Szent István University
Postgraduate School of Veterinary Science

Genetic analysis of the sturgeon adenovirus and fish herpesviruses

Brief Summary of the PhD Thesis

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2011

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Introduction

At the beginning of my work, all herpes-like viruses isolated from lower vertebrates were classified into the family *Herpesviridae* by their morphological features and cytopathic effects. However, phylogenetic calculations showed that these viruses did not belong to any of the existing subfamilies *(Alpha-*, *Beta-* and *Gammaherpesvirinae*). The *Ictalurid herpesvirus 1* (IcHV-1) was the first fish herpesvirus (HV), the complete genome of which was sequenced. A novel unclassified genus (*Ictalurivirus*) was established for it. At that time, from other fish and amphibian HVs there were only short DNA sequences in public databases. It seemed to be interesting to sequence and analyze the genome of a HV (*Acipenserid herpesvirus 2*, AciHV-2) isolated from an ancient chondrostean fish. Further on, DNA samples from *Ictalurid herpesvirus 2* (IcHV-2) isolated in Italy and from Siberian sturgeon herpesvirus (SbSHV) isolated in Russia were sent to our lab. Previous serological studies indicated that IcHV-1 and 2 were different virus species. The SbSHV was identified as an HV by the morphological features and cytopathic effects. The partial genome analysis of these two viruses was taken into our study.

Before of my work, the family *Adenoviridae* contained four genera. The genus *Mastadenovirus* comprises only mammalian adenoviruses (AdVs), the genus *Aviadenovirus* is composed of avian AdVs. Different reptilian, avian, ruminant and marsupialian AdVs belong to the genus *Atadenovirus*. It seems that the atadenoviruses have co-evolved originally with the squamate reptiles, and host switches occurred to species of other vertebrate classes. The genus *Siadenovirus* contains a frog, several avian and turtle AdVs. Formerly it had been hypothesized that the siadenoviruses had co-evolved with the amphibians. Considering the newer results, however, the host origin of the siadenoviruses can not be determined. The genome sequencing of the white sturgeon AdV (WSAdV-1) was started in our lab earlier. The sequence of the conserved middle part of its genome has been determined by random cloning and PCR. The phylogenetic calculations have implied that the WSAdV-1 represents an independent lineage within the family *Adenoviridae*. The genome organization of the AdVs belonging to different genera shows significant differences at the genome ends. It seemed to be very interesting to analyze the full genome of the WSAdV-1, the first member of a putative new genus.

Aims of the study

- Partial genome sequencing and analysis of the white sturgeon herpesvirus
- Full genome sequencing and analysis of the white sturgeon adenovirus
- Genetic study of other fish herpesviruses
- Detection of fish herpesviruses by PCR in Hungary

Materials and Methods

Virus strains and fish samples

The genetic content of four viruses was examined in this study. Two American strains, WSAdV-1 and AciHV-2, were isolated from adult, healthy, wild-living white sturgeons. The IcHV-2 was isolated from black bullhead in Italy. The SbSHV strains (SK1/0406, SK2/0506 and BK/0506) were isolated in Russia from Siberian sturgeon.

In Hungary, samples (liver, gill, kidney and epithelium) were collected from a carp showing typical carp pox symptoms, and a pool of several internal organs from Prussian carp was collected after mass mortality.

The purification of the viral DNA

The virions of AciHV-2 and WSAdV-1 were concentrated by ultracentrifugation from the cell culture supernatant. Subsequently DNA extraction was carried out by phenol.

A lyophilized sample of IcHV-2 and the extracted DNA of SbSHV fixed on Whatman-paper were used as target in PCRs.

The carp and Prussian carp samples were homogenized and digested with proteinase K and treated by guanidin-hidrochloride. Subsequently the DNA was precipitated by ethanol.

