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EXPERIMENTAL WORKS

Structural and Functional Characteristics of the *LMP1* Oncogene in Patients with Tumors Associated and Not Associated with the Epstein–Barr Virus

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Abstract—Epstein–Barr virus (EBV) is an etiological agent of a number of human benign and malignant tumors, including infectious mononucleosis (IM), Burkitt lymphoma (BL), Hodgkin lymphoma (HL), non-Hodgkin lymphomas (NLHs), nasopharyngeal carcinoma (NPC), among others. Latent membrane protein 1 (LMP1) encoded by the gene of the same name (*LMP1*) is the main oncoprotein of EBV. LMP1 is a transmembrane protein capable of activating many signaling pathways and transcription factors of the cell, which leads to its transformation. Molecular analysis of LMP1 of various clinical origins identified many gene variants with different types of mutations that are the causes of the change in its biological activity. Since the role of LMP1 in the development of NPC is still not fully understood, it was important to determine the difference between LMP1 samples from patients with EBV-associated forms of NPC and patients with other tumors also located in the oral cavity (OTOC) not associated with this virus. In contrast a single works of this kind conducted in endemic regions, the present work aimed at a comparison of the genetic structure and transforming activity of LMP1 variants from NPC and OTOC patients was carried out in a nonendemic region, Russia, where NPC is rarely diagnosed. The obtained data show the structural and functional similarity of LMP1 variants in two groups of patients and, therefore, the genetic relationship of EBV strains persisting in these patients. Our work suggests that there is no special virus variant that causes NPC in nonendemic regions: any EBV strain with any *LMP1* structure may, it seems, become the etiological agent of NPC. However, according to the modern understanding, cancer may develop in EBV-infected persons only given the presence of a unique HLA pattern associated with a high sensitivity to the NPC development combined with exposure to harmful environmental factors, which contribute to the accumulation of a certain number of mutations necessary for EBV-associated initiation of carcinogenesis in infected epithelial cells.

Keywords: Epstein–Barr virus (EBV), nasopharyngeal carcinoma (NPC), other tumors of the oral cavity (OTOC), latent membrane protein 1 (LMP1), polymorphism, transforming activity of LMP1

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INTRODUCTION

Epstein–Barr virus (EBV) is a ubiquitous lymphotropic human gamma herpes virus type 4, which infects 95% of the world population's during their lifetime. Remarkably, the ease of transmission of this virus may be partially determined by its ability to set different programs of gene expression, which allow the virus to adapt to different types of host cells. The wide range of diseases associated with the virus can be also explained by expression variability of viral genes. EBV genomes and its gene products are regularly found in various human neoplasms, including the endemic form of Burkitt lymphoma (BL), nasopharyngeal carcinoma, posttransplant lymphoma, AIDS-related lymphomas, certain variants of Hodgkin lymphoma, nasal T/NK-cell lymphoma, peripheral T-cell lymphoma, lymphoepithelioma-like gastric adenocarcinoma, and others [1].

The ubiquity of EBV has prompted many researchers to look for its causal relationship with other human malignancies, such as breast cancer [2], lung cancer [3], tumors of the hematopoietic system [4–7], tumors of the central nervous system [8], and so on. Despite the detection of some features of the EBV relationship with several pathologies, the etiological role of the virus in most cases did not meet the criteria formulated in *Fields Virology* [9], which consist in obligate detection of EBV in almost every cell and always in the form of monoclonal plasmids that would indicate the origin of the tumor from the single cell infected by the virus. In the case of tumors of epithelial origin, viral DNA must be present already in dysplastic lesions, confirming the involvement of the virus at the early stages of carcinogenesis. Moreover, at least one gene of the latent infection must be expressed, which would indicate an active role of the virus in mainte-

nance of the tumor process. In any case, confirmation of the etiological role of EBV is a difficult objective, since the virus modifies its functional activity for the induction of each tumor, including depending on cofactors involved in carcinogenesis, without which EBV-associated tumors never arise in the majority of virus carriers.

