72 Rai I-IV untreated, 73 Rai I-IV treated and 10 unclassifiable subjects). Ethical approval from each participating centre (Central IRB# IORG0003220, study code 2008/3123/I) and subject informed consents were obtained.

Seroreactivities against the following viral proteins were determined. Capsid protein VP1 was measured for all human polyomavirus (MCPyV, BKPyV, JCPyV, KIPyV, WUPyV, HPyV-6, HPyV-7 and trichodysplasia spinulosa-associated polyomavirus; TSPyV). Additionally, the polyomavirus oncoprotein LT-Ag for MCPyV, JCPyV and TSPyV, as well as small T-antigen (sT-Ag) for MCPyV were measured. Seroreactivity against African green monkey lymphotropic polyomavirus (LPyV) VP1, closely related and highly cross-reactive with HPyV-9 (Nicol et al., 2012), was determined. We also measured the structural proteins glycoprotein B (gB) for herpes simplex virus 1 (HSV-1) and pp150 (also known as UL32) for cytomegalovirus (CMV) within the herpesvirus family as a reference of immunological behaviour of common latent or persistent infections, like polyomaviruses, and known to potentially reactivate among CLL subjects.

Measurement was performed using multiplex serology, a glutathione S-transferase capture immunosorbent assay combined with fluorescent-bead technology, as described elsewhere (Kjaerheim et al., 2007; Waterboer et al., 2005). Bead sorts, each carrying a different antigen, were mixed and incubated with human sera at 1:1000 dilutions. Antibodies bound to the beads via the viral antigens were stained by biotinylated anti-human-IgG and streptavidin-
R-phycocerythrin. Beads were examined in a Luminex 100 analyser that identified the bead colour of each bead sort and quantified the antibody bound to viral antigen via the median R-phycocerythrin fluorescence intensity (MFI) of at least 100 beads of the same internal colour. Polyomavirus seroprevalence cut-off values were defined arbitrarily by visual inspection of frequency distribution curves (percentile plots) of antibody reactivities (MFI) from all sera tested in a previous study (Teras et al., 2014) using stringent criteria to increase specificity. Cut-offs were set to 400 MFI for LT-Ag, 200 MFI for sT-Ag of MCPyV and 250 MFI for VP1 proteins, except for BKPyV VP1, which was adjusted to 100 MFI because of decreased reactivity in comparison to other polyomavirus VP1 seroactivities. Seroprevalence cut-offs for gB and pp150 were set at 50 MFI and 150 MFI, respectively, using the same methodology (Brozy, 2009).

To study the association between antigen seroprevalence and categorized seroreactivity (tertiles according to control distribution among seroprevalent subjects) with CLL, odds ratio (OR) and 95% confidence intervals (95% CI) were estimated using unconditional logistic regression adjusted by sex, region and age (quartiles according to control distribution). Statistical significance level was established at 0.05 and all tests were two-sided. Analyses were conducted with Stata software, version 10.1.

CLL cases showed a 1.6:1 male/female ratio, median age of 69 years (range=41–88) and more frequently had a relative with history of haematological neoplasm (P=0.04). In the control population, at increasing level of education, a lower seroprevalence of HSV-1 and CMV antigen seroprevalences were observed (P-value<0.001), as well as a decreased HSV-1 gB seroprevalence in smokers (P-value=0.01). Description of study population and antigen seroprevalences are detailed in Tables S1 and S2, respectively, available in the online Supplementary Material.

The estimated associations between CLL and viral seroprevalences and seroactivities are shown in Table 1. Overall, VP1 polyomaviruses seroprevalences in the control population ranged between 69% and 99%, except for LPyV (44%). Cases consistently showed lower seroprevalences than controls, translated into statistically significant lower OR of CLL (OR range=0.21–0.70) for all polyomaviruses except for MCPyV (OR=0.79, P-value=0.23). Regarding viral seroactivity, we obtained a significantly decreasing OR of CLL (P-trend<0.005) for all polyomaviruses at increasing seroactivity. Further adjustment by education level and smoking status did not substantially change the associations obtained. As expected, we also observed differences in the total number of polyomavirus infections (24.5% cases versus 8.7% controls were seropositive for five infections or less). These decreasing ORs with viral seroprevalence and seroactivity were also observed for the herpesviruses structural proteins explored.

