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EPSTEIN–BARR VIRUS LMP1 ONCOGENE POLYMORPHISM IN TATAR AND SLAVIC POPULATIONS IN RUSSIAN FEDERATION IMPACTING ON SOME MALIGNANT TUMOURS

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Abstract. Objective: To compare genetic structure of the main Epstein–Barr virus (EBV) oncogene, latent membrane protein 1 (LMP1), in EBV strains circulating in two genetically distinct ethnic populations in Russian Federation, Tatars and Slavs, as well as assess an impact of diverse LMP1 variants on incidence and mortality rate for some malignant tumors partially associated with EBV infection. *Materials and methods.* Oral washing samples were collected from 60 ethnic Kazan Tatars and 65 ethnic Moscow Slavics. Carboxy-terminal nucleotide sequences (41 and 40 sequences, respectively) derived from hypervariable LMP1 gene region were amplified from EBV DNA samples. Next, final nucleotide sequences were translated into amino acid sequences and analyzed according to classification by Edwards et al. *Results.* Analysis of 41 and 40 LMP1 samples obtained from ethnic Kasan Tatars and ethnic Moscow Slavics, respectively, revealed significant difference in relevant amino acid structures. In particular, all LMP1 samples derived from Moscow Slavics were found to belong to the four protein variants: B95.8/A, Med–, China1 and NC. Among them, low-transforming variant B95.8/A was dominant (82.5%). In contrast, solely 21 out of 41 LMP1 samples derived from ethnic Tatars were classified as B95.8/A, Med– and China1 variants. Importantly, the percentage of low-transforming B95.8/A variant among ethnic Tatar samples was significantly lower compared to that one found in Moscow Slavics (29.3% vs. 82.5%). On the other hand, seven (17.1%) out of 20 other samples formed a unique protein mono group characterized by LMP1 amino acid sequence differed from that one available in the GenBank database. Such group of variants was designated as LMP1-TatK. The remaining 13 samples (31.7%) did not match either protein variants, thereby forming the "beyond classification" (LMP1-TatBC) group. *Conclusion*. The data obtained suggest that various LMP1 variants exist in EBV strains persisting in ethnic Tatrs and ethnic Slavics examined in Russian Federation. It was also found that EBV strains isolated from ethnic Tatars contained a unique LMP1 gene variant encoding protein LMP1-TatK lacked in EBV strains derived from ethnic Moscow Slavics. Taking into account the genealogy of Tatars, it cannot be ruled out that EBV strain bearing LMP1-TatK variant represented ethnically specific EBV strain that might circulate many centuries ago among their historical human predecessors called Mongol-Tatar tribes. In addition, it was shown that the LMP1 variants in EBV strains isolated from ethnic Kazan Tatars and ethnic Moscow Slavics did not affect the incidence and mortality of different forms of cancer consisting of EBV-associated cases.

Key words: *Epstein–Barr virus, latent membrane protein 1, ethnic tatars and slavs, sequence analysis, phylogenetic analysis, real-time PCR, Epstein–Barr virus DNA copies.*

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ПОЛИМОРФИЗМ ОНКОГЕНА LMP1 ВИРУСА ЭПШТЕЙНА–БАРР В ДВУХ ЭТНИЧЕСКИХ ГРУППАХ РОССИИ, ТАТАР И СЛАВЯН, И ЕГО ВЛИЯНИЕ НА РАЗВИТИЕ НЕКОТОРЫХ ЗЛОКАЧЕСТВЕННЫХ ОПУХОЛЕЙ

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Резюме. Цель работы: выяснить, отличается ли генетическая структура основного онкогена вируса Эпштейна–Барр (ВЭБ), латентного мембранного белка 1 (LMP1), у штаммов ВЭБ, циркулирующих в двух российских генетически разных популяциях, этнических татар и этнических славян, и каково влияние различных вариантов LMP1 в этих популяциях на уровень злокачественных патологий, часть которых ассоциирована с ВЭБ. *Материалы и методы.* Смывы полости рта были получены от 60 этнических казанских татар и 65 этнических московских славян. Сорок одна и сорок (соответственно) С-концевых нуклеотидных последовательностей наиболее вариабельной части LMP1 были амплифицированы из образцов ДНК ВЭБ, экстрагированных из соответствующих смывов. Нуклеотидные последовательности были переведены в аминокислотные последовательности и классифицированы в соответствии с классификацией Edwards и соавт. *Результаты.* Анализ 41 образца LMP1, полученного от этнических татар, и 40 LMP1 образцов, полученных от этнических славян, выявил существенные различия в структуре их аминокислот. Все образцы LMP1 московского происхождения принадлежали к четырем белковым вариантам, B95.8/A, Med–, China1 и NC. Среди них вариант с низким уровнем трансформации, B95.8/A, был доминирующим (82,5%). Из 41 образца LMP1 татарского происхождения только 21 был классифицирован как B95.8/A, Med– и China1 варианты. Процентное содержание низко трансформирующего варианта B95.8/A среди казанских татар было значительно ниже, чем у московских славян (29,3% против 82,5%). Из других 20 образцов семь (17,1%) образовали уникальную моногруппу белков с аминокислотной структурой последовательностей LMP1, отличной от имеющейся в GenBank. Эта группа вариантов была обозначена как LMP1-TatK. Остальные 13 образцов LMP1 (31,7%) не соответствовали ни одному из вариантов в вышеуказанной классификации, формируя, таким образом, группу «вне классификации» (LMP1-TatBC). *Заключение.* Полученные данные указывают на разнообразие вариантов LMP1 в штаммах ВЭБ, персистирующих у этнических татар и славян. Также было обнаружено, что штаммы ВЭБ татарского происхождения содержат уникальный вариант гена LMP1, кодирующий онкобелок LMP1-TatK, который отсутствует в штаммах ВЭБ славянского происхождения. Принимая во внимание генеалогию татар, нельзя исключить, что штамм ВЭБ, кодирующий вариант LMP1-TatK, является этнически специфическим штаммом, который, возможно, циркулировал среди их исторических предшественников, монголо-татарских племен, много веков назад. Было также показано, что варианты LMP1 в штаммах ВЭБ татарского и славянского происхождения не оказывают какого-либо влияния на показатели заболеваемости опухолями и смертности от них, которые включают и случаи, ассоциированные с этим вирусом.

