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Application of a nanoflare probe specific to a latency associated transcript for isolation of KHV latently infected cells

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Abstract

One of the unique features of herpesvirus infection is latent infection following an initial exposure, which is characterized by viral genome persistence in a small fraction of cells within the latently infected tissue. Investigation of the mechanisms of herpesvirus latency has been very challenging in tissues with only a small fraction of cells that are latently infected. Cyprinid herpesvirus 3, also known as koi herpesvirus (KHV), is an important and deadly pathogen of koi and common carp, *Cyprinus carpio*. Acute infection can cause up to 100% mortality in exposed fish, and fish that survive the infection become latently infected. KHV becomes latent in a small percentage of B lymphocytes and can reactivate under stressful conditions. During latency, KHV ORF6 transcript is expressed in the latently infected B lymphocytes. In order to study KHV latent infection in cells that are only latently infected, a nanoflare probe specific to ORF6 RNA was used to separate KHV latently infected cells from total peripheral white blood cells (WBC). Using the ORF6 nanoflare probe, less than 1% of peripheral WBC was isolated from KHV latently infected koi. When this enriched population of WBC was examined by real-time PCR specific for KHV, it was estimated that about 1 to 2 copies of viral genome persists in the sorted cells. In addition, KHV ORF6 transcript was shown to be the major transcript expressed during latency by RNA-seq analysis. This study demonstrated that an RNA nanoflare probe could be used to enrich latently infected cells, which can subsequently be used to investigate the molecular mechanisms of KHV latency.

Keywords

nanoflare probe; ORF6; KHV; latent infection; B cell; herpesvirus

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Introduction

Cyprinid herpesvirus 3 (CyHV-3), also known as koi herpesvirus (KHV), is a recently emerged pathogen of koi and common carp, *Cyprinus carpio* (1). The KHV belongs to the family *Alloherpesviridae*, which includes herpesviruses of fish and amphibians (2-4). KHV infection causes millions of dollars in loss and damage to the aquaculture industry as well as the ornamental koi trade (5, 6). Fish that do not succumb to a fatal infection from KHV become latently infected following an initial infection (7, 8). During herpesvirus latency there is no production of virus particles and the viral genome alone persists inside the latently infected cell with limited gene transcription. Upon physiologic stress, however, the latent viral genome can enter productive replication and begin making infectious particles. Reactivation of latent KHV can lead to transmission to naïve hosts and result in morbidity or mortality in naïve fish as well as reactivated hosts (7, 9).

Although it is known that many herpesviruses can lead to latent infection following an initial exposure, the mechanisms of latent infection and reactivation of latency remain largely unknown. The major obstacle of studying herpesvirus latent infection is that for any given herpesvirus, the virus becomes latent in a small percentage of cells within the recovered host (10). To understand how latency is established or how reactivation of latency is regulated, it requires the investigation of the cell population that directly harbors the latent herpesvirus. During human simplex virus type 1 (HSV-1) latency, the latent HSV-1 genome resides in 1-10% neurons within the trigeminal ganglia (10, 11), which can only be collected post-mortem (12-14). It is very challenging to investigate the molecular mechanism of latency in tissue where latency occurs in such a small, relatively inaccessible portion of cells.

Recently, nanoflare technology capable of detecting intracellular mRNA in live cells at the single-cell level has been developed and made commercially available by EMD Millipore (EMD Millipore Corporation) (15-17). The nanoflare consists of a spherical gold nanoparticle densely coated with a monolayer of single-stranded DNA (ssDNA) that is complementary to mRNA of a target gene. The ssDNA “recognition sequence” or “capture sequence” is prehybridized to a shorter complement DNA oligo, or flare strand, containing a fluorescent reporter (the “reporter flare”) whose fluorescence is quenched based on its proximity to the gold particle. When target mRNA binds the capture sequence, the reporter flare strand is displaced, providing a fluorescent signal. These constructs have been shown to enter over 60 cell lines and primary cells tested to date, and they are believed to do so by engaging scavenger receptors that facilitate caveolin-mediated endocytosis (15). They are also stable to nuclease degradation (18), lack cytotoxicity (19), and are effective for single-gene or multigene detection from a single nanoconstruct (20). Nanoflares, when coupled with flow cytometry, can be used to isolate cells with a target gene in the context of a greater cell population. They will allow for the enrichment of a cell population that has a specific RNA expression.

