ISSN 0006-2979, Biochemistry (Moscow), 2007, Vol. 72, No. 7, pp. 766-771. © Pleiades Publishing, Ltd., 2007.
Original Russian Text © G. V. Volgareva, D. A. Golovina, N. M. Gasparjan, V. D. Ermilova, L. S. Pavlova, A. A. Petrenko, M. N. Fedorova, L. K. Kurbatov, I. B. Cheglakov, K. N. Jarigin, F. L. Kisseljov, 2007, published in Biokhimiya, 2007, Vol. 72, No. 7, pp. 941-947.

STAT1 Gene Expression in Cervical Carcinomas

G. V. Volgareva[1], D. A. Golovina[1], N. M. Gasparjan[1], V. D. Ermilova[1], L. S. Pavlova[1], A. A. Petrenko[1], M. N. Fedorova[1], L. K. Kurbatov[2], I. B. Cheglakov[2], K. N. Jarigin[2], and F. L. Kisseljov[1]*

[1]Blokhin Cancer Research Center, Russian Academy of Medical Sciences, Kashirskoe Shosse 24, 115478 Moscow, Russia; fax: (495) 324-1205; E-mail: f_kiss@crc.umos.ru
[2]Orekhovich Institute of Biological and Medical Chemistry, Russian Academy of Medical Sciences, ul. Pogodinskaya 10, 110121 Moscow, Russia

Received February 1, 2007
Revision received March 22, 2007

Abstract—Expression of the *STAT1* gene belonging to the group of interferon-regulated genes was analyzed in cervical tumors and cell lines harboring the genome of human papilloma viruses (HPV) of so-called high risk group. Expression of this gene in invasive carcinomas was maintained on a definite level that was not significantly distinct from that in adjacent normal (control) tissue. Tumors from different patients differ from each other by expression level of the *STAT1* gene. These variations can be attributed to the heterogeneity of tumor cell population and different ratio between normal and tumor cells, as well as to putative persistence of intra-individual variability of *STAT1* expression in normal cell population. It was demonstrated that viral genome status (episomal or integrative) did not influence *STAT1* gene transcription. In conclusion, these data demonstrate that the *STAT1* gene is expressed in an individual and specific manner both in HPV-positive cervical tumors and cell lines harboring transforming genes of these viruses.

DOI: 10.1134/S0006297907070115

Key words: cervical cancer, human papilloma viruses, interferon-regulated genes

Etiological agents of human cervical cancer are human papilloma viruses (HPV) of so-called high risk group (HPV types 16 and 18 and related ones). The key role in development of tumor progression belongs to the viral transforming genes *E6* and *E7*, which disturb function of tumor suppressor genes *p53* and *RB*, respectively [1, 2].

The specified viral genes fulfill initiating functions during malignant transformation, but complete manifestation of oncogenic potential requires a number of additional mutational and epigenetic changes. Among the latter are those linked to alterations in gene activity without mutational changes in their structure. Many of these changes are described, such as elevated production of p16ink4a protein (the inhibitor of cyclin-dependent kinases) correlating with the transcription activation of its gene in tumor [3-9]; elevation of telomerase activity [10]; transcription activation of genes involved in epithelium differentiation (*S100A9* and *ANX8*) [11]; methylation of some genes (implicit participants of tumorigenesis) and, as a consequence, decrease in their transcription activity [8, 12]. More detailed analysis using expression microchips has demonstrated an increase in activity of 240 genes and decrease in activity of 265 genes among 14,500 studied genes in tumors [13]. Besides, expression microchips demonstrate a decrease in activity of the *STAT1* gene from the interferon-regulated family (IRG) in cultures of normal human keratinocytes after their transfection with HPV type 31 DNA (high risk type) [14]. This phenomenon was more clearly exhibited for *STATα/β* gene encoding the transcription factor (signal transducer and activator of transcription), responsible for interferons' intracellular signal transduction.

