FAMILIAL CASE OF INHERITED HUMAN HERPESVIRUS 6A WITH PHYLOGENETIC ASSESSMENT

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The paper reports a familial case of HHV-6A chromosomal integration being an important and relevant issue of genetics and medicine. The study was aimed to test the hypothesis of HHV-6A chromosomal integration and vertical transmission in patient with persistent virus detection during recurrent respiratory diseases and the asymptomatic period when there were no health complaints. Sequencing of the patient's father genome DNA was performed, and a phylogenetic tree was constructed by aligning 270 HHV-6A/B genome assemblies from the GenBank database. As a result, a familial case of ciHHV-6A transmission was identified. It was found that the detected ciHHV-6A observed on the phylogenetic tree was closely related to other two chromosomally integrated HHV-6A sequences reported by Moscow researchers. The study confirmed HHV-6A chromosomal integration. Further precise chromosome mapping of ciHHV-6A would be useful in terms of excluding probable somatic disorders associated with the chromosome structure alteration following HHV-6, particularly HHV-6A, integration, as well as for identification of insertion sites specific for various geographic locations.

Keywords: human herpesvirus 6A/B (HHV-6A/B), chromosomal integration, ciHHV-6A/B, inherited herpesvirus, phylogenetics

Author contribution: Goleva OV, Babachenko IV, Tian NS — study planning, data acquisition, analysis and interpretation, manuscript draft; Danilov LG — bioinformatics analysis, search for analytical papers; Kusakin AV — study planning, literature review, data acquisition, analysis and interpretation, bioinformatics analysis, constructing a phylogenetic tree, manuscript draft; Eismont YuA, Chukhlovin AB — study planning, data acquisition, analysis and interpretation; Krylov AV — data acquisition, analysis and interpretation; Glotov OS — research supervision, data analysis and interpretation, manuscript draft.

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СЕМЕЙНЫЙ СЛУЧАЙ НАСЛЕДУЕМОЙ ХРОМОСОМНОЙ ИНТЕГРАЦИИ ВГЧ-6А С ПРОВЕДЕНИЕМ ФИЛОГЕНЕТИЧЕСКОГО АНАЛИЗА

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В статье рассмотрен семейный случай хромосомной интеграции ВГЧ-6А, которая является важной и актуальной темой в области генетики и медицины. Целью исследования было проверить гипотезу о хромосомной интеграции ВГЧ-6А и его вертикальной передаче у пациента с длительным обнаружением вируса во время рекуррентных респираторных заболеваний, а также в бессимптомный период, при отсутствии жалоб на здоровье. Проведено секвенирование геномной ДНК отца пациента, построено филогенетическое дерево путем выравнивания 270 геномных сборок ВГЧ-6А/В из базы данных GenBank. В результате исследования установлен семейный случай передачи хиВГЧ-6А. Показано, что обнаруженный хиВГЧ-6А, наблюдаемый на филогенетическом древе, находится в тесном контакте с двумя другими хромосомно-интегрированными последовательностями ВГЧ-6А, о которых сообщали московские исследователи. Исследование подтвердило хромосомную интеграцию ВГЧ-6А. Дальнейшее точное хромосомное картирование хиВГЧ-6А/В было бы полезно для исключения вероятных соматических заболеваний, связанных с изменением структуры хромосом при интеграции ВГЧ-6, в частности ВГЧ-6А, а также для идентификации участков инсерции, специфичных для различных географических точек.

