PATTERN OF β–DEFENSIN–2 (hBD–2) AND EGFR mRNAs EXPRESSION IN CERVICAL AND VULVAE CANCER CELLS

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ОСОБЕННОСТИ ЭКСПРЕССИИ мРНК β–ДЕФЕНСИНА–2 (hBD–2) И РЕЦЕПТОРА ЭФР В ЗЛОКАЧЕСТВЕННЫХ КЛЕТКАХ ШЕЙКИ МАТКИ И ВУЛЬВЫ ЧЕЛОВЕКА

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The present work continues the study of β-defensin gene expression in malignantly transformed human cells of genital origin. We have shown that in A431 and M-HeLa cells the hBD-2 mRNA expression may be induced by either EGF or bacterial (but not viral) stimulation and is stable for at least 12 h. It was shown also that 24-h long challenge of A431 cells with LPS, bacteria or EGF caused significant decrease of the level of EGFr autophosphorylation and didn't affect the level of expression of EGFr mRNA. The research of surgical specimens has shown that the expression of hBD-2 mRNA and overexpression of EGFr mRNA are typical only for tumor cells of cervix and vulvae, but not for normal ones, and those patterns are associated with the presence of papillomavirus infection.
***Key Words*: defensin, epidermal growth factor receptor, tumor of cervix and vulvae, papillomavirus.**

Дана робота продовжує дослідження експресії генів β-дефенсинів у злоякісно трансформованих клітинах, що походять з тканин вульви та шийки матки людини. Встановлено, що в клітинах А431 та M-HeLa експресія мРНК hBD-2 може бути індукована внаслідок інкубації клітин з епідермальним фактором росту чи мікробними тілами (але не вірусами) та залишається стабільною протягом 12 год. Виявлено також, що 24-годинна інкубація клітин А431 з ЛПС, бактеріями чи ЕФР призводить до значного зниження рівня аутофосфорилювання рецептора ЕФР, але не впливає на рівень експресії його гена. Дослідження хірургічного матеріалу встановило, що експресія мРНК hBD-2 та гіперекспресія мРНК ЕФРР є характерними для клітин первинних пухлин вульви та шийки матки, але не для нормальних тканин; ці властивості пухлинних клітин асоційовані з наявністю в їх геномі ДНК папіломатозних вірусів.
***Ключові слова*: дефенсин, рецептор епідермального фактора росту, пухлини шийки матки та вульви, папіломавірус.**

Human antimicrobial peptides — defensins — are playing an important role in host defense. Up to date 6 α–defensins (HNP–1–4, HD–5 and HD–6) and 4 β–defensins (hBD1–4) have been identified in humans [1–4]. The expression of three of them — β–defensin–2–4 (hBD–2–4) — was shown to have an inducible character and may be recorded in epithelial cells after challenge with inflammatory agents or bacteria [3–5]. Our resent results have shown that hBD–2 mRNA expression may be induced *in vitro* also by growth factors — EGF or TGF–α and that it is linked to malignant phenotype of epithelial cells originated from human vulvae and cervix [6]. At the present time the possible involvement of defensins in tumor progression remains unclear; just few publications reported the pattern of expression of defensins in human oral neoplasms [7–9]. Human papillomavirus (HPV) infection has been shown to be implicated in human neoplasms, including cervix, vulvae, skin, esophagus, head and neck [10]; thus, one may expect that the expression of defensin genes may be linked to the viral (particularly, HPV) infection of respective epithelium. The aim of the present study was to assess the possible relation between expression of hBD–2 and epidermal growth factor receptor (EGFr) mRNAs and its correlation with HPV status in squamous cell carcinoma of human cervix and vulvae.

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Abbreviations used: EGF — epidermal growth factor; EGFr — epidermal growth factor receptor; FCS — fetal calf serum; G3PDH — glyceraldehyde 3–phosphate dehydrogenase; hBD–2 — human beta–defensin–2; HPV — human papillomavirus; LPS — lipopolysaccharide; TGF–α — transforming growth factor–α.