Ключевые слова: *вирус Эпштейна–Барр, латентный мембранный белок 1, этнические татары и славяне, анализ последовательности, филогенетический анализ, ПЦР в реальном времени, копии ДНК вируса Эпштейна–Барр.*

Introduction

Epstein–Barr virus (EBV; Human herpesvirus 4) is a double-stranded DNA virus with a genome size of about 170 kb, belonging to the Herpesviridae family, Gammaherpesvirinae subfamily and Lymphocryptovirus genus. EBV is estimated to infect up to 95% of the global adult population over their lifetime [11]. Infection with the virus usually occurs when someone comes into contact with the saliva of an infected person, but can also occur through blood transfusion and organ transplantation from infected individuals [19, 40]. In early childhood, infection with the virus is usually asymptomatic; however, infection of young people who have not previously encountered the virus can lead to the development of infectious mononucleosis [14].

In a healthy person, B lymphocytes (mainly memory B cells) and epithelial cells are the main targets of the virus. EBV primarily infects epithelial cells of the mucous membrane that lines the nasopharynx and lymphoid formations surrounding the entrance to the respiratory and digestive tracts (Valdeyer's ring, consisting of the tonsils and adenoids) [43]. Cells that are infected with the virus, other than B lymphocytes, can also determine the development and pathogenesis of a number of EBV-associated pathologies [45]. Through the Valdeyer's ring, the virus enters the peripheral blood and infects memory B cells, in which EBV persists for life [29]. There is no expression of latent proteins in these cells, as only non-translated RNAs are transcribed from episomal viral DNA. The recognition of a "related" antigen by the receptor on memory B cells induces

reactivation of EBV in the cell pool, and the differentiation of plasma cells leads to the development of a lytic infectious virus. In the bloodstream, the virus is implanted by circulating memory B cells into all parts of the peripheral lymphoid system, and then returns to the oral cavity through the lymphoid ring. In this case, the number of infected memory B cells in the population of lymphoid elements of the Valdeier's ring and the peripheral blood are similar, according to Laichalk et al. For every 10 million B cells in the lymphoid formations of the Valdeyer's ring and peripheral blood, the number of infected memory B cells averages 175 and 110, respectively, although only 1% of infected B cells are in the peripheral blood [7]. In an infected person, the virus avoids recognition by the immune system. This is due to the expression of a limited number of viral genes in memory B cells, as well as interruption of the mechanism of viral antigen expression on the surface of these cells [38]. In the body of a healthy person, the virus is under strict immunological control [37]. Weakening of the immune system for various reasons allows the virus to actively multiply, and the restoration of immunocompetence suppresses the replication of the virus, reducing it back to the background level.

Being a ubiquitous virus, EBV is able to simultaneously initiate a number of benign and malignant pathologies. Benign neoplasms are the infectious mononucleosis mentioned above and hairy leukoplakia of the oral cavity. The malignant neoplasms include Burkitt's lymphoma, nasopharyngeal carcinoma, certain histological variants of classical Hodgkin's lymphoma, a number of non-Hodgkin's lymphomas and certain variants of stomach cancer, among others [42, 45].

Numerous studies have shown that one of the latent EBV infection genes, latent membrane protein 1 (LMP1), which encodes the oncoprotein LMP1, plays an active role in the development of EBV-associated pathologies. This protein has the ability to stimulate cell growth, inhibit apoptosis in various cells types [27, 34] and induce tumours in transgenic mice [28]. LMP1 reduces the immunogenicity of viral proteins and enhances their signalling activity [8, 23]. Studies have shown that nucleotide substitutions in LMP1 sequences are usually located in regions that regulate transcription or translation [16, 25].

The LMP1 protein consists of 386 amino acids that affect functional activity, immunogenicity, half-life and the transforming potential, which can vary significantly in viral isolates [42]. Unlike the prototype variant LMP1-B95.8 of American origin, LMP1 "Cao" (LMP1-Cao), of Chinese origin with high tumourigenic activity, contains a characteristic 10-member deletion (30 bp) in the C-terminal domain, adjacent to the CTAR2 region, as well as three 11-amino acid repeats, numerous point mutations [13, 24].