Ключевые слова: вирус герпеса человека 6А/В (ВГЧ-6А/В), хромосомная интеграция, хиВГЧ-6А/В, унаследованный герпесвирус, филогенетика

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Human betaherpesvirus 6A/B (HHV-6A/B) is widely spread in human population. In 1986, the research team of the laboratory at the National Cancer Institute (USA) isolated the virus from patients with lymphoproliferative diseases and identified it as human B-lymphotropic virus, however, later its affinity for T cells and belonging to the family *Herpesviridae*, subfamily *Betaherpesvirinae*, genus *Roseolovirus*, were determined [1]. The virus is primarily transmitted through contact with saliva or less often by airborne droplets, sexual contact, and transplanted organs. $CD4^+$ T cells are the main target cells for the virus. HHV-6 enters the cells via receptor-mediated endocytosis followed by virus replication. After primary infection viral DNA persists in mononuclear peripheral blood cells [2, 3]. HHV-6A/B can trigger immunosuppression and chronic autoimmune processes [4].

In 2012, International Committee on Taxonomy of Viruses (ICTV) ratified HHV-6A division into two distinct taxonomic variants: HHV-6A and HHV-6B [5, 6]. Despite the fact that the genomes of these viruses demonstrate 90% homology, the viruses show phenotypic differences, are tropic for different cellular receptors, and in the majority of cases have different clinical manifestations [7]. HHV-6A is a less explored virus, it is acquired later in life and more often detected in immunocompromised individuals. It is assumed that this virus is associated with such neurodegenerative disorder as Alzheimer's disease [3, 8]. HHV-6B is common everywhere, more than 90% of the population get infected during the first three years of life, while reactivation can occur at any age [3]. The viruses show different tropism against immunocompetent cells. Thus, HHV-6A uses CD46 receptors for cell entry, it is capable of affecting T helper cells, cytotoxic T cells, and natural killers. By contrast, HHV-6B uses CD134 receptors and fails to persist in cytotoxic T cells [9, 10].

HHV-6A/B genome consists of a double-stranded DNA with an average length of about 160 kbps. It is noteworthy that the genome of HHV-6A is shorter than that of HHV-6B, it is about 159 kbps vs. 162 kbps, respectively [11]. The majority of genes are located in the unique segment flanked by direct repeats (DR). DRs, in turn, are surrounded by pac1 and pac2 being the cis-acting packaging signals [11, 12]. The number of open reading frames (ORF) depends on the virus type (A/B) and the detection method. A total of 115–119 ORFs were earlier predicted based on the sequence [11, 13], however, the researchers managed to identify 268 ORFs in HHV-6A and 216 in HHV-6B using advanced Ribo-seq and RNA-seq methods [8]. The average sequence similarity of HHV-6A and HHV-6B is 90%. U39 and U48 that encode gB and gH envelope glycoproteins, respectively, are the most conservative genes. Their nucleotide sequences show 94% similarity, and amino acid sequences show 96% similarity [11, 14]. Moreover, the most variable genes that are located close to the genome termini primarily encode proteins that are likely to be involved in immunomodulation, signaling (chemotaxis), and viral entry [12].

Polymerase chain reaction (PCR) is considered to be the main method of HHV-6A/B diagnosis, however, to date no clear boundary between identification of latent and active viral infection based on PCR results has been determined. Absence of HHV-6A/B DNA in blood plasma or serum does not mean that there is no persistent virus in low concentrations in the tissues (for example, in the heart, thyroid gland, brain). Detection of specific IgM and IgG antibodies in blood serum is also of some diagnostic significance [16]. The researchers have proposed the test systems considering different reading frames for HHV-6A (U11, p100) and HHV-6B (101K) [17].

HHV-6 capability of integration into subtelomeric region of the cellular chromosome was found in 1993 [18]. Today, it is known that viral integration most often occurs in the telomeric regions of chromosomes 1q, 6q, 9q, 10q, 11p, 17p, 18p, 19q, 22q, Xp, however, the mechanisms are poorly understood [19–23]. HHV-6 integration into the germ cell genome enables transmission of the virus to the next generations and formation of the inherited chromosomally integrated HHV-6A/B (inherited ciHHV-6A/B) in accordance with the Mendel's laws [24]. CiHHV-6A/B can be also transmitted with the transplanted cells, organs, and tissues. CiHHV-6A/B abundance varies between 0.2% in Japan, 0.6% in Canada and 1–3% in Europe, it depends on geographic factors and the assessed population of patients [25, 26].

