Silver-enhanced in situ hybridization for detection of polyomavirus DNA in patients with BK virus nephropathy

Fritzsche, F R; Pianca, S; Gaspert, A; Varga, Z; Wang, L; Farrell, M P; Chen, X B; Hirsch, H H; Springer, E; Fehr, T; Myles, J; Tubbs, R; Moch, H

Postprint available at:
http://www.zora.uzh.ch

Posted at the Zurich Open Repository and Archive, University of Zurich.
http://www.zora.uzh.ch

Originally published at:
Fritzsche, F R; Pianca, S; Gaspert, A; Varga, Z; Wang, L; Farrell, M P; Chen, X B; Hirsch, H H; Springer, E; Fehr, T; Myles, J; Tubbs, R; Moch, H (2011). Silver-enhanced in situ hybridization for detection of polyomavirus DNA in patients with BK virus nephropathy. Diagnostic Molecular Pathology, 20(2):105-110.
Silver-enhanced in situ hybridization for detection of polyomavirus DNA in patients with BK virus nephropathy

Abstract

BK virus nephropathy is not an infrequent complication of renal transplantation associated with high rates of graft loss. Although antibodies against SV40 antigen detect different viruses of the polyomavirus family, immunohistochemistry is widely used to confirm the diagnosis of BK virus nephropathy in renal biopsies. Here we aimed to validate the novel silver-enhanced in situ hybridization (SISH) technique for the automated detection of BK virus in renal transplant biopsies. Two different patient cohorts were included. Twenty-nine consecutive patients suspicious for BK virus infection were investigated by SISH and chromogenic in situ hybridization. An additional 26 renal biopsies positive by SV40 immunohistochemistry from 19 patients were analyzed by SISH. Polyomavirus DNA serum levels, as determined by nested PCR analysis, were available for all of these patients. The presence of BK virus DNA in renal tubular cells was identified in 5 of the suspicious cases by both, SISH and chromogenic in situ hybridization. One additional patient was only positive in the SISH. In the second cohort, SISH was positive in all SV40 positive biopsies, but SISH signals were less extensive than SV40 immunohistochemistry. Our results show that the BK virus SISH is an ancillary tool for the detection of polyomavirus DNA in renal biopsies using bright-field microscopy. However, its diagnostic value in comparison with standard immunohistochemistry seems to be limited.
Silver enhanced in situ Hybridization for Detection of Polyomavirus DNA in Patients with BK Virus Nephropathy

Florian R. Fritzche\textsuperscript{1}, Silvio Pianca\textsuperscript{2}, Ariana Gaspert\textsuperscript{1}, Zsuzsanna Varga\textsuperscript{1}, Lin Wang\textsuperscript{3}, Michael P. Farrell\textsuperscript{4}, Xiao-Bo Chen\textsuperscript{4}, Hans H. Hirsch\textsuperscript{5}, Erik Springer\textsuperscript{6}, Thomas Fehr\textsuperscript{2}, Jonathan Myles\textsuperscript{3}, Raymond Tubbs\textsuperscript{3}, Holger Moch\textsuperscript{1}

\textsuperscript{1}Institute of Surgical Pathology and \textsuperscript{2}Division of Nephrology, University Hospital Zurich, Zurich, Switzerland
\textsuperscript{3}Departments of Molecular and Anatomic Pathology, Pathology and Laboratory Medicine Institute and The Cleveland Clinic Lerner College of Medicine, Cleveland, Ohio, USA
\textsuperscript{4}Roche Ventana Medical Systems International, Tucson, Arizona, USA
\textsuperscript{5}Institute for Medical Microbiology, University Hospital Basel, Basel, Switzerland
\textsuperscript{6}Institute of Pathology, University Hospital Mainz, Mainz, Germany

Corresponding author:
Holger Moch, MD
Institute of Surgical Pathology, University Hospital Zurich
Schmelzbergstr. 12, 8091 Zurich, Switzerland
E-Mail: holger.moch@usz.ch
Phone: +41 44 255 2500
Fax: +41 44 255 4440

Disclosure
Michael P. Farrell and Xiao-Bo Chen are employees of Roche Ventana Medical Systems. Raymond Tubbs received research support and speaking honoraria by Roche Ventana Medical Systems. Holger Moch received research grants from Ventana Medical Systems and unrestricted research funds of the University of Zurich. There was no conflict of interest for any of the other authors.
Abstract

BK virus nephropathy is not an infrequent complication of renal transplantation associated with high rates of graft loss. Although antibodies against SV40 antigen detect different viruses of the polyomavirus family, immunohistochemistry is widely used to confirm the diagnosis of BK virus nephropathy in renal biopsies. We here aimed to validate the novel silver-enhanced in situ hybridization (SISH) technique for automated detection of BK virus in renal transplant biopsies. Two different patient cohorts were included. Twenty-nine consecutive patients suspicious for BK-virus infection were investigated by SISH and chromogenic in situ-hybridization (CISH). Additional 26 renal biopsies positive by SV40 immunohistochemistry from 19 patients were analyzed by SISH. Polyomavirus DNA serum levels as determined by nested PCR analysis were available for all of these patients.

