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Epstein–Barr virus (Orthoherpesviridae: *Lymphocryptovirus*) among Russian ethnic groups: Prevalence of EBV types (EBV-1 and EBV-2), *LMP1* gene variants and malignancies

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Abstract

Introduction. The discovery of two EBV types (EBV-1 and EBV-2) has stimulated the study of their prevalence in populations and association with malignancies.

Objective. To study the prevalence of EBV-1 and EBV-2 types among ethnic groups in Russia, to analyze PCR products of the *LMP1* gene in virus isolates, and to evaluate the contribution of EBV types to the incidence of malignant neoplasms.

Materials and methods. EBV isolates from oral lavages of the Republics Adygea, Kalmykia, Tatarstan and the Moscow Region (MR) representatives were studied by nested PCR for the belonging to EBV-1 and EBV-2 types. *LMP-1* amplicons obtained by real-time PCR from viral isolates DNA were classified and sequenced on an automatic DNA sequencer ABI PRISM 3100-Avant (USA). The sequencing results were analyzed using Chromas 230 and Vector NT programs (Invitrogen, USA). The reliability of the obtained data was assessed using statistical packages Statistica for Windows, 10.0.

Results. The prevalence rates of EBV-1 and EBV-2 in representatives of four ethnic groups were compared with the incidence rates of some tumors in the population of three Republics and MR.

The dominant persistence of the transforming *in vitro* EBV-1 type in representatives of the Republic of Tatarstan and MR correlated with a high incidence of gastric cancer and lymphomas in the population of these territories. On the contrary, predominant infection of the non-transforming *in vitro* EBV-2 type and both types of the virus in approximately the same percentage of representatives of Adygea and Kalmykia, respectively, correlated with a lower level incidence of above tumors in populations of these Republics. The differences between the incidence rates of neoplasms in the compared ethnic populations were statistically insignificant ($p > 0.05$). *LMP1* variants of viral isolates did not reflect either the level of EBV persistence types or the incidence of tumors.

Conclusion. Infection of ethnic groups with EBV-1 and EBV-2 may vary significantly under the influence of various factors. The predominance of the *in vitro* transforming EBV-1 type in the population did not increase the incidence of tumors due to cases associated with the dominant virus type.

Keywords: Epstein-Barr virus (EBV); EBV-1 and EBV-2 types; latent membrane protein 1 (*LMP1*); sequence analysis; Adygeians; Kalmyks; Tatars; Slavs; real-time PCR; malignant tumors

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ОРИГИНАЛЬНОЕ ИССЛЕДОВАНИЕ

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Вирус Эпштейна–Барр (Orthoherpesviridae: *Lymphocryptovirus*) у этносов России: распространенность типов ВЭБ (ВЭБ-1 и ВЭБ-2), варианты гена *LMP1* и злокачественные опухоли

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Резюме

Введение. Открытие двух типов вируса Эпштейна–Барр (ВЭБ) – ВЭБ-1 и ВЭБ-2 – стимулировало изучение их распространенности в популяциях и связи со злокачественными опухолями.

Цель исследования. Изучить персистенцию ВЭБ-1 и ВЭБ-2 среди этносов России, проанализировать ПЦР-продукты гена *LMP1* в изолятах вируса и оценить вклад типов ВЭБ в заболеваемость злокачественными новообразованиями.

Материалы и методы. Изоляты ВЭБ, амплифицированные из смывов ротовой полости представителей республик Адыгея, Калмыкия, Татарстан и Московской области (МО), изучали методом гнездовой ПЦР на принадлежность к ВЭБ-1 и ВЭБ-2. Ампликоны *LMP1*, полученные с помощью ПЦР в реальном времени из ДНК вирусных изолятов, подвергали классификации и секвенированию на автоматическом секвенаторе ДНК ABI PRISM 3100-Avant (США), а результаты секвенирования анализировали с помощью программ Chromas 230 и Vector NT (Invitrogen, США). Достоверность полученных данных оценивали с помощью статистических пакетов Statistica for Windows 10.0.