The cases of integrated ciHHV-6A/B reactivation up to clinically manifested forms in individuals with immunodeficient conditions and pregnancy have been reported [2, 27, 28]. Reactivation of the chromosomally integrated virus during pregnancy can result in the increased risk of spontaneous abortion [29]. The British study conducted in 2020 showed that women with fetuses infected with ciHHV-6A/B had a 2.5–3 times higher risk of preeclampsia [30]. Biological and medical effects of HHV-6A and HHV-6B chromosomal integration are currently being studied. For example, telomeres linked to endogenous HHV-6A/B are often prone to sudden deletions, which lead to telomere shortening. As a result, premature cell ageing and impaired tissue homeostasis are observed [31–33]. Genome instability can cause cancer.

The study was aimed to test the hypothesis of the HHV-6A chromosomal integration and vertical transmission in patient with persistent virus detection during recurrent respiratory diseases and the asymptomatic period when there were no health complaints.

METHODS

Five family members, mother (36 years old), father (39 years old), three sons (4 years, 6 years, and 14 years old), were the research objects. The family lived in the town of Kirishi (Leningrad region).

Nucleic acid isolation and HHV-6A/B detection

Nasopharyngeal smears and venous blood were collected during the study for further molecular genetic tests and enzyme-linked immunoassay. Specific fragments of nucleic acids of influenza viruses A and B, respiratory syncytial virus, type 1–4 parainfluenza viruses, seasonal coronaviruses, metapneumovirus, rhinoviruses, as well as DNA of group B, C, E adenoviruses and bocaviruses were detected in the nasopharyngeal smears using AmpliSens Influenzavirus A/B-FL and AmpliSens ARVI-screen-FL kits (Rospotrebnadzor; Russia) for multiplex PCR with fluorescent hybridization detection of amplification products. DNA of Epstein–Barr virus (EBV), HHV-6A/B and cytomegalovirus (CMV) was detected in blood and oropharyngeal mucosal smears by real-time PCR (RT-PCR) using AmpliSens EBV/CMV/HHV6-screen-FL kit (Rospotrebnadzor; Russia). The HHV-6A/B viral load in the studied biomaterials was assessed within the range of 22–38 amplification cycles (Ct) and expressed in genome equivalents per 1 mL (gEq/mL) of native sample after preanalytical processing. The results obtained within the range of 35 cycles (10^3–10^4 gEq/mL) were considered to be of diagnostic significance. Venous blood collected into K2-EDTA blood sampling tubes was used to extract DNA. Oropharyngeal smears were placed into Transport Medium with Mucolytic Agent (ILS; Russia). DNA was extracted from venous blood

Table 1. Laboratory markers of herpesvirus infections at admission

Biomaterial	Markers of herpesvirus infection	Patient assessment results	
		S.T., 4 years old	S.A., 6 years old
Blood	EBV DNA EBV IgG (VCA)	Positive Positive	Positive Positive
Blood	HHV-6A DNA	Negative	105*
Oropharyngeal smear	HHV-6A DNA	Negative	104*
Blood	HHV-6B DNA	103*	Negative
Oropharyngeal smear	HHV-6B DNA	104*	Negative
Blood	HHV-6 IgG	Negative	6.9**

Note: * — gEq/mL; ** — AU.

using MagNa Pure automated nucleic acid extraction system (Roche; Switzerland) by standard sample preparation method. Biomaterial from the oropharyngeal smear was purified using RealBest Extraction 100 kit (Vector-Best; Russia). Sample preparation and extraction were carried out in accordance with the manufacturers' instructions. Extraction was controlled with NanoStar spectrophotometer (BMG; Germany). The extracted material was quantified using Quantus fluorimeter (Promega; USA).

