Sequence Variants of LMP1 Oncogene in Patients with Oral Cavity Tumors Associated and Not Associated with Epstein–Barr Virus

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Abstract—The role of Epstein–Barr virus (EBV), ubiquitous lymphotropic human herpesvirus 4, in etiology of nasopharyngeal carcinoma (NPC) has not been completely clarified. The mechanism of carcinogenesis in this disease (closely associated with EBV) is also unclear. The aim of the present study was to compare the structure of the LMP1 oncogene of EBV in isolates of the virus obtained from patients with two types of oral cavity tumors, including (a) associated (NPC) and (b) not associated (other tumors of the same anatomical region, OTOC) with EBV. A comparative analysis of the deduced C-terminal amino acid sequences of the LMP1 variants was carried out based on the LMP1 sequence data from samples of the tumor, blood, and oropharynx lavages from patients with NPC and OTOC. It was demonstrated that, in the compared groups of patients, all structural characteristics of LMP1 were close, and existing differences between the compared parameters were statistically insignificant. Thus, it was demonstrated for the first time that genetically related EBV strains with structurally similar LMP1 variants persist in patients with NPC and OTOC in Russia, which most likely reflects the polymorphism of EBV strains that circulate in the population. Based on the data obtained, it is possible to assume that the risk of the occurrence of NPC in NPC non-endemic world regions (including Russia) depends not so much on the EBV strain (and on the variant of the LMP1 that it contains) as on the genetic predisposition to the disease of individuals infected by this virus and the effect of other (still unknown) agents.

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INTRODUCTION

Epstein–Barr virus (EBV) refers to the family of herpesviruses and infects more than 90% of the adult population worldwide. Epstein–Barr virus is ubiquitously distributed and does not cause any clinical manifestations in most virus carriers, but in some cases it can become an etiological agent for a wide range of malignant neoplasms of lymphoid or epithelial origin, including Burkitt’s lymphoma, Hodgkin’s lymphoma, nasopharyngeal carcinoma (NPC), certain cases of stomach cancer, etc. [1]. Some EBV-associated neoplasms (in particular NPC) are characterized by limited geographical and racial distribution [2, 3]. A rare phenomenon in most countries in the world (including Russia), NPC is found with an average frequency in only some countries and is considered to be an endemic disease in the populations of southern provinces in China and some other countries of Southeast Asia [4]. The reasons for the high morbidity of EBV-associated NPC in certain geographic regions cases have still not been determined. The influence of a number of factors on this process (namely, circulation on highly pathogenic EBV strains in endemic regions, genetic predisposition to the development of NPC in the population of these territories, and its susceptibility to the effect of a number of deleterious environmental factors) cannot be excluded. The reasons of the appearance of NPC in nonendemic regions are an even greater mystery. In particular, it is not clear what role EBV plays in the pathogenesis of this disease.

It was demonstrated that the unique ability of EBV to transform infected cells is realized due to the functioning of one of the main virus proteins (latent membrane protein 1 (LMP1) [5]. It is also known that the transformation mechanism is carried out through the interaction of LMP1 with the other virus protein (EBNA2), which has a nuclear localization [6].

Structurally, the LMP1 is an integral membrane phosphoprotein with a molecular mass of 63 kDa, which consists of 23-membered N-terminal cytoplasmic domain, six hydrophobic transmembrane

Abbreviations: LMP1, latent membrane protein 1; EBV, Epstein–Barr virus; OTOC, other tumors of oral cavity; NPC, nasopharyngeal carcinoma; CTAR, C-terminal transactivating region; VCA, viral capsid antigen; EA, early antigen; EBNA, Epstein–Barr virus nuclear antigen.
domains (TMD) (163 amino acid residues), and a long cytoplasmic C-terminal domain (CTD) (200 amino acid residues) [7]. The LMP1 CTD includes three C-terminal transactivating regions (CTAR). Both proximal CTAR1 (amino acid residues 194–232) and distal CTAR2 (amino acid residues 351–386) regions are responsible for the activation of a number of cellular signal cascades, which determines the significance of these LMP1 regions in transforming potential

[8, 9]. The CTAR3 region (amino acid residues 275–330), located between the CTAR1 and CTAR2, presumably recruits JAK3, which results in the STAT3 activation. This LMP1 region (as opposed to the CTAR1 and CTAR2) is involved in transforming growth of B lymphocytes [7, 10, 11]. It was demonstrated that the LMP1 expression results in transformation of the rodent fibroblasts and human B lymphocytes in vitro, as well as in the development of lymphomas in transgenic mice [12].