Результаты. Показатели распространенности ВЭБ-1 и ВЭБ-2 у представителей четырех этносов сравнивали с уровнями заболеваемости некоторыми опухолями у населения трех республик и МО. Доминирующая персистенция трансформирующего *in vitro* ВЭБ-1 у представителей Татарстана и МО коррелировала среди населения этих территорий с высокой заболеваемостью раком желудка и лимфомами. Напротив, преобладающее инфицирование не трансформирующим *in vitro* ВЭБ-2 представителей Адыгеи и обоими типами вируса примерно у одинакового процента представителей Калмыкии коррелировало с более низкой заболеваемостью вышеуказанными опухолями населения этих республик. Различия между показателями заболеваемости указанными новообразованиями в сравниваемых этнических популяциях были статистически недостоверными ($p > 0,05$). Обнаруженные варианты *LMP1* не отражали ни уровень персистенции типов ВЭБ, ни частоту возникновения опухолей.

Заключение. Инфицированность этносов ВЭБ-1 и ВЭБ-2 может существенно различаться под влиянием разных факторов. Преобладание в популяции трансформирующего *in vitro* ВЭБ-1 не увеличивает заболеваемость опухолями за счет случаев, ассоциированных с доминирующим типом вируса.

Ключевые слова: вирус Эпштейна–Барр (ВЭБ); типы ВЭБ (ВЭБ-1 и ВЭБ-2); латентный мембранный белок 1 (*LMP1*); секвенный анализ; адыгейцы; калмыки; татары; славяне; ПЦР в реальном времени; злокачественные опухоли

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Introduction

Epidemiological data suggest that the Epstein-Barr virus (EBV) is involved in the occurrence of approximately 200 thousand tumors per year worldwide [1]. Moreover, the biological peculiarity of EBV lies in its ability to induce neoplasia of various cellular origins, while tumors caused by other oncogenic viruses arise only in the cells of their target tissues. On the other hand, it is known that EBV infects more than 90% of the world's population, but its permanent presence in the human body is not a prerequisite for the occurrence of a tumor. To realize the oncogenic potential of the virus, the participation of additional factors, both common and different for different types of tumors, is necessary. In particular, it has been proven that the risk of developing endemic Burkitt's lymphoma in the countries of Equatorial Africa is associated with a high burden of EBV infection in children at birth, coupled with *Plasmodium falciparum* infection, which is mutagenic on B cells. [2]. The risk of developing nasopharyngeal carcinoma (NPC) is also contributed by high infection with EBV in infancy and dietary habits from the first years of a child's life, characteristic of the Southern China culture – feeding children salted fish, rich in precarcinogens (nitrosamines) [3]. Impaired immunity, changing the balance between the virus and the host in favor of the virus, is also an important prerequisite for the occurrence of both types of tumors.

In the occurrence of some tumors, for example, NPC, the genetic element also plays an important role. So-called familial cancer cases account for approximately 10% of all NPC cases reported for the population in the NPC-endemic southern provinces of China. [4]. Several allelic susceptibility determinants most closely associated with the HLA class I region have also been found in patients with NPC outside of endemic regions [5].

With the discovery of two EBV types, EBV-1 and EBV-2, differing in the genes encoding nuclear antigens (EBNA-2, -3A, -3B, -3C) and biological properties, investigations began to be carried out aimed at elucidation the role of each type in EBV-associated carcinogenesis. These studies were based on the fact, that EBV-1, unlike EBV-2, is able to transform B cells *in vitro* [6] and on the results of experimental studies showing slower growth of lymphoblastoid cell lines infected with EBV-2, compared to those with EBV-1. It turned out, however, that both types of the virus were capable of causing B-cell lymphomas in CBH mice [7].

Both types of virus also differ in the degree of prevalence among different populations. In particular, it has been shown that the Caucasian population is more often infected with type 1 virus (~74%), and its even greater predominance is observed in healthy individuals of Asian origin (~85%) [8, 9]. There are also data indicating a fairly wide spread of EBV-2 among certain population groups, for example, in persons asymptotically infected with HIV (50%) [10], patients with progressive form of HIV infection (62%), AIDS patients with non-Hodgkin's lymphoma (53%), and even among American donors in certain US states (50%) [11]. However, the question of

whether EBV-1 and EBV-2 are tumor-specific remains unanswered to this day.