MATERIALS AND METHODS

Bacterial and viral strains and growth conditions. The strain of *Bacillus subtilis* ATCC 6633 was kindly provided by Dr. E. Kiprianova from Ukrainian Collection of Microorganisms (Kyiv, Ukraine). The strain was maintained from the frosen stock by seeding on LB agar plate at 37°C for 2 days. The single colony was harvested from the plate and suspended in 3 ml of LB broth and grown in liquid culture at 37°C overnight with vigorous shaking. Then the overnight culture was centrifugated, the bacterial pellet was redissolved in DMEM and boiled in the water bath for 15 min.

The human adenovirus (*Adh*) of type 5 was received from the collection of the Institute of Microbiology (Medical University, Budapest, Hungary). The strain was cultivated in the cultured HEp–2 cells and its titer was assessed by cytomorphological method [11]. HIV capsid proteins GAG120 and ENV1 were received from company "DiaProphMed" (Kyiv, Ukraine).

Cell lines. A431 (human epidermoid carcinoma) and M–Hela (human cervical carcinoma) cell lines were received from the Institute of Molecular Biology (Russian Academy of Sciences, Moscow, Russia).

Challenge with bacterial cells, viruses and EGF. A431 cells were cultivated in 30 mm dishes in complete medium consisting of DMEM supplemented with 10% FCS at 37°C in an atmosphere with 5% CO_2 until cells reached 80% confluency. Then the culture medium was changed to 1 ml of fresh DMEM without FCS. 0.025 ml of *B.subtilis* ($2.5 \cdot 10^9$ CFU/ml) in DMEM or 0.025 ml of DMEM, containing $5 \cdot 10^{-6}$ g of human EGF were added to the medium in 30–mm dishes and incubation continued for 0–12 h. The experiments with *Adh5* culture or with HIV capsid proteins GAG120 and ENV1 were carried out on A431 cells. The incubation of A431 cells with *Adh5* or with GAG120 or ENV1 lasted for 1 h, then the cells were washed with fresh DMEM and were grown up in DMEM without FCS for 1, 6 or 24 h.

Surgical specimens. Surgically resected specimens were obtained from 10 patients who had been diagnosed with squamous cell carcinoma of vulvae (cases 1–5) or cervix (cases 6–10). The clinical features of the cases were as following: Case 1, 69–year–old female ($T_2N_XM_0$); Case 2, 61–year–old female ($T_3N_0M_0$); Case 3, 56–year–old female ($T_2N_0M_0$); Case 4, 67–year–old female ($T_3N_1M_0$); Case 5, 65–year–old female ($T_3N_2M_0$); Case 6, 83–year–old female ($T_2N_1M_0$); Case 7, 57–year–old female ($T_1N_0M_0$); Case 8, 33–year–old female ($T_1N_0M_0$); Case 9, 35–year–old female ($T_1N_1M_0$); Case 10, 49–year–old female ($T_1N_0M_0$).

RNA preparation and reverse transcription (RT)–PCR. Total RNA was extracted with acid guanidinum thiocyanate/phenol–chlorophorm according to the method [12]. The quality of extracted RNA was estimated by electrophoresis in 1% agarose gel that contained formaldehyde. Primers for genes of human β–defensin–2 and EGFrs (primers which flanc the homology sequences for 4 genes of EGF receptor family) were designed according to nucleotide database of NCBI with the use of computer program "Oligo". The primers used for amplification were: for hBD–2 — 5′–gaagctcccagccatcagcc/5′–gtcgcacgtctctgatgaggga; for EGFrs — forvard–5′–ctgct(c/g/t)aactgg(t/g)g(t/a)(g/a)t(g/a/c)ca(g/a)at(a/t/c)gc(t/c/a) aaggg; reverse — 5′–atcatcca(a/g)ca(c/t)ttgaccat(g/c)a(t/c)catgta(a/g)ac (g/a)tc. For detection of HPV DNA the commercial universal primers for all HPV types were used: forward — 5′–cgtccmarrggawactgatc ("Perkin Elmer" № 808–0011) and reverse — 5′–gcm–cagggwcataayaatgg ("Perkin Elmer" № 808–0012), where m–a/c, r–a/g, w–a/t, y–c/t. The RNA was reverse transcribed (M–MulV reverse transcriptase, MBI Fermentas), using reverse primers mentioned above. The RT products were amplified using a PCR kit (MBI Fermentas). The primer for G3PDH (5′–tgaaggtcggagt–caacggatttggt/5′–catgtgggccatgaggtccaccac) was used as a positive control. The PCR consisted of 40 cycles in Perkin Elmer 2400 amplificator. The products of amplification and their restriction fragments were detected by electrophoresis in 2% agarose gel or 8% polyacrylamide gel (29:1) in Tris–borate 0.089 M pH 7.4 containing 2 mM EDTA.