Some of EBV-associated tumors, such as BL and nasopharyngeal cancer (NPC), are characterized by a limited geographical and racial prevalence [10, 11]. In particular, NPC is quite rare in most countries of the world. Endemic regions include the southern provinces of China, the countries of Southeast Asia and the Mediterranean, where the NPC incidence ranges from 5 to 30 cases per 100 thousand residents. A high incidence of NPC is also found among the Eskimo Indians and Aleuts of Alaska [12]. The etiological role of EBV in NPC pathogenesis is confirmed by the presence of clonal genomes of the virus in precancerous and cancerous lesions in the nasopharynx and expression of a spectrum of viral markers in the tumor cells on the level of DNA or RNA [13, 14]. The fact that NPC is accompanied by high titers of humoral IgG and, especially, IgA antibodies to EBV proteins detected at the early or even preclinical stages of the disease are evidence that the virus plays an etiological role [15]. In addition, at the early stages of tumor development, significantly increased levels of DNA fragments of EBV have also been found in the plasma of most patients [16]. All these facts relating to a close EBV association with NPC were obtained when studying in geographical areas that are endemic for the disease.

Among the latent proteins encoded by the EBV genome, which are responsible to different degrees for the transforming potential of the virus, a crucial role in NPC carcinogenesis is played by latent membrane protein 1 (LMP1). Being the primary oncogene encoded by EBV, LMP1 functions as a viral mimic of the TNFR family member, CD40, which distorts the transmission of cellular signals that lead to morphological and phenotypical changes in epithelial cells [17]. LMP1 also activates the epithelial–mesenchymal transition (EMT) and contributes to the high metastatic activity of NPC [18]. Furthermore, EMT caused by LMP1 functioning is accompanied by expression of markers of cancer stem cells (CSC)/cancer progenitor cells (CPC) (CD44high/CD24low) and acquisition of the properties of stem cells/progenitor cells [19]. BART miRNAs encoded by the BamHI-A region of the viral genome are the most common transcripts in NPC cells [20]. They modulate apoptosis and innate immune defense mechanisms. LMP1 secreted by exosomes is introduced into the cells uninfected by EBV through endocytosis and affects the tissues surrounding the tumor. Furthermore, it has been shown that LMP1 suppresses the intercellular adhesion and activates cell motility by the activation of ETS-1 and c-Met and ezrin expression [21–23]. It has also been shown that LMP1 induces mucin 1 (MUC1) expression, which plays an important role in tumor invasion and metastasis [24]. LMP1 affects not only the tumor cell, but also the degradation of stroma surrounding the tumor through the enhancement of the activity of various MMP and suppression of RECK1, an inhibitor of MT1-MMP [25–28]. Induction of MMP by LMP1, as has been shown, is performed via cell signaling systems such as NF-kB, AP-1, ETS-1, and ERK MAPK [25, 27, 28]. Recently, the role of LMP1, IL-6, and laminin in the enhancement of NPC metastatic activity was also reported [29].

Despite the comprehensive investigation that has taken place of the transforming potency of EBV and its main oncogene *LMP1*, the role of the latter in the NPC development has not been fully clarified. The mechanism of EBV-associated NPC carcinogenesis in nonendemic regions is especially unclear. Hence, the aim of this work was to carry out a comparative study of the features of the genetic structure and transforming activity of *LMP1* in patients with EBV-associated tumors (NPC) and other tumors not associated with the virus (OTOC), but also localized in the oral cavity, in Russia, a nonendemic region.

MATERIALS AND METHODS

Groups of patients and DNA samples. Samples of tumor tissue and blood, as well as oropharyngeal swabs from 21 patients with NPC, 14 patients with OTOC, and 19 blood donors were the object of the study (all participants in the study were residents of Russia). OTOC patients included patients with cancer of the oral mucosa, tongue, or lingual tonsil or who suffered from other malignant lesions of the oral cavity. The study that was conducted, in which patients with NPC and OTOC were included with their consent as a result of a random sample, was approved by the Ethics Committee of the Blokhin Russian Cancer Research Center.

DNA extraction and sequencing of PCR products. DNA from the biological material collected for the study was extracted by the method of phenol-chloroform deproteinization. The presence and concentration of the DNA preparations were analyzed by real-time PCR as described previously [30]. A panel of ABI PRISM Big Dye Terminator, v.3.1 reagents was used for DNA sequencing followed by analysis of the reaction products on an ABI PRISM 3100-Avant automated DNA sequencer. Data processing was performed using the Chromas 230 and Vector NTI software.