The search for tumor-specific EBV strains regardless of the type of virus was also carried out based on the genetic diversity of the *LMP1* genes, classified according to the generally accepted classification of Edwards et al. [12]. In particular, almost all EBV isolates of Chinese origin belonging to the virus type 1 were found to contain a 30-bp deletion with characteristic amino acid (aa) substitutions in its protein variant, LMP1 [13]. In populations from other geographic regions, such as Japan, the 30-bp deletion found in LMP1 was associated predominantly with type 2 virus. [14]. It is important to note that the majority of molecular polymorphisms found in EBV isolates from healthy virus carriers are also found with equal frequency in virus-associated patient's tumors from in the same geographic region. [15].

The question arises whether EBV types and/or LMP1 virus variants can influence the incidence of certain forms of tumors or whether the frequency of the latter is determined mainly by the genetic characteristics of the population and/or other factors. In this regard, it would be important to study the molecular profile as well as the tumor-inducing properties of EBV isolates among ethnic groups representing genetically distinct populations and exposed to different environmental factors. Based on the above, the purpose of this study was to analyze the prevalence of EBV types and LMP1 variants in representatives of four genetically distinct ethnic groups (Adygeans, Kalmyks, Tatars and Slavs), residents of different climatic zones and geographical territories of Russia with their own lifestyle and culture. It was also important to find out whether there is a correlation between the dominance of one of the EBV types and/or LMP1 variant and the incidence of malignant tumors.

Materials and methods

Objects of research

Oral lavages from representatives of four ethnic groups – Adygeans, Kalmyks, Tatars and Slavs, residents of three Republics of the Russian Federation and the Moscow region (MR), respectively, were studied. Detailed characteristics of the individuals participating in the study are presented in **Table 1**. All participants were practically healthy people and representatives of the above ethnic groups no less than in three generations. Each lavage was a cell suspension obtained individually after rinsing the mouth for 30 s with 15 mL of sterile saline solution. Lavage samples collected in hermetically sealed plastic tubes were stored at a temperature of +4 °C for no more than two days before the study. Informed consent was obtained from all individuals examined. The research protocol was approved by the Ethics Committee of the N.N. Blokhin National Medical Research Center of Oncology, Ministry of Health of Russia (Protocol No. 512 of November 10, 2021).

DNA extraction and LMP1 gene amplification

Samples of total DNA from oral cells lavages collected after centrifugation were isolated by phenol-chloroform

Table 1. Characteristics of ethnic group representatives involved in the study

Таблица 1. Характеристика представителей этносов, участвовавших в исследовании

Representatives of ethnic groups Представители этносов	Religion Религия	Geographical region Географический регион	Number of investigated persons Число обследованных лиц	Gender ratio: M/F Гендерное соотношение: М/Ж	Average age, years Средний возраст, лет
Adygeans Адыгейцы	Islam Ислам	Maykop town, R. Adygea г. Майкоп, Р. Адыгея	59	24/34	41,4
Kalmyks Калмыки	Buddhism Буддизм	Elista town, R. Kalmykia г. Элиста, Р. Калмыкия	50	19/31	21,4
Tatars Татары	Islam Ислам	Kazan city, R. Tatarstan г. Казань, Р. Татарстан	60	15/45	21,5
Slavs Славяне	Orthodoxy Православие	Small towns, Moscow region Московская область, районы	40	21/19	47,5

deproteinization. The presence and concentration of EBV DNA amplified from total DNA samples was analyzed by real-time polymerase chain reaction (PCR), which we described previously [16]. Amplification of the *LMP1* gene from EBV DNA was carried out in two stages with external and internal primers according to our previously adopted method [17]. Each PCR product was purified on a QIAGEN mini-column (QIAquick PCR Purification kit, cat. 28104, Germany) according to the manufacturer’s instructions. Approximately 100-200 ng of PCR product was used for the reaction, and the DNA concentration was assessed visually in an agarose gel. DNA isolated from the B95-8 cell line was used as a positive control, and water was used as a negative control.

