Closing report of PD109108 "Study of pathogenic role of new human polyomaviruses" project

Aim of the project:

The aim of the project was to collect prevalence data about novel human polyomaviruses (PyV) KI, WU, 9, MW, MX and STL. These viruses were discovered recently, some of them in that year when the project starts. Little or hardly anything was known about these viruses at the beginning, the questions about the way of transmission, the site of replication, the ability to establish latency, the pathogenesis, the possible oncogenic potential, the importance in immunocompromised patients were opened. By collecting valuable clinical samples from different patient groups, hunting for viral DNA these questions were studied. Since it was a new research field, publications of other research teams were followed up and the project (methods, materials, new samples) was adapted to the new results.

Methods developed and used:

Different PCR methods were tested, developed and optimized for DNA prevalence studies. In connection with prevalence studies it has high importance to be able to compare data from different research projects. Hence, we adapted primers and methods from publications, but we also developed new primers, methods to achieve better sensitivity, to amplify the complete genomes and to examine viral RNA. To study KI and WU virus (KIPyV, WUPyV) a multiplex, real-time, quantitative TaqMan assay, a nested PCR, inverse primed PCRs (back-to-back primers are used to amplify both strands of the whole, circular genome) amplifying the complete viral genome and RT-PCRs for examination of viral RNAs were used. Plasmids containing KI and WU viral sequences were obtained from the research teams who discovered the viruses. In order to study human polyomavirus 9 (HPyV9) we adapted a nested and a real-time PCR method, then developed two new, quantitative, TaqMan assays and an inverse PCR, as well. The positive control (a plasmid containing partial sequence of the virus) was obtained from the discoverers. Viral DNA positivity were proved by sequencing. MW and STL polyomaviruses (MWPyV and STLPyV) were studied by quantitative real-time PCRs, and positive controls were synthesized (the required target sequence could not be obtained from the discoverers). Based on GeneBank sequences the target viral DNAs were synthesized, then cloned into plasmid vectors and used as positive controls for PCRs. A quantitative, real-time, TaqMan assay were optimized to study BK virus,

the complete viral DNA of Dunlop reference strain was available as positive control.

Nucleic acid isolation methods were optimized for different sample types (detailed later).

Although our original aim was to collect also seroprevalence data, but the project grant could not afford it. The original plan was to perform it in collaboration, but only the antigen coding vectors could have been obtained. To produce antigens, to examine and test them, to optimize ELISA, then test serum samples for these five novel polyomaviruses is beyond the budget of the project. Most of the budget was spent for DNA prevalence study, high number of PCRs were performed. During the last year of the project we asked budget correction: miscellaneous cost was planned for sequencing, but less number of viral DNA positive samples was found than expected. The remaining money was used to develop an ELISA method to study the seroprevalence of human polyomavirus 9. The major viral capsid protein (VP1) coding sequence was synthesized, tagged with 6xHis, cloned into inducible pTRIEX4Neo vector, and protein was expressed in Origami™ B(DE3) *Escherichia coli* using IPTG induction. The His-tagged protein was purified by metal-chelate affinity chromatography, concentrated, then analysed. Western-blot revealed that the protein quality is excellent, pure VP1 protein of HPyV9 was produced and the quantity is good for ELISA.

Samples collected and analyzed:

Tumour tissue samples to study the possible oncogenic potential of the human polyomaviruses studied:

Formalin-fixed paraffin-embedded tissue samples from Department of Pathology, University of Debrecen were collected. Tissue sections from renal adenoma, renal angiomyolipoma, renal oncocytoma, leiomyosarcoma renis, carcinoma renocellulare renis, uroepithelial carcinoma, renal biopsy from renal transplant patients and lung carcinoma (small cell, non-small cell, mixed and non-differentiated carcinoma), altogether 290 samples from 278 patients were analysed. The number of the tissue samples is enormously high comparing it with other publication up to date, and fulfil the plans of the project.