Regarding the polyomavirus oncoproteins, none of the subjects were seropositive for TSPyV LT-Ag or MCPyV LT-Ag and only two subjects (one control and one CLL) were MCPyV sT-Ag seropositive. Forty-five study subjects were JCPyV LT-Ag seropositive, and as observed for the structural viral proteins, an inverse association with CLL (7.0% controls versus 5.3% cases; OR=0.74, 95% CI=0.40–1.36) was obtained (data not shown).

To study if the seroreactivity decline could be related to disease stage, we plotted the cumulative percentile distribution of seroreactivity values stratified by disease stage (Fig. 1) and compared the median seroreactivity levels among seroprevalent subjects using the Mann–Whitney U test (Table 2). When compared to the control population, irrespective of disease stage, categorized CLL cases showed lower median seroactivities for all the viruses under evaluation. CLL Rai I-IV showed lower median seroactivity values (stronger significant differences) than CLL Rai 0 subjects, except for HPyV-6. However, when median values were compared between Rai 0 and Rai I-IV subjects, significant differences were only observed for CMV pp150 antigen (P-value=0.005) and of borderline significance for BKPyV (P-value=0.068).

Seroactivity levels among treated subjects compared to untreated Rai I-IV untreated CLL subjects increased for most polyomaviruses except for JCPyV, TSPyV and HPyV6, although only the latter showed a significant increase in median values. Further, most polyomaviruses seroprevalences were higher among treated compared with untreated CLL subjects, except for LPyV and WUPyV.

Polyomaviruses low seroactivity values in CLL subjects might reflect a decline in total IgG values in CLL subjects rather than a viral specific one. We did not test our samples for total IgG levels as to look into this theory in detail. However, in a retrospective search we retrieved data on 30 IgG levels and 66 gammaglobulin fractions, determined in peripheral blood of CLL cases collected on the study sample date from one of the participating centres. Within 6 months of the study sample date, 57 subjects had both parameters measured on the same date and showed a good correlation (Pearson correlation coefficient r=0.684). Therefore, due to a larger number of samples available, gammaglobulin fractions were used as a proxy for total IgG values. We used Spearman correlation coefficient to study the relationship between a potentially impaired immune response and decreased viral seroreactivity. Although some CLL subjects might be categorized as seronegative subjects due to a decreased seroreactivity, we restricted our analyses to only seropositive subjects to ensure previous viral exposure and therefore specific viral seroreactivity presence.

As expected, the proportion of subjects with gammaglobulin fraction values below the reference laboratory range (11.1–18.8%) increased with disease stage from 10.5% (n=6) in CLL Rai 0 to 42.9% (n=9) in CLL Rai I-IV untreated subjects. However, no association was observed between viral seroreactivity and gammaglobulin fraction among CLL subjects (Spearman coefficient range=−0.15
Table 1. Association between polyomaviruses and CLL; results for seroprevalence and seroreactivity (tertiles) among seroprevalent subjects

<table>
<thead>
<tr>
<th>Seroprevalence</th>
<th>Seroreactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>All cases (n=370)</td>
<td>N* %</td>
</tr>
<tr>
<td>MCPyV (VP1)</td>
<td>310 (83.8)</td>
</tr>
<tr>
<td>BKPyV (VP1)</td>
<td>338 (91.4)</td>
</tr>
<tr>
<td>JCPyV (VP1)</td>
<td>255 (68.9)</td>
</tr>
<tr>
<td>HPyV-6 (VP1)</td>
<td>329 (88.9)</td>
</tr>
<tr>
<td>KIPyV (VP1)</td>
<td>283 (76.5)</td>
</tr>
<tr>
<td>HSV1 (pp150)</td>
<td>309 (83.5)</td>
</tr>
</tbody>
</table>

*Number (and %) of seroprevalent subjects for each specific virus among controls (n=370) and cases (n=359).

