PhD thesis

IN VITRO STUDY OF THE INTERACTION BETWEEN HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 AND HUMAN HERPESVIRUS 6

Eszter Csoma

Supervisors: Dr. Ferenc D. Tóth
Dr. Lajos Gergely

University of Debrecen
Faculty of Medicine
Medical and Health Science Centre
Department of Medical Microbiology
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INTRODUCTION

Human herpesvirus 6 (HHV-6) frequently reactivates in patients infected with human immunodeficiency virus 1 (HIV-1). HHV-6 may influence the replication of HIV-1, hence it is thought to be an important cofactor in AIDS progression. The interaction between these viruses are studied in a number of *in vitro* cell cultures, although it is not clarified in macrophages and also not in syncytiotrophoblast (ST) cells which have key importance in vertical transmission of HIV-1.

Vertical transmission of HIV-1 from mother to child is one major problem in the transmission of the virus. Transplacental infection of the foetus in utero is an important mechanism of transmission. The placental ST cells form a continuous, multinucleated epithelial barrier in direct contact with maternal blood in the intervillous space. As a result of this, only traversing the ST would HIV-1 be exposed to underlying foetal cells. Different studies have proved that HIV-1 is able to infect the ST cells: HIV-1 specific proviral DNA has been detected in the ST layer *in situ*, on the other hand these cells could be infected by cell-free HIV-1. In ST cells after in vitro contact with cell-free HIV-1, the replication of the virus is abortive, there is no virus production or only low level of productive viral replication is achieved. However, productive infection of trophoblast in vivo might occur in defined, transient conditions. Observations in support of this assumption include detection of HIV-1 p24 antigen in the placental trophoblastic cells of HIV-1-positive gravidae. These findings suggest that multiple cofactors are likely to be involved in the transplacental transmission of the virus.

One possible mechanism for induction and stimulation of HIV-1 replication is coinfection with HHV-6. HHV-6 is highly prevalent in the general population and the seropositivity in the population exceeds 90 %. It has been shown that HHV-6 – generally variant A of HHV-6 (HHV-6A) – reactivates to active infections early in the course of HIV-1 infection. Reactivation of HHV-6A during pregnancy is also frequent, which enable the ST cells to be infected. HHV-6 is able to activate CD4 gene, as a result of this, it may increase the number of cells susceptible to HIV-1 infection. In addition, tranascriptional transactivator genes of HHV-6 can activate HIV-1 from latency and enhance its transcription acting on the long terminal repeat (LTR).

However, congenital infection with HHV-6 is not frequent, as low as 1-1,6 %, in accordance with the above-mentioned it may have importance in the transplacental transmission of HIV-1. Based on epidemiological studies, in utero transmission of HIV-1

seems to occur either late in pregnancy or in early pregnancy. Several studies provide indirect evidence that late *in utero* infection accounts for the vast majority of transplacental transmission of HIV-1. Hence, the interaction between HHV-6A and HIV-1 was investigated in human term ST cells cultured in vitro.

During acute phase of HHV-6 infection, monocyte/macrophage cells are major targets for the virus, in addition HHV-6 establish latency in monocyte-macrophage lineage. Macrophages in vivo serve as primary targets and reservoirs for HIV-1, and as a result of their ability to migrate, they play an important role in virus dissemination and the pathogenesis of the infection.

Primarily, these cells are susceptible to R5 variants of HIV-1 for which CCR5 is the main coreceptor. The sensitivity and cell surface expression of this receptor may be influenced by several factors. Chemokines as a natural ligand of these receptors binding to their receptors act as a competitor of HIV-1, consequently they inhibit HIV-1 replication at the first step. In addition, binding of a chemokine to its receptor results in not only the loss of the sensitivity and internalization of the receptor, but also heterologous desensitization and internalization of other chemokine receptors.

Interaction between HHV-6 and HIV-1 was examined in different cell types. It is demonstrated that HHV-6 was able to suppress HIV-1 replication *in vitro* in CD4+ lymphocytes, peripheral blood mononuclear cells, dendritic cells and *ex vivo* lymphoid tissue. Presumably, soluble factors have an important role in this process.

Despite the fact that macophages have key importance in HHV-6 and also in HIV-1 infection, the interaction of these viruses in these cells are not clarified.