Our previous studies discovered that ORF6 mRNA is expressed in the peripheral IgM⁺ B cells from koi latently infected with KHV (21). In this study, a nanoflare probe is designed to specifically target ORF6 mRNA and used to separate KHV⁺ cells from peripheral WBC of latently infected koi. This study demonstrates that KHV latently infected cells can be

enriched by ORF6-specific nanoflare probe. The enriched cells can then be used to investigate the gene expression during KHV latency.

Materials and Methods

Koi and sampling

Koi 1-3 were donated by a distributor in Eugene, Oregon in March 2014 with unknown history of KHV exposure and were negative for KHV antibodies when tested by ELISA. Koi 4-6 were acquired in July 2011 from a local distributor in Corvallis, Oregon with a history of KHV exposure. All koi were screened for KHV by nested PCR as reported previously (22). Koi from each distributor were kept separately and maintained at 12°C in 4-foot diameter tanks at Oregon State University Salmon Disease Research Lab (OSU-SDL) in accordance with the Animal Care and Use Committee regulations. No fish in this study have had any clinical signs since their arrival at the OSU-SDL. All blood samples were collected via venipuncture of the caudal vein after anesthetizing the koi with 90 ppm MS-222 buffered with an equal amount of sodium bicarbonate. Whole blood was immediately transferred into tubes containing 3.2% sodium citrate to prevent both coagulation and erythrocyte lysis.

WBC isolation

Peripheral white blood cells (WBC) were isolated on a gradient of Ficoll-Paque PLUS (GE Healthcare). Briefly, 2 ml of anticoagulated whole blood was diluted with an equal amount of Hank's Balanced Salt Solution (HBSS) and then gently layered onto 3 ml Ficoll-Paque in 15 ml conical tubes. Tubes were centrifuged at $400 \times g$ at 20°C for 40 minutes. WBC formed a visible layer on the Ficoll-Paque and were removed and washed twice with HBSS at $200 \times g$ at 20°C for 10 minutes.

IgM⁺ and IgM⁻ WBC isolation

Both IgM⁺ and IgM⁻ WBC were isolated similarly as previously reported (21). Briefly, total WBC were stained first with anti-carp IgM monoclonal antibody (Aquatic Diagnostics Ltd) at 1:100 dilution on ice for 60 min and washed twice with HBSS, and then stained with anti-mouse IgG microbeads (Miltenyi Biotec) at a 1:4 dilution at 4°C for 30 min and washed once with HBSS. Following antibody staining, WBC were passed through an LS column on a magnet according to manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). The non-selected cells that flowed through the magnetized column were collected and labeled as "IgM⁻ WBC"; selected cells were washed off the column, collected, and labeled as "IgM⁺ WBC".

RNA nanoflare probe design

The ORF6 nanoflare probe made by EMD Millipore has a KHV capture sequence designed to target a 27 bp region of the KHV ORF6 transcript (5'-CGCACACTCTGACTGCTGAGGAGAAGT-3') and flare strand complementary to the capture sequence at 14 bp long to the 5' region of the capture strand (EMD Millipore). An Uptake-Cy5 probe (Cat. No. SF-137, EMD Millipore) that is always fluorescent was used to assess the cells' ability and efficiency to endocytose the probe. An additional control probe, Scramble-Cy5 probe (Cat. No. SF-102, EMD Millipore) that is always quenched was used

to determine background fluorescence. Additionally, WBC were treated in parallel with no added probe as a negative control. The lyophilized probes (Uptake-Cy5 probe, Scramble-Cy5 probe, and ORF6-Cy5 probe) were reconstituted according to manufacturer's instructions by slowly adding 50 μ l sterile water in a drop-wise fashion. Each probe was diluted at 1:20 with PBS and then added separately to cells at 1 μ l per 3×10^5 cells in RPMI 1640 media with 5% FBS (Life Sciences). The mixtures were incubated at room temperature in the dark for 20 hours before analyzing.