EBV typing with nested PCR of the EBNA-2 gene

Typing of EBV isolates into EBV-1 and EBV-2 types was carried out using nested PCR, following the previously described method [18] with minor modifications. The primers used demonstrated high specificity and lack of cross-reactivity with the human genome and other viruses or microorganisms [19].

Sequencing of LMP1 PCR products

LMP1 amplicons were sequenced in both directions. Sequencing was performed using the ABI PRISM® Big-Dye Terminator v. 3.1 reagent kit (USA), followed by analysis of the reaction products on an automatic DNA sequencer ABI PRISM 3100-Avant (USA). Sequencing results were processed using Chromas 230 and Vector NT programs (Invitrogene, USA).

LMP1 classification

The nucleotide *LMP1* sequences amplified from EBV isolates obtained from oral lavages were translated into LMP1 amino acid sequences and analyzed using the literature-accepted classification of Edwards et al. [12].

Statistical analysis

The number of EBV-1 and EBV-2 copies in individual’s oral lavages of the study groups was assessed using the Mann–Whitney U test. Fisher’s exact test was used to

calculate the *p* value when comparing the number of individuals infected with EBV type 1 or 2; differences were considered statistically significant at $p \leq 0.05$. Calculations were carried out using statistical packages Statistica for Windows, 10.0.

Results

EBV types

EBV samples amplified from cell suspensions of four ethnic groups representatives were tested for the EBV-1 and EBV-2 virus types. The results obtained indicate (**Figure 1**) that EBV-1 dominates among representatives of the Slavs and Tatars (81%, 30/37; and 83%, 43/52, respectively), and EBV-2 dominates among representatives of the Adygeans (81%; 48 /59 respectively). Representatives of the Kalmyks were infected with both types of the virus in approximately equal proportions (with EBV-1 – 51%, 25/49 and with EBV-2 – 49%, 24/49). Simultaneous infection with both types of the virus was found per one case in the groups of Kalmyks, Slavs and Tatars.

Polymorphism of EBV LMP1 gene samples

The nucleotide sequences of the *LMP1* gene samples were translated into amino acids, followed by determination of the variant for each LMP1 sample using Edwards et al. classification [12]. Detected LMP1 variants, as well as the results of their sequencing, are presented in **Table 2**. It follows from the table that the low transforming *in vitro* LMP1-95.8 variant was characteristic for 100% of Adygean’s viral isolates. This LMP1 variant was also widely presented among Kalmyk’s and Slav’s viral isolates (75.9% and 82.5%, respectively), and to a lesser extent – among Tatar’s ones (34.1%). The LMP1-China-1 variant, an analogue of the highly transforming *in vitro* Chinese variant LMP1-Cao, was identified among viral isolates of Kalmyks (17.2%), Slavs (7.5%), and Tatars (9.8%). The LMP1-Med- (minus) variant among viral isolates of the above ethnic groups representatives was found in 3.4%, 2.5% and 14.6% of cases, respectively), and the LMP1-NC variant was