The standard *GAPDH* cellular gene was used to test the extracted sample for the presence of DNA and its quality [34]. Amplification was performed in CFX-96 PCR system (BioRad; USA) using qPCRmix-HS kit (Evrogen; Russia).

IgM and IgG antibodies against the listed above herpesviruses were detected by qualitative enzyme-linked immunosorbent assay (ELISA) using VectoEBV-VCA-IgM/G and VectoCMV-IgM/G kits (Vector-Best; Russia) and semi-qualitative ELISA using the HHV-6-IgM/G-ELISA-BEST kit (Vector-Best; Russia) in the open type Lazurite unit (Dynex Technologies Inc.; USA) within the framework of standard laboratory testing. The results were represented by positivity rate (PR) expressed in arbitrary units (AU) according to the test system manufacturer's instructions.

The semen sample collected from the father was used as supplementary material. Spermatozoa were obtained by density gradient centrifugation using SupraSperm System (ORIGIO; USA) for extraction of viable sperm. DNA was isolated from spermatozoa by phenol chloroform extraction. The quality of isolated DNA was estimated using 4200 TapeStation System and Genomic DNA ScreenTape kit (Agilent Technologies; USA), concentration was measured using QuantiFluor dsDNA System (Promega; USA).

Other laboratory tests

Standard diagnostic tests were supplemented by differentiation of HHV-6A/B variants using the reported primers [34]. Alignment of primers to the HHV-6 reference sequences was checked using the BLAST tool (NCBI; USA):

HHV-6A/B FP: 5'- GACAATCACATGCCTGGATAATG-3';
HHV-6A RP, 5'- TGGTAATGTAATTGTGTGTTGTTTTA-3';
HHV-6B RP, 5'- TGGTAATGTAAGTGTGCGTTATTTTC-3';
HHV-6 probe, 5'-FAM- AGCAGCTGGCGAAAGCTGTGC-TAMRA-3'.

NGS library preparation

The sequencing libraries were prepared for two instruments in order to obtain long and short reads. Long reads were acquired using MinION system (Oxford Nanopore Technologies; UK). Libraries were prepared in accordance with the whole-genome sequencing protocol using SQK-LSK109 sample preparation kit (Oxford Nanopore Technologies; UK) and NEBNext module (New England Biolabs Inc.; USA) for preparation of Oxford Nanopore Technologies libraries (NEBNext). Short reads were acquired by sequencing in the MGISEQ 2000 system (MGI Tech Co.; China). Libraries were prepared in accordance with the guidelines [35].

The quality of resulting libraries was assessed using D1000 ScreenTape and High Sensitivity D1000 ScreenTape kits (Agilent Technologies; USA); concentration was measured with Quantus fluorimeter using QuantiFluor dsDNA System kit (Promega; USA).

DNA sequencing

To perform whole-genome sequencing in the MinION system, the R10 (FLO-MIN111) flow cell for nanopore sequencing (Agilent Technologies; USA) was used.

Whole-genome sequencing in the MGISEQ 2000 system was performed using the DNBSEQ-G400 CoolMPS High-throughput Sequencing Set (PE100, 320 G) (MGI Tech Co.; China). One lane was selected for whole-genome sequencing.

Table 2. Laboratory markers of herpesvirus infections obtained during re-examination eight months later

Biomaterial	Markers of herpesvirus infection	Patient assessment results	
		S.T., 4 years old	S.A., 6 years old
Blood	EBV DNA	Negative	Negative
Blood	HHV-6A DNA	Negative	106*
Oropharyngeal smear	HHV-6A DNA	Negative	104*
Blood	HHV-6B DNA	Negative	Negative
Oropharyngeal smear	HHV-6B DNA	Negative	Negative
Blood	HHV-6 IgG	5.4**	6.1**

Note: * — gEq/mL; ** — AU.