Of the three LMP1 domains identified, the C-terminal domain is the most studied [31]. Analysis of this domain in different geographic regions allowed Edwards et al. in 1999 to propose a widely used classification, according to which all studied LMP1 samples were subdivided into variants that differed with respect to their key amino acid substitutions compared to the prototypic LMP1-B95.8 variant [15]. In this classification system, LMP1 variants are named according to their geographic origin. However, the above classification of LMP1 was created for EBV strains circulating among a population inhabiting of a limited number of territories, thus it is unclear whether it can be applied for the analysis of LMP1 from other geographic regions [17, 36]. Unfortunately, a classification uniting all possible variants of the gene has not yet been suggested, and it would be very difficult to achieve. It is known that the genetic structure of the population, environmental hazards and the spectrum of EBV-associated diseases in different regions can differ significantly, and it cannot be ruled out that LMP1 variants for each population will contain unique structural changes that are not related to the proposed classification.

The goal of this study was to find out whether EBV strains spread in representatives of two Russian ethnic groups, Volga Tatars and Moscow Slavs, differ in their EBV LMP1 oncogene structure, and influence LMP1 variants onto the incidence of some tumors in above ethnic groups, part of which are EBV-associated.

Materials and methods

Sampling. Throat washing samples were collected from 65 ethnic Slavs and 60 ethnic Tatars. The latter were students at the Kazan State Medical University (KSMU), no less than third generation Tatars. This group consisted of 15 males and 45 females, with an average age of 21.5 years old. Similar washing samples were obtained from ethnic Slavs (21 males and 19 females) with an average age of 47.5 years old. This group consisted of Moscow city residents, who were no less than third generation Russian Slavs. Each throat washing sample was a cell suspension obtained from each person after rinsing their mouth with 15 ml of sterile PBS for 30 seconds. Washing samples were collected in hermetically sealed plastic vials and stored at +4°C before the study. The Ethics Committee of the FSBI "N.N. Blokhin National Medical Research Center of Oncology" approved this study, which included randomly selected individuals in Moscow city and the R. Tatarstan who had given their informed consent.

Extraction and amplification of the LMP1 gene. DNA from the throat washing samples was isolated by phenol-chloroform deproteinisation. The presence of viral DNA in the isolated samples was evaluated by real-time PCR, as described previously [20].

Amplification of LMP1 was carried out in two stages with the corresponding external and internal primers, according to a previously described technique [22]. Each PCR product was purified using a mini-column (Qiagen, Germany) according to the manufacturer's instructions. Approximately 100–200 ng of PCR product was used for each reaction, and the DNA concentration was assessed visually in an agarose gel. As a positive control, 100 ng of DNA isolated from the B95–8 cell line was used. Each sample was analysed in duplicate. Negative water blanks were included in every analysis.

Sequencing of LMP1 PCR products. LMP1 amplicons were sequenced in both directions. DNA sequencing was performed by means of a panel of ABI PRISM BigDye Terminator v3.1 reagents (Thermo Fisher Scientific, USA), with subsequent analysis of the reaction products on an automatic DNA sequencer (ABI PRISM 3100-Avant, Thermo Fisher Scientific, USA). Data processing was carried out using Vector NTI software (Thermo Fisher Scientific, USA).

LMP1 classification. The nucleotide sequences of LMP1 samples amplified from DNA extracted from throat washing samples and translated into amino acid sequences were classified according to the presence of mutations resulting in amino acid substitutions, deletions and duplications in the primary protein structure. The LMP1 variants were determined according to the Edwards et al. classification published in 1999. In this classification, the name of LMP1 variants reflected their geographic origin: Alaskan (Ala), China1 (Ch1), China2 (Ch2), China3 (Ch3), Mediterranean-plus (Med+), Mediterranean-minus (Med–), and North Carolina (NC). The variant LMP1-China1 has been reported to be an analogue of the highly tumourigenic variant LMP1-Cao [15, 33].

Quantitative determination of viral DNA. The number of copies of viral DNA in 1 ml of throat washing sample was determined by real-time PCR. To construct the calibration curves, the DNA of diploid Namalwa cells containing two integrated viral genomes was used. This was based on the ratio of 3.3 pg of genomic DNA per copy of viral DNA [30]. For real-time PCR, primers for the 76-bp fragment in the BamHI-W region of viral DNA (GenBank ID no. V01555) were used: sense primer W-44F (5'-CCCAACACTCCACCACACC-3'), antisense primer W-119R (5'-TCTTAGGAGCTGTCCGA GGG-3'); fluorescent probe W-67T (5'-FAM-CAC ACACTACA-CACACCCACCCGTCTC-RTQ1) [32]. The DNA extracted from throat washings can be used as a template for PCR when the unique K-RAS gene is used as a control, as described previously [9]. The reaction was conducted in 96-well plates on a CFX96 device (Bio-Rad Laboratories, USA). The 50-μl reaction mixture (Synthol, Russia) contained 0.3 μM of each of the primers, 25 nM of the fluorescent probe, 4 mM of $MgCl_2$ 200 mM of each dNTP, 1 unit of Taq polymerase, 10 μl of DNA solution in 10 mM Tris-HCl buffer (pH 8.0) and 1 mM EDTA (corresponding to 50 μl of wash sample). Each analysis included two negative controls that did not contain DNA. The PCR conditions were denaturation for 5 min at 95°C, followed by 40 cycles at 95°C for 15 s then at 56.5°C for 30 s. The real-time PCR was analysed using CFX Manager software (BioRad).