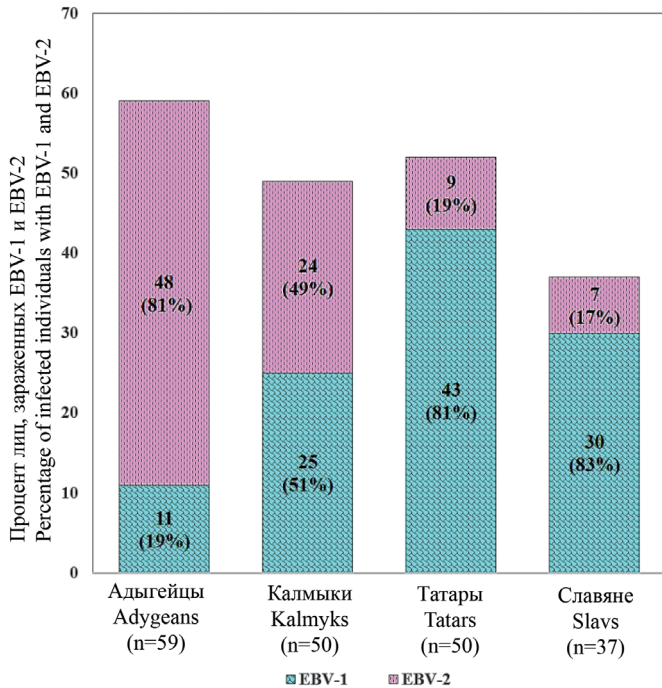


Fig. 1. The ratio of EBV-1 and EBV-2 in oral lavages of four ethnic groups' representatives: Adygeans, Kalmyks, Tatars and Slavs.

Рис. 1. Соотношение ВЭБ-1 и ВЭБ-2 в смывах полости рта представителей четырех этносов: адыгейцев, калмыков, татар и славян.

found only among representatives of Kalmyks (3.4%) and of Slavs (7.5%).

Out of 41 LMP1 samples of Tatar's representatives according to the classification of Edwards et al., 24 LMP1

variants were identified. The remaining 17 samples (41.5%) could not be interpreted using the above mentioned classification, which allowed us to designate these samples as "out of classification" (OC). Among the 17 unidentified LMP1 samples, a group of eight samples was characterized by a combined content of 5 aa deletions in codons 312–316 and 382–386, which are not characteristic of any of the LMP1 variants known to us. It is apparent that this group, related to the ethnic Tatars and designated as LMP1-TatK (Tatarstan-Kazan), deserves further investigation.

Sequencing of LMP1 samples obtained revealed the presence of important key mutations in the C-terminal region of the trans-activating domains (CTARs). In particular, in the CTAR1 domain of LMP1 samples belonging to representatives of the Adygeans, Kalmyks and Tatars, a mutation in codon 229 (S→T) was detected in 6.9%, 6.9% and 19.5% of cases, respectively. In the CTAR2 domain, LMP1 samples from representatives of all four ethnic groups contained mutations at codon 366 (S→A/T) in a range from 3.4% to 53.6% of cases. In the CTAR3 domain, LMP1 samples also obtained from representatives of all ethnic groups contained a mutation at codon 309 (S→T/N) with a frequency of 4.9% to 16.9% of cases. In the same domain, among representatives of the Kalmyks, Tatars and Slavs, the mutation (Q→N/E/T) at codon 322 was found in 24.1%, 22.0% and 17.5% of cases, respectively, and the mutation at codon 328 (E→Q) was detected only among representatives of the Slavs in 30% of cases (4/12). Based on the results of sequencing LMP1 samples from representatives of the Adygeans, Kalmyks and Tatars, we can conclude that the EBV strains circulating among these ethnic groups are genetically re-

Table 2. LMP1 polymorphism in EBV isolates from oral lavages of four ethnic groups representatives

Таблица 2. Полиморфизм LMP1 в изолятах ВЭБ из смывов полости рта представителей четырех этносов

Investigated ethnic groups (number of oral lavages) Этническая группа (количество промываний полости рта)	Number of amplified LMP1 samples Число амплифициро- ванных образцов LMP1	LMP1 variants by classification of Edwards et al. [12] Варианты LMP1 по классификации R. Edwards и соавт. [12]					Mutations in CTAR regions of the LMP1 gene Мутации в CTAR-областях гена LMP1		
		B95.8	China-1	Med-	NC	BK* OC*	CTAR 1 191-232	CTAR 2 351-386	CTAR 3 275-330
Adygeans Адыгейцы (n = 59)	29/59 (49,2%)	29/29 (100%)	0 (0%)	0 (0%)	0 (0%)	–	S229T 2/29 (6,9%)	S366T 1/29 (3,4%)	S309T/N 2/29 (6,9%)
Kalmyks Калмыки (n = 50)	29/50 (58,0%)	22/29 (75,9%)	5/29 (17,2%)	1/29 (3,4%)	1/29 (3,4%)	–	S229T 2/29 (6,9%)	S366A/T 6/29 (20,7%)	S309N; 8/29 (16,9%) Q322E/T 7/29 (24,1%)
Slavs Славяне (n = 40)	40/40 (100%)	33/40 (82,5%)	3/40 (7,5%)	1/40 (2,5%)	3/40 (7,5%)	–	S229T 8/41 (19,5%)	S366A/T 22/41 (53,6%)	S309N; 2/41(4,9%) Q322N/E; 9/41(22,0%)
Tatars Татары (n = 60)	41/60 (68,3%)	14/41 (34,1%)	4/41 (9,8%)	6/41 (14,6%)	0 (0%)	17/41 (41,5%)	0 (0%)	S366T 19/40 (47,5%) L338S 7/40 (17,5%)	S309N; 4/40 (10,0%) Q322N/E/T 7/40 (17,5%) E328Q; 4/12 (30,0%)