Table 3. Laboratory markers of herpesvirus infection in other family members

Biomaterial	Markers of herpesvirus infection	Patient assessment results		
		S. A., 14 years old	Mother, 36 years old	Father, 39 years old
Blood	HHV-6A DNA	106*	Negative	106*
Oropharyngeal smear	HHV-6A DNA	104*	Negative	105*
Semen	HHV-6A DNA	–	–	106*
Blood	HHV-6B DNA	Negative	Negative	Negative
Oropharyngeal smear	HHV-6B DNA	Negative	Negative	Negative

Note: * — gEq/mL.

Genome assembly

The data obtained from the Nanopore platform were used for viral genome assembly. Genome was assembled using the customized assembly line: the herpes virus-associated reads were extracted with the Cookiecutter tool [36] using Moscow strain (GenBank ID: MK630134, MK630133) as reference [37], since it was characterized by larger depth (500x). Later only a fragment of gene *gB* (U39) was used in the study, which was completely assembled in these sequences. The reads were assembled with the SPAdes tool [38]; the assembled contigs were configured manually by searching for complete reference sequence in BLAST [39].

Phylogenetic analysis

The glycoprotein B (*gB*, U39) HHV-6A gene (Gene ID: 1487917) nucleotide sequence was used for phylogenetic analysis. All sequences of 270 herpes virus assemblies (both 6A and 6B) available from the GenBank database were included in the analysis. MAFFT v7.505 algorithm with Kimura 1 parameter substitution model were used for sequence alignment [40]. Then the resulting alignments were arranged to construct the tree using the Neighbor-Joining method (Jukes-Cantor, Bootstrap resampling = 100) [41].

RESULTS

In December 2018, the child S. A., 6 years old, with his brother S. T., 4 years old, were admitted to the Pediatric Research and Clinical Center for Infectious Diseases of FMBA of Russia with the primary diagnosis of “acute nasopharyngitis, tonsillitis of moderate severity”. PCR revealed no markers of respiratory viruses in oropharyngeal smears, no bacterial pathogens were detected by bacteriological method. Considering a positive PCR test for herpes viruses, the