Molecular cloning

The viral and plasmid DNA was digested with *Hin*dIII and *Pst*I enzymes. The purified DNA fragments were ligated into plasmid (pBluescript KS; Stratagene Ltd., Santa Clara, CA, USA). Subsequently *E. coli* strains were transformed by heat shock or electroporation. The bacteria were spread on LB agar containing ampicillin. The plasmid DNA was purified by alkaline mini preparation method.

The DNA fragments amplified by PCR were cloned using the CloneJet kit (Fermentas AG., Vilnius, Lithuania) according to the manufacturer's instructions.

Polymerase chain reaction (PCR)

Two types of primers specific and consensus were used. The specific primers were designed on the known sequences. The AciHV-2, IcHV-2 and SbSHV DNA polymerase and terminase genes were amplified by consensus primers. The degenerate primers were designed by amino acid (aa) alignments of the gene products. For amplifying the DNA polymerase of CyHV-1 and 2 consensus primers were used designed for the DNA polymerase of all known fish HVs.

For the PCRs, Phusion® High-Fidelity DNA polymerase enzyme (Finnzyme Ltd., Espoo, Finland) was used. The following PCR mix was found to be appropriate: 35 µl distilled water, 10 µl Phusion® 5X HF buffer, 1.5 µl dNTP (10 mM), 1 µl of each primer, 0.5 µl enzyme and 1 µl target DNA.

Determination of the ends of the WSAdV-1 genome

The terminal protein (TP) attached to the 5' ends of the DNA of AdVs makes difficult to clone the genome ends as blunt-ended fragments. Therefore, unidirectional PCRs were carried out with one primer from the known region toward the genome ends. To confirm that the genome ends were gained, the 5'/3' RACE Kit (Roche Ltd., Basel, Switzerland) was applied according to the manufacturer's instructions.

DNA sequencing, analysis and phylogenetic calculations

The DNA sequencing reactions with BigDye^{TM} Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Ltd., Warrington, United Kingdom) were carried out according to the manufacturer's instructions. The electrophoresis was performed on an automated sequencing machine (ABI Prism® 3100), by a commercial supplier (MTA SZBK).

For DNA sequence identification BLASTX and BLASTN were used. The sequences were joined by Staden program package.

Phylogenetic calculations were carried out by maximum likelihood, distance matrix and Bayesian statistics.

Results

White sturgeon herpesvirus

A 66,037-bp-long fragment was sequenced from the middle of the AciHV-2 genome. The fragments gained by random cloning and consensus primers were joined by 7 PCR products, and submitted to the GenBank (accession number: FJ815289). The G+C content of the fragment proved to be 38%. It contains 46 putative ORFs. Thirty-eight ORFs show

convincing homology to the corresponding ORFs of IcHV-1. The position and orientation of these ORFs are the same in the two genomes. Eight ORFs did not show homology to any herpesviral ORFs. The sequenced genome part of the AciHV-2 contains 12 ORF, which are shared in all known alloherpesviruses.

Phylogenetic calculations were carried out with these conserved genes. Using different statistical methods, the topology of the phylogenetic trees was essentially identical, supported by high statistical values. The trees showed that the AciHV-2 and the two ictalurid HVs (IcHV-1 and 2) are close relatives forming a monophyletic clade. Additionally, the trees showed that the family *Alloherpesviridae* has three main clades, the cypriniviruses, the batrachoviruses, and the ictaluriviruses.

IcHV-2 and SbSHV

A 7982-bp-long fragment was sequenced from the black bullhead HV (IcHV-2) and submitted to the GenBank (FJ815290). The G+C content of the fragment proved to be 51.9%. This genome part contains the homologues of ORF(57+58), 59, 60, 61 and 62 of IcHV-1.

The nucleotide (nt) sequences of the three Siberian sturgeon HV (SbSHV) strains (SK1/0406, SK2/0506 and BK/0506) proved to be identical, therefore we examined only the strain SK1/0406 further. The DNA fragment between the genes of the DNA polymerase and terminase (ORF57-62) was amplified, the length of the fragment is 7048 bp (GenBank accession number: GU253908). The G+C content of the fragment was found to be 38.1%.