STAT1α and STAT1β are two proteins (84 and 91 kD, respectively) belonging to the numerous protein family STAT. They are encoded by the same gene *STAT1* and arise as products of alternative splicing and mRNA polyadenylation at two sites; the proteins STAT1α and STAT1β differ from each other in 38 amino acid residues

Abbreviations: APOT) amplification of papilloma oncogenic transcript; dNTP) deoxynucleoside triphosphates; HPV) human papilloma virus; IRG) interferon-regulated genes.

* To whom correspondence should be addressed.

on the COOH-terminus of the molecule [15]. The *STAT1* gene encodes proteins functioning in the regulatory *Jak–STAT* pathway, by which signals from different cytokines including interferons are transmitted into the cell nucleus [15, 16].

STAT1 expression in keratinocytes infected by HPV type 31 was 3.5-fold decreased [14], and these data were confirmed by another group of authors who demonstrated the responsibility of genes *E6* and *E7* of HPV of high risk for the observed phenomenon [17], whereas the transcription of *STAT1* gene was not suppressed when genes *E6* and *E7* from the low risk group (HPV types 6 and 11) were used for the transfection [18]. It must be emphasized that one report is known about antipodal effect – enhancement of several IRG activities during *in vitro* cultivation of HPV-containing cervical keratinocytes [19].

The HPV genome is known to persist in infected cells in either episomal or integrated state, but both DNA forms can persist together [1, 20]. The system of human keratinocytes transfected by HPV type 31 is characterized by the feature that at the first steps of the cultivation the viral DNA is present in the cells in episomal form, and a higher level of suppression of *STAT1* expression is therewith observed. The amount of episomal viral DNA drastically decreases and the integrated forms appear in the course of *in vitro* cultivation, which is generally accompanied by moderation of the transcription activity suppression [21]. A similar phenomenon of the transition of episomal viral DNA into the integrated one is described for W12 cells as well. It was also associated with alterations in transcription of several IRGs [19].

Thus, the IRG group in tumor cells of cervical cancer cultivated *in vitro* and bearing a high risk HPV genome can present interest for understanding of the carcinogenesis mechanism in cervical epithelium and possible involvement of immunological components in the process. Thus, the aim of the present work was to study the expression of one representative IRG, the *STAT1* gene, whose protein product realizes the transmission of interferon signal into the cell nucleus, in primary epidermoid (squamous cell) cervical carcinoma containing integrated or episomal genome of HPV type 16.

MATERIALS AND METHODS

Samples of 21 invasive epidermoid (squamous cell) cervical carcinomas obtained from patients receiving surgical treatment at the Blokhin Cancer Research Center, Russian Academy of Medical Sciences, were used in the study. Morphologically normal tissues (eight cases cervical, the rest fallopian tubes and uterus) were also taken from all these patients. All the materials, received with patient consent, were stored in liquid nitrogen.

SiHa, CaSki, and HeLa cell lines were obtained from the American Type Culture Collection. Cells of SiHa and CaSki lines contain the genetic material of HPV type 16, and HeLa cells contain HPV type 18. The cells were cultivated on DMEM medium supplied with 10% fetal calf serum.

DNA and RNA from frozen tissues were isolated by the guanidine isothiocyanate method with subsequent ultracentrifugation through a CsCl layer [22]. Total RNA preparation (1 μg) was treated with DNase (DNase I Amp. Grade; Invitrogen, USA) and used for reverse transcription with hexaprimers (Lytech, Russia) and reverse transcriptase Super Script II (Invitrogen) in accordance with the manufacturer's protocol.

A sample stored in liquid nitrogen was transferred into a freezing microtome pre-cooled to –20°C for microdissection. Sections 5 μm thick were placed on microscope slides, fixed with ethanol, and stained with Mayer's hematoxylin according with the standard protocol. A homogeneous island of tumor cells, wherein their portion was no less than 95% and stroma cells were virtually absent, was chosen on a fixed and dried section. This site was marked and a drop of guanidine isothiocyanate-containing lysing buffer from the RNeasy Micro Kit microcolumn (Qiagen, USA) was applied onto the site. Similarly, the microdissection of a morphologically normal pair sample was carried out. RNA was isolated in accordance with the instruction of the manufacturer of the columns.

