# Cellular Expression of *INK4a* Gene in Cells of Bladder Cancer Associated with Human Papilloma Virus-16

**G. M. Volgareva, L. E. Zavalishina*, D. A. Golovina, Yu. Yu. Andreeva*, V. D. Ermilova, O. B. Trofimova**, D. A. Kuevda**, O. Yu. Shipulina**, V. A. Glazunova, Shuling Cheng***, L. S. Pavlova, N. L. Cheban, V. B. Matveev, and G. A. Frank***

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Hyperexpression of $p16^{INK4a}$ protein is an early marker of cervical cancer. Hyperexpression of *INK4a* gene encoding this protein at the level of mRNA and $p16^{INK4a}$ was detected in tumor cells of some patients with bladder cancer associated with human papilloma virus-16. However, in contrast to cervical cancer, this phenomenon in urothelial carcinomas does not correlate with expression of human papilloma virus-16 oncogenes *E6* and *E7*.

**Key Words:** *carcinogenesis; urothelium; papillomaviruses; $p16^{INK4a}$*

Human papillomaviruses (HPV) are epitheliotropic DNA viruses. The so-called high risk HPV are etiological agents of cervical cancer and other cancer forms [6,7]. Oncoproteins E6 and E7 encoded by HPV gene interact with various cell proteins impairing their functioning and inducing malignant transformation of cells. HPV16 is the most prevalent carcinogenic papillomavirus.

The list of cancer forms, for which the etiological role of HPV is proven, was significantly supplemented in recent years [7]. Etiological heterogeneity of these cancer forms is a known fact. Papillomaviruses induce just some of oropharyngeal, vulval, and perianal carcinomas. Differences in the molecular profiles of HPV-positive and negative carcinomas, their histological and clinical heterogeneity were detected. Clinical heterogeneity manifests in different severity of the disease course in patients with HPV-positive and negative forms of cancer and sometimes in more difficult diagnosis of HPV-negative cancer [5,10].

We previously detected HPV16 DNA in bladder cancer cells [2,15]. In some patients, the virus genome was expressed in tumor cells at the level of mRNA and E7 oncoprotein [1,15]. The search for markers of HPV-associated bladder cancer for clinical diagnostic laboratory is therefore an important problem. Experience gained in the search for these markers for cervical cancer seemed to be useful for this problem. Attempts at detecting high risk HPV DNA by PCR did not lead to creation of a reliable test for prediction of cervical cancer. Just few women with HPV-positive dysplasia of the cervix uteri develop *in situ* cancer and invasive cervical cancer [6,7]. Hence, despite high sensitivity of PCR, the specificity of this approach proved to be low. Attempts at improving early diagnosis of cervical cancer are now focused on the search for genes of HPV-infected host cell, the expression of these genes irreversibly changing in dysplasia and carcinoma under the effect of the virus oncoproteins.

One of these genes is *INK4a* encoding $p16^{INK4a}$ protein (Cdk 4/6 cyclin-D-dependent kinase inhibitor) [12]. Expression of E7 virus oncoprotein results in

N. N. Blokhin Cancer Research Center, Russian Academy of Medical Sciences; *P. A. Hertzen Institute of Oncology, Ministry of Health Care and Social Development; **Institute of Epidemiology, Moscow, Russia; ***Neodiagnostic Labs Inc. 47853 Warm Springs BLVD, Fremont, CA 94539, USA. ***Address for correspondence:*** gmvolgareva@front.ru. G. M. Volgareva

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many-fold increase of transcription of *INK4a* in cervical epithelial dysplastic and cancer cells infected with high risk HPV in comparison with normal epithelium, this being paralleled by a drastic increase in $p16^{INK4a}$ protein content [8,9,13]. The immunohistochemical test, widely used in early diagnosis of cervical cancer, is based on this phenomenon.

The factors determining $p16^{INK4a}$ expression in bladder cancer cells can be basically different from those in HPV-positive cervical cancer. In contrast to cervical cancer, *INK4a* gene in bladder cancer is often subjected to deletions and point mutations and methylated in the promotor region [3,4,11]. Any of these events can completely or partially suppress *INK4a* expression at the protein level. Homozygotic deletions of *INK4a*, as a result of which the cell is no longer able of producing $p16^{INK4a}$, were detected in ~30-50% specimens of bladder cancer [3,4]. It was never studied whether hyperexpression of $p16^{INK4a}$ is characteristic of HPV-positive urothelium

We evaluated the possibility of using $p16^{INK4a}$ cell protein for indication of HPV-positive bladder cancer.