AIMS

1. Can ST cells be infected with HHV-6A?
2. Has the coinfection with HHV-6A and HIV-1 any effect on the replication of these viruses in ST cells?
3. If yes, how they affect the replication of each other?
4. Does coinfection of macrophages with HHV-6A and HIV-1 influence the replication of these viruses?
5. If yes, what mechanisms are involved in it? Does HHV-6A and / or HIV-1 infection alter the production of soluble factors in macrophages?
6. Does soluble factors any effect on the replication of these viruses in macrophages?

MATERIALS AND METHODS

Isolation, culture and differentiation of cytotrophoblast cells

Cytotrophoblast cells were separated by immunomagnetic method from human term placental samples. For *in vitro* differentiation of trophoblast keratinocyte growth medium supplemeted with 2 mM glutamine, 15 % foetal bovine serum (FBS) and antibiotics was used. We used 5-day-old ST cultures consisting of multinucleated differentiated cells in experiments.

Human macrophage cultures

Peripheral blood mononuclear cells (PBMCs) of healthy blood donors were isolated by Ficoll-Hypaque density gradient centrifugation from buffy coat preparations. For HIV-1 propagation adherent cells of PBMCs were used. For experiments monocytes were separated by Monocyte Negative Isolation Kit according to the manufacturer's instruction.

Monocytes were suspended at 10^6 cells/ml in RPMI medium with 20 % FBS, 2mM and antibiotics. 7-day-old monocyte-derived macrophage (MDM) cultures were used in all experiments.

Viruses and virus titrations

The GS strain of HHV-6A was propagated in HSB-2 cells. X4 variant IIIB were grown in H9 cells, R5 variant Ada-M and Ba-L were propagated in MDMs. Cultures were maintained in RPMI medium supplemented with 10 % FBS, 2mM glutamine and antibiotics. Culture supernatants were collected, clarified and in case of HHV-6A concentrated by ultracentrifugation. Virus stocks were stored at -70 °C.

Infectivity titers of HHV-6A were determined as focus-forming units by using HSB-2 monolayers, adhered chemically. HIV-1 production was assayed by ELISA for extracellular p24 antigen and syncytium-forming assay in case of HIV-1_{IIIB} and HIV-$1_{Ba\text{-}L}$. Ada-M titer was determined by measuring the reverse transcriptase activity (RT) of the virus.

Infection of ST cells

5-day-old ST cells were infected simultaneously with HHV-6A and HIV-1 or one virus was added to the cells earlier than the other. Single virus controls were used. Cells were incubated with virus for 1 h at 37 °C.

Infection of macrophage cultures

7-day-old MDM cultures were infected in 24 well plates with HHV-6A or HIV-1. Cells were incubated with virus for 2 hours at 37 °C. In case of simultaneous infection, macrophages were incubated with HHV-6A for two hours, following three wash cells were inoculated with HIV-1 for two hours. After three wash, cells were fed with fresh, completed medium.

Immunofluorescence Assay

For analyses of expression of viral proteins, indirect cytoplasmic immunofluorescence assay (IFA) was used. Coexpression of HHV-6 early (E) antigen and HIV-1 p24 protein in dually infected ST cells was demonstrated by double indirect IFA.

Cell surface expression of CD14 was investigated by indirect membrane IFA.

Transfection of ST cells

ST cells were transfected by plasmids containing HHV-6A open reading frames (U16-17, U18-19, U86 and U89) using lipofectin procedure.

Polymerase chain reaction (PCR)

DNA from cells and clarified culture supernatants were isolated by High Pure Viral Nucleic Acid Kit according to the manufacturer's instruction. Amplification of DNA was carried out with nested PCR.

Measurement of chemokines

Production of IL-8 and RANTES from infected and uninfected macrophage cultures were measured by ELISA kits according to the manufacturer's guidelines.

Intracellular Calcium Measurement

RANTES activated Ca^{2+} mobilization was carried out in two ways.

Intracellular calcium concentration ($[Ca^{2+}]_i$) change after RANTES addition was examined in single cells and macrophage populations. HHV-6A-infected and uninfected MDMs were cultured on coverslips, loaded with 10 μM fura-2AM and 0,02 % pluronic in RPMI-1640 medium for 60 min at 37 °C. Following wash with PBS, coverslips were positioned on an inverted fluorescence microscope. Excitation wavelength was altered between 340 and 380 nm by a dual-wavelength monocromator and an on-line connected

microcomputer. The emission was monitored at 510 nm by a photomultiplier at an acquisition rate of 10 Hz per ratio. $[Ca^{2+}]_i$ was calculated.