Plasmids. pSG5-LMP1-B95.8 and pSG5-LMP1-Cao vector constructs were kindly provided by F. Grasser (Hamburg, Germany). The studied variants of the *LMP1* gene were recloned from the pGEM-T Easy system (Promega, United States) into the eukaryotic expression vector pSG5 and retroviral vector pBabe-puro (pBabe) using appropriate restriction endonucleases.

Cell cultures, obtaining of retroviral stock, and transduction. All cell lines were cultured on DMEM medium supplemented with 10% inactivated fetal calf serum (GIBCO), 2 mM L-glutamine, 100 U/mL gentamicin and 5% CO_2 at 37°C. The cells of the Phi-NX-AmphoPhoenix-A cell line (HEK293 cell derivative) were transfected with genetic constructs based on pBabe-puro using LipofectAMINE Plus (Invitrogen, United States) according to the manufacturer's instructions. Transduction of the Rat-1 cell line with the obtained viral particles was performed as described previously [31].

Cell lysates and Western blot analysis. Cell lysates of the Rat-1 line, which continuously express different variants of LMP1, were prepared by a method described previously [31]. Monoclonal antibodies against β-catenin (Sigma, United States) and LMP1–S12 (provided by F. Grasser, Hamburg, Germany) were used to perform Western blot analysis. The method was also described previously [31].

Analysis of the ability to form transformation foci. Four hundred cells of each culture were plated onto a 35-mm Petri dish (three dishes per line). DMEM medium was supplemented with 10% FBS. The cells were cultured for 1–1.5 weeks. After the formation of colonies, the medium was decanted and the cells were washed with PBS. The cells were then fixed with 70% ethanol for 2 min, after which they were stained with aqueous solution of Crystalviolet dye for 2 min. The solution was then decanted, and the cells were air dried. The number of colonies was counted using a magnifying glass.

Statistical analysis. Fisher's exact test was used to assess the significance of differences between the percentage content of LMP1 variants in similar biological samples of two groups of patients, NPC and OTOC. The values were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Structural features of the *LMP1* oncogene in patients with NPC and OTOC. To determine whether LMP1 variants differ in patients with NPC and OTOC, the C-terminal region of the gene was examined in samples of tumor tissue and blood and oropharyngeal swabs (OS) from these patients. Analysis of nucleotide and predicted amino acid (a.a.) sequences of the obtained amplicons showed (Table 1) that they vary, but, in general, are the same as the variants described in the literature: B95.8/A, Cao/China1, Mediterranean+ (Med+),Med–, and North Carolina (NC) [32–34]. The amino acid sequences found in four LMP1 samples, which differed from each other and from all known variants, were specified as "out of variant" (OV) samples. All of them were obtained from NPC patients: two samples of tumor tissue, one of blood and one of OS. It also follows from Table 1 that low divergent variant $LMP1^{B95.8/A}$, which is characterized by a small number (three or four) of a.a. substitutions (compared with prototype variant $LMP1^{B95.8}$), dominated among donor blood samples, reaching 78.9% (15/19). The frequency of this variant did not exceed 30% in NPC and OTOC patients. The highly tumorigenic variant $LMP1^{China1}$, belonging to the Chinese variant Cao in its structural and functional characteristics, was most often detected in blood samples (58.3%, 7/13), tumor tissue (35.7%, 5/14), and OS (33.3%, 3/9) from OTOC patients in the present study. In NPC patients, by contrast, a highly tumorigenic variant $LMP1^{Med+}$ was more often detected in tumor samples (33.3%, 7/21) and OS (30%, 6/20). The total content of the variants China1 and Med+ in tumor tissue of patients in both groups did not differ significantly (42.8% (9/21), 57.1% (8/14), respectively; $p > 0.05$). Less tumorigenic variants, in particular, Med–, were detected in a higher percentage of cases in blood samples of NPC patients (43.8%, 7/16), while the NC variant was relatively uniformly distributed in all the studied samples and ranged from 4.8% in tumor tissue of NPC patients to 15% in OS samples of the same patients. The conducted study showed that, in general, the LMP1 variants described in the literature are typical for patients with tumors associated and unassociated with EBV (NPC and OTOC, respectively) and their spectrum in two groups did not differ fundamentally. It was also revealed that, in these patients, there were no LMP1 variants such as China 2, which is often found in patients with NPC and healthy individuals from southern provinces of China and Southeast Asia, or the variant Alaskan, which is found in Alaska [32, 35, 36]. NPC-specific LMP1 variants have not been identified in Russia or in endemic regions.