Autophosphorylation of EGF–receptor. Autophosphorylation assay was performed as following: A431 and M–HeLa cells were grown up at 37°C, 5% CO_2 on 24–well plates for 48 h in RPMI–1640 culture medium supplemented with 5% of FCS. When the density reached $2 \cdot 10^5$ cells per well the cells were washed with serum–free medium and incubated in this medium with respective effector (EGF, heat–killed or living bacteria, LPS) for 2–24 h. Then the plates were transferred on the ice and the culture medium was replaced by phosphorylation buffer (0.2 ml/well) (20 mM HEPES, pH 7.4; 145 mM NaCl; 5.4 mM KCl; 1.8 mM $CaCl_2$; 0.8 mM $MgSO_4$; 2 mM $MnCl_2$; 0.02 mM $ZnCl_2$; 2 mM $NaVO_4$; 0.005 mM ATP) and incubation was continued for 2 min on ice. Then phosphorylation buffer was aspirated and the cells were immediately lysed by hot Laemmly sample buffer. Then the samples were subjected to 7–22% SDS–PAGE gradient electrophoresis and Western–blot analysis was carried out by standard schedule using antiphosphotyrosine antibodies.

RESULTS AND DISCUSSION

Induction of hBD–2 mRNA expression by EGF in vitro. Earlier [6] we have demonstrated by RT–PCR that 6–h–long incubation of A431 cells with $5 \cdot 10^{-6}$g of EGF resulted in the induction of hBD–2 mRNA expression. Now we have shown that the time–course of EGF–dependent hBD–2 mRNA expression didn't differ from that caused by *Bacillus subtilus* cells and is stable for at least 12 h after single induction (Fig. 1). For determination of the time–course of EGF–dependent hBD–2 mRNA expression A431 cells were incubated with $5 \cdot 10^{-6}$g of EGF for 0, 2, 6 and 12 h. It was demonstrated (see Fig. 1) that whilst in the point 0 hBD–2 gene expression is absent, after 2 h of incubation with EGF hBD–2 mRNA expression reached maximum and remained stable during next 10 h of incubation. The similar data were obtained when M–HeLa cells were incubated with EGF.

We checked also the influence of viral infection on β–defensin–2 mRNA expression *in vitro*. A431 cells were

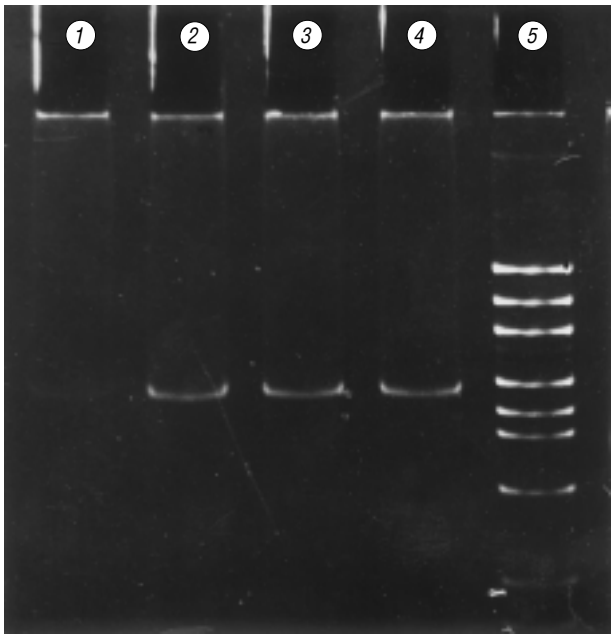

Fig. 1. Time–course of hBD–2 gene expression in A431 cells, undergoing stimulation with $5 \cdot 10^{-6}$g EGF. Lanes *1–4* — 0, 2, 6, 12 h of incubation, respectively. Lane *5* — molecular weight markers (pUC19/HaeIII)

incubated for 1 h with vital culture of human adenovirus or with HIV capsid proteins GAG120 and ENV1. Then the levels of expression of hBD–1 and hBD–2 mRNA were checked by RT–PCR. We demonstrated that *Adh5* particles as well as HIV capsid proteins didn't influence the expression of human β–defensins–1 and –2.