The increased incidence of Merkel cell carcinoma in CLL cohorts, and vice versa (Howard et al., 2006; Kaae et al., 2010; Tadmor et al., 2011), could be explained by (i) a shared underlying immunosuppression setting required to develop both diseases (such as a potential immunosenescence in the elderly population) or (ii) the immunosuppression/hypogammaglobulinemia linked to CLL leading to MCPyV reactivation and subsequent Merkel cell carcinoma development. The reactivation of MCPyV in the setting of an asymptomatic but not yet diagnosed CLL could be mistakenly interpreted as CLL being developed after Merkel cell carcinoma development.

Therefore, although overall seroprevalence against structural proteins from common persistent infections was high in our control population, CLL subjects showed decreased seroprevalences and seroreactivities independent of disease stage and treatment. As per the seroprevalence against the tested polyomaviruses alleged oncoproteins, it is either null or low, both in controls and CLL subjects.

A lack of association of MCPyV with CLL is consistent with the low DNA and LT-Ag presence in CLL subjects (Shuda et al., 2009; Toracchio et al., 2009; Teman et al., 2011; Comar et al., 2012; Imaoh et al., 2012; Cimino et al., 2013), but a plausible explanation is needed for the previous findings encouraging an association (Haugg et al., 2011; Pantulu et al., 2010). The mutated sequence of MCPyV LT-Ag in the nucleus of CLL cells was only detected in six (8%) out of 70 samples previously purified to contain mainly CD19+/CD5 CLL cells. In contrast, a truncated MCPyV LT-Ag sequence was detected in nine (100%) Merkel cell carcinoma samples and to date, no further data have been published on MCPyV mutations in CLL cells.
Fig. 1. Cumulative percentile distribution of polyomaviruses seroreactivity (log scale) stratified by disease stage.

Table 2. Viral seroprevalence and median seroreactivity levels; by disease stage and treatment status

<table>
<thead>
<tr>
<th></th>
<th>Control (n=370)</th>
<th>Rai 0 (n=204)</th>
<th>Rai I-IV untreated (n=72)</th>
<th>Rai I-IV treated (n=74)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prevalence N (%)</td>
<td>Median</td>
<td>P value*</td>
<td>Prevalence N (%)</td>
</tr>
<tr>
<td>MCPyV (VP1)</td>
<td>310 (83.8)</td>
<td>5720.8</td>
<td>166 (81.4)</td>
<td>4796.6</td>
</tr>
<tr>
<td>BKPyV (VP1)</td>
<td>338 (91.4)</td>
<td>1747.7</td>
<td>158 (77.5)</td>
<td>1233.0</td>
</tr>
<tr>
<td>JCPyV (VP1)</td>
<td>255 (68.9)</td>
<td>2547.7</td>
<td>115 (56.4)</td>
<td>1822.7</td>
</tr>
<tr>
<td>LPyV (VP1)</td>
<td>162 (43.8)</td>
<td>1618.3</td>
<td>62 (30.4)</td>
<td>904.8</td>
</tr>
<tr>
<td>KIPyV (VP1)</td>
<td>329 (88.9)</td>
<td>2324.0</td>
<td>157 (77.0)</td>
<td>1433.5</td>
</tr>
<tr>
<td>WUPyV (VP1)</td>
<td>365 (98.7)</td>
<td>4302.1</td>
<td>196 (96.1)</td>
<td>2529.1</td>
</tr>
<tr>
<td>HPyV-6 (VP1)</td>
<td>345 (93.2)</td>
<td>4809.3</td>
<td>181 (88.7)</td>
<td>3569.8</td>
</tr>
<tr>
<td>HPyV-7 (VP1)</td>
<td>297 (80.3)</td>
<td>2669.4</td>
<td>141 (69.1)</td>
<td>1785.9</td>
</tr>
<tr>
<td>TSPyV (VP1)</td>
<td>283 (76.5)</td>
<td>2568.1</td>
<td>89 (43.6)</td>
<td>2097.1</td>
</tr>
<tr>
<td>HSV1 (gB)</td>
<td>346 (93.5)</td>
<td>1885.6</td>
<td>192 (94.1)</td>
<td>1137.3</td>
</tr>
<tr>
<td>CMV (pp150)</td>
<td>309 (83.5)</td>
<td>2031.4</td>
<td>158 (77.5)</td>
<td>1663.9</td>
</tr>
</tbody>
</table>