LMP1 deletion variants and characteristic a.a. substitutions in the tumors of NPC and OTOC patients. It was shown that tumorigenic and transforming properties of LMP1 are associated with the deletion of ten amino acids [37], which is often part of an overlapping 23 a.a. deletion (amino acid residues 346–355 and 334–355, respectively). The study that was carried out showed (Table 2) that, in tumor biopsy material from NPC patients, samples with 10 a.a. deletion occurred in 14.3% (3/21) of cases and 23 a.a. deletion in 28.6% (6/21) of cases, while in OTOC patients these values were 50% (7/14) and 7.1% (1/14), respectively. However, the total values of the content of both LMP1 deletion variants in tumors of the compared groups were close—42.9% (9/21) for patients with NPC and 57.1% (8/14) for OTOC patients—and the differences were not statistically significant ($p > 0.05$). Although an increased content of LMP1 deletion variants in blood samples from OTOC patients compared with NPC patients (61.5% (8/13) and 12.5% (2/16), respectively) was close to a statistically significant value ($p = 0.074$), the difference in the content of these LMP1 variants in oropharyngeal swabs from these patients (44.4% (4/9) and 30% (6/20), respectively) turned out to be statistically unreliable ($p > 0.05$). Based on the

Table 1. Spectrum of LMP1 variants in samples of tumor, blood, and oropharynx swabs from patients with NPC and OTOC, as well as peripheral blood lymphocytes of donors

Tested samples	Groups of patients	LMP1[1] variants					
		B95.8/A, %	China 1, %	Med+, %	Med–, %	NC, %	OV[2], %
Tumor	NPC, $n = 21$	6 (28.6%)	2 (9.5%)	7 (33.3%)	3 (14.3%)	1 (4.8%)	2 (9.5%)
	OTOC, $n = 14$	2 (14.3%)	5 (35.7%)	3 (21.4%)	2 (14.3%)	2 (14.3%)	0 (0%)
Blood	NPC, $n = 16$	4 (25.0%)	0 (0.0%)	2 (12.5%)	7 (43.8%)	2 (12.5%)	1 (6.3%)
	OTOC, $n = 13$	2 (16.7%)	7 (58.3%)	1 (8.3%)	1 (8.3%)	1 (8.3%)	0 (0%)
	Blood donors, $n = 19$	15 (78.9%)	1 (5.3%)	0 (0.0%)	1 (5.3%)	2 (10.5%)	0 (0%)
Oropharynx swabs	NPC, $n = 20$	6 (30.0%)	0 (0.0%)	6 (30.0%)	4 (20.0%)	3 (15.0%)	1 (5.0%)
	OTOC, $n = 9$	2 (22.2%)	3 (33.3%)	1 (11.1%)	2 (22.2%)	1 (11.1%)	0 (0%)
Total	111	37	18	20	20	12	4

LMP1[1] samples amplified from biological materials of NPC and OTOC patients, as well as blood donors, were divided by the variants according to the classification [32, 34], which is based on the geographical origin of the EBV isolates; OV[2]—LMP1 samples, which do not correspond to any variant described in the literature in a set of specific mutations, are in the group "out of variants."

Table 2. Amino acid deletions (10 and 23 a.a.) in LMP1 isolates from samples of tumor, blood, and oropharyngeal swabs of patients with NPC and OTOC

LMP1 isolates	Tumor of NPC patients ($n = 21$)	Tumor of OTOC patients ($n = 14$)	Blood of NPC patients ($n = 16$)	Blood of OTOC patients ($n = 13$)	Oropharyngeal swabs of NPC patients ($n = 20$)	Oropharyngeal swabs of OTOC patients ($n = 9$)
Deletion of 10 a.a.	3 (14.3%)	7 (50.0%)	1 (6.3%)	8 (61.5%)	1 (5.0%)	4 (44.4%)
Deletion of 23 a.a.	6 (28.6%)	1 (7.1%)	1 (6.3%)	0 (0.0%)	5 (25.0%)	0 (0.0%)
Sum of deletions	9 (42.9%)	8 (57.1%)	2 (12.5%)	8 (61.5%)	6 (30.0%)	4 (44.4%)

data obtained, it can be stated that, in the studied material from patients with NPC and OTOC, the content of LMP1 deletion variants was approximately the same, suggesting a genetic relationship between the EBV strains in these patients.