cDNA samples were normalized in PCR with primers to the *GAPDH* gene. The following primers were used for *GAPDH*: direct (5′-ACC ACA GTC CAT GCC ATC AC-3′) and reverse (5′-TCC ACC ACC CTG TTG CTG TA-3′). The length of PCR product was 450 base pairs (bp). The reaction mixture consisted of PCR buffer (0.016 M $(NH_4)_2SO_4$, 0.06 M Tris-HCl, pH 8.8, 0.1% Tween-20, 2 mM $MgCl_2$, 200 μM of each dNTP, 8 pmol of each the primer, and 0.7 U of Taq-DNA polymerase (manufactured at the Institute of Bioorganic Chemistry, Russian Academy of Sciences). Amplification conditions: 94°C for 5 min followed by 28 cycles: 94°C for 30 sec, 58°C for 30 sec, and 72°C for 60 sec, then the final elongation at 72°C for 2 min.

Direct (5′-AGT GGA AGC GGA GAC AGC AGA GC-3′) and reverse (5′-CCG AAA TTC AGC CGC CAG ATC-3′) primers to *STAT1* gene were kindly provided by Dr. E. Chang (Northwestern University, USA). Identical products have been synthesized in PCR with cDNA corresponding to two isoforms of *STAT1* (*STAT1α* and *STAT1β*) in the presence of these primers (personal communication of Dr. E. Chang). The length of the amplification product is 503 bp. The reaction mixture consisted of PCR buffer, 200 μM of each dNTP, 8 pmol of each the primer, 1.5 mM $MgCl_2$, and 0.7 U of Taq-DNA polymerase. Amplification conditions: 94°C for 5 min followed by 31 cycles: 94°C for 30 sec, 60°C for 30 sec, and 72°C for 2 min, then the final elongation at 72°C for 5 min.

PCR was carried out in a Tercyc PCR Cycler amplifier (Russia). The amount of the amplification product for the both genes corresponded to linear PCR zone under the above-indicated conditions (28 cycles for *GAPDH* and 31 cycles for *STAT1*). To compare samples by amounts of *GAPDH*- and *STAT1*-specific products, the products were separated in agarose gel, visualized with ethidium bromide, and the data were processed thereafter using Image Quality software.

The status of HPV type 16 genome was also determined by PCR with primers to *E2* frame as well as by the APOT method [20]. PCR to *E2* gene was carried out with direct (5′-ATG AAA ATG ATA GTA CAG AC-3′) and reverse (5′-CCA GTA GAC ACT GTA ATA G-3′) primers. The length of PCR product is 1026 bp. The reaction mixture consisted of PCR buffer, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 16 pmol of each the primer, 1 U of Taq-DNA polymerase, and 10% glycerol. Amplification conditions: 94°C for 4 min followed by 40 cycles: 94°C for 30 sec, 48°C for 30 sec, and 72°C for 2 min, the final elongation at 72°C for 6 min.

RESULTS

The DNA samples from tumors were analyzed for the presence of HPV of various types. Genetic material of HPV from high risk group (in 20 of them – type 16, in one of them – type 33 (sample 445)) was revealed in all carcinomas.

The data on expression of *STAT1*-specific RNA in normal–tumor pair samples and in cell culture are presented in Figs. 1 and 2. It follows from the data that the gene *STAT1* is expressed and a distinct level of its transcriptional activity is observed in most cases in normal tissue obtained from the same patient. This level is either negligibly altered (samples 444, 577) or slightly elevated (samples 445, 466) (Fig. 1). In cell lines the transcriptional activity is maintained on a stable level (with minor variations for each cell line), the variability in expression of *GAPDH* gene being substantially lower (Fig. 1).