Flow cytometry and cell sorting with nanoflare probes

WBC incubated with nanoflare probes were analyzed and sorted directly on the BD InFLEX cell sorter at Oregon Health and Sciences University (OHSU) Core Flow Lab in Portland, OR. For initial analysis, WBC were pooled from two fish with known KHV exposure and sorted together. For RNA-seq analysis, WBC were collected from 3 fish with unknown KHV status (Koi 1-3) and from 3 fish with known KHV exposure (Koi 4-6) and each sample was sorted independently. Cells were excited by a red laser (640 nm) and analyzed by Cy5 mean fluorescence intensity. To assess probe uptake efficiency, cells with no probe were analyzed first to set a gate for the assessment of the cells incubated with Uptake-Cy5. To assess flare signaling and estimate background fluorescence (Gate A), cells were incubated with both uptake probe and Scramble-Cy5, which is always quenched. In addition, to assess the ORF6-Cy5 background staining (Gate B), both IgM⁺ cell and IgM⁻ cells were incubated with ORF6-Cy5 probe and Scramble-Cy5 to set a gate for sorting KHV⁺ cells by ORF6-Cy5 probe (Gate C).

Confocal Microscopy

KHV latently infected WBC were incubated with nanoflare probes as described above and nuclear staining was performed with Vectashield Mounting Media with DAPI (Vector Labs, Burlingame, CA). Stained cells were then examined on a Zeiss LSM510 META with Axiovert 200 motorized microscope using LSM software v3.2. Confocal images were analyzed with ImageJ software v1.46r.

Real-time Quantitative PCR

WBC from two KHV⁺ fish were pooled before incubating with nanoflare probes and sorted by flow cytometry. After 1000,000 cells were analyzed for determining total events in each gate described above, 5,000 were sorted and collected from each Gate A, B, and C. Total DNA was extracted from 5,000 cells from each gate using a DNA extraction kit (Omega BioTek) according to the manufacturer's instructions and eluted in 40 μ l TE buffer. KHV DNA was quantified in each sample by real-time PCR using primers KHV 86F and KHV 163R and TaqMan probe KHV 109P (23). Amplification was performed on the BioRad CFX96 thermocycler (BioRad Laboratories, Hercules, CA) using a 25 μ l reaction consisting of 12.5 μ l Platinum qPCR Supermix-UDG with ROX (Invitrogen, Carlsbad, CA), 0.5 μ l of each primer (20 nM) and probe (10 mM), and 10 μ l (approximately 500 ng total) of DNA template; the reaction was subjected to 50°C for 2 min, 95°C for 2 min, followed by 60 cycles of 95°C for 15 sec and 60°C for 60 sec; a slower ramp time was adjusted to 2°C/sec to facilitate higher sensitivity. Data analysis was performed by the associated CFX Management Software suite (BioRad Laboratories, Hercules, CA). A standard curve that

was previously established for this assay was used to calculate genome copy numbers based on C_T values ($Y = -3.296X + 44.301$) (21). The total genome copy numbers for the entire 5,000 sample (in 40 μ l) was calculated by the value from the 10 μ l sample (1/4 of the DNA sample).