Two problems were to be solved: is hyperexpression of *INK4a* characteristic of HPV16-positive bladder cancer DNA and, if so, is there a correlation between *INK4a* hyperexpression at the level of $p16^{INK4a}$ protein and expression of *E6* and *E7* oncogenes.

## MATERIALS AND METHODS

The study was carried out on 50 specimens of transitional cell bladder cancer and 5 specimens of morphologically normal bladder urothelium, collected in 5 of the same patients. According to the previous PCR screening, all specimens of cancer and morphologically intact urothelium contained HPV15 DNA [15]. The expression of HPV16 oncogenes *E6* and *E7* at the level of mRNA and/or protein was detected in 12 of these specimens of bladder cancer (Table 1) [1,15]. The expression of *INK4a* was studied at two levels (mRNA and protein) by reverse transcription PCR (RT-PCR) and by immunohistochemical staining of $p16^{INK4a}$ by specific monoclonal antibodies.

Total RNA for RT-PCR was isolated from frozen tissue by the guanidine isothiocyanate method with ultracentrifugation through cesium chloride pad (30,000 rpm, 16 h, 19°C). Total RNA (1 μg) was treated with DNase (DNase I Amp. Grade, Invitrogen) and used in RT-PCR with hexaprimers (Litech) and Super Script II reverse transcriptase (Invitrogen) according to the instruction. The cDNA samples were standardized in PCR with primers to *GAPDH* gene. The following primers were used: forward, 5'-ACC ACA GTC CAT GCC ATC AC-3'; reverse, 5'-TCC ACC ACC CTG TTG CTG TA-3'. The length of the resultant amplification product was 450 b.p. The following primers to *INK4a* were used: sense, 5'-AGC CTT CGG CTG ACT GGC TGG-3' (exon I); antisense, 5'-GCG CTG CCC ATC ATC ATG AC-3' (exon II). The size of amplification product was 140 b.p. It was detected by electrophoresis in 2% agarose gel with ethidium bromide (0.5 μg/ml). Southern blot hybridization was carried out for more demonstrative visualization of the products. The PCR product was transferred from agarose gel onto Hybondand™-N+ Nylon membrane (Amersham Bioscience) and visualized using $^{32}$P-labeled radioactive probe to the internal sequence of this product. The nucleotide sequence of the probe was 5'-CTG CCC AAC GCA CCG AAT AGT TAG G-3'. The procedures were carried out according to instructions of the membrane manufacturer.

The levels of *INK4a*-specific amplification products in different samples were compared by optical densities of these products using Image Quality software.

Protein $p16^{INK4a}$ was detected in cells of tumor and morphologically normal bladder urothelium by immunohistochemical staining of preparations from these specimens with $p16^{INK4a}$-specific monoclonal antibodies (CINtec™ Histology Kit, Dako Cytomation). The details of the reaction and interpretation of the results were described previously [13].

## RESULTS

The content of *INK4a* mRNA in morphologically normal urothelium from different patients varied within a great range: A/N differed from B/N and A/N differed from C/N by 30 and 70 times, respectively; no *INK4a* specific transcript was found in D/N specimen (Fig. 1). This variability can reflect the initial genetic polymorphism of these patients by the level of *INK4a*

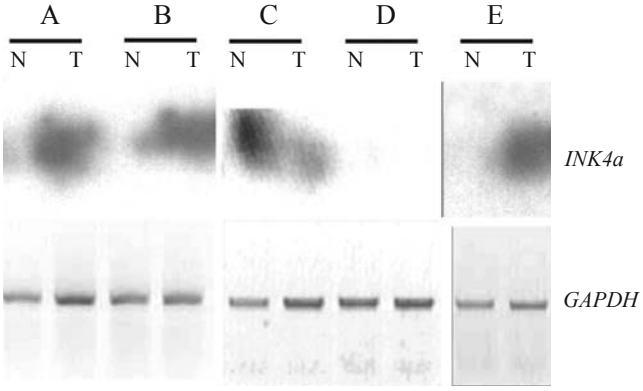


**Fig. 1.** RT-PCR analysis of *INK4a* expression in transitional cell bladder cancer. T: tumor tissue; N: morphologically normal urothelium adjacent to the tumor. A, B, C, D, E: patients with bladder cancer. Upper row of products: *INK4a*-specific transcripts after visualization with radioactive test. Lower row: results obtained with *GAPDH*-specific primers (RNA stability and quality control).

transcription activity in the bladder urothelium irrespective of bladder cancer which developed later; or mutation and/or epigenetic disorders in *INK4a* in the somatic urothelial precursor cells, which could stimulate malignant degeneration of bladder epithelial cells; or location of these morphologically normal tissue in the immediate vicinity of bladder cancer, specifically, the presence of micrometastases in the studied tissue, the content of *INK4a*-specific transcripts in these metastatic cells differing from that in intact urothelial cells; or a combination of these facts, which can be different in the patients with bladder cancer. Analysis of the causes of this variability is beyond our present task. It is obvious that it would be correct under these conditions to compare transcription activity of *INK4a* in bladder cancer with that in morphologically normal bladder urothelium of the same patient.