RANTES-elicited $[Ca^{2+}]_i$ increase in HHV-6A-infected and uninfected macrophage populations was also measured by FluoroMax Spectrofluorimeter. Measurements were carried out with fura-2AM loaded cells in cuvettes. The ratios of the fluorescence intensities measured at 340 and 380 nm excitation wavelengths (R340/380) were compared.

Fluorocytometric analysis

CCR5 receptor on HHV-6A infected and uninfected macrophages were immunofluorescence stained. Both infected and uninfected cells were fixed in cold PBS containing 4 % paraformaldehyde. Fluorocytometric analysis was performed by using FACScan analyzer and CellQuest Software.

Statistic

Data were analyzed with Students' t-test using SPSS version 11.

RESULTS

Lack of infective HHV-6A and HIV-1 production in singly infected ST cultures

To investigate whether ST cells could be productively infected with HHV-6A, indirect cytoplasmic IFA was used. We detected E antigens as early as 24 hr postifection in HHV-6A-infected ST cells which persisted 10 days, but no L antigens were seen during 22 days of culture. Consistent with the absence of L antigens, production of infectious virus was not observed. These results suggest that HHV-6A can enter ST cells, but its replication is restricted at early times in these target cells.

During the examination period, no infectious virus was found in the supernatants of ST cultures infected with HIV-$1_{Ba\text{-}L}$ or HIV-1_{IIIB} alone. We did not detect HIV-1 Tat and p24 proteins in singly infected cells. However, in our previous study we proved that Ba-L and IIIB strains of HIV-1 were able to enter ST cells but their replication was blocked in a very early step of the virus life cycle.

Effect of dual infection on HIV-1 and HHV-6A replication in ST cells

Coinfection of ST cells with HHV-6A turned HIV-1 infection from an abortive to a productive one. HIV-1 p24 antigen was detected in the supernatants of the coinfected cell cultures, and titration of infectious virus also indicated a significant level of virus release. The maximum value of p24 antigen and infectious virus production was detected in cultures preinfected with HIV-1 and 3 days later superinfected with HHV-6A. Heat-inactivated HHV-6A particles failed to induce any HIV-1 production, suggesting that active HHV-6A expression was necessary for HIV-1 replication.

Expression of Tat protein in cells coinfected simultaneously was observed first at 32 hr postinfection, suggesting a stimulatory effect of HHV-6A on HIV-1 in an early step of HIV-1 replication.

Two-colour IFA for detection of HHV-6A E antigen and HIV-1 p24 showed the simultaneous presence of both viruses in the same cells of dually infected ST cultures.

At the same time, no stimulatory effect of HIV-1 on HHV-6A replication was found in dually infected cell cultures independently of the time of coinfection. Neither L antigen, nor infectious HHV-6A release were detected in dually infected ST cells. These results suggest that HIV-1 infection does not influence the rarely occurred vertical transmission of HHV-6.

Receptor expression and function on ST cells

Next we examined whether HHV-6A influences the replication of HIV-1 altering the expression of the cell surface receptors, increasing the number of cells susceptible to HIV-1. Uninfected ST cells were found to express CXCR4 and CCR3 but not CD4 and CCR5 on their surface as revealed by indirect membrane IFA. HHV-6A infection did not induce CD4 or CCR5 surface molecules on infected ST cells and did not alter CXCR4 or CCR3 expression.

To obtain information on the functional capacity of CXCR4 and CCR3 receptors to bind HIV-1 on ST cells, the ability of anti-CXCR4 and anti-CCR3 antibody to inhibit HIV-1 infection was studied in HHV-6A infected ST cultures. Neither the anti-CXCR4, nor the anti-CCR3 had any inhibitory effect upon infection of ST cells with HIV-$1_{\text{Ba-L}}$ or HIV-1_{IIIB}. Data of these blocking experiments indicated that CXCR4 and CCR3 were unnecessary for infection of ST cells with HIV-$1_{\text{Ba-L}}$ or HIV-1_{IIIB}.