Non-cancerous, respiratory tract tissue sample collection in order to reveal the possible site of viral replication:

Nucleic acid was isolated from 47 formalin-fixed paraffin-embedded lung tissue samples. 100 fresh tonsil and 100 adenoid tissue samples were also collected and analysed from children and adults. Kidney biopsy samples from renal transplant patients were analysed together with cancerous tissue samples of the urinary tract. It was not planned for the project

but based on the literature data (published during the project period) it seemed to be valuable sample in which viral replications are suggested. The other explanation for collection of these samples was that the original collaborator partner, Department of Infectious and Paediatric Immunology, University of Debrecen was dissolved, sample collection from paediatric patients (e.g. the valuable respiratory and stool samples from children) was not available anymore (ethical approval was obtained for this collaboration). As soon as possible, we established new cooperation with Department of Otolaryngology and Head and Neck Surgery, University of Debrecen. New ethical approval was asked, it took more than a year to obtain, which was an explanation for modifying the closing date of the project. At the same time, these samples seemed to be one of the most valuable among the samples collected and analysed (detailed later).

Blood, urine, respiratory, stool and cerebrospinal fluid samples from different patient groups:

1850 samples (blood, urine, respiratory) from 77 renal transplant patients, 720 samples (blood, urine, respiratory) from 150 patients with different haematological disorders, 200 cerebrospinal fluid samples from 200 patients (children and adult) taken for diagnosis of encephalitis causative agents, 190 respiratory samples from 190 immunocompetent individuals (adults and children) and 250 blood samples from adults and children were collected.

Results:

The taxonomy of the polyomaviruses was changed during the project, MW and MX are not distinct viruses now, MWPyV is the representative isolate of human polyomavirus 10. Hence, in our project MWPyV was examined. MW and STL polyomaviruses were examined, but not detected in any samples tested.

WUPyV was detected in throat swab and blood samples from renal transplant patients, in throat swab, in middle ear discharge and in adenoid tissue samples from children, and in tonsillar tissues from children and a young adult, as well. Based on seroprevalence data from literature, childhood primary infection is suggested. Our results strengthen the hypothesis that the virus may spread via respiratory route during childhood: we detected WUPyV DNA in 7.87% of upper respiratory samples from children, but the virus was not found in the same sample types from healthy, adult individuals. Since up to date, middle ear samples were not examined, a new and interesting result is that we detected WUPyV in middle ear discharge

samples from three patients with positive upper respiratory tract samples, suggesting that the virus may spread into to the middle ear. Two publications are available about survey of WUPyV in adenoid tissues. We collected higher number of samples (100 vs. 51 and 83), and detected higher DNA prevalence (29% vs. 7.8 and 27.7%). Tonsils were examined also in high number (n=100) and the virus positivity rate was found to be 4 %. Although WUPyV was neither detected in upper respiratory samples, nor in lung tissues from healthy adults, but it was found in the upper respiratory samples from immunocompromised patients. The virus was detected in respiratory samples from 9.1 % of the immunocompromised patients, suggesting that these patients might be more susceptible for this virus or reactivation can occur under immunosuppression, if latency is established. Statistical analysis revealed that WUPyV infection is more frequent within the first two month after the transplantation than later. Summer seasonality of viral infection is suggested based on our results. Based on the statistical analysis of clinical data consequences of WUPyV infection were not revealed, as we did not find relationship between WUPyV infection and other virus (human cytomegalovirus) reactivation. Interestingly, WUPyV was detected also in blood samples from 5.3 % of renal transplant patients, suggesting that the virus might spread via the blood. The virus was examined, but not detected in lung cancers, in bladder cancers and in renal neoplasia. We did not find evidence that WUPyV may be aethiologic agent in tumourigenesis. At the same time, BK virus were also studied, but not found in cancerous tissue samples, BKPyV was detected in a kidney biopsy sample.

KIPyV was also detected in 2.5 % of the upper respiratory samples, in 11 % of the adenoid tissues and in 3 % of the tonsils from children, but not in respiratory samples and lung tissues from adults. At the same time, 14.3 % of the renal transplant patients examined had KIPyV positive upper respiratory samples mainly during the first month after the transplantation. Statistical analysis revealed that viral infected patients were significantly younger than those from whom viral DNA negative respiratory samples were taken. KPyV was detected in blood and urine samples from 3.9 % and 4.1 % of the immunocompromised patients, suggesting that the virus may spread within the body, and it can be secreted by urine. Although clinical consequences of KIPyV infection were not revealed, the childhood primary infection via respiratory route and higher risk for the infection due to immunosuppression were also suggested. The tumour tissues collected and analysed were all negative for KIPyV DNA, hence no association of tumour and KIPyV infection were found.