The HPV16 DNA was isolated from the D/T and E/T specimens and expression of HPV16 oncoprotein E7 was detected in these specimens (Nos. 11 and 12, respectively, in Table 1; Fig. 1) [1]. Significant intensification (30-100 times) of *INK4a* transcription in comparison with transcription activity of these gene in normal urothelial cells was detected in cancer cells of 3 (A, B, E) of 5 patients. More intense transcription of *INK4a* in some HPV16 DNA-containing specimens of bladder cancer (one of these with verified expression of the virus oncogene) is similar to the phenomenon we previously described for invasive cervical cancer [13]. However, $p16^{INK4a}$ was not detected in all specimens of bladder cancer with *INK4a* transcripts. For example, we observed a negative reaction with $p16^{INK4a}$-specific antibodies not only in the D/T, but also in E/T specimen. This latter result suggests the mechanisms of posttranscription regulation of *INK4a* activity in bladder cancer cells, which are not intrinsic of cervical cancer. On the other hand, *INK4a* transcription in some specimens of bladder cancer was weaker than in morphologically normal urothelium or was zero (C/T and D/T specimens, respectively). Presumably, mutation and/or epigenetic disorders of *INK4a* characteristic of bladder cancer underlie these events [3,4,11].

The results of immunochemical detection of $p16^{INK4a}$ were taken into consideration only in cases with adequate effects of antibodies in the control (Fig. 2, *a*, *b*). Only 5 of 50 HPV16 DNA-positive specimens of bladder cancer were $p16^{INK4a}$-positive (10%) (Fig. 2, *c*). The reaction of $p16^{INK4a}$-specific antibodies was negative in all specimens of morphologically normal urothelium. The protein was not found in any of 12 specimens of bladder cancer expressing HPV16 *E6* and/or *E7* (Table 1; Fig. 2, *d*, *e*). It was previously reported that $p16^{INK4a}$ hyperexpression is not a highly specific marker of HPV presence. This hyperexpression was described for HPV-negative *RB* mutant cervical cancer cells [8,9]. Presumably, *RB* mutations were responsible for the expression of $p16^{INK4a}$ in some bladder cancer specimens in our study.

Complete absence of correlation between the expression of HPV16 oncogenes *E6* and/or *E7* and hyperexpression of $p16^{INK4a}$ indicate that this cell protein

**TABLE 1.** Immunohistochemical Detection of $p16^{INK4a}$ in Specimens of Bladder Cancer with Expression of HPV16 Genome

| Bladder cancer specimen No. | HPV DNA | | Expression of HPV16 oncogenes *E6* and *E7** | | $p16^{INK4a}$ |
|---|---|---|---|---|---|
| | present | virus type | *E6*/*E7* mRNA | HPV16 protein E7 | |
| 1 | + | 16 | + | + | – |
| 2 | + | 16 | + | + | – |
| 3 | + | 16 | + | + | – |
| 4 | + | 16 | + | + | – |
| 5 | + | 16 | + | – | – |
| 6 | + | 16 | + | – | – |
| 7 | + | 16 | + | – | – |
| 8 | + | 16 | – | + | – |
| 9 | + | 16 | – | + | – |
| 10 | + | 16 | – | + | – |
| 11 | + | 16 | – | + | – |
| 12 | + | 16 | – | + | – |

**Note.** *Differences in the results of evaluation of HPV16 *E7* expression at the mRNA and protein levels in specimens Nos. 5-12 can be due to frequent focal expression (not in all tumor cells, but just in solitary ones) of *E7* oncoprotein in bladder cancer [1]. Cells containing HPV16 *E7* can be present in the stained section but not in the tissue specimen from which RNA was isolated and vice versa.