Effect of transactivator genes of HHV-6A on HIV-1 replication

On the basis of the results from the above experiments, we hypothesized that the stimulator effect of HHV-6A on HIV-1 replication may be mediated by its transacting gene products. To address this question, ST cells were transfected with plasmids containing different transactivator genes of HHV-6A, then infected with HIV-1. In all experiments HIV-1 replication was productive as revealed by syncytium-forming assay. U16-U17, U18-U19, U86 and U89 were able to stimulate the replication of HIV-1, in addition U86 has synergistic effect together with U89 gene product. U16-U17 and U18-U19 are transcribed under immediate early and early conditions in HHV-6A infected cells, as a result of this, HIV-1 replication could be stimulated at both IE and E times of HHV-6A replication in coinfected ST cells.

HHV-6A suppresses HIV-1 replication in human macrophages

To examine the effect of HHV-6A on HIV-1 replication, 7-day-old MDMs were coinfected with both viruses. Our results revealed that CCR5-tropic HIV-1 variant, Ada-M replication was significantly (n=5, p<0.05) suppressed in simultaneously coinfected cultures compared with macrophages infected with HIV-1 alone. HIV-1 RT activity was first detected 6 days after infection in cultures infected with HIV-1 alone and also in dually infected macrophages. The viability of coinfected cells was not different from macrophages infected with Ada-M alone.

To investigate that the suppressive effect is related to infectious HHV-6A, live HHV-6A was replaced by the same amount of heat inactivated (1h, 56 ºC) HHV-6A in coinfection experiments. Inactivated HHV-6A failed to suppress HIV-1 replication, suggesting that infectious HHV-6A is required to mediate anti-HIV-1 effect in coinfected macrophages.

Lack of infective HHV-6 production in macrophages

Productive HHV-6A infection was not observed in macrophages infected with HHV-6A alone or in coinfected cultures. During 14 days of cultures, there was no infectious HHV-6A release as determined by titration on HSB-2 monolayers. We also did not observe p150 capsid polypeptide of HHV-6 by IFA. Moreover, there was no accumulation of HHV-6A genome in culture supernatants as determined by nested PCR. However, we detected HHV-6 specific DNA in macrophages by nested PCR, proving that HHV-6A could infect macrophages. These results suggested that the suppressive effect of HHV-6A did not depend on productive HHV-6A infection.

HHV-6A enhances IL-8 and RANTES production in macrophages

To investigate whether HHV-6A can alter chemokine production of macrophages we assessed the secretion of RANTES and IL-8, which may have effects on CCR5 receptor susceptibility to HIV-1. Clarified supernatants of uninfected, dually and singly infected cultures were harvested and assayed.

As a result of HHV-6A infection, large amount of RANTES was secreted by macrophages. In cultures infected with GS — alone or in combination with Ada-M — the peak value was observed on day 2 after infection. Then the production decreased and from day 10 of infection, the level of the chemokine was not different in HHV-6A infected macrophages from uninfected, control cultures. Infection of macrophages with HIV-1 alone also resulted in increased RANTES secretion which was first detected on day 6 after infection and was maintained until day 14. Whereas the maximum amount of RANTES in HIV-1 infected cultures was 10-fold, HHV-6A induced chemokine secretion was approximately 100-fold higher compared with control cultures.

Next, we measured the production of IL-8. During 14 days of examination we observed increased IL-8 release in cultures infected with HHV-6A alone or along with HIV-1. Until day 6 after infection, the level of IL-8 in HHV-6A infected cultures were approximately 10-fold compared with uninfected cells after which the rate of secretion decreased. The

secreted IL-8 in supernatants of macrophages infected with Ada-M alone was not different from that of the control cells.

According to previous publications, RANTES is able to suppress HIV-1 replication in macrophages, but also to enhance the virus production depending on the stage of maturation and culture conditions. RANTES — as a natural ligand of CCR5 — binding to its receptor acts as a competitor of R5 variants HIV-1, consequently at the time of HIV-1 infection RANTES can inhibit virus replication in macrophages.

Effects of exogenous IL-8 and RANTES on HIV-1 replication

To evaluate whether RANTES and IL-8 evoked by HHV-6A infection may alter HIV-1 replication in macrophages, we studied the effects of exogenous chemokines. The same amount of RANTES and IL-8 as we measured in coinfected cultures were added to macrophages infected with HIV-1 alone after the infection. We mimicked the temporal variation in the amount of HHV-6A elicited chemokine secretion of coinfected cultures.