The pattern of EGFr autophosphorylation and expression in vitro. Since it became evident that EGF–dependent system of growth control is somehow implicated in the regulation of *hBD–2* gene expression it seems reasonable to check the pattern of EGF–receptor mRNA expression in A431 and M–HeLa cells. For that purpose the common primers for genes of 4 members of EGFr family were choosed and applied to RT–PCR schedule. We have shown that A431 and M–HeLa cells are constantly expressing EGFr–1 mRNA (Fig. 2). When A431 or M–HeLa cells were incubated for 0–12 h with the heat–killed *B.subtilis* cells or EGF, the level of EGFr mRNA expression remained unaffected by those agents.

Next, we checked the relation between EGF–receptor phosphokinase activity and hBD–2 expression. For that purpose A431cells were exposed to a) EGF, b) LPS of *P.aeruginosa*, c) heat–killed *B.subtilis* cells for 30 min (Fig. 3). After this autophosphorylation assay was carried out. We show that short–term challenge of A431 cells by EGF, LPS or bacterial cells resulted in immediate increase of EGFr autophosphorylation (see Fig. 3).

The next experiment was carried out by the same schedule, but the incubation of A431 cells with EGF, LPS or *B.subtilis* cells lasted for 24 h; in the end of 24 h–long incubation the cells were stimulated by EGF for 30 min and autophosphorylation assay was carried out. We have shown that prolongated challenge of cells by EGF, LPS or heat–killed *B. subtilis* cells resulted in

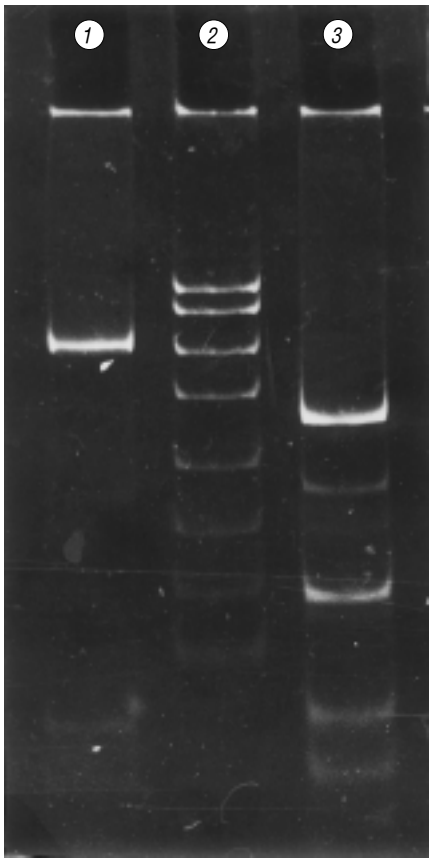

Fig. 2. EGFr mRNA expression in A431 cells. EGFr cDNA fragment (lane *1*) and the product of its restriction with HaeIII (lane *3*). Lane *2* — molecular weight markers (pUC19/MspIII)

the accumulation of the protein phosphorylated at tyrosin residues with molecular weight approximately 35–40 kDa (Fig. 4). The level of autophosphorylation of EGFr in the cells which were incubated with LPS or *B. subtilis* and then stimulated by EGF for 30 min was found to be significantly decreased in comparison with the control cells.

Thus, the challenge of A431 cells by gram–positive bacteria, LPS or EGF caused an immediate increase in the level of autophosphorylation of EGF–receptor, but prolongated incubation of the cells with those agents resulted in its 2–fold decrease and accumulation of the phosphorylated intermediate with molecular weight 35–40 kDa. Taking to account our previous results [13] we hypothetize that the inhibition of kinase activity of EGFr may be caused by hBD–2 production.