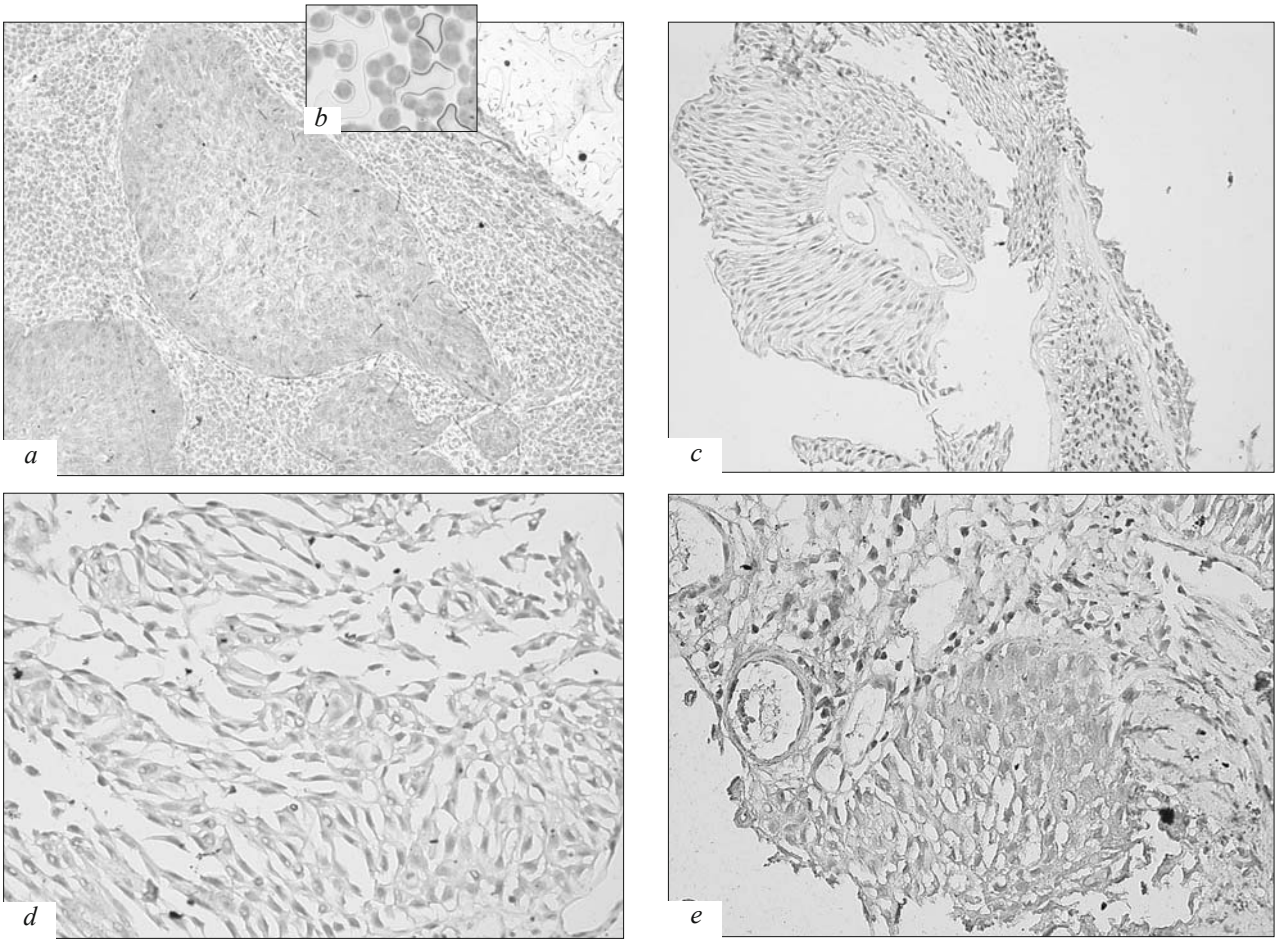


**Fig. 2.** Results of immunohistochemical detection of $p16^{INK4a}$ (*a*-*d*) and HPV15 oncoprotein E7 (*e*). *a*) squamous-cell cervical cancer (positive control): diffuse staining with $p16^{INK4a}$-specific antibodies, ×100; *b*) HCT cells, smear (negative control): negative reaction with $p16^{INK4a}$-specific antibodies, ×400; *c*) transitional-cell bladder cancer, diffuse staining with $p16^{INK4a}$-specific antibodies, ×200; *d*) transitional-cell bladder cancer, negative reaction with $p16^{INK4a}$-specific antibodies, ×400; *e*) the same specimen as in *d*, diffuse reaction with HPV16 E7-specific antibodies (details of the reaction were described [1]), ×400.

cannot serve as an indicator of HPV-associated bladder cancer variant.

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