Statistical data processing on the expression of *STAT1* gene in carcinomas was carried out in relation to the mean expression level of *STAT1* gene in six samples of morphologically normal cervical epithelium adjacent to the tumor, on *GAPDH* for 17 carcinoma samples, whose cDNAs were normalized to the given gene (Fig. 2). Due to the particularity of tumor material sampling, we failed to obtain fragments of normal tissue for many samples. Hence, average data were used for the statistical processing, in which the results of analysis of normal uterus or fallopian tube tissues were included. In additional experiments we have found that these data did not radically differ from that obtained in the studies of normal cervical tissue.

Insignificant variations of values around one were observed in the vast majority of normal–tumor pair samples, that is, the expression of *STAT1* in a carcinoma was close to the expression of this gene in the pair sample of arbitrary normal tissue. Like other authors [14], we assumed that the gene expression in carcinoma is altered when two-fold or more difference was observed in comparison with normal tissue. The results of this method of statistical data processing are presented in the diagram (Fig. 2). Mean value for normal tissue in this case was 1.00 ± 0.60, and *STAT1* expression levels in the samples 443, 444, 448, 585, 593, 244, 445, 454, 558, 559, 560, 564, and 577 did not differ significantly from the mean. More than two-fold decrease in *STAT1* expression in sample 574 revealed under the given conditions of the treatment is a consequence of very low absolute absorption values for the *STAT*-specific product for both carcinoma and its pair normal tissue ("weak" products). More than two-fold increase in *STAT1* expression in carcinoma in comparison with that in normal tissue was revealed for samples 466 (Fig. 1), 550, and 594 with this method of the data processing. Thus, we did not detect regular depression of *STAT1* expression in invasive cervical carcinoma, which was typical for this gene in the case of human keratinocytes infected *in vitro* with HPV type 31 [14].

Due to the heterogeneity of the tumor population, the expression of *STAT1* was studied on tumor cell popu-

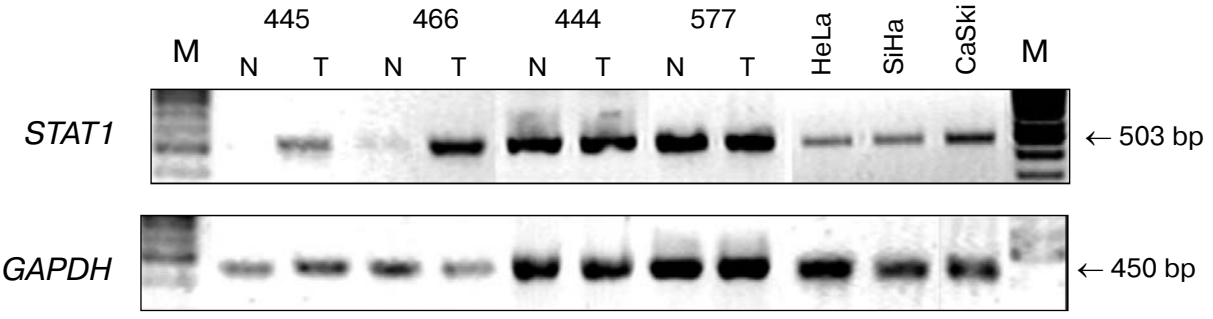

Fig. 1. Expression of *STAT1* and *GAPDH* genes in cervical carcinomas and in stable cell lines from cervical tumors. Arrows indicate molecular mass markers. T, tumor tissue; N, arbitrary normal tissue adjacent to the tumor.