Note. *OC – out of classification.

Примечание. *BK – вне классификации.

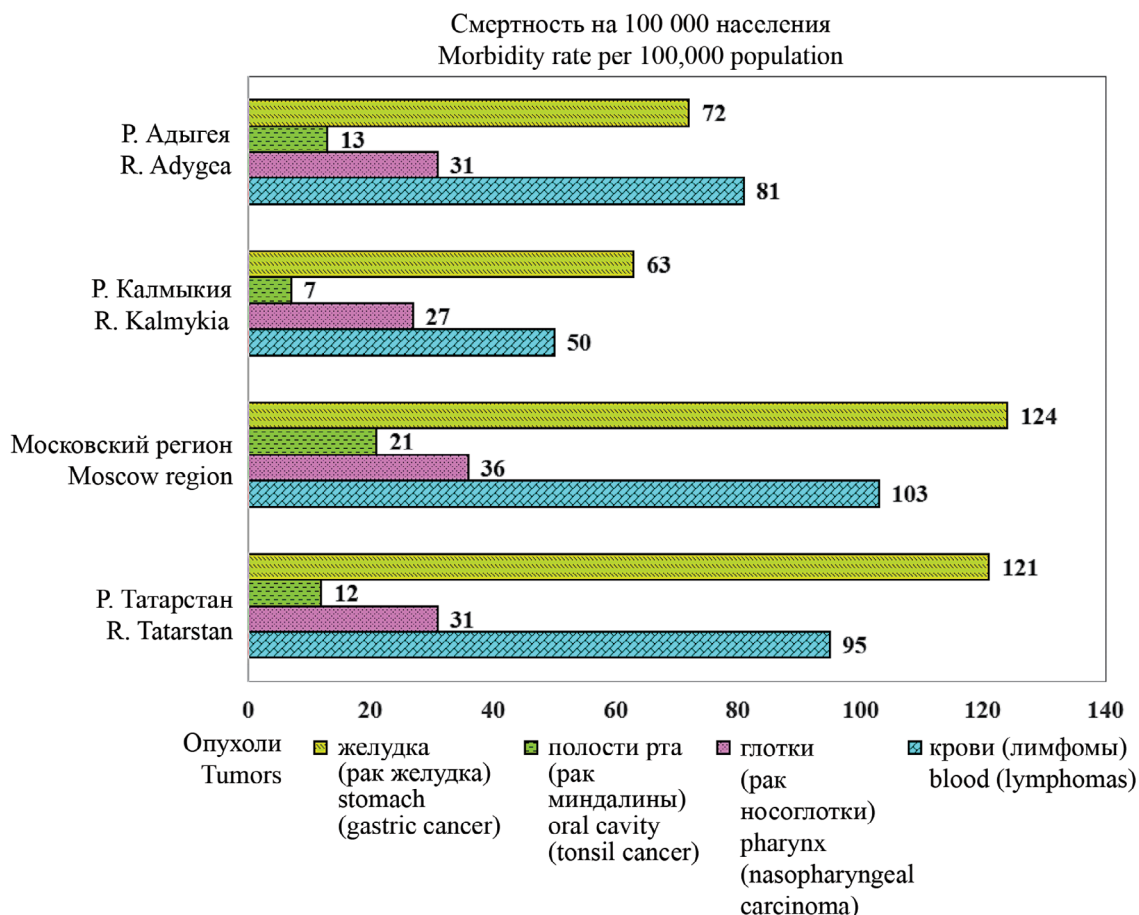


Fig. 2. Incidence rates of malignant neoplasms with EBV-associated cases among the population of the Republics of Adygea, Kalmykia, Tatarstan and the Moscow region.