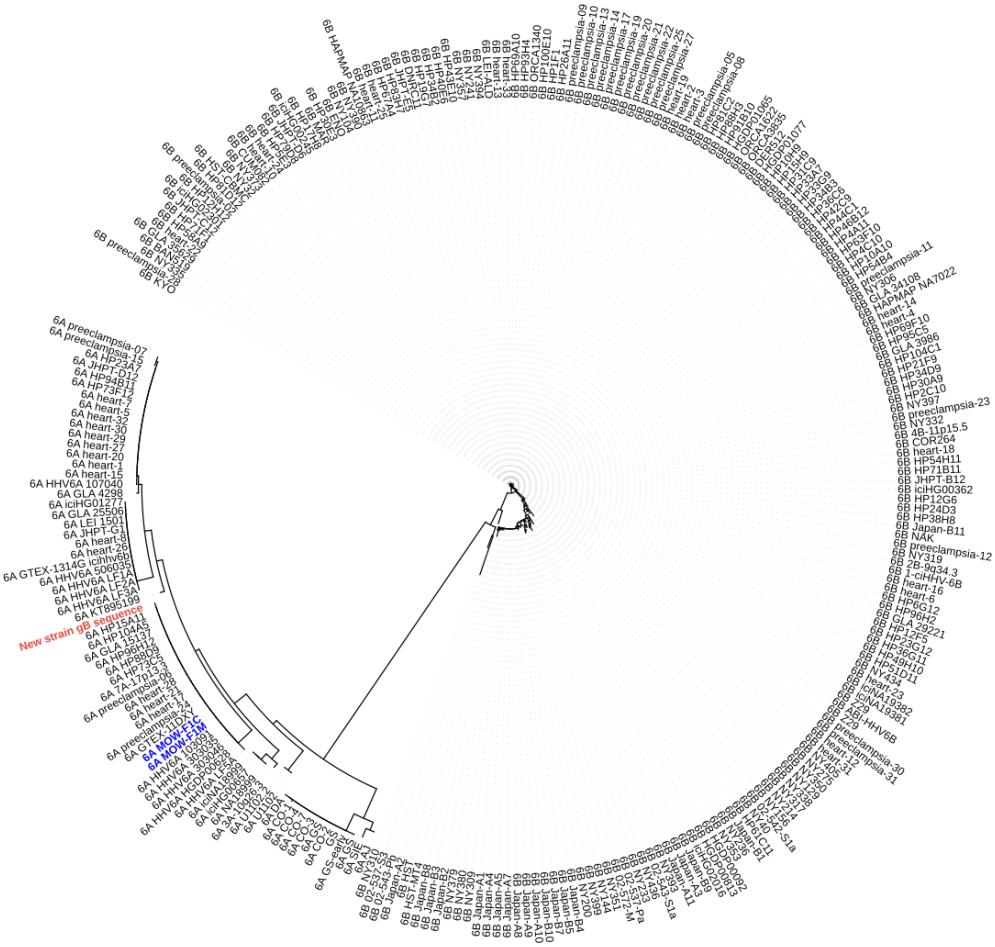

Fig. Phylogenetic position of novel type 6 herpesvirus relative to other herpes strains based on gene gB: position of new virus is highlighted in red in the tree, two viral strains from Moscow are highlighted in blue

diagnosis was clarified as "mixed etiology herpesvirus infection (HHV-6 + EBV), acute rhino-tonsillopharyngitis of moderate severity". HHV-6A/B and EBV DNA was found in peripheral blood of both patients. Positive tests for EBV DNA in blood cells, along with late IgG against EBV capsid antigen (VCA) in blood serum, proved virus reactivation. The data of laboratory tests provided in Table 1 were obtained during assessment in the first days after admission.

No CMV markers (DNA, IgM, IgG) were found in patients. Meanwhile, HHV-6 genotyping in blood and oropharyngeal smears confirmed the presence of HHV-6A variant in S.A. and diagnostically significant concentrations of HHV-6B in his brother S.T. However, no antibodies against HHV-6A/B were found in blood of S.T., which could be due to early stage of acute viral infection, before the start of antibody synthesis, or low concentrations of antibodies being outside the limits of the diagnostic test system sensitivity; high concentrations of IgG against HHV-6A/B (6.9 AU) were found in the 6-year-old patient, which could be indicative of longer infection duration.

Re-examination of these patients was performed during the follow-up visit on August 14, 2019 (Table 2).

When assessing both patients eight months later, no EBV DNA was found in blood. There were diagnostic concentrations of IgG against HHV-6A/B in blood, however, persistent HHV-6A viral load in blood and oropharyngeal smear was reported in the child S.A. over time, while no HHV-6B DNA was found in blood and oropharyngeal smear of the child S.T. Both children were clinically healthy at the time of re-examination. The fact of persistent HHV-6A isolation from blood and oropharyngeal smear of the followed-up 6-year-old patient could be associated with the viral genome integration into DNA of human cells, which required further confirmation.

To prove the HHV-6A chromosomal integration, we invited parents (mother, 36 years old, father, 39 years old) and the followed-up patients' elder brother (S.A., 14 years old), having no health complaints at the time of screening tests, for examination (Table 3).

Thus, it was found that the clinically asymptomatic father and elder brother of the patient were also characterized by high viral load represented by diagnostic concentrations of HHV-6A in blood and oropharyngeal smears. However, no HHV-6A or HHV-6B DNA was found in the mother's biomaterials. Since equally high levels of HHV-6A DNA were found in the samples of two elder brothers and the father, we suspected hereditary transmission of ciHHV-6A/B from the father to his children. It was decided to collect the parent's biomaterial other than blood with no leukocytes and cytoplasmic DNA, i.e., sperm as in the study [42], to answer the question concerning possible vertical transmission of ciHHV-6A due to technical impossibility of testing hair follicles or nail plates. DNA of spermatozoa was subjected to RT-PCR, separated from other ejaculate. After that DNA was extracted. Then we revealed the HHV-6A load of 106 gEq/mL, which was equivalent to virus concentrations in other biomaterials. This also confirmed chromosomal integration.