In our experiments, the same amount of exogenous RANTES as we measured in coinfected MDM cultures inhibited virus replication significantly ($p<0.05$; n=3) in HIV-1 infected MDMs. However, besides homologous desensitization and down-regulation, chemokine receptors cross-regulate the function of each other. This prompted us to seek other possible mechanisms by which HHV-6A is able to suppress HIV-1 replication in macrophages.

Experiments with exogenous IL-8 revealed that the same amount of IL-8 as we measured in coinfected cultures was able to significantly inhibit virus replication in macrophages infected with HIV-1 alone.

Effects of HHV-6A on CCR5 sensitivity to RANTES

Binding of RANTES to CCR5 results in receptor phosphorylation, homologous desensitization and internalization.

Effects of receptor-ligand coupling are activation of phospholypase, intracellular Ca^{2+} mobilization, generation of inositol-1,4,5-triphosphate and diacylglycerol. Activation of G protein-coupled receptor kinases leads to carboxyl-terminal phosphorylation and homologous desensitization of CCR5 through which the chemokine receptor becomes refractory to agonist stimulation. Owing to the phosphorylation, CCR5 is able to bind to β-arrestin which initiates rapid endocytosis of receptor through clatrin-coated pits.

Activation of CXCR1 chemokine receptor by its ligand IL-8 lead to cross-desensitization of CCR5. At least two mechanisms contribute to this heterologous desensitization. One of them is cross-phosphorylation of CCR5 by second messengers activated protein kinase C, which is accompanied by receptor-G-protein uncoupling. The other one is inhibition of phospholypase C. Moreover, activated CXCR1 cross-internalized CCR5 and macrophages express CXCR1

We hypothesized that HHV-6A infection, the chemokines which are elicited by the infection may alter the susceptibility of CCR5 receptor to its ligands. Hence, we investigated the sensitivity of the chemokine receptor to its natural ligand, RANTES. For this purpose we compared RANTES-elicited intracellular Ca^{2+} mobilization in uninfected and HHV-6A infected macrophages 6 days after infection. $[Ca^{2+}]_i$ increase after RANTES addition was smaller in HHV-6A infected than in uninfected MDM, it was measured as calcium transients in single cells. Our results revealed that in infected macrophage populations the relative R340/380 change following RANTES addition was significantly (p=0.032; n=6) lower, 22 %, as compared with uninfected populations.

HHV-6A influences CCR5 expression on macrophages

However, beside desensitization, the decreased sensitivity also might result from low-level expression of CCR5, which prompted us to investigate receptor expression. Additionally, HIV-1 infection via CCR5 coreceptor is not blocked by desensitization of the chemokine receptor, receptor-internalization is required.

To determine whether HHV-6A affects not only the sensitivity of CCR5, but also the expression level, we detected receptor expression on uninfected, control cells, and HHV-6A infected macrophages using flow cytometry analysis. HHV-6A infection resulted in marked decrease of CCR5 expression compared with chemokine receptor expression on uninfected macrophages.

DISCUSSION

1. ST cells in vitro could be infected with HHV-6A and certain HIV-1 strains, but the replication of these viruses was abortive, production of infectious virus particles was not observed.
2. Coinfection of HIV-1 infected ST cells with HHV-6A turned the replication of HIV-1 from an abortive to a productive one, while HIV-1 did not influence the replication of HHV-6A.
3. HHV-6A infection did not alter the expression of CD4, CXCR4, CCR3 and CCR5 surface receptors on ST cells. The stimulatory effect of HHV-6A on HIV-1 replication in ST cells was not mediated by an increase uptake of HIV-1.
4. Transactivator gene products of HHV-6A were able to stimulate the replication of HIV-1 in ST cells.
5. HV-6A significantly suppressed the replication of HIV-1 in dually infected macrophages.
6. The replication of HHV-6A was abortive in macrophages, coinfection with HIV-1 did not stimulate the replication of HHV-6A.
7. HHV-6A infection enhanced the IL-8 and RANTES production in macrophages.
8. These chemokine were able to significantly inhibit the replication of HIV-1 in macrophages.
9. HHV-6A infection resulted in decreased sensitivity and expression level of CCR5 receptor on macrophages.