HPV DNA in cell lines. It is well recognized now that the tumors of female genital tract are associated with papilloma virus infection (HPV) [10]. The cell lines A431 and M–HeLa were originated from malignant neoplasms of vulvae and cervix, respectively. So it seems reasonable to check the presence of HPV DNA in the genomes of those cells. Using PCR technique we demonstrated that A431 and M–HeLa cells are HPV–positive (Fig. 5). RT–PCR revealed that under standard conditions of cultivation as well as upon the challenge of cells with LPS or EGF viral DNA is not transcribed in A431 and M–HeLa cells. Thus, one may conclude that the expression of hBD–2 mRNA isn't linked to HPV mRNA expression *in vitro*.

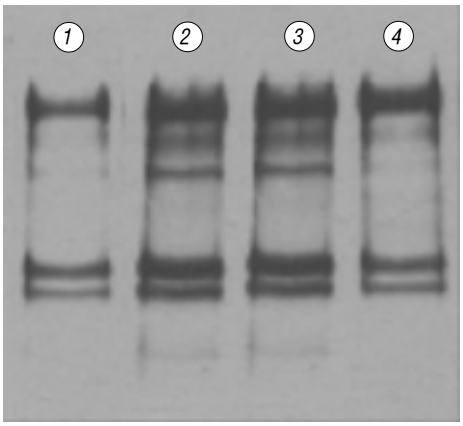

Fig. 3. Autophosphorylation of EGFr in the lysate of A431 cells. A431 were grown to 90% confluency, and were incubated in the serum–free medium for 30 min with: *1* — control, *2* — heat–killed *B. subtilis* cells, *3* — LPS, *4* — EGF

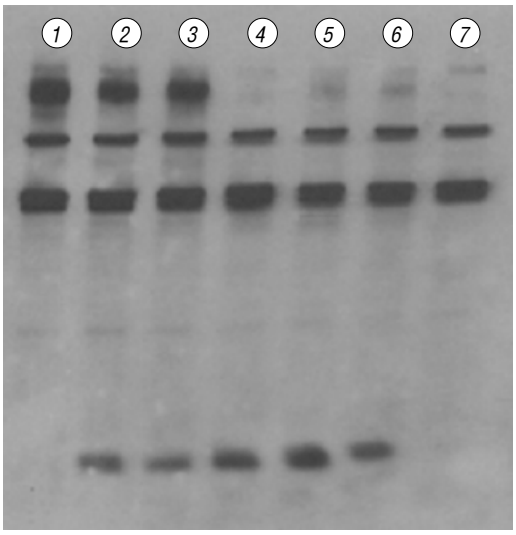

Fig. 4. Autophosphorylation of EGFr in the lysate of A431 cells. A431 were grown to 80% confluency, and were incubated in the serum–free medium for 24 h with heat–killed *B. subtilis* cells (lane *2*), LPS (lanes *3* and *4*), EGF (lane *5* and *6*). Probes *1, 2, 3, 6* were incubated with EGF for 10 min before removal of cells. *1, 7* — control probes

Expression of hBD–2 and EGFr mRNAs in human cervical and vulval tumors. The study of expression patterns of *hBD–2* and *EGFr* genes was carried out on 5 samples of resected tumors of human vulvae and 5 samples of resected cervical tumors in comparison with the samples of adjoining normal tissues. Our data, as well as previous ones [6], have demonstrated that the expression of *hBD–2* gene, which may be detected in 40–cycle RT–PCR, is characteristic only for cancerous tissues; this property of cervical and vulval tumors was found to be highly associated with the existence of HPV infection (Table). At the same time in the samples of normal tissues surrounding tumors no expression of hBD–2 mRNA was found in 40–cycle RT–PCR, as well as no HPV DNA was detected in 8 control samples from 10 samples of normal tissues tested in our research (Table). The study of resected tissues demonstrated also the significant overexpression of EGFr mRNA in the samples of cervical and vulval tumors in comparison with respective controls (Fig. 6). So the investigation of the surgical specimens demonstrated that the expression of β–defensin–2 is typical for tumor tissues of cervix and vulvae and is associated with the presense of HPV DNA and overexpression of EGFr mRNA.