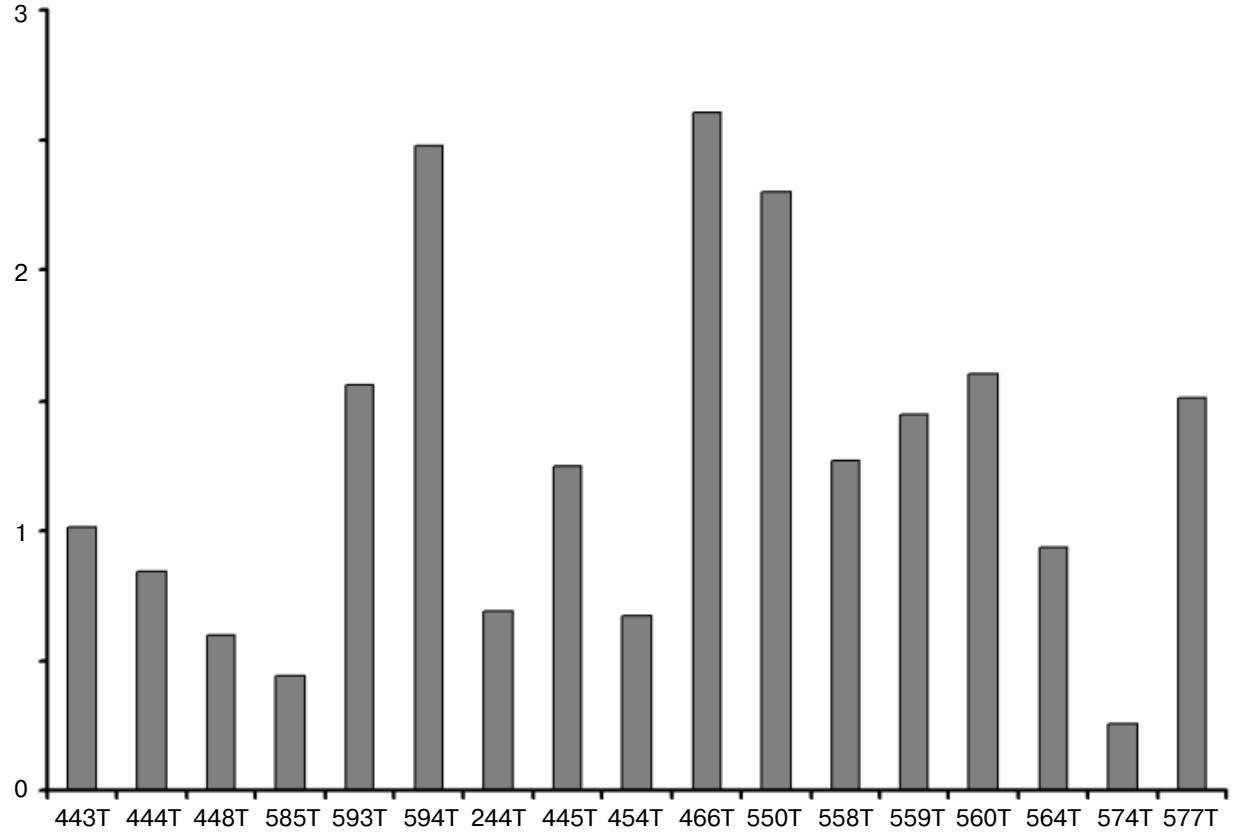

Fig. 2. Transcription level of *STAT1* gene in tumors in comparison with "averaged" expression level in normal tissue. Along the abscissa are numbers of tumor samples (T), along the ordinate – expression level.

lation enriched by the microdissection method. The data for one of the samples (577) are given in Fig. 3. The expression of *STAT1* was evaluated in parallel in the pair normal sample also subjected to microdissection. In this case, three-fold enhancement of STAT1 expression in comparison with that of morphologically normal tissue was detected in the carcinoma.

The status of HPV type 16 genome was determined for five carcinoma samples (table). With this aim, we used the amplification of full-length HPV *E2* gene, which is present in cells with episomal form of viral genome, and the method without amplification, when integrated form of viral genome with *E2* deleted at 5′-terminus persisted in the cells. The APOT method is based on amplification of expressing sequences of the viral genome with subsequent identification in the product of the viral *E4* gene, which can be revealed in episomal DNA and deleted after its integration [20].