*P value for median comparison with control subjects.
†P value for median comparison between treated and untreated Rai I-IV subjects.
Hypogammaglobulinemia presence, associated with increased Binet stage (Nataša et al., 2001), is theoretically consistent with the generalized decreased seroreactivity against the polyomavirus antigens, as observed with herpesviruses among CLL subjects (Vanura et al., 2013). The significantly lower seroreactivities against polyomaviruses already observed in CLL Rai 0 cases is also supported by previous studies suggesting that an immune impairment might be present several years prior to the actual diagnosis of CLL (Hamblin & Hamblin, 2008; Tsiodras et al., 2000). However, the fact that viral seroreactivities were not related to gammaglobulin fractions and no differences in median levels were observed between disease stages, suggest that hypogammaglobulinemia does not fully explain the decline in polyomavirus seroreactivity. We speculate that polyomaviruses might reactivate due to a poor immune response in CLL subjects and therefore disrupt on the initially proportional decline in comparison to the total IgG reduction.

Differences from our previous results in MCPyV seroprevalence (Robles et al., 2012), in which we observed an increased seroprevalence, might be explained by control selection, due to the previous use of hospital controls versus the current use of general population ones. The decreased seroprevalences against all polyomaviruses in our CLL subjects is somehow consistent with the findings from Teras et al. (2014), in which none of the polyomaviruses showed a significant association with CLL, but half of them showed a decreased risk of CLL among seroprevalent subjects. Increased nonsignificant risks might be explained by sample size, time from sample to diagnosis and differences in viral immunogenicity between polyomaviruses.

Interestingly, we did not observe significant differences in median seroreactivity between treated and untreated Rai I-IV subjects, except for HPyV-6. However, JCpV and TSPyV, both reported as opportunistic diseases in subjects with haematologic disorders (Amend et al., 2010; Kazem et al., 2013), showed higher seroprevalences at lower seroreactivity levels among treated subjects when compared to untreated ones. This effect might be explained by a sustained poor immune response independent of treatment status. The same behaviour was observed for HPyV-6, potentially suggesting a viral reactivation in treated subjects.

This is the largest CLL case-control study to date looking for a potential association with polyomaviruses and the first to measure the seroreactivity against the MCPyV oncoproteins. However, we cannot discard chance findings by multiple testing. On the other hand, the use of serological biomarkers reduces the potential misclassification of exposure, a frequent limitation in retrospective studies, but a prospective evaluation of the seroresponse prior to disease development may provide additional conclusive information.

We conclude that MCPyV and the other polyomaviruses are unlikely to be associated to CLL development. The lower seroreactivity values observed in CLL. Rai 0 cases when compared to the control population suggest that low seroactivities against polyomaviruses, and potentially other common persistent viral infections, may be an early marker of CLL development.

Acknowledgements

We thank all the subjects who participated in the study and all MCC-Spain collaborators (the list can be found in Supplementary documentation). This work was partially supported by the Spanish Ministry Council (Accion Transversal del Cancer, approved on the 11 October 2007); the Instituto de Salud Carlos III (ISCIII) – Spanish Government [PI08/1770, PI08/0533, PI08/1359, PS09/0073, PS09/01662, PI11/01810, PI11/02213, PI14/01219, RCEPS C03/09, RTICEPS C03/10, RTIE RD06/0020/0095, RTIC RD12/0036/0056, Rio Hortega CM13/00232, SV-09-CLINIC-1 and CIBERESP] the Fundacion Marques de Valdecilla [API 10/09] Obra Social CAJASTUR [SV-CAJASTUR-1] Recercaixa [2010ACUP 0010], the Spanish Association Against Cancer (AECC) Scientific Foundation and the Agencia de Gestió d’Ajuts Universitaris i de Recerca (AGAUR) – Generalitat de Catalunya (Catalonian Government) [2009SGR1026, 2009SGR1465 and 2014SGR756]. The IGCC CLL-Genome Project is supported by Spanish Ministerio de Economía y Competitividad (MINECO) and the Red Tematica de Investigacion del Cancer [RTIC RD12/0036/0036]. Sample collection and storage was partially supported by the ISCIII [FEDER RD09/0076/00036] and Xarxa de Bancs de Tumours de Catalunya sponsored by Pla Director d’Oncologia de Catalunya (XBTC).

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