RNA extraction and Illumina library preparation and RNA-seq

To investigate gene expression in ORF6⁺ WBC, 2×10^5 cells were sorted and collected from Gate C using the ORF6-Cy5 probe from each Koi 1-6 ($n = 6$), with the exception of Koi 5 which only yielded 1.7×10^5 cells from Gate C from the total WBC. Cells were pelleted and kept on ice for 2-hours before RNA extraction. Total RNA was isolated with PureLink RNA mini kit according to manufacturer's instructions (Ambion Life Technologies). RNA was then treated with DNase (Invitrogen) as described previously (21) and purified by ethanol precipitation with sodium acetate and glycerol according to manufacturer's instruction (Ambion Life Technologies). Ribosomal RNA was depleted by the Ribo-Zero Magnetic Gold kit (Epicentre) according to manufacturer's instructions. A cDNA library was prepared by using the Smarter Stranded RNA-seq kit (Clontech Laboratories) with random hexamer primers according to manufacturer's instructions, which recommend an optimum final fragment length to be around 300 bases. For RNA-seq, paired-end reads of 100 bases were obtained by the Illumina HiSeq 2000 at the Center for Genome Research and Biocomputing (CGRB, Oregon State University, Corvallis Oregon) (24).

Qualitative and quantitative analysis of RNA and cDNA library

RNA was analyzed on the NanoDrop spectrophotometer (Thermo Scientific) as well as by the Qubit fluorometer (Life Technologies). qPCR was used by the CGRB with primers for Illumina adapters to quantify the cDNA as well as to assess average size of cDNA fragments in each sample. Each cDNA sample was also analyzed by the Agilent 2100 Bioanalyzer to further obtain electropherograms on a High Sensitivity DNA chip.

Bioinformatics analysis of Illumina reads

A spliced and truncated reference genome was prepared from the CyHV-3 genome (NC_009127.1) using Geneious software. The second terminal repeat was eliminated to minimize a bimodal assembly of genes in the terminal repeat regions. Additionally, introns were removed at predicted splicing sites according to the annotated genome from all ORFs with predicted introns, which are ORF6, ORF7, ORF10, ORF90, ORF131, ORF134, ORF139, and ORF155, except ORF33. Returned reads were parsed by sample Koi 1-6 according to their indexed barcode. Illumina adapters were removed with the cutadapt script using stringency of minimum cut-length 45 bases; reads shorter than 45 bases were removed from analysis. Because of the overall suboptimal quality, the reads were not trimmed based on quality or phred scores. Reads were aligned to the reference KHV genome using Bowtie2 software (25), and resulting .sam files were converted to .bam files using samtools. Alignment files were visualized by Geneious software v 7.1.5 (Supplemental Fig. 1).

Results

Evaluation the endocytosis of the nanoflare probe in koi peripheral WBC

To determine whether the nanoflare probe can be endocytosed in the koi peripheral WBC, total WBC were incubated with Uptake-Cy5 probe (EMD Millipore), which is a positive control probe designed to evaluate the efficiency of endocytosis. Following incubation with the probe, cells were analyzed by flow cytometry and evaluated for mean fluorescence intensity (MFI). As shown in Figure 1A, the cells incubated with the Uptake-Cy5 probe had a higher MFI compared to cells that were incubated with no probe. These results demonstrate that the nanoflare probe can be effectively endocytosed by koi peripheral WBC.

Evaluation the endocytosis of the ORF6-Cy5 nanoflare probe in koi peripheral WBC

To determine whether ORF6-Cy5 nanoflare can be endocytosed as the uptake probe in koi WBC, IgM⁺ cell and IgM⁻ cells sorted by magnetic beads were incubated with uptake-Cy5 probe or ORF6-Cy5, the specific probe to ORF6 transcript, and then analyzed by flow cytometry. As shown in Figure 2, both IgM⁺ cells and IgM⁻ cells had similar staining following uptake-Cy5 or ORF6-Cy5 probe staining. It suggests that both cell populations can internalize the nanoflare probe. In addition, both IgM⁺ or IgM⁻ cells stained by the ORF6-Cy5 probe had a higher fluorescence staining than cells stained by the uptake-Cy5 probe (Figure 2). It suggests that ORF6-Cy5 had a higher fluorescence staining than the uptake probe. To further confirm the ORF6-Cy5 is internalized following the staining, the cells were also viewed by confocal microscopy. A few cells incubated with ORF6-Cy5 (Fig. 3, bottom panel) displayed Cy5 signal in the cytoplasm whereas no Cy5 signal was visualized from cells incubated with Scramble-Cy5, which is a negative control probe provided by the manufacture (Fig. 3, top panel) (Cy5 seen as red, DAPI as cyan).