It is important to note that, in tumor LMP1 samples of both groups of patients, common a.a. substitutions localized between the LMP1 codons 309 and 366: S309N, Q322E/N/D, E328Q/A, Q334/R, L338S/P, H352R/N, and S366T/A, as well as a.a. substitutions in functionally important codons (not shown) were identified. Thus, the a.a. substitution S → T in codon 366, which is typical for patients with NPC analysed in endemic regions, was also found in 100% of cases, but only in patients with OTOC. The a.a. substitution S → A was often found in NPC patients from Russia: in tumor samples, 40.9%; in blood samples, 26.7%; and in oropharyngeal swabs, 47.4%. Another feature of Russian LMP1 samples was complete absence of a.a. substitution G → S in the codon 212, which is quite typical for different EBV-associated pathologies described in various countries.

Thus, these data suggest that the occurrence of NPC in Russia is not associated with the spread of a certain EBV strain, carrying in its genome highly oncogenic variants of *LMP1* with a 30 bp/10 a.a. deletion (China 1 also or Med+). Viral strains with these gene variants were detected in biological material from OTOC patients and even in blood samples of donors. This idea is consistent with the findings of other researchers who showed that the high incidence of NPC in the southern provinces of China is not associated with the persistence in the population of these regions of EBV strains, carrying a highly divergent LMP^{Cao} strain with the deletion of 30 bp/10 a.a. and possessing the most pronounced transforming activity.

Transforming activity of *LMP1* variants from patients with NPC and OTOC. To determine different characteristics of *LMP1* variants obtained from patients with NPC and OTOC, PCR products of the selected *LMP1* variants were obtained at the first stage of the study. The studied gene variants were cloned into the eukaryotic expression vector pSG5, and, then, preparative quantities of plasmid DNA, which were used in further experiments, were isolated. Thus, we obtained *LMP1* variants from the tumor tissue of patients with NPC (M6, M28, M33, M37, M42) and OTOC (M19, M25, M32, M36).

To obtain cell lines with stable expression of the studied *LMP1* variants, the gene variants were cloned into the retroviral vector pBabe-puro at the appropriate sites. After obtaining the studied *LMP1* gene variants recloned into pBabe-puro, as well as its control variants $LMP1^{B95.8}$ (low tumorigenic) and $LMP1^{Cao}$ (highly tumorigenic), transfection of the Phi-NX-Ampho cell line (Phoenix-A, HEK293 cell derivative) with these variants was carried out. In addition, indicator cells of Rat-1, rat embryonic fibroblasts, were transduced with the obtained retroviral stocks. The choice of this cell line was primarily determined by the sensitivity of these cells to the transforming properties of *LMP1* and their reactivity to the transforming action of the carboxyl domain of the protein, as well as by the results of previous studies, which showed their lower sensitivity to the cytotoxic effect of the LMP1 molecule.

The transforming activity of LMP1 variants of EBV obtained from patients with NPC and OTOC was studied using the method of phase contrast by the ability of cell lines constantly expressing the studied gene variants to form colonies. As can be seen from Table 3, cells containing the empty control vector pBabe did not form colonies during prolonged cultivation, whereas, in the remaining lines, colonies of different sizes and morphologies were formed under the expression of LMP1 variants B95.8, M6, M28, M33, M37, M42, M19, M25, M32, and M36. The cells expressing the $LMP1^{Cao}$ variant formed a significantly greater number of colonies than did other lines. At the same time, attention is drawn to the fact that the number of gene variants determined by the total of key a.a. substitutions, as well as the absence or presence of the 10 a.a. deletion in LMP1 samples of both groups, was almost the same and did not affect the number of formed transformation foci.