Presence of BK virus DNA in renal tubular cells was identified in five of the suspicious cases by both, SISH and CISH. One additional patient was only positive in the SISH. In the second cohort, SISH was positive in all SV40 positive biopsies, but SISH signals were less extensive than SV40 immunohistochemistry.

Our results demonstrate that BK virus SISH is an ancillary tool for the detection of polyomavirus DNA in renal biopsies using bright-field microscopy. However, its diagnostic value in comparison to standard immunohistochemistry seems to be limited.

**Key words:** BK virus, polyomavirus, SISH, renal transplantation, PVAN
Introduction

BK-, JC- and Merkel cell-polyomavirus belong to the human polyomavirus family. In 1995, BK virus nephropathy has been described as cause of polyomavirus-associated nephropathy (PVAN) in renal transplant recipients.\(^1\) Other BK virus associated urological complications include ureteral obstruction, hemorrhagic cystitis and hydronephrosis.\(^2\) In renal transplant patients, the prevalence rate of PVAN varies from 1-10%.\(^3\) In earlier years a functional decline of renal allografts due to PVAN occurred in 60% to 90% of cases.\(^1\)\,\(^4\)-\(^6\) Nowadays, early diagnosis and timely reduction in immunosuppression lead to preservation of graft function.\(^6\)\,-\(^9\) Reduction of immunosuppression implies a higher risk of rejection due to the lowered immunosuppression. To avoid consequences and side effects of therapy, the diagnosis of a PVAN is of utmost clinical importance.

Primary infection with BK virus occurs via droplets during childhood and is clinically either unapparent or unspecific. Following the primary infection, the virus remains intracellular in a latent state in renal tubular and urothelial cells.\(^10\) Eighty percent of adults have antibodies against BK or JC virus. In immunocompetent individuals, reactivation of the virus is usually asymptomatic.\(^11\) Meanwhile, reactivation is more frequent and clinically important in immunocompromised patients. The diagnosis of PVAN in renal biopsies is essential to reliably exclude other causes of a decline in renal function such as allograft rejection, drug toxicity, and infection with other viruses including other polyomaviruses (JC virus) but also Herpes simplex virus, adenovirus, and cytomegalovirus (CMV).\(^12\)
The current “gold standard” for the diagnosis of PVAN requires light microscopic detection of the characteristic virus-induced tubular changes in a renal biopsy.\textsuperscript{13} Diagnostic confirmation of the infected tubular epithelial cells can be achieved by polymerase chain reaction or by immunohistochemistry with antibodies directed against SV40. The latter is possible due to high homology between SV40 antigen (present in kidneys of monkeys) with BK and JC-virus in humans.\textsuperscript{14}

Virus screening tests, especially the detection of so called decoy cells (virus-infected cells with nuclear inclusions) in the patients’ urine or polymerase chain reaction (PCR)-based virus detection in the blood plasma, help to identify patients at risk for PVAN.\textsuperscript{15} Although both methods have high sensitivity rates, the latter one outbalanced the mere morphology-based one in terms of specificity and positive predictive value.\textsuperscript{15}

In situ-hybridization technologies, such as chromogenic in situ-hybridization (CISH) have been developed for DNA detection using bright field microscopy in a setting familiar to all histopathologists. Recently, a novel high-sensitivity in situ-hybridization technique based on enzymatic metallography and metallic silver deposition (silver in situ hybridization; SISH) has been developed for \textit{HER2} gene testing in breast cancer and other tumor entities.\textsuperscript{16-24} This method is fully automated, ensuring consistency of methodology and results. In this study, we determined the reliability of SISH to identify BK-virus DNA in kidney transplant biopsy specimens. Our results suggest that BK virus SISH is an ancillary tool for the detection of BK virus DNA in renal tubular cells, using an automated staining system and bright field microscopy.
Materials and Methods

Patients

Two different cohorts of kidney transplant patients were examined. The first cohort included 29 kidney transplant biopsies from the archives of the Institute for Pathology, Cleveland Clinic, Ohio. These cases were either demonstrating suspicious nuclear inclusions by light microscopy or were clinically suspicious for PVAN. BK-virus infection was determined in parallel by chromogenic in situ-hybridization (CISH) and silver in situ hybridization (SISH).