Isolation of live KHV⁺ latent cells from total WBC with ORF6 nanoflare probe

To isolate the KHV⁺ cell from total WBC directly, WBC were stained by the ORF6-Cy5 probe first, and then analyzed by flow cytometry. As shown in Fig. 1B, cells incubated with ORF6-Cy5 had two major peaks of MFI which are higher than cells incubated with the Scramble-Cy5, and can be seen as a right-shift in MFI for the majority of cells compared to cells that were incubated with Scramble-Cy5 (Fig. 1B). Since the peak (Gate B) had similar fluorescence staining as seen in Fig. 2, it is considered as the background staining (Table 1, n=6, paired t-test, p = 0.0002), while the peak (Gate C) has significant higher fluorescence staining, it is considered as the specific staining for ORF6 transcript (Fig. 1B; Table 1, n=6, paired t-test, p < 0.0001). Cell sorted in gate C accounted for about 0.6% of total white blood cells sorted.

Assessment of the KHV genome presence in the enriched KHV⁺ population

To determine the specificity of ORF6-Cy5 and its ability to identify KHV⁺ cells from latently infected koi, WBC from Koi 4-5 were pooled and incubated with the probe before sorting by flow cytometry into three Gates A, B, and C (Fig. 1B). DNA from 5,000 cells from each gate (Gates A, B, and C) was extracted and KHV genome copy numbers were estimated by real-time qPCR. Based on the standard curve made with a known concentration

of KHV DNA (21), about 101 copies and 1240 copies of the genomes were detected in 5000 cells from Gate B and Gate C, respectively. The amount of KHV genomes detected in 5000 cells from gate C is about 12 fold higher than the number of genome copies detected in the same number of cells from Gate B (Table 2). There was no detection of KHV genomes in 5,000 cells from Gate A. This confirms that the majority of latent KHV genomes reside in cells from Gate C with a few KHV genomes residing in cells from Gate B. To estimate the efficiency of DNA recovery from 5,000 cells, KHV genomic DNA at a known concentration was added to Vero cells and extracted similarly as the peripheral WBC. As shown in Supplemental Table 1, about 13-25% of the spiked DNA was recovered by the DNA extraction method used in this study. Based on the DNA recovery rate (Supplemental Table 1), approximately 4958 to 9535 copies of KHV genome is predicted to exist in 5,000 latently infected cells, which corresponds to 1 to 2 KHV genome copies per cell in Gate C. Since only about 101-252.5 copies KHV genome was found in the 5000 cells from Gate B, it suggests not all the KHV⁺ cells were stained and sorted into Gate C.

Qualitative and quantitative analysis of RNA, cDNA, and raw reads

To determine if the KHV⁺ cells can be used to investigate the latent KHV transcriptome, total RNA was extracted from 170,000 to 200,000 cells that were sorted into Gate C using ORF6-Cy5. Ribosomal RNA was depleted using the Ribo-Zero Magnetic Gold kit (Epicentre) according to manufacturer's instructions. Following ribosomal RNA depletion, 20 µl of RNA at 7.05 – 15.75 ng/µl was obtained from each fish, which is less than the amount of RNA recommended for cDNA library synthesis. cDNA libraries were made from those RNA as templates using the Smarter Stranded RNA-seq kit (Clontech Laboratories), which gave rise to cDNA fragments at average between 365-422 bases at 165.2 – 648.0 pg/µl (Agilent Bioanalyzer). Samples were subsequently sequenced by the Illumina HiSeq 2000. Post-run analysis of reads for each sample yielded a range of 3792 to 6463 M bases from 51,935,260 to 79,018,010 individual paired-end reads of 100 bases each (Table 3). The mean quality score of reads ranged from 23.36 to 31.87, with the percent of bases with a quality score over 30 ranged from 61.17% to 80.07% for each sample.