CyHV-1 and 2

A 464-bp-long fragment of the DNA polymerase gene was amplified from the carp HV (CyHV-1) and the Prussian carp HV (CyHV-2), respectively. Every examined sample from both fish was positive for HV, except the liver sample of the carp proved to be negative.

White sturgeon adenovirus

The full genome of the WSAdV-1 was found to consist of 48,395 bp. It was deposited to the GenBank with accession number AY082701. The G+C content was found to be 42.6%. The inverted terminal repeats of WSAdV-1 were 126 bp long. Out of the 48 ORFs, found in the genome, 16 genes occupied in the middle part of the genome, the homologues of which are present in every known AdV genome. At the left end, in an unusual location, four putative fiber genes were found. Rightward from the conserved genes, 28 novel ORFs were found. Two of them show homology to genes coding parvovirus non-structural (NS) proteins. The

predicted protein coded by another ORF contains two Ig superfamily domains. The putative gene products of two other ORFs comprise sulphotransferase domains. The remaining 23 ORFs did not show any homology to any known genes.

The phylogenetic calculations showed that the WSAdV-1 is separated from the members of the other four genera. Using different methods, the topology of the trees was almost identical. However, using different conserved genes for the calculations, the topology of the trees showed slight differences.

Discussion

White sturgeon herpesvirus

The genome organization of AciHV-2 with that of IcHV-1 showed high co-linearity. All genes of the IcHV-1 with known function were identified also in the genome of AciHV-2.

To date, five complete genomes of AHVs have been sequenced, namely the *Ictalurid herpesvirus 1*, *Cyprinid herpesvirus 3*, *Anguillid herpesvirus 1*, *Ranid herpesvirus 1* and *2*. Comparative analyses of these genomes led to radical changes in the taxonomy of HVs in 2008. A novel family (*Alloherpesviridae*) was established for the HVs of fish and amphibians. For the only known mollusk HV, the family *Malacoherpesviridae* was created. The HVs of higher vertebrates remained in the family *Herpesviridae*. The three families were classified into the novel order *Herpesvirales*.

Nowadays, the family *Alloherpesviridae* contains four accepted genera. The genus *Batrachovirus* contains the amphibian HVs. The genus *Cyprinivirus* comprises three carp HVs and soon the AngHV-1. The genus *Salmonivirus* comprehends the HVs of salmonids. The genus *Ictalurivirus* contains HVs of ictalurid and acipenserid fish. The classification of the latter two viruses into this genus was proposed by us. Eventually the Herpesvirales Study Group's official proposal to the ICTV was also based on our results.

The phylogenetic calculations, based on an alignment prepared from the concatenated full sequence of 11 ORFs, showed that the family *Alloherpesviridae* is composed of three main clades. One of these comprises CyHV-3 and AngHV-1. This lineage corresponds to the genus *Cyprinivirus*. AngHV-1 is not yet classified officially as a member of this genus, but a proposal for it is pending. The second clade contains the viruses of the genus *Batrachovirus*, namely RaHV-1 and 2. The third group includes the genus *Ictalurivirus* with IcHV-1 and AciHV-2. Based on earlier published phylogenetic trees, additional viruses can be assigned to two of the clades. These include the other members of the genera *Cyprinivirus* and *Ictalurivirus*. Moreover, the genus *Salmonivirus* with three members (SalHV-1, 2 and 3) clusters together with the lineage of *Ictalurivirus*.

The number of the conserved genes supports the three clade theory as well. The genomes of the RaHV-1 and 2 contain only 40 homologous genes. The two ictaluriviruses (AciHV-2 and IcHV-1) share 38 homologous ORFs, and only 46 are known from the genome of AciHV-2. If the AngHV-1 will be classified into the genus *Cyprinivirus*, the members share only 28 convincingly homologous genes. In the family *Herpesviridae*, all members have 43 homologous genes, whereas in the family *Alloherpesviridae*, the number of the homologous genes is only 12. Supposedly the HVs of lower vertebrates are more ancient than the members of the family *Herpesviridae*, and had a longer time for the diversification of their genomes.