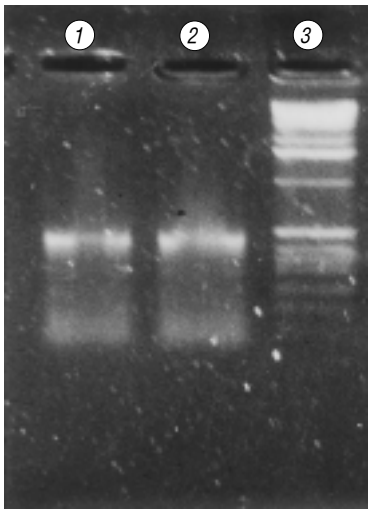

Fig. 5. The presense of HPV DNA detected by PCR in M–HeLa (lane *1*) and A431 cells (lane *2*). Lane *3* — molecular weight marker (1 kb ladder)

Earlier the fact was established that the overexpression of EGFr mRNA is typical for the different types of human carcinomas [14]. The newest data about mechanism of action of papillomaviruses may partially explain the mechanisms of EGFr overexpression in cervical cells. It was shown that major oncogenes of papillomaviruses (E6 and E7) are able to impair normal expression of EGFr in human keratinocytes and both contribute to increasing EGFr levels [15]. So, those results point to the possibility that EGFr overexpression

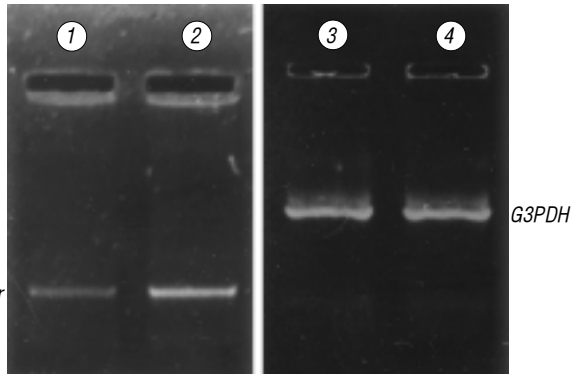

Fig. 6. Expression of EGFr mRNA in clinical specimen (Case 2). Lane *1* — normal tissue; lane *2* — tumor tissue. Expression of G3PDH mRNA in normal (lane *3*) and tumor (lane *4*) tissues, case 2

Table. Expression of hBD–2 and EGFr mRNAs and the presense of HPV DNA in clinical specimens, found by 40–cycle RT–PCR and 30–cycle PCR, respectively

Gene	Case 1		Case 4		Case 3		Case 4		Case 5		Case 6		Case 7		Case 8		Case 9		Case 10	
	N	T	N	T	N	T	N	T	N	T	N	T	N	T	N	T	N	T	N	T
hBD–2	–	+++	–	++	–	++	–	+++	–	+++	–	+++	–	++	–	++	–	+++	–	+++
EGFr1	+	+++	++	+++	+	++	++	+++	+	++	++	++	+	+++	++	+++	+	+++	+	++
HPV–L1	–	++	–	+++	+	+++	–	++	–	++	–	+++	–	+++	+	++	–	++	–	+++

N — normal tissue. T — tumor tissue.

in tumor cells of cervix or vulvae may be HPV–dependent.

The regulation of hBD–2 mRNA expression in tumor cells is far from being clear. Our data obtained *in vitro* show that in transformed cells of cervix and vulvae, which carry HPV DNA, hBD–2 mRNA is not expressed in the absence of bacteria or growth factor stimulation. Moreover, the expression of hBD–2 mRNA *in vitro* seems to be unrelated to the expression of HPV genes, but is somehow implicated in EGF–dependent signalling pathway and, possibly, in the regulation of EGFr phosphokinase activity. At the same time our research of clinical samples revealed the strong association between hBD–2 mRNA expression and presense of HPV DNA in tumor tissues. We tried to assess the levels of EGFr autophosphorylation in the surgical specimens but the results were unsucsessfull. The study of expression of hBD–2 and EGFr on the protein level, carried out on the surgical specimens with the use of respective monoclonal antibodies, is the appropriate tool for solving this problem; this study is underway now.

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