The second cohort included 26 renal biopsies from 19 patients. These biopsies were retrieved from the archives of the Institute of Surgical Pathology Zurich between 2001 and 2008. Sixteen patients were men, 3 women, the median patient age was 42 years (range 15 to 66 years). Time after transplantation was 14 months (range 1.2 to 98 months). Diagnosis of BK-virus nephropathy was confirmed by SV40 immunohistochemistry. Serum levels for BK-virus as determined by nested PCR analysis were available in all of these patients.

Staining procedures: Silver enhanced in situ hybridisation (SISH)

The recently developed SISH technology for the detection of HER2 signals in breast cancer was adopted for identification of BK virus DNA in renal biopsies. A plasmid containing the complete BK-virus (BKV) genome (pBKV (35-1)) was obtained from the American Type Culture Collection (ATCC catalog number 45026). BK virus DNA was released from the vector, pBR322, by digestion with BamHI. After agarose gel electrophoresis the BKV DNA was excised from the gel and purified. Double digestion with AluI and DpnI was followed by exposure to T4 DNA polymerase in the presence of dATP, dTTP, dCTP and dGTP to render the ends flush. Phenol extraction and ethanol precipitation was followed by ligation in a small volume to
generate concatenated permuted BKV DNA. This product was subjected to repeated sequential amplification using random hexamer primers (terminated by 2 phosphorothioates at the 3’ end) to generate milligram quantities of DNA. This amplification product was labeled with DNP (dinitrophenol) by the MIRUS Label IT kit (catalog number MIR3800). The resulting labeled DNA was used as a probe on the Ventana Benchmark XT. Following a pretreatment with protease2 for four minutes, probe and target tissue were codenaturated at 90°C for 8 minutes. After this followed the hybridization for one hour at 37°C. One drop of the custom-made BK virus probe was applied (2.5 ng/μl). After several stringency washes at 37°C the slide was incubated with SISH DET HRP complex for 2 hours. For detection one drop of SilverA, SilverB and SilverC was added. The chemistry of the SISH reaction, briefly described, is driven by the sequential addition of silver A (silver acetate), silver B (hydroquinone), and silver C (H₂O₂). Here, the silver ions (Ag⁺) are reduced by hydroquinone to metallic silver atoms (Ag). This reaction is fuelled by the substrate for horseradish-peroxidase, hydrogen-peroxidase (silver C). The silver precipitation is deposited in the nuclei and a single copy of the BK-virus is visualized as a black dot. The specimen is then counterstained with Ventana hematoxylin 2 for interpretation by light-microscopy. The SISH technique is illustrated in Figure 1.

**Staining procedures: Chromogenic in situ hybridisation (CISH)**

Unstained paraffin sections of formalin fixed renal biopsy tissue were evaluated by automated in situ hybridization (Discovery XT; Ventana Medical Systems, Tucson, AZ, USA) as previously described. Pretreatment, denaturation and hybridization were equal to the BK virus SISH as described above. After several stringency washes at 37°C detection was via streptavidin alkaline phosphatase and NBT/BCIP. The BK virus probe was a biotin labeled genomic probe (Enzo; Farmingdale). All components of the procedure, including deparaffinization, cell conditioning,
probe/target denaturation and hybridization, duplex detection, stringency washing, and counterstaining with nuclear fast red, were automated.\textsuperscript{25} The slides were mounted as permanent preparations using Cytoseal (Richard-Allan Scientific; Kalamazoo), and evaluated using conventional bright field microscopy.

**Staining procedures: SV40 Immunohistochemistry**

Immunohistochemistry was conducted with the Ventana Benchmark automated staining system (Ventana Medical Systems, Tucson, AZ) and with Ventana reagents. For the SV40 immunohistochemistry we used the mouse monoclonal antibody (PAb416, Abcam plc, Cambridge, UK, dilution 1:600). Primary antibodies were detected using the UltraVIEW DAB detection kit after heat-induced epitope retrieval. The slides were counterstained with hematoxylin, dehydrated and mounted.