In conclusion, data presented above suggest that HHV-6A may have importance in the vertical transmission of HIV-1, while HIV-1 may not influence the rearly occurred transmission of HHV-6 from mother to child. We assume that IL-8 and RANTES evoked by HHV-6A affect the sensitivity and surface expression of CCR5, hence HHV-6A infection is able to decrease the susceptibility of macrophages to R5 variants of HIV-1. Low-level CCR5 sensitivity and expression might be a selective pressure in favour of CXCR4 utilizing X4 variants, which might result in rapid progression of AIDS. Consequently, HHV-6 can be a cofactor in AIDS progression not only by decreasing the number of lymphocytes, but also by altering cell susceptibility to R5 variants of HIV-1.

PUBLICATIONS

This thesis is based on the following publications:

Attila Bácsi, **Eszter Csoma**, Zoltán Beck, István Andirkó, József Kónya, Lajos Gergely, and Ferenc D. Tóth: Induction of HIV-1 Replication in Latently Infected Syncytiotrophoblast Cells by Contact with Placental Macrophages: Role of Interleukin-6 and Tumor Necrosis Factor-α.
Journal of Interferon and Cytokine Research 21: 1079-1088 (2001)

Impact factor: 2.629, citation: 3

Attila Bácsi, Peter Ebbesen, Judit Szabó, Zoltán Beck, István Andirkó, **Eszter Csoma**, and Ferenc D. Tóth: Pseudotypes of Vesicular Stomatitis Virus-Bearing Envelope Antigens of Certain HIV-1 Strains Permissively Infect Human Syncytiotrophoblasts Cultured In Vitro: Implications for In Vivo Infection of Syncytiotrophoblasts by Cell-Free HIV-1.
Journal of Medical Virology 64: 387-397 (2001)

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Other publications:

Attila Bácsi, **Eszter Csoma**, Zoltán Beck, István Andirkó, József Kónya, Lajos Gergely, and Ferenc D. Tóth: Induction of HIV-1 Replication in Latently Infected Syncytiotrophoblast Cells by Contact with Placental Macrophages: Role of Interleukin-6 and Tumor Necrosis Factor-α.
Journal of Interferon and Cytokine Research 21: 1079-1088 (2001)

Impact factor: 2.281, citation: 8

Attila Bácsi, Peter Ebbesen, Judit Szabó, Zoltán Beck, István Andirkó, **Eszter Csoma**, and Ferenc D. Tóth: Pseudotypes of Vesicular Stomatitis Virus-Bearing Envelope Antigens of Certain HIV-1 Strains Permissively Infect Human Syncytiotrophoblasts Cultured In Vitro: Implications for In Vivo Infection of Syncytiotrophoblasts by Cell-Free HIV-1.
Journal of Medical Virology 64: 387-397 (2001)

Impact faktor: 2.881, citation: 0

Etelka Nagy, Zoltán Beck, Attila Kiss, **Eszter Csoma**, Béla Telek, József Kónya, Éva Oláh, Kálmán Rák, Ferenc D. Tóth: Frequent methylation of *p16*^{INK4A} and *p14*^{ARF} genes implicated in evolution of chronic myeloid leukemia from its chronic phase to acceleration.
Europen Journal of Cancer 39: 2298-305 (2003)

Impact factor: 3.694, citation: 5

Zoltán Beck, Attila Bácsi, Xiangdong Liu, Peter Ebbesen, István Andirkó, **Eszter Csoma**, József Kónya, Etelka Nagy, and Ferenc D. Tóth: Differential Patterns of Human Cytomegalovirus Gene Expression in Various T-Cell Lines Carrying Human T-Cell Leukemia-Lymphoma Virus Type 1: Role of Tax-Activated Cellular Transcription Factors.
Journal of Medical Virology 71: 94-104 (2003)

Impact factor: 2.371, citation:0

Etelka Nagy, György Veress, Krisztina Szarka, **Eszter Csoma**, Zoltán Beck: Frequent methylation of p16INK4A/p14ARF promoters in tumorigenesis of Epstein-Barr virus transformed lymphoblastoid cell lines.
Anticancer Research 25: 2153-60 (2005)

Impact factor: 1.395, citáció: 0