Phylogenetic analysis. To analyse the phylogenetic relationship among ethnic Tartar EBV isolates, the LMP1 C-terminus sequences were compiled. All sequences were aligned, and the distances between each sample were calculated with the ClustalW program using the Kimura two-parameter model. A phylogenetic tree was constructed from these matrices by the neighbour-joining method using MEGA software. The main LMP1 EBV variants (Alaskan, NC, B95.8/A, Med–, China1, China2) available in GenBank were used for phylogenetic comparison.

Statistical analysis. Standardized rates (SR) for the incidence and mortality and their standard errors (SE) in 2015–2017 years for some malignant tumours among which EBV-associated cases are diagnosed in Moscow and the R. Tatarstan, were obtained from the publication "Malignant neoplasms in Russia in 2015–2017 (incidence and mortality)", Eds.: A.D. Kaprin, V.V. Starinskiy, G.V. Petrova; Moscow, 2018; Mean values of SR for incidence and mortality and their SE in 2015–2017 for both sexes were analysed. 95% confidence intervals (CI) for SR were calculated as SR±1.96 SE. Absence of overlapping of CI for Moscow and the R. Tatarstan proves statistically significant difference between SR at $P = 0.05$ level, the overlapping of CI shows that the difference is not significant.

Results

The multinational population of the Russian Federation is known to consist of numerous ancient ethnic groups that have preserved their own culture and customs, and have occupied certain regions of the country for centuries. Therefore, it was important to perform a comparative analysis of the LMP1 oncogene structure of EBV strains persisting in these groups (which we assume have been transmitted from generation to generation since historical times) and to clarify the effect of these strains on the incidence of tumours of certain localizations due to the cases associated with the virus included in these tumours.

Tatars represent one such ethnic group, the second largest one in the Russian Federation after Slavic people (fig. 1, A and B). Representatives of Slavic nation are dominant in Moscow (91.5%), while Tatars occupy only the third position (1.38%) after Ukrainians (1.42%; fig. 1A). In the R. Tatarstan,

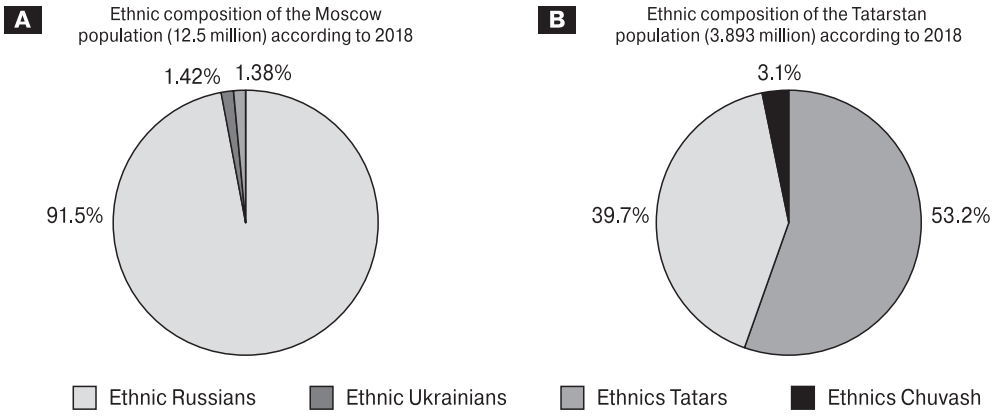


Figure 1. Leading ethnic nations in Moscow city (A) and the Republic of Tatarstan (B)

the Tatar population is slightly higher than the Slavic population (53.2% vs. 39.7%, respectively), and significantly exceeds the third ethnic nation, Chuvash (3.1%; fig. 1B).

LMP1 polymorphism

The investigation of LMP1 in the EBV strains circulating among ethnic Tatars was carried out by studying the throat washings from students of the KSMU, representatives of all administrative territories of the R. Tatarstan (tabl. 1). Analysis of the nucleotide and translated amino acid sequences of the 41 LMP1 amplicons obtained from 60 samples revealed significant polymorphism. According to the Edwards et al. classification [21] of the 41 sequences only 21 could be classified. Of these, 12 sequences corresponded to B95.8/A (29.3%, 12/41) variant, 6 — to Med– (14.6%) and 3 — to China1 (7.3%) variants. LMP1 sequences homologous to Alaskan, Med+, China2, China3 and NC variants were not detected. Among the other 20 LMP1 sequences that did not correspond to any variants of the above classification, seven (17.1%) formed a mono group consisting of sequences which differed not only from the sequences of Moscow Slavs, but also from sequences of other Kazan Tatars. This group was designated as LMP1-TatK. The remaining 13 unclassifiable Tatar sequences (31.7%) formed a group that was designated as LMP1 outside the classification (LMP1-TatBC). In contrast to the low percentage of the low-transforming variant LMP1-B95.8 found in the Tatar group, this variant was dominant among the representatives of the Slavs (82.5% and 29.3%, respectively). Among representatives of the Moscow Slavs, the percentage of LMP1-Med-variant was lower than in the Kazan Tatars (2.5% vs. 14.6%, respectively); however, the difference between these values was not statistically significant. The percentage of LMP1China1 variant was practically identical in both ethnic groups (7.5% vs. 7.3%, respectively) (tabl. 1).