Later we tried to assembly the genome of this HHV-6A isolate. The HHV-6A genome sequencing involving acquisition of short and long reads of viral gene regions was carried out to confirm HHV-6A chromosomal integration and perform phylogenetic analysis.

Genome structure and position on a phylogenetic tree

We obtained a HHV-6A genome assembly, however, coverage of the reads did not exceed 3–4 reads per nucleotide, that is why genome assembly for certain genes was performed manually.

To determine the novel viral isolate phylogenetic position, we selected the gB gene, which was conventionally used to compare phylogenetic trees of herpesvirus [43]. For that the search for similar sequences of this gene among related genome assemblies using the BLAST local alignment tool was performed, and manual assembly was performed based on the results obtained. The sequences of 270 herpes virus genome assemblies were used to assess phylogenetic identity. According to phylogeny constructed for gene gB, the resulting virus strain turned out to be very similar to two strains presented by the Moscow group (GenBank ID: MK630134, MK630133) (Figure). The fact that these strains are integrated into human genome is their important feature. This conclusion can confirm our findings showing integration of novel reported strain into the host genome.

DISCUSSION

The first case of HHV-6A/B chromosomal integration was reported in early 1990s. After that the virus was often found in a number of human chromosomes: 1q, 6q, 9q, 10q, 11p, 17p, 18p, 19q, 22q, and Xp [19–23]. It is acknowledged that this is typical for both HHV-6A and HHV-6B, it is observed in telomeric chromosome regions. The paper [43] shows that the integrated HHV-6A remains inactive throughout human lifespan. The integrated virus can re-activate under exposure to various factors, which is more common for HHV-6B, and trigger infection. It has been shown that ciHHV-6A splits into clades characterized by certain chromosome and locus, in which the virus is integrated.

During the study we came across the case of prolonged HHV-6A DNA detection in biomaterials (venous blood and nasopharyngeal smears) of the patients, when performing testing at admission and during follow-up, after eight months, during the period of having no health complaints. However, viral load in venous blood and nasopharyngeal smear remained high (10^5–10^6 gEq/mL and 10^4 gEq/mL, respectively). We suspected HHV-6A chromosomal integration based on the findings. Subsequent clinical and laboratory assessment of other family members made it possible to revealed comparable high viral load in similar biomaterials of the patient's elder brother and father. Furthermore, HHV-6A was detected in the father's germ cells. Thus, it was hypothesized that the virus could be not only integrated into chromosome, but also passed to the followed-up child paternally.

We performed phylogenetic analysis based on the sequence of gene gB encoding one of the viral envelope glycoproteins to clarify the origin of HHV-6A detected in the father's germ cells. It was found that the studied HHV-6A was closely related to two assembled sequences of ciHHV-6A isolated by the research team [37] in Moscow in 2017 (GenBank ID: MK630134, MK630133). The findings confirmed the relationship of the virus we had studied with other ciHHV-6A included in the GenBank database.

CONCLUSIONS

Determination of exact position of ciHHV-6A in the chromosome locus by FISH aimed at excluding probable somatic disorders caused by chromosome structure impairment after the virus integration over time and determining the pattern of integration depending on the geographic locations of the cases revealed is an important direction of further research. Further studies will also allow us to accept or reject the earlier hypothesis that the viral genome sequence corresponds to the site of integration into human chromosome. This will make it possible to avoid using the expensive and time-consuming FISH method and adapt the tests for clinical practice.

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