We made an attempt to prove that adenoid and/or tonsil, as important lymphoid organs might be the site for KIPyV and WUPyV replication: RT-PCRs were developed targeting the

possible mRNAs. Since up to date, no the virion itself has been isolated, there is no in vitro model for replication, and there is no positive control for these RT-PCRs. It was proved that RNA isolation was successful, but viral RNAs have not been detected yet. More complex methods, e.g. transcriptome analysis may facilitate the survey of viral RNAs, but the cost of this method is over the budget of this project. These organs might serve place for latency. Complete genome sequences were amplified and sequenced, the analysis of the sequences is in progress.

HPyV9 is an unexplained virus: up to date, beside the paper about the discovery of the virus, two work teams (we and another one) published the detection of the virus. Today nothing about the pathogenesis of this virus is known. We planned to collect and analyse different clinical samples (blood, urine, throat swab, cerebrospinal fluid, stool, adenoid, tonsil and lung tissue, bladder, kidney and lung tumour tissue samples) from patients. Altogether 3000 samples were analysed by nested PCR and quantitative real-time PCRs targeting the VP1 and LT regions of the virus. The sensitivity of the methods was determined by serial dilutions of a plasmid control, and although the sensitivity of the nested PCR was found to be the best, but each sample was tested by all methods. The samples originated from different anatomical sites and contained various quantities of nucleic acids from normal flora and human genome, which affect the effectiveness of the PCRs. The frequency of HPyV9 was found to be low, as the viral DNA load was also low in samples. HPyV9 was detected in respiratory samples from patient with haematological disorder and from renal transplant patients; the frequencies were 1.3% and 5.8 %, but it was not found in respiratory samples from immunocompetent children and adults. Interestingly, the virus was detected in a tonsil tissue sample from a child (1/100). Blood samples were positive for viral DNA from 10.1 % of immunocompromised patients, and the virus was detected in urine samples from 5.8 % of the kidney transplant patients, as well. Human polyomavirus 9 was not detected in any of the other samples tested. Our results suggest the respiratory transmission of the virus and the spread within the body. Because of the low number of the viral positive samples, clinical consequences were not revealed.

Seroprevlance data about HPyV9 infections have been published, suggesting that seropositivity increases with age, and immunosuppression might result in higher susceptibility. Since our DNA prevalence results strengthen the last, we decided to test the HPyV9 seropreavlence in the Hungarian population. During the last year of the project an indirect ELISA was developed and optimized: VP1 protein (expressed as detailed above) was coated on the surface, the blocking buffer, the dilution of the serum samples (possibly

containing IgG antibody against HPyV9 VP1) and the secondary antibody, hence the ELISA method is optimized. It is proved that the VP1 protein produced is immunologically active. The specificity of the ELISA method is tested by pre-incubation of an HPyV9 seropositive serum sample with VP1 proteins before adding it to the wells containing the antigens bound. This revealed that the ELISA method is specific and suitable for seroprevalence study. Serum samples from immunocompetent children, adults, from patients with haematological disorders and from immunocompromised patients are collected. The ELISA tests are in progress.

Summary:

More than 3000 samples were collected from patients representing different ages and immunostatus. PCR reactions were developed and tested for DNA prevalence studies. Thousands of PCR reactions were performed; valuable prevalence data were obtained and published. Our results gave new data to a developing, new research field, high – in many cases higher number of samples than previously– were collected and analysed. Analysis of clinical data did not reveal the clinical consequences of the infections, but our data add new results to the knowledge about the pathogenesis of the novel polyomaviruses studied. Three papers are published, and two others are in preparation. Working with clinical samples not retrospectively, organizing the sample collections from patients are challenging. Based on the results published during the project and also the availability of samples, we should have modified our original plan focusing in lymphoid tissue related to the respiratory tract. Although it resulted in delay, and genome sequence analysis and seroprevalence studies are in progress, the remaining, not published data of the project are valuable. We plan to publish these in good quality (Q1) journals. These will be the followings:

Human polyomavirus KI and WU in lymphoid tissues and respiratory samples

DNA and seroprevelance of human polyomavirus 9

The leader of the study worked on the project, collaborators from clinics helped the sample and clinical data collections. As a supervisor I tutored graduated students, four of them prepared their thesis for the degree from topics related to the project. During the project period, a PhD student obtained her PhD degree, the PhD thesis is not overlapping with these results, however the topic, study of novel polyomaviruses was related. Most of the samples collected are archived, frozen nucleic acids and samples are stored, which ensure a good basis for further research.

Debrecen, 2017. szeptember 29.

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