LMP1 sequence analysis

Analysis LMP1 samples of Tatar origin showed that many of them contained so-called Cao-associated mutations (tabl. 2). One such mutation, a deletion of 5 aa (276–280) observed in 16 samples

Table 1. Polymorphism of LMP1 in the EBV strains circulating among Moscow Slavs and Kazan Tatar

Number of examined individuals	Number of amplified LMP1 samples	LMP1 variants according to the Edwards et al. classification* Total: 21 (51.2%)				LMP1 variants outside the Edwards et al. classification Total: 20 (48.8%)	
		B95.8/A	Med–	China1	NC	Beyond Edwards' et al. classification (TatBC)	Unique to the ethnic Tatars (TatK)
Ethnic Moscow Slavs							
65	40 (100%)	33/40 (82.5%)	1/40 (2.5%)	3/40 (7.5%)	3/40 (7.5%)	0/40 (0%)	0/40 (0%)
Ethnic Kazan Tatars							
60	41 (69.5%)	12/41 (29.3%)	6/41 (14.6%)	3/41 (7.3%)	(0%)	13/41 (31.7%)	7/41 (17,1%)

* Edwards et al. 1999 (20)

Table 2. C-terminal domain mutations in the LMP1-Tat^K variants in comparison with low (LMP1-B95.8) and highly transforming (LMP1-Cao) LMP1 variants

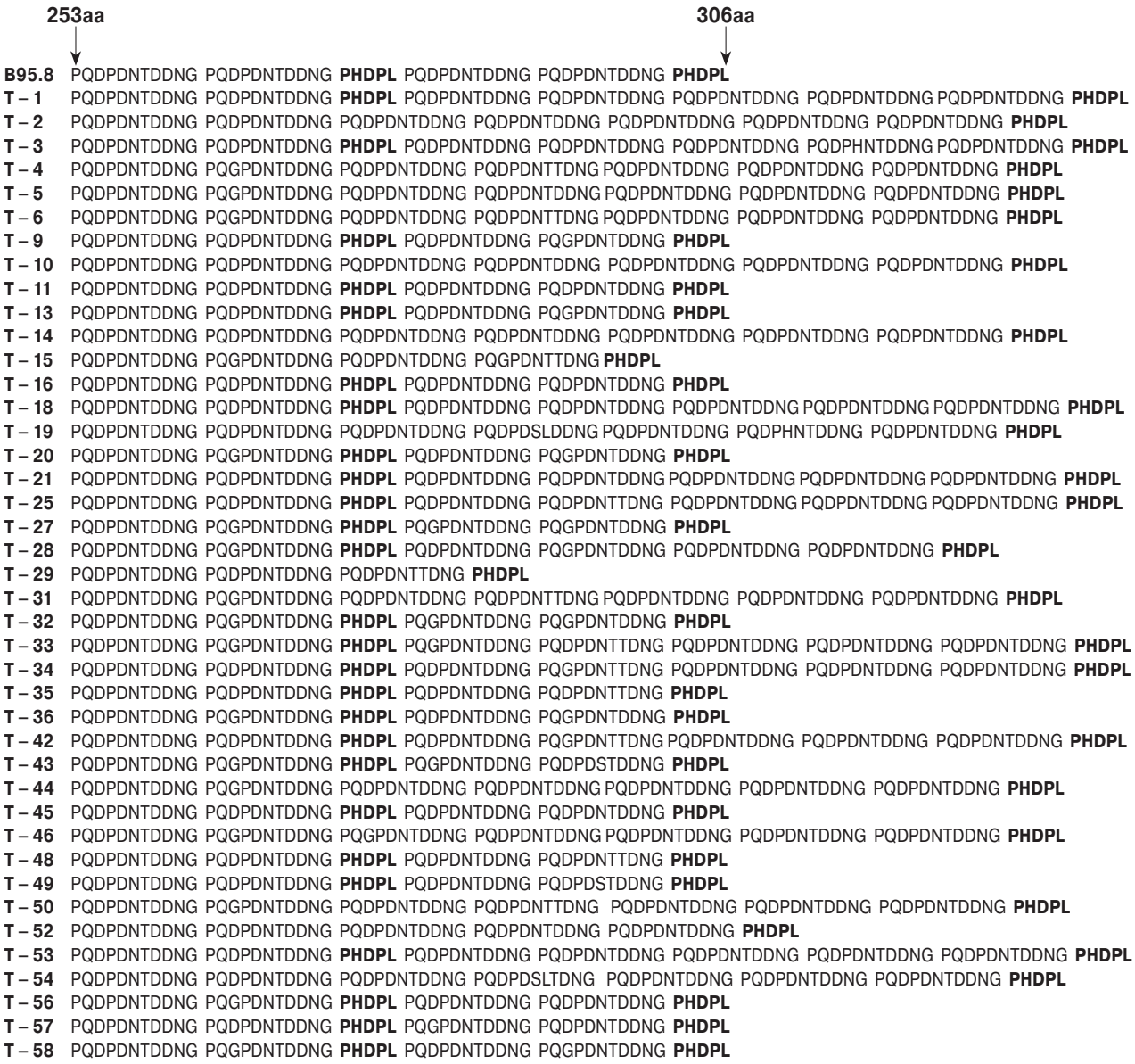
LMP1 variants	Mutations in CTAR and other regions				Cao-associated LMP1 mutations			LMP1 mutations unique to the ethnic Tatars	
	Q322N	CTAR 1 191–232	CTAR 2 351–386	CTAR 3 275–330	Deletion 276–280	Insertion 302–303	Deletion 346–355	Deletion 312–316	Deletion 382–386
LMP1-B95.8 & LMP1-Cao variants									
B95.8	–	–	–	–	–	–	–	–	–
Cao	Q322N	G212S	S366T	276–280	5aa	(11aa x3)	10 aa	–	–
LMP1-Tat^K variants									
T-2	–	–	–	276–280	5aa.	11aa x3	–	5aa	5aa
T-4	–	S229T	–	276–280	5aa	11aa x3	–	5aa	5aa
T-5	–	S229T	S366T	276–280	5aa	11aa x3	–	5aa	5aa
T-6	–	S229T	–	276–280	5aa	11aa x3	–	5aa	5aa
T-33	–	S229T	S366A	–	–	11aa x3	–	5aa	5aa
T-44	–	–	S366T	276–280	5aa	11aa x3	–	5aa	5aa
T-45	Q334R	S229T	–	–	–	–	–	5aa	5aa