Bioinformatics analysis of RNA-seq reads

Bioinformatics analysis of reads from each sample identified few reads that mapped to the unique region of KHV genome (47,624 - 230,667 mapped reads), the majority of which mapped to repeated regions within the KHV genome or had higher identity to the *Cyprinus carpio* genome (Supplementary Table 2). After visual inspection of each read that mapped, reads that were determined as artificially mapped to the KHV genome were disregarded. The resulting reads mapped consistently to ORF6 for every sample (Table 3). The number of reads from each sample that mapped to ORF6 are 28 reads, 121 reads, 68 reads, 46 reads, 236 reads, and 162 reads for Koi 1-Koi 6, respectively. Koi 1 had two reads that mapped to ORF81 and two reads that mapped to ORF114; Koi 2 had 4 reads that mapped to ORF79; Koi 3 had 8 reads that mapped to ORF92; and Koi 5 had 2 reads that map to TNFR family gene ORF4. There are also reads aligning to the repeat region outside the coding region of KHV. The Illumina data suggests that ORF6 transcript is the major RNA present in KHV positive latently infected WBC.

Discussion

This study demonstrates that KHV latently infected WBC can be identified by an ORF6-specific nanoflare probe and subsequently sorted by flow cytometry. The enriched KHV⁺ cells from each fish can be used to investigate the gene expression by RNA-seq. Sorting with nanoflare probes is based on the difference in mean fluorescence intensity (MFI) staining between two different cell populations that are known to be KHV positive and KHV negative. The background staining (Gate B) is determined by incubation of the ORF6-Cy5 probe with IgM⁻ cells from koi, which are known to be KHV negative in our previous study (21). Although ORF6-Cy5 probe used in our study had a higher non-specific staining, cells with ORF6 RNA specific staining (Gate C) had an average fluorescence that is about six fold greater than the background staining (Gate B). The higher background staining may be caused by the sequence selected for the target gene and non-specific binding to cellular targets. The qPCR analysis further demonstrated cells from Gate C carry KHV genome, which is estimated at 1-2 copies per cell; while cells from Gate B contain very few copies of KHV genome. Detection of KHV genome in cells from Gate B may result from that not all KHV⁺ cells were stained by the probe and sorted into Gate C. Although a very small fraction of cells, less than 1% of peripheral WBC, was sorted into Gate C, they have a much higher MFI than cells in Gate B. This suggests that reporter flares were capable of identifying KHV⁺ WBC containing ORF6 transcripts (Table 2).

In humans, acquisition of primary EBV infection results in a life-long persistent infection in memory B cells. It is estimated that 1 to 50/10⁶ B cells were latently infected with EBV in healthy seropositive individuals (26, 27). Using the ORF6-Cy5 nanoflare probe, about 200,000 KHV⁺ cells were sorted from about 1-2×10⁸ cells from 2 ml whole blood. This suggests that, about 1-2/10³ cells are latently infected by KHV. Our results demonstrated that KHV latency frequency in koi is greater than EBV latency in humans (about 500-1,000 fold greater). When the KHV genomes were quantified in the cells from Gate C, only about 4958 to 9535 copies were detected from DNA extracted from 5,000 WBC (Table 2) when 13-25% DNA recovery efficiency was considered, which suggests that about 1-2 copies of KHV genome persists in each latently infected cell. This result is similar to HSV-1 latency in the ganglion neurons, which has 1-10 copies of viral genome per neuron (28).