Levels of *STAT1* gene expression in cervical carcinoma containing episomal or integrated genome of HPV type 16

Sample	Status of HPV type 16 genome	Method	*STAT* expression
443	integrated forms	PCR to *E2*	unchanged
444	episomal forms, the presence of integrated forms is not excluded	PCR to *E2*	unchanged
466	integrated forms	APOT	increase
574	integrated forms	PCR to *E2*	decrease
577	episomal forms, the presence of integrated forms is not excluded	PCR to *E2*	unchanged

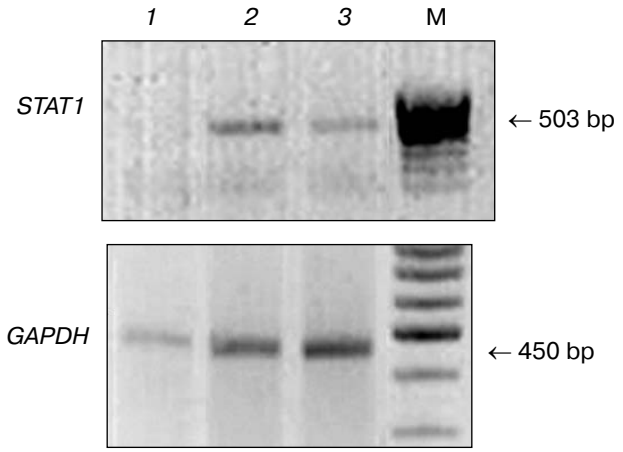

Fig. 3. Expression of *STAT1* and *GAPDH* genes in cervical carcinoma sample No. 577 subjected to microdissection. Arrows indicate molecular mass markers. *1*) Normal tissue; *2*) tumor tissue; *3*) cDNA of SiHa cells.

Under the comparative analysis of *STAT1* gene expression in cervical carcinoma containing viral DNA in episomal or integrated form, we did not observe deviations from normal tissue in tumors either with integrated (sample 443) or with episomal forms (samples 444 and 577). Regarding two other cases with the integrated form, we have observed either the increase in *STAT1* expression (sample 466), or its decrease (sample 574). Thus, transcriptional activity of this gene hardly depends on HPV genome status in tumor cells.

DISCUSSION

The results of the present work and reports cited above suggest that the expression of one of the IRG genes, *STAT1*, can substantially vary in various cell systems containing high risk HPV genome. Generally, the transcription of this gene is maintained at a distinct level not differing substantially from arbitrarily normal tissue in invasive cervical carcinoma cultivated *in vitro*. The carcinomas from various patients can differ from each other in the expression level. This may be associated with the inter-individual variability of *STAT1* expression level in normal tissues as well as with heterogeneity of tumor population with various ratios between tumor and normal cells in various cervical carcinomas, which is partially confirmed by our data on samples subjected to microdissection. The status of viral genome (episomal or integrated) does not apparently determine the level of *STAT1* gene expression. Another situation is observed in cells cultivated *in vitro* and carrying the HPV genome. The transcriptional activities are maintained on a sufficiently high level and insignificantly differ from each other in stable cell lines obtained from tumors. Another situation exists in cell lines infected with either whole virus or transfected with transforming HPV genes (*E6* and *E7*) [14, 17]. A notable suppression of transcriptional activity of the *STAT1* gene is observed even at the initial stages of appearance of viral genes in cells, when viral DNA persists in episomal form. Thus, the control of *STAT1* gene expression is individual and specific for each cervical carcinoma containing papilloma viral genome and for each cell culture carrying transforming genes of these viruses.

The authors are indebted to N. P. Kiseleva for consultations.