(39.0%), belongs to the CTAR-3 region of the LMP1. This region is required for activation of the Jak3/STAT signalling pathway, and is located between the CTAR-1 and CTAR-2 carboxy-terminal LMP1 regions. In 22 samples (53.7%), rare LMP1 insertions of 33 aa (302–303) were detected, which were found to be located within the same CTAR-3 region. Single point mutations that do not occur in known LMP1 variants were also identified. Among them, a mutation in codon 252 (G → A) was detected in seven samples (17.1%). This mutation is within the CTAR-2 region, which is the region that recruits the TNFR-associated death domain protein (TRADD) and the so-called receptor-interacting protein (RIP). In six samples (14.6%), LMP1 mutations were found in codon 317 (D → E) within the CTAR-3 region, as well as in codon 229 (S → T) in five samples (12.2%). The latter substitution is located in the CTAR-1 region, which actively interacts with TNFR proteins (TRAF1, 2 and 5). It can be assumed that the detected mutations may change the intracellular activity profile of a number of signalling pathways, as well as their biological activity. Sequence analysis of LMP1 samples from representatives of Slav group showed a similar result, which is not surprising, given that mutual enrichment of gene pools, as well as the exchange of EBV strains, between the Slavic and Tatar populations took place over the centuries.

However, particular interest are the seven LMP1-TatK samples of Tatar origin, which are structurally different not only from the other LMP1 samples of Tatar origin and those of Slav origin, but also from LMP1 samples of healthy people and patients with head and neck tumours from different regions of Russia [41]. This group of samples was characterized by the combined content of 5-amino acid deletions in codons 312–316 and 382–386, which were absent in all other LMP1 samples that we have analysed so far (tabl. 2). Given the genealogy of the ethnic Tatars studied (at least the third generation) it can be assumed that the LMP1-TatK variant probably represents an evolutionarily ancient strain of the virus.

Amino acid repeats and insertions

A number of studies have shown that the C-terminal LMP1 domain contains a different number of repeats consisting of 11 aa (PQDPDNTDDMG), localised between aa 253 and 306. The prototype variant LMP1-B95.8 contains four such repeats and two inserts consisting of 5 aa (PHDPL), one of which is located between the second and third repeats (275–279), and the second after the last repeat (302–306). Box 5 aa is a JAK3 motif within the CTAR3 domain (aa 275–330), which presumably participates in the JACK3/STAT signalling pathway [31, 33]. To characterise LMP1 of ethnic Tatars, the C-terminal domains were analysed for repeats and insertions (fig. 2). Forty-one samples denoted by the letter T were studied. A structure of 11-aa repeats and 5-aa inserts, similar to the prototype strain LMP1-B95.8, was found for 16 samples. Seven 11-aa repeats were observed in 21 samples, 13 of which lacked the 5-aa insert between the second and third repeats, as present in the prototype variant LMP1-B95.8. In addition, point mutations (D → G) were detected in 17 samples in the region of 11-aa repeats. Similar mutations were found in patients with nasopharyngeal carcinoma, who were residents of the North Caucasus of Russia [41]. The findings suggest that EBV isolates from ethnic Tatars contain LMP1 variants which possess both a B95.8-like structure of repeating elements and a structure characteristic of LMP1 variants of African and Japanese origin [26]. However, unlike EBV isolates of Japanese origin,



T — Number of throat flushing samples from ethnic Tatars;
PQDPDNTDDNG — 11 aa repeating elements;
PHDPL — 5 aa sequences insertion;
D → G — point mutations in the region 11 aa

Figure 2. 11 aa repeats and 5 aa insertions in the C-terminal domain of LMP1 isolates from ethnic Tatars

point mutations D→G in LMP1 were often encountered in isolates from Tatar and Slavic samples. The difference in the number of repeats and 5-aa insertions between repeats for the JAK3 motif could be explained by recombination events that arise during the process of viral replication [33]. However, others have hypothesised that the mutational changes are related to the geographical origin of EBV [26].

EBV concentration in throat washing samples

From the literature and the results of own investigations, the saliva of even healthy individuals can contain EBV particles [12, 21]. In general, the concentration of viral particles in saliva is much higher than in peripheral blood. These particles, which are mixed with saliva, appear in the oral cavity as a result of EBV-induced lytic destruction of infected epithelial cells of the mucous membrane lining the nasopharynx and the Valdeier's lymphoid ring, in particular. In our study, the median number of EBV DNA copies in 15 ml of throat washing samples varied from 833 in the group of Moscow Slavs to 3538 in Tatar students from KSMU (tabl. 3). However, the median number of viral DNA copies per cell in identical volumes of throat washing samples was 0 in students and 0.01 in Moscow Slavs. The data obtained probably indicates moderate replication of the virus in the oral mucosa of individuals tested, as we detected less than 1 copy of the viral genome per cell in the cell suspension, which is in line with other studies [7, 10].