**Scoring Criteria**

SISH and CISH of the Cleveland cohort were analyzed by one pathologist (RT). For the Zurich cohort, SISH signals and SV40 immunohistochemistry were evaluated by two pathologists (FRF; AG). Percentage of tubules with positive cells were scored using a four-tiered approach (negative: 0%, weak: 1-25%, moderate: 26-50% and strong: >50% positive tubular cells). Only nuclear staining signals were considered positive.

**Statistical analysis**

Statistical analysis was performed using SPSS, version 18.0. P values $< 0.05$ were considered significant.
Results

SISH and CISH

Presence of BK virus DNA was identified by both methods in nuclei of epithelial cells in the renal medulla and in cortical renal tubules (Figure 2). The Cleveland cohort was used to compare CISH and SISH in 29 renal transplant biopsies clinically suspicious for PVAN. BK-virus DNA was detected in five out of 29 cases with the CISH. All of these cases were also positive by BK-virus SISH. One BK-virus positive SISH patient was CISH negative.

BK-virus SISH and SV40 immunohistochemistry

The SV40 IHC staining was easy to evaluate in most cases with a distinct nuclear staining ranging from weak to strong. The SV40 antigen was detected in more than 50% of the tubules in 6 out of 26 biopsies. Moderate positivity (25 - 50% of tubules) was seen in 7 biopsies and weak positivity (<25% of tubules) in 13 biopsies.

BK-virus SISH was also positive in all 26 immunohistochemically positive cases. The grading of the SISH signal intensity revealed strong positivity in 2 of 26 biopsies, moderate positivity in 3 biopsies, and weak positivity in 21 biopsies. In comparison to SV40 staining, BK-virus SISH displayed more extensive signals in 2 of 26 biopsies (7.7%), equal signal intensity in 6 biopsies (23.1%) and weaker signal intensity in the remaining 18 biopsies (69.2%). Nonetheless, the BK-virus SISH staining intensity was significantly correlated with SV40 staining (p=0.001, correlation coefficient 0.631). In summary, the BK-virus SISH signals were weaker than the immunohistochemical staining product. A truly positive signal (e.g. in single cells) was sometimes not equivocally discernable from artifacts on the slide, resulting in time-consuming microscopic focusing.
Polyomavirus DNA serum levels

Polyomavirus DNA serum levels as determined by nested PCR analysis were available for 16 of the Zurich patients, allowing correlation of BK virus serum levels with staining intensities by SV40 immunohistochemistry and BK virus SISH. The median serum levels (closest date to renal biopsy) were 574222 copies/ml (range 3287 – 4020000 copies/ml). There were significant correlations between the serum virus load and both, the SV40 (p=0.01; correlation coefficient: 0.513) and the BK-virus SISH (p=0.026; correlation coefficient: 0.455) staining intensity.

There were 3 patients with JC-virus DNA in the patient’s serum. Only JC virus was present in one of these patients. In the other two patients, both JC- and BK-virus DNA were detected in the serum. In these 3 patients, staining intensities of SV40 immunohistochemistry and SISH BK virus in the tissue were compared. The 2 biopsy samples from the patient with only JC virus DNA in the serum were weakly and strongly positive for SV40 (< 25% and > 50% of the tubules, respectively). The BK-virus SISH was weakly positive in both biopsies (< 25% of the tubules). A subsequent PCR analysis of the biopsy tissue confirmed that only JC virus DNA was present in this patient. The two other patients with JC and BK virus DNA in the serum demonstrated weak SV40 immunohistochemistry and BK-virus SISH signal intensity (<25% of tubules) with BK-virus SISH being weaker than SV40 immunostaining. These data suggest potential cross reactivity of the BK SISH probe with JC virus.
Discussion

In the present study, BK virus DNA was identified in renal biopsies of patients with BK virus nephropathy by a novel silver-enhanced in situ-hybridization technology. The results of the study indicated good efficacy and robustness of the SISH assay in comparison with CISH and immunohistochemistry.

SISH is a recently developed method that offers the advantages of bright field in situ hybridization coupled with automation. SISH testing for HER2 gene amplification in breast cancer has recently been developed to enable diagnostic laboratories to perform this assessment in the routine work process. In breast cancer diagnostics, the SISH approach has advantages compared to fluorescence in situ hybridization (FISH). The main problem of FISH is represented by the impossibility of archiving the slides because of signal decay. Further, FISH is time-consuming and requires the technical infrastructure of a fluorescence microscope. Her2-SISH has already been suggested as an alternative for the FISH-based Her2 amplification analysis in breast cancer patients and has even been successfully tested in cytological specimens. In these studies, SISH showed a high concordance with FISH and a remarkable interobserver reproducibility.