REFERENCES

1. Zur Hausen, H. (2000) *J. Natl. Cancer Inst.*, **92**, 690-698.
2. Langworth, M. S., and Laimins, L. A. (2004) *Microbiol. Mol. Biol. Rev.*, **68**, 362-372.
3. Sano, T., Oyama, T., Kashiwabara, K., Fukuda, T., and Nakajima, T. (1998) *Am. J. Pathol.*, **153**, 1741-1748.
4. Klaes, R., Friedrich, T., Spitkovsky, D., Ridder, R., Rudy, W., Petry, U., Dallenbach-Hellweg, G., Schmidt, D., and von Knebel-Doeberitz, M. (2001) *Int. J. Cancer*, **92**, 276-284.
5. Volgareva, G. M., Zavalishina, L. E., Andreeva, Y. Y., Frank, G. A., Krutikova, E. I., Golovina, D. A., Bliev, A. Y., Spitkovsky, D. D., Ermilova, V. D., and Kisseljov, F. L. (2004) *BMC Cancer*, **4**, 58.
6. Wang, J. L., Zheng, B. Y., Li, X. D., Angstrom, M. S., and Wallin, K. L. (2004) *Clin. Cancer Res.*, **10**, 2407-2414.
7. Murphy, N., Ring, M., Heffron, C. C. B. B., King, B., Killalea, A. G., Hughes, C., Martin, C. M., McGuinness, E., Sheils, O., and O'Leary, J. J. (2005) *J. Clin. Pathol.*, **58**, 525-534.
8. Kisseljov, F. L., Mazurenko, N. N., Volgareva, G. M., and Kisseljova, N. P. (2004) *Mol. Biol. (Moscow)*, **38**, 183-190.
9. Volgareva, G., Zavalishina, L., Golovina, D., Andreeva, Y., Petrov, A., Petrenko, A., Bateva, M., Ermilova, V., Kisseljova, N., and Frank, G. (2006) in *New Research on Cervical Cancers* (Rolland, G. Z., ed.) Nova Science Publishers, N. Y., pp. 129-147.
10. Skvortsov, D. A., Gasparian, N. M., Rubtsova, M. P., Zvereva, M. E., Fedorova, M. D., Pavlova, L. S., Bogdanov, A. A., Dontsova, O. A., and Kisseljov, F. L. (2006) *Dokl. Akad. Nauk*, **408**, 556-559.
11. Chao, A., Wang, T. H., Lee, Y. S., Hsuch, S., Chao, A. S., Chang, T. H., Kung, W. H., Huang, S. H., Shao, F. Y., Wei, M. L., and Lai, C. H. (2006) *Int. J. Cancer*, **119**, 91-98.
12. Ivanova, T., Petrenko, A., Gritzko, T., Vinokourova, S., Eshilev, E., Kobzeva, V., Kisseljov, F., and Kisseljova, N. (2002) *BMC Cancer*, **2**, 4-11.
13. Santin, A., Zhan, F., Bignotti, E., Siegel, E., Cane, S., Belloni, S., Palmieri, M., Anfossi, S., Thomas, M., Burnett, A., Kay, H., Roman, J., O'Brien, T., Tian, E., Cannon, M., Shaughnessy, J., and Pecorelli, S. (2005) *Virology*, **331**, 269-291.

14. Chang, Y. E., and Laimins, L. A. (2000) *J. Virol.*, **74**, 4174-4182.
15. Darnell, J. E., Jr., Kerr, I. M., and Stark, G. R. (1994) *Science*, **264**, 1415-1421.
16. Der, S. D., Zhou, A., Williams, B. R. G., and Silverman, R. H. (1998) *Proc. Natl. Acad. Sci. USA*, **95**, 15623-15628.
17. Nees, M., Geoghegan, J. M., Hyman, T., Frank, S., Miller, L., and Woodworth, C. D. (2001) *J. Virol.*, **74**, 4283-4296.
18. Thomas, J. T., Oh, S. T., Terhune, S. S., and Laimins, L. A. (2001) *J. Virol.*, **74**, 7564-7571.
19. Alazawi, W., Pett, M., Arch, B., Scott, L., Freeman, T., Stanley, M., and Coleman, N. (2002) *Cancer Res.*, **62**, 6959-6965.
20. Kisseljov, F. L., Kisseljova, N. P., Kobzeva, V. K., Gritsko, T. M., Semenova, L. A., Pavlova, L. S., Klaes, R., and von Knebel Doeberitz, M. (2001) *Mol. Biol. (Moscow)*, **35**, 399-404.
21. Chang, Y. E., Pena, L., Sen, G. C., Park, J. K., and Laimins, L. A. (2000) *J. Virol.*, **74**, 8864-8874.
22. Samoylova, E., Shaihaev, G., Petrov, S., Kisseleva, N., and Kisseljov, F. (1995) *Int. J. Cancer*, **61**, 337-341.