The significant difference in the size range of the genomes of the members of these three main groups further supports the separation. The genome of IcHV-1 is 134 kbp, that of the ranid HVs is 220-230 kbp. The AngHV-1 and CyHV-1-2-3 have a genome of 245-295 kbp long. The estimated genome size of SalHV-1 is 174 kbp long. In former studies, phylogenetic calculations showed that AHVs of different salmonid fish species are more closely related to ictaluriviruses than to other AHVs.

By right of these findings we proposed the subdivision of family *Alloherpesviridae* into three subfamilies. One would contain the genus *Cyprinivirus* (CyHV-1, 2 and 3) and the AngHV-1, the second would comprise the genus *Batrachovirus* (RaHV-1 and 2), and the third subfamily would include the genera *Ictalurivirus* and *Salmonivirus*.

It seems that the main lineages of the AHVs similarly to those of HVs do not divide according to the host taxa. One can speculate that the ancestors of these different viruses diverged before or at the beginning of the separation of the different fish species. Later the viruses of the different lineages have been evolving independently and appeared in representatives of evolutionarily distinct taxa. Thus, the AHVs of the two genera have a wide host range. They can infect fishes from different superorders (Elopomorpha, Ostariophysi) in case of cypriniviruses or from different subclasses (Chondrostei, Neopterygii) in case of ictaluriviruses.

Another explanation for the high similarity between the viruses of these evolutionarily distant fish species could be that host switches have occurred. If we accept this scenario, the question remains whether originally which host the ictalurivirus lineage co-evolved with. The G+C content of AHVs with known full sequence ranges between 52.8 and 59.2%. Interestingly the G+C content of the AciHV-2 genome part, sequenced to date, is as low as 38%. This finding could suggest that the ictaluriviruses have co-evolved originally with the ictalurid fish species and some of them got into the acipenserid fish species by host shift. According to a prevailing hypothesis, decreasing G+C content of a viral genome might reflect an adaptation process to a new host. This phenomenon has been described in case of feline immunodeficiency virus and canine parvovirus. Similarly, the biased base composition of

atadenoviruses and siadenoviruses has also been attributed to hypothesized host switches. The evolutionary advantages of such changes in the genomes are not understood yet. Presumably the virus tries to escape the defence mechanism of the immune system of the new host in this way, reducing the number of the CpG dinucleotides. The host switch theory is further supported by the observation that AciHV-2 can cause severe disease outbreaks with much higher mortality (80%) than AciHV-1 (40%). Moreover, the Siberian sturgeon herpesvirus, which is a very close relative of AciHV-2 (or perhaps the same virus species), causes 100% mortality among sturgeon fingerlings. These findings suggest that these viruses might have not spent long time with the host and did not adapt to it. Former phylogenetic calculations have shown that the AciHV-1 is not a member of the genus *Ictalurivirus*. We presume, by virtue of G+C content (47.4%) of AciHV-1 and the lower mortality connected to it, that the AciHV-1 has co-evolved originally with acipenserid fish species.

IcHV-2 and SbSHV

A gene block between the gene of the DNA polymerase and the terminase was studied from the IcHV-2 and SbSHV genomes. In all examined ictaluriviruses (AciHV-2, IcHV-1 and 2, SbSHV), this gene block seems to be conserved. The identity/similarity between the corresponding gene products of the two IcHVs is 56-80%. It suggested that the IcHV-2 belongs to the genus *Ictalurivirus*, but, in accordance with the results of serological tests, is not identical species with IcHV-1. In 2009, the ICTV accepted the proposal of the Herpesvirales Study Group, and accepted the IcHV-2 as a separate species, and classified into the genus *Ictalurivirus*.