A global interest to the LMP1 was largely stimulated by a number of reports about the detection of the protein variants that determine aggressive, geographically limited EBV phenotype. Thus, several mutations that are absent in the wild (prototype) EBV strain (B95-8) were found during the LMP1 cloning and sequencing from EBV isolated from NPC patients in China [13]. The protein (that is encoded by this found in nature LMP1 variant) was called Cao (404 amino acid residues). It has a ten-membered deletion in the region that is adjacent to the CTAR2 region and 26 amino acid substitutions in all LMP1 wild-type domains. Later, highly transforming LMP1 variants (like Chinese Cao) were isolated from NPC patients in Taiwan (C15) and Mediterranean (TS10) [14–16].

The study of the polymorphism of the LMP1 gene and its possible association with EBV-associated diseases yielded important information about the existence of different isoforms of this gene and allowed it to be classified into groups [17–20]. Sandejev et al. [18] distributed all samples of gene isolates (that they studied) by four main groups and called them A, B, C, and D. The members of group A carry six single substitutions in the gene and a single substitution in the promoter. Thus, several mutations are absent in the wild (prototype) EBV strain (B95-8). The number of mutations is larger and more diverse in members of other groups. It is important to note that even variants of group A, as well as members of other groups, contain several Cao-specific amino acid substitutions, such as I85L, F106Y, G212S, and S366T. In addition, most isolates that belong to any of the groups have additional random mutations.

Based on the LMP1 samples amplified from patients with EBV-associated pathology and healthy individuals from different geographic regions, Edwards et al. [20] suggested their own classification of the LMP1 variants based on the frequency of geographical detection [Alaskan, China 1 (Ch1), China 2 (Ch2), China 3 (Ch3), Mediterranean + (Med+), Mediterranean – (Med–), New York City (NC)]. Subsequent studies demonstrated that the frequency of these variants also varies considerably among different pathologies associated with EBV.

Taking into account that the role of EBV in the origin of NPC in nonendemic regions remains poorly studied, the aim of the present work was to conduct a comparative study of the gene peculiarities of the LMP1 samples in EBV strains persisting in patients with EBV-associated cases of NPC and in patients with other tumors (OTOC) that are not associated with this virus but are also localized in oral cavity.

EXPERIMENTAL

**Patient groups and DNA samples.** Objects of the study included samples of the tumor tissues, as well as of oropharynx lavages from 23 patients with NPC, 20 patients with OTOC, and 20 blood donors (all participants were Russian citizens). Patients with cancer of the oral mucosa, tongue, sublingual tonsil, and some other malignant affections of the oral cavity were included in the composition of patients with OTOC. The study (in which patients with NPC and OTOC were included with their consent as a result of the random sample) was approved by the Ethical Committee of the Blokhin Russian Cancer Research Center, Russian Academy of Medical Sciences.

**DNA extraction and PCR product sequencing.** DNA was isolated from the biological material collected for the study by phenol–chloroform deproteinization. The presence and concentration of DNA preparations was analyzed by real-time PCR using the following pair of primers to the GAPDH gene region (S′-AGTCCAGTGAGCTTCCCGTTCAGC–3′ and 5′-TGGTATCGTGGAAGGACTCATGAC-3′). Intercalating SYBR Green I dye (in dilution 1 : 75000) was used to detect amplification products, and the melting of amplification products was conducted after the end of PCR. Amplification was conducted according to the following scheme, including denaturation at 94°C (2 min), then 50 cycles of 94°C (20 s), 64°C (20 s), and 72°C (10 s). The reaction was conducted in 96-well plates using the iQ5 Real-Time PCR detection system (Bio-Rad Labs, CA). Each DNA sample was then analyzed for the presence of the LMP1 using nested PCR with the following primers (constructed for amplification of the gene fragment, which encodes the C-terminal protein region (amino acid residues 212–387)):

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including external (Eco3′: 5′-TCCAGGGAGAATTCCCATCCTCGAGAGTG-3′ and 8785: 5′-CGACCCCCAATCTGGATGTATTATGATG-3′)
and internal (Bam3′: 5′-GCTACCGATTCTCGCCATGCATGAC-3′ and 8702: 5′-GCTACCGATTCTGAGCGACGAC-3′) as described previously [21].
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