In agreement with our previous study, ORF6 transcript is also found to be the major transcript expressed during latency by the RNA-seq analysis. The RNAseq reads agree with analysis of previous 5'RACE and 3'RACE analysis and confirmed that ORF6 transcript starts -127 bp upstream of the ATG of ORF6 and ends at 189 nt untranslated region downstream of the stop codon (21). In addition, RNAseq data also revealed an intron within ORF6 gene which is in agreement with previous gene annotation (4). Searching against Conserved Domain database (www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml), a conserved domain (aa 342-472) was identified in the predicted 725 aa of CyHV-3 ORF6, which has homology to consensus sequences of EBNA-3B conserved domain (residues 683 to 808) and the N-terminal regulator domain of ICP4 (residues 103 to 225). It suggests that ORF6 may serve as transcription regulator as ICP4 of HSV-1 or EBNA of EBV. In addition to ORF6 transcripts, a few reads that mapped to other regions of the KHV genome such as ORF4, ORF 79, ORF 81, ORF92 and ORF 114, were obtained from only one or two koi.

ORF4 is predicted to encode a protein involved in immune regulation and may possibly play a role in latency maintenance. Both ORF 81 and ORF 114 encode a small membrane protein, which has an unknown function. ORF 79 encodes a DNA polymerase catalytic subunit, involved in DNA replication. ORF 92 encodes a major capsid protein, which only present during productive infection. It is possible that KHV may undergo reactivation within a few cells during incubation with the ORF6 probe and its replication may be incomplete. Therefore, detection of those transcripts was random.

In addition, there were several regions within the KHV genome that tends to be artificially mapped by non-specific repeating reads. These regions containing several repeating nucleotides were often aligned to by single read at about 100 bp, which has an abundance of coverage. An example is the 74 base-long repeat of “GTGTGT” that mapped to bases 37880 – 37943 of the KHV genome (has 116, 924× coverage. Another example is the 162 bp of “GAGAGA” nucleotides that mapped to KHV genome positions 177,568-177,729 has 12,632× coverage. These single-read alignments were considered an artifact due to the repeating nature of the KHV genome and were not considered true viral transcription. This type of alignment artifact accounts for the majority of the reads mapping to the KHV genome from the Bowtie2 alignment (Table 3). Another source of non-specific mapping artifact is the homology between the *C. carpio* genome and the KHV genome (GenBank DQ657948). Some reads that had 99% homology to the *C. carpio* genome also had 90-95% homology to the KHV genome due to the co-evolution of the herpesvirus with its host and therefore erroneously mapped to the KHV genome.

In this study, KHV exposure from Koi 4-6 was previously known, while Koi 1-3 were from a facility with no previous history of KHV exposure and tested negative by ELISA (unpublished data) and real-time PCR (23). It is interesting that the latency associated transcript ORF6 was identified in Koi 1-3, but with generally fewer reads and less coverage for ORF6 than in Koi 4-6. It is possible that these fish are KHV latently infected but their infection was not detected due to the low sensitivity of the ELISA test and real-time PCR of the total WBC (9). Our previous study detected KHV in 17 of 24 wild common carp with no clinical signs of KHV from two different natural ponds in Oregon (22), demonstrating a widespread prevalence of latent KHV in natural waterways. It suggests that fish from facility without KHV outbreak history may also have KHV latent infection.

In conclusion, this study describes successful employment of a nanoflare probe specific to the KHV latency associated transcript ORF6 for isolation of KHV⁺ WBC from latently infected koi. The latent infection frequency of KHV was estimated to be 1-2/1000 WBC. Only 1 to 2 copies of viral genome is estimated to be present in the latently infected cell. RNA-seq analysis of viral transcripts from the enriched KHV⁺ WBC revealed ORF6 transcripts to be the major latency associated transcripts expressed during KHV latency. The ability to obtain a population of exclusively latent-infected cells will allow further investigations into the molecular program of KHV latency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- RNA nanoflare probe can be used to isolate cells with specific mRNA expression.
- ORF6 specific nanoflare probe can be used to isolate cells latently infected by KHV.
- WBC with KHV latency can be sorted based on ORF6 expression using the nanoflare probe.
- The cells sorted based on ORF6 mRNA expression can be used for RNA-seq analysis.
- ORF6 transcript was also found to be the major mRNA expressed in the latently infected cells.
- 1-2 copies of KHV genome was detected in ORF6⁺ cells.