Рис. 2. Показатели заболеваемости опухолями с ВЭБ-ассоциированными случаями среди населения республик Адыгея, Калмыкия, Татарстан и Московской области.

lated. EBV strains of Slavic origin, although characterized by the absence of mutations in the CTAR1 domain and an increased number of mutations in the CTAR2 and CTAR3 domains, taking into account the other detected mutations, can also be considered genetically close to EBV strains circulating in other representatives of the studied ethnic groups.

EBV types and malignant tumors

To find out whether each type of EBV affects the incidence of malignant tumors the prevalence rates of EBV-1 and EBV-2 among representatives of the Adygeans, Kalmyks, Tatars and Slavs were compared with the incidence rates of certain malignant neoplasms among the population of three Republics and the Moscow region. The incidence rates of tumors of the stomach, pharynx, oral cavity and blood were analyzed, in which corresponding EBV-associated neoplasms occur, such as gastric adenocarcinoma, tonsil cancer and nasopharyngeal carcinoma, Hodgkin's lymphoma, and non-Hodgkin's lymphomas (**Figure 2**). According to standardized values (per 100,000 population), the incidence of the oral cavity and pharynx tumors among the population of three Republics and the

MR was low and ranged from 7 to 36 [20]. The incidence of gastric cancer, lymphomas were significantly higher. Their values for R. Tatarstan (121 and 95, respectively) and MR (124 and 103, respectively) correlated with the dominance of the *in vitro* transforming type EBV-1 in representatives of these regions. On the contrary, lower incidence rates of the same tumors were observed in the population of the R. Adygea (72 and 81, respectively), whose representatives were infected predominantly with a non-transforming virus type, EBV-2, and the population of the R. Kalmykia (63 and 50, respectively), whose representatives were infected with both types of the virus in approximately equal proportions. Statistical analysis, however, showed that the differences between the incidence rates of gastric cancer and lymphomas among the populations of the R. Tatarstan and the MR, on the one hand, and the R. Adygea and the R. Kalmykia, on the other hand, were statistically insignificant ($p > 0.05$).

Discussion

We studied the prevalence of EBV types, EBV-1 and EBV-2, in representatives of four ethnic groups that are genetically distinct and inhabit different geographical

and climatic regions of Russia. It has been shown that the ratio of individuals infected with EBV-1 and EBV-2 among representatives of each ethnic group was different, which is likely due to the genetic characteristics of the ethnic groups and, above all, the diversity of their MHC types. Likely for the same reason, EBV-1 is dominating in the Caucasian population, while the population of certain African countries is more often infected with EBV-2 [21]. It also seems impossible to exclude the influence of climate and living conditions on the spread of virus types.

Attempts to discover the existence of tumor-specific EBV strains have been made by many researchers, but have so far been unsuccessful. In this study, the dominance of the transforming *in vitro* EBV-1 type among representatives of the R. Tatarstan and the MR correlated with a high incidence of gastric cancer and lymphomas in the population of these territorial entities. On the contrary, lower incidence rates of the same neoplasms among the population in other studied Republics were combined with the predominant spread of the non-transforming EBV-2 type among representatives of the R. Adygea and both types of the virus in almost equal ratio among representatives of the R. Kalmykia. However, the differences between the incidence rates of these tumors in the ethnic groups studied turned out to be statistically insignificant ($p > 0.05$). Nevertheless, the data obtained are of interest and require additional research. It can be assumed that in order to clarify the effect of EBV infection on the incidence of malignant tumors, it would be important to determine the frequency of EBV-associated cases among the tumors of studied location. Establishing the ratio of tumors associated with transforming and non-transforming types of EBV, EBV-1 and EBV-2 could also make an important contribution to the study of EBV-associated carcinogenesis.

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