The transforming ability of the tested cell lines that continuously express the studied LMP1 variants was further evaluated by characterization of their growth without substrate in a liquid agar. The study showed that all the analyzed LMP1 variants formed colonies of different sizes and morphologies when cultured in a liquid agar. In this case, as in the case of the experiments described above, the cell line expressing a highly tumorigenic variant $LMP1^{Cao}$ formed much larger number of colonies than the cell line expressing the tested gene variants and $LMP1^{B95.8}$ (Table 4). Moreover, the cell lines expressing LMP1variants from patients of both groups did not differ in their ability to form colonies in liquid agar.

It is known that the induction of stress-activated fibril formation and filopodia formation is observed in target cells under LMP1 expression. To determine the effect of a minimum set of LMP1 mutations on this process, we analyzed the movement of the cells in a "wound." The "wound" was applied onto a cell monolayer and the movement of the cells was fixed after 24 and 48 h. In the case of the expression of a highly tumorigenic variant Cao almost completely, wound healing was observed after 48 h due to the absence of contact inhibition, typical for normal cells. As for the studied LMP1 variants, although a slight difference in the rate of wound healing was observed among them, all of them, including the prototype variant B95.8, caused movement of cells at a faster rate

Table 3. Number of transformation foci formed by cell lines expressing LMP1 variants from patients with NPC and OTOC, as well as control LMP1 variants

Cell lines	Number of transformation foci
Control variants	
Rat-1-pBabe-puro	0
Rat-1-pBabe-B95-8	>100
Rat-1-pBabe-Cao	>260
LMP1 from tumor tissue of patients with NPC	
Rat-1-pBabe-M6 (B95.8)	>90
Rat-1-pBabe-M28 (Med+)	>80
Rat-1-pBabe-M33 (Med+)	>120
Rat-1-pBabe-M37 (Med–)	>120
Rat-1-pBabe-M42 (Med+)	>110
LMP1 from tumor tissue of patients with OTOC	
Rat-1-pBabe-M19(B95.8)	>100
Rat-1-pBabe-M25(B95.8)	>100
Rat-1-pBabe-M32 (Med+)	>90
Rat-1-pBabe-M36 (Med–)	>100

Table 4. Number of colonies formed by cell lines expressing LMP1 variants from patients with NPC and OTOC, as well as control LMP1 variants

Cell lines	Number of colonies
Control variants	
Rat-1-pBabe-puro	0
Rat-1-pBabe-B95-8	>25
Rat-1-pBabe-Cao	>55
LMP1 from tumor tissue of patients with NPC	
Rat-1-pBabe-M6 (B95.8)	>45
Rat-1-pBabe-M28 (Med+)	>30
Rat-1-pBabe-M33 (Med+)	>40
Rat-1-pBabe-M37 (Med–)	>35
Rat-1-pBabe-M42 (Med+)	>30
LMP1 from tumor tissue of patients with OTOC	
Rat-1-pBabe-M19(B95.8)	>25
Rat-1-pBabe-M25(B95.8)	>30
Rat-1-pBabe-M32 (Med+)	>35
Rat-1-pBabe-M36 (Med–)	>30

than that of control cells carrying the empty vector pBabe-puro.

Currently, the mechanism of EBV-associated carcinogenesis leading to the development of NPC is not completely understood. The ubiquity of the virus and differing incidence of EBV-associated tumors in different geographical regions make to look for the cause of the possible existence of specific EBV strains, contributing to the development of certain types of EBV-associated malignancies. In the present study, which was conducted in a geographic region (Russia) nonendemic for NPC, the EBV strain with the LMP1 variant typical for NPC was not found. However, according to some publications, several specific variants of EBV (a variant having an additional site BamHI in the Bam HIF fragment, F/V29A/SPM or V-val subtype of EBNA1) were detected for NPC in endemic regions that were absent in other EBV-associated pathologies [38]. Obtaining such a result for nonendemic regions would indicate the universality of the NPC relationship with these variants of the virus, regardless of the geographical region and would allow one to determine the structural and functional features of the *LMP1* oncogene in these virus strains compared to the *LMP1* oncogenes of EBV strains circulating in the healthy population.

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Conflicts of interest. We declare that there is no conflict of interest.

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