Fig. 1.

The nanoflare probe endocytosis in koi WBC. Flow cytometry analysis of WBC incubated with nanoflare probes. A: Top: WBC incubated with no probe; Bottom: WBC incubated with Uptake-Cy5 probe. B: Top: WBC incubated with Scramble-Cy5 probe; Bottom: WBC incubated with ORF6-Cy5 probe. Gate A: set to MFI of Scramble-Cy5 cells. Gate B: set to MFI of majority of ORF6-Cy5 stained cells (both IgM⁺ and IgM⁻). Gate C: set to MFI of top 1% of ORF6-Cy5 stained cells. X axis: log MFI [mean fluorescence intensity of Cy5 at 640-1 (670/630)], Y axis: cell counts.

Fig. 2.

Flow cytometry analysis of WBC incubated with nanoflare probes. A: IgM⁺ and IgM⁻ WBC incubated with uptake-Cy5 probe. B: IgM⁺ and IgM⁻ WBC incubated with ORF6-Cy5 probe. The fluorescence intensity is on X-axis by a red laser at 640 nm (670/30). The B is the fluorescence intensity (Gate B) set for background staining.

Fig. 3. Confocal micrographs of live WBC incubated with nanoflare probes. **A.** WBC stained with Scramble-Cy5 probe. **B.** WBC stained with ORF6-Cy5 flare probe. White arrow shows positive Cy5 signal. Magnification 40X. Bar = 5 μ m. Cy5 appears red, DAPI appears cyan.

Table 1

Averaged mean fluorescence intensity (MFI) of Cy5 fluorophore (640-1 670/30) for WBC incubated with each probe (n = 6 fish).

	Unstained	Scramble (Gate A)	Uptake	Flare (Gate B)	Flare (Gate C)
Koi 1	3	19	40	313	1846
Koi 2	3	16	33	362	2034
Koi 3	3	29	48	444	1845
Koi 4	4	13	24	213	2191
Koi 5	4	15	27	394	2434
Koi 6	4	19	36	308	1751
Average MFI	3.5	18.5	34.67	339	2016.83
P value			0.0004	0.0002	<0.0001

Table 2

Quantitative Taqman PCR results for WBC in each gate by ORF6-Cy5 probe for latently infected koi peripheral WBC, using previously established standard $Y = -3.296X + 44.301$

DNA source	Ct value	Copy number in ¼ of DNA sample extracted from 5000 WBC	Total number of KHV genome per 5000 WBC
KHV DNA (pos)	16.31	3.11E+08	n/a
Gate A	0	0	0
Gate B	39.68	25.22	100.88
Gate C	36.09	309.88	1239.52
Negative	0	0	n/a

Table 3

Post-run data from 100 base reads from Illumina HiSeq 2000 and KHV reads

	Koi 1	Koi 2	Koi 3	Koi 4	Koi 5	Koi 6
Yields (Mbases)	3,792	5,042	6,463	5,274	6,029	4,216
No. of reads	51,935,260	68,889,138	79,018,010	66,387,952	74,901,846	58,854,060
Mean quality score	23.36	25.76	31.49	30.82	31.87	25.65
%>30 quality bases	61.17	62.67	78.93	77.03	80.07	62.06
Reads aligned from Bowtie2	47,624	69,209	149,929	121,134	230,667	88,374
Reads aligned to KHV ORF6	28	121	68	46	236	162
Reads aligned to other KHV genes						
ORF4					2	
ORF79		4				
ORF81	2					
ORF92			8			
ORF114	2					