Table 3. Oral cavity EBV infection in representatives of ethnic Tatars and ethnic Slavs

Groups	Number of observations	Average age (years)	Number of EBV DNA copies in 15 ml of throat washings		Number of EBV DNA copies per 1 cell of throat washings	
			Median	IQR*	Median	IQR**
Students of the KSMU* ethnic Tatars	60	21.5	3538	0–183792	0.00	0–0.009
Healthy adults, ethnic Slavs	65	47.5	833	0–3281025	0.01	0–0.257

* KSMU — Kazan State Medical University. ** IQR — interquartile range.

Phylogenetic analysis

The 41 LMP1 sequences of Tatar origin were used to generate a phylogenetic tree to elucidate the genetic relationship between EBV isolates of ethnic Tatars. The neighbour-joining tree, constructed with additional LMP1 C-terminus sequences from GenBank (Alaskan, NC, B95.8/A, Med–, China1 and China2), showed that the LMP1 sequences of the tested throat washing samples represent a heterogeneous group. Based on their sequence variations, the LMP1 alleles under investigation could be classified into five variants, for which corresponding clusters were created in the phylogenetic tree: B95.8/A, China1, Med–, BC, and TatK (fig. 3). The LMP1 B95.8/A and TatBC variants were represented with the same frequency (both 29.3%). The estimated frequencies of the LMP1 Med– and China1 variants were lower (14.6% and 7.3%, respectively). As seen in the phylogenetic tree, part of the investigated LMP1 sequences formed a separate phylogenetic group, TatK one, which most likely had a different EBV ancestor. It was also shown that LMP1 TatBC samples, which did not relate to any variant in the phylogenetic tree, were located quite close to the branches belonging to previously reported LMP1 variants with increased transforming activity (NC, Alaska, Med–) and the new TatK variant, but they were genetically distant from sequences of the prototype LMP1 B95.8/A variant.

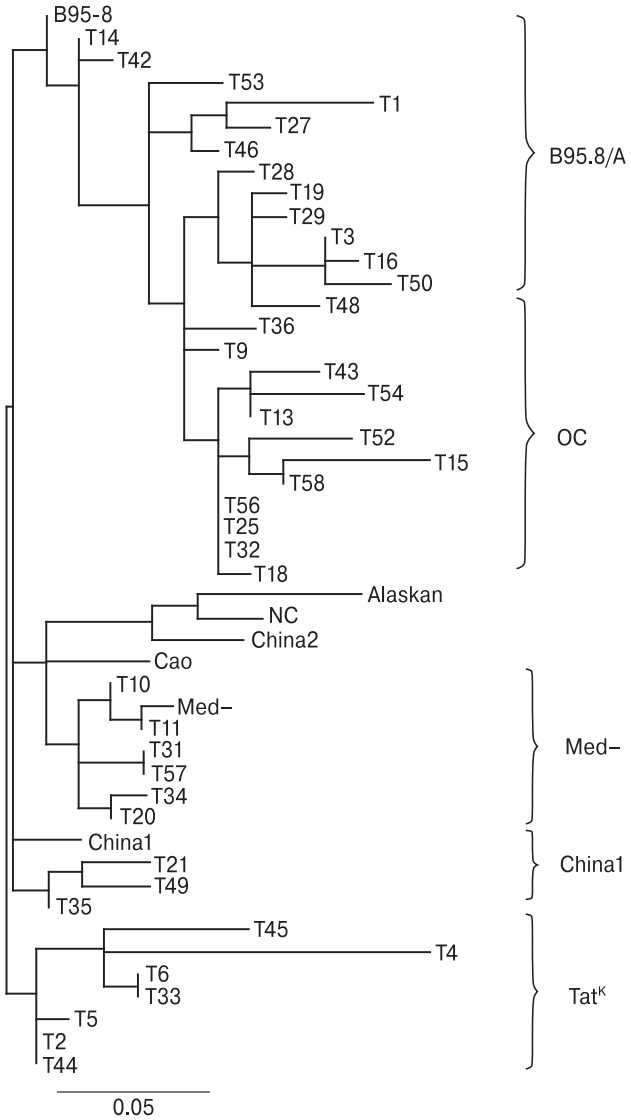


Figure 3. Phylogenetic analysis of the LMP1 samples from throat washing of ethnic Tatars

The higher polymorphism of the LMP1 gene identified for EBV strains among ethnic Tatars compared to that of the Slavic group (tabl. 1) allow us to suggest a higher rates of incidence and mortality among the Tatar population for some malignant neoplasms in which cases associated with EBV are included. To find out the validity of our assumption the tumours of the oropharynx, nasopharynx, gastric cancer and Hodgkin's lymphoma were included in the list of analysed pathologies (tabl. 4). It is known, that EBV-associated cases of tonsil and lymphoepithelial salivary gland cancers are part of the oral cavity tumours [44]. Among tumours located in the pharynx, nasopharyngeal carcinoma associated with EBV is often diagnosed [6]. Among stomach tumours some cases are associated with EBV [35]. And among the Hodgkin's and non-Hodgkin's lymphomas EBV-associated cases can be also detected [2, 4, 5].