Studying the same gene block of the SbSHV, the overall identity/similarity between the deduced aa sequences of the examined ORFs of SbSHV and that of the AciHV-2 was 89/95%, respectively. The nt sequences of the DNA polymerase and terminase gene fragments showed 99% identity with the Canadian strain of AciHV-2 originating from short-nose sturgeon. For a final decision, whether the AciHV-2 and SbSHV are of the same virus type (species), the results of serological comparison would be essential.

CyHV-1 and 2

We reported the first PCR-based detection of CyHV-1 and 2 in Hungary. For the very first time, the occurrence of CyHV-2 was described in the Prussian carp instead of the usual host the goldfish. A 464 bp-long fragment of the DNA-dependent DNA polymerase gene was amplified by PCR from both viruses. The nt sequence of the amplicons was determined and analyzed. In the case of both viruses, the nucleotide sequences exhibited only slight (1%)

discrepancies when compared to their counterparts published abroad previously. However, none of these differences resulted in alterations of the aa sequences.

White sturgeon adenovirus

We sequenced the complete genome of WSAdV-1 (the only known AdV isolated from fish). From other adeno-like particles detected in fish species, sequence data are not available.

The genome organization of the WSAdV-1 remarkably differs from that of all known AdVs. The genome (48,395 bp) of the WSAdV-1 proved to be the largest among AdVs. The genome ends are flanked by ITRs. The first 17 nt of the ITR did not contain guanine in formerly studied AdVs, but in the WSAdV-1 genome the ITR's first and fifth nt is guanine.

At the left end of the genome the E1 region lacks. Instead of it, at the left end of the genome of WSAdV-1 four putative fiber genes were identified by virtue of the sequence analysis. For lack of good quality EM photos about the free virions, the real number of fibers on the vertexes is unknown. Surprisingly, the fiber genes are located left from the E2B region, the first four ORF at the left end of the genome. So far, fiber gene or genes of all known AdV located at right from E3 or right from pVIII gene. From the vertex of aviadenoviruses two fibers protrude. The FAdV-1 genome codes two fiber genes. The other fowl AdVs genomes contain only one, but their gene products are duplicated on the vertexes. Some human and non-primates monkey AdV genomes comprise two fiber genes, but only one fiber protrudes from the vertexes.

The tail region of the fiber attaches to the penton base with the FNPVYP motif. The putative fiber-1, -2 and -3 of the WSAdV-1 contain this motif. The shaft region contains repetitions, the fiber-1 and -2 comprise 14 repetitions, the fiber-3 contains 12. The fiber-1 and -3, and the fiber-2 and -4 supposedly come into existence by gene duplications (in case of fiber-4 inversion occurred, as well). The fiber-4 supposedly does not function as a fiber, it does not contain conserved motifs and repetitions, moreover it is coded on the other stand.

The size and location of the genes of the E2 region, the DBP, pTP, DNS polymerase, IVa2 and from the late genes the 52K, pIIIa, penton base, hexon, protease, 100K, 33K and 22K are similar to other AdVs.

Exceptions are the pVII, which proved to be very short in the WSAdV-1. The deduced polypeptide consists of only 24 aa, usual size of the pVII in different AdVs is between 72 and 160 aa. We could identify only one proteolitic cleavage site by analyzing the aa sequences.

The pX proved to be also short, only 34 aa (compared to 71-214 aa in other AdVs genome). However, the size of the mature protein, after proteolitic cleavage, seems to be average.

The size of the pVI is average, a type II proteolitic cleavage site was found at the N-terminus. At its C-terminus another proteolitic cleavage site was identified, with a sequence (FCGR'G) slightly differs from the usual consensus sequence. However, in case of cleavage at this site, the 11 aa-long pVIc cofactor peptid arises, which was described in all AdVs.