The histologic diagnosis of PVAN in a renal biopsy should be confirmed by a molecular method. This is the first study evaluating the SISH technology for BK virus DNA detection in formalin-fixed paraffin-embedded tissue. According to our data, there is a very high concordance between the BK-virus SISH, CISH and conventional SV40 immunohistochemistry for the identification of polyomavirus in renal transplant biopsies. PCR is the most sensitive method for virus detection,
but PCR results should be interpreted with caution. To distinguish productive forms (clinically significant) from non-productive latent infections (clinically insignificant) only strong PCR signals of BK virus DNA are clinically relevant.\textsuperscript{26-27} It has been reported that 50\% of healthy native kidneys and 40\% of ureters harbor foci of latent BK virus.\textsuperscript{28} In addition, PCR is technically demanding, does not produce a permanent archival slide and requires specialized equipment.

In situ methods, like SISH, CISH or immunohistochemistry allow an unequivocal identification of the virus in infected cells compared to conventional H&E morphology with sometimes equivocal nuclear changes, especially in tubular regeneration. Interpretation of SV40 immunohistochemistry was easier than SISH interpretation, due to a higher staining intensity and a lower background staining. However, SV40 immunohistochemistry is unspecific and detects all viruses of the polyomavirus family. This explains that both cases with JC virus infection and absence of BK virus were strongly positive by SV40 immunohistochemistry. Unfortunately, these cases also revealed a weak SISH signal. For the SISH assay, a plasmid containing the complete BK-virus (BKV) genome (pBKV (35-1) was used, but this probe might have some cross reactivity with JC virus. It has been reported that the genome sequence shares 75\% homology in BK and JC virus, causing this cross reactivity with JC virus.\textsuperscript{14} Considering the higher costs of in situ hybridization techniques in comparison to immunohistochemistry, the BK virus SISH can therefore not be recommended as a primary but as an ancillary tool for the assessment of PVAN.

We observed an almost similar sensitivity using CISH and SISH. Recent data suggest that like SISH, CISH could be utilized like as a FISH equivalent with comparable accuracy. Automation of CISH is possible and has been described.\textsuperscript{25, 29} However, most studies use CISH still with a
manual protocol. While processing times for CISH and SISH are similar, the BK virus SISH is specially designed for automation offering a good integration into laboratory processes. In principle, a manually performed BK SISH would be possible if all components of the respective kit would be ordered separately. From a practical point of view this is unrealistic and Ventana platforms constitute an essential prerequisite for the automated SISH analysis.

We were able to compare BK virus serum levels with the level of BK virus SISH staining in renal tissue of PVAN patients. It is well established that high copy numbers of BK virus in the serum are a reliable surrogate marker for PVAN. Our data show significant correlations of PCR values with the quantified histological results. Nonetheless such correlations could be missing reflecting the time difference between serum analyses and biopsies as well as the heterogenic character of the disease. In fact, Drachenberg et al. found no direct correlation between level of viremia and extent of tissue involvement.

In conclusion, SISH has a high diagnostic concordance with CISH and SV40 immunostaining, fulfils the requirements for diagnostic testing and allows automation. Due to the stable light opaque metallic silver signal, SISH can be used with conventional light-microscopy in a setting familiar to all pathologists.
Acknowledgements

We are indebted to Mrs. Silvia Behnke for excellent technical support.
References


18. Dietel M, Ellis IO, Hofler H, et al. Comparison of automated silver enhanced in situ hybridisation (SISH) and fluorescence ISH (FISH) for the validation of HER2 gene status in


Figure Legends

Figure 1 Illustration of the BK-SISH technology
A/B: Cell with BK virus DNA integrated in the host cell DNA. C: The host cell DNA is degraded. C: The BK virus specific DNA probe with its antibody binding element (green) binds to the BK virus DNA in the host cell. D: The primary antibody binds to the binding element of the probe. E: The secondary antibodies with the silver elements bind to the primary antibody. F: The black silver staining is detectable with conventional light microscopy.

Figure 2 BK-SISH in comparison to the SV40 staining
A-D Cases with frequent strong specific positivity of SV40 (A/C) and BK-SISH (B/D) in corresponding tubules. E/F In the majority of cases the SV40 staining (E) was more frequent and easier to detect than the BK virus SISH (F). The only positive nucleus in the BK virus SISH and the corresponding one in the SV40 staining are marked with the arrow. Note the other positive nuclei in the SV40 staining without a corresponding SISH signal.