An analysis of the standardized incidence and mortality rates per 100,000 populations in 2015–2017 years revealed that no statistically significant differences between above rates for nasopharyngeal and oropharyngeal tumours and Hodgkin's lym-

phomas, were found for geographical regions compared. Statistically significant differences for both the incidence and mortality rates were obtained only for gastric cancer. However, given the fact that EBV-associated gastric cancers make up only a small percentage of tumours of this localization (up to 10%), their effect on morbidity and mortality rates can hardly be taken into consideration.

Discussion

Since the discovery of EBV in 1964, numerous studies have been devoted to its study. The interest in EBV has not waned due to the unique properties of the virus. EBV, which is present in virtually every human body without harming it, has a powerful transforming potential. Due to this ability EBV is aetiologically associated with a wide range of benign and malignant human neoplasms.

Molecular biological studies revealed that the transforming and oncogenic (under certain conditions) properties of EBV are due to the function of nine latent viral proteins, comprising six nuclear and three membrane proteins. Each of these proteins contributes to virus-associated carcinogenesis, but the transforming capacity of LMP1 prevails. It has also been found that LMP1 samples from various geographical regions are characterised by a wide variety of polymorphisms, upon which several LMP1 classifications have been based. Among these classification systems, the most widely used in the literature is the Edwards et al. classification [15]. However, in some geographical regions, LMP1 samples do not fit into the "Procrustean bed" of the above classification [18, 39] and additional efforts should be made to create a more universal classification system.

It is important to note that, according to our knowledge, not a single study has been devoted to the investigation of ancient EBV strains. As latency genes are characterised by a large nucleotide diversity, particularly LMP1, it cannot be ruled out that mutations that occurred in the virus genome over the course of evolution led to the formation of virus strains with new structures and properties. In this regard, it is important to compare so-called "modern" EBV strains with strains that circulated among our ancestors several centuries ago.

This study represents an attempt to clarify this issue. To this end, the genetic structures of LMP1 from representatives of ethnic Tatars and Slavs, possible carriers of ancient virus strains, were compared. The genetic succession of Kazan Tatars to their ancient predecessors is based on the following historical facts, "... after the conquest of the Volga Bulgaria in 1236 by the Mongols and a number of Bulgarian uprisings of 1237 and 1240, the Volga Bulgaria becomes a part of the Golden Horde. Later on, after the collapse of the Golden Horde and the emergence of a number of independent khanates in its place, the Kazan Khanate was formed on the Bulgarian lands. As a result of the consolidation of part of the Bulgars with another Kypchak and partly Finno-Ugric population of the region, the Kazan Tatars are formed" [1]. The ethnic Slavs in our study were represented by native inhabitants of Moscow. It should be noted, however, that Moscow was captured by the Mongol-Tatar troops in 1238 [3] and, consequently, there was mutual enrichment

Table 4. Standardized incidence and mortality rates for malignant tumors in Moscow and the R. Tatarstan per 100,000 populations in 2015–2017, among which EBV-associated cases are diagnosed (both sexes)

Region	Nasopharynx (C11*)	Oropharynx (C10)	Gastric cancer(C16)	Hodgkin's Lymphoma (C81)	Lip, oral cavity, pharynx (C00–14)
Incidence per 100,000 population					
Moscow	0.16 (0.03**)	0.94 (0.7)	10.67 (0.22)	1.64 (0.12)	–
R. Tatarstan	0.25 (0.075)	0.84 (0.11)	15.08 (0.48)	1.85 (0.22)	–
Statistically significant differences	No	No	Yes***	No	–
Mortality per 100,000 population					
Moscow	–	–	9.39 (0.19)	0.36 (0.5)	3.33 (0.12)
R. Tatarstan	–	–	11.22 (0.42)	0.38 (0.8)	4.09 (0.25)
Statistically significant differences	–	–	Yes	No	No

*C11 — The section in the book "Malignant neoplasms in Russia in 2015–2017 (incidence and mortality)", eds.: A.D. Kaprin, V.V. Starinskiy, G.V. Petrova; Moscow, 2018 [45]. **Standard error. ***Yes — The difference between the standardized incidence and mortality rates was statistically significant at P = 0.05 level.

of the gene pools of the Slavic and Tatar populations for centuries, as well as the exchange of EBV strains. Therefore, it is not surprising that among representatives of the Tatar and Slav ethnic groups studied, the EBV strains contained the same variants of LMP1. On the other hand, detection among ethnic Tatars, i.e. Tatars no less than in the third generation, unique LMP1 variant (TatK), which does not comply with Edwards et al. classification and was not found among LMP1 variants of representatives of ethnic Slavs, raises the question of the origin of the EBV strain carrying this variant of LMP1. It can be assumed that this viral strain is related either geographically to the territory of Tatarstan, or ethnically to the Tatar ethnic group. In the latter case, why it is impossible to assume that this EBV strain has an ancient origin? Further investigations involving increased number of ethnic Tatars, as well as a detailed analysis of the structure and molecular properties of the TatK LMP1 variant, will probably allow us to clarify the question of whether an ancient EBV strain exists indeed and whether it affects the morbidity and mortality rates of certain tumours in the Tatar population.

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