At the right end of the genome, rightward from the pVIII, 28 ORFs were found. Most of them did not show homology to any other known genes. There are only five exceptions. The predicted products of ORF5 and 6 show homology to parvovirus non-structural proteins (NS). Among AdVs, only aviadenoviruses contain similar genes. Their function is unknown in aviadenoviruses. The putative protein coded by ORF25 comprises two immunoglobulin domains. Similar genes were found also only in aviadenoviruses. The ORF4 and 9 show homology to genes coding sulphotransferase. So far, such genes were not found in the genome of viruses. The origin and function of the other 23 ORFs remain unknown.

The genome size of the AdVs varies between 26-46 kbp, and is generally characteristic for the members of the different genera. We hypothesized, that the genome of viruses of higher vertebrate hosts is longer. The more complex genome allows a better response to the defense mechanism of the more developed immune system. At that time, the lineage of siadenoviruses was thought to be the AdVs of amphibians. Accordingly, we expected that the genome of fish AdV would be the shortest.

It is hypothesized that the AdVs have been co-evolving with their vertebrate hosts for millions of years. Mastadenoviruses were isolated only from mammals, whereas aviadenoviruses only from birds. Supposedly these viruses have co-evolved with these classes of vertebrates. The atadenoviruses were described first from ruminants and birds. These viruses have a high A+T content in the genome, and cause severe diseases. AdVs of squamate reptiles have balanced G+C content but their genome organization resembles to that of atadenoviruses. It is likely that atadenoviruses have co-evolved originally with squamate reptiles, and host switches occurred to other vertebrate classes. The nt bias could be the result of the host switch. We do not know the original host of the siadenoviruses, because the avian, frog and sulavezi turtle siadenoviruses have a very low G+C content, implying that siadenoviruses got into these species also by switching host.

The phylogenetic calculations showed that the WSAdV-1 did not belong to any of the existing four genera. Using different genes for the calculations, the topology of the trees showed slight differences. Based on these calculations and genome organization we proposed the establishment of the *Sturgeon adenovirus A* species for the WSAdV-1. Morover, the establishment of a novel genus for fish AdV, the *Ichtadenovirus* was also proposed. The ICTV accepted our proposal in 2009, so the number of genera in the family *Adenoviridae* grew to five.

New scientific results

1. A large genome part (66 kbp) of the AciHV-2, isolated from white sturgeon (*Acipenser transmontanus*), was sequenced and analyzed.
2. DNA sequences of black bullhead (*Ameiurus melas*) herpesvirus (IcHV-2) were determined foremost.
3. The AciHV-2 and IcHV-2 were classified into the genus *Ictalurivirus* within the family *Alloherpesviridae* on the grounds of our proposal.
4. The first sequences of the SbSHV, isolated from Siberian sturgeon (*Acipenser baeri*), implying that the SbSHV is a very close relative of AciHV-2 were published.
5. In Hungary, the CyHV-1 and 2 were detected by PCR foremost. This is the very first report of CyHV-2 in Prussian carp (*Carassius gibelio*).
6. The full genome of WSAdV-1 (the only known fish adenovirus) was sequenced.
7. Upon our proposals a novel genus, the *Ichtadenovirus*, was established for fish adenovirus within the family *Adenoviridae*.

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Acknowledgements

I am very thankful for my supervisor Dr. Mária Benkő, and for Dr. Balázs Harrach for their support and intensive efforts in supervising my scientific work.

I am grateful to Dr. György Csaba and Dr. Kálmán Molnár for providing me samples from Hungarian fish farms.

I would like to express my appreciation to the foreign scientists, Scott LaPatra, Giuseppe Bovo, Igor Shchelkunov, they provided me the virus strains.

Many thanks for all members of the Molecular Virology and Comparative Virology groups of HAS, Veterinary Medical Research Institute.

This work was supported by a grant (OTKA K61317 and NKTH-OTKA K67781) from the Hungarian Scientific Research Fund.

Thanks for the Kőbányai and Soproni Breweries, their products helped me bring through the hard times of the writing of the thesis.