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HUMAN BETA–DEFENSIN 3 (hBD–3) EXPRESSION IN A431 CELL LINE AND HUMAN VULVAL TUMORS

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The present work was *aimed* on the study of human beta-defensin 3 (*hBD-3*) gene expression in A431 cell line and human vulval tumors. *Materials and methods*: Twenty surgical specimens of both malignant and conventionally normal human vulval tissues have been analyzed for the presence of hBD-3 mRNA by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). *In vitro* experiments have been carried out on A431 cells. *Results:* It has been shown that *hBD-3* gene expression in A431 cells was induced upon EGF stimulation. In human vulval tissues, in 8 cases the expression of *hBD-3* gene was registered only in tumor tissue and was absent in paired controls, and 7 cases were characterized by significant increase of *hBD-3* gene expression in malignant transformed epithelium in comparison with normal ones. Remaining surgical specimens showed either equal levels of *hBD-3* expression or even moderate elevation of *hBD-3* expression in normal tissue. In 2 specimens of tumor tissue hBD-3 mRNA was not observed. *Conclusion:* In human vulval epithelium, the increase in hBD-3 mRNA expression was predominantly associated with malignant phenotype.

***Key Words*: human beta-defensin 3, vulval tumor, malignant transformation, epidermal growth factor, A431 cell line, reverse transcription polymerase chain reaction.**

This work continues the study of possible involvement of human beta–defensins (hBDs) in the processes of malignant transformation. In previous investigations the expression of human beta–defensin–2 (hBD–2) in human vulval tumors has been demonstrated [1, 2]. According to the data [3] recently discovered human beta–defensin 3 (hBD–3) has similar regulatory mechanisms of expression as hBD–2 has. In particular, the expression of hBD–2 and –3 is induced by TNF–alpha, lipopolysaccharides, IL–1 and PMA [4, 5]. Additionally, it has been shown, that the expression of *hBD–2* gene can be induced upon activation of MAPK cascade by growth factors [6]. The resemblance in hBD–2 and –3 expression mechanisms lead us to examine hBD–3 expression in A431 cell line upon epidermal growth factor (EGF) stimulation and in human vulval tumors by semi–quantitative reverse transcription polymerase chain reaction (RT–PCR).

A431 cells were grown in RPMI 1640 medium (Gibco, USA) with 10% fethal bovine serum (Gibco, USA) at 37 °C to 70% confluency. Then the cells were incubated for 2 h in serum–free medium and stimulated with 3 μg/ml of EGF (Sigma, USA) for 6 h.

20 surgically resected samples of human vulval tumor tissue and pair–matched samples of conventionally normal vulval epithelium were obtained from the of Deparment of Oncogynecology, Institute of Oncology of AMS of Ukraine (Kyiv, Ukraine). All samples were immediately frozen in liquid nitrogen. According to histologic verification, the studied tumors (Table) were verified as squamous cell carcinoma.

Table. The expression of hBD–3 gene in surgical specimens of vulval carcinomas in comparison with adjacent normal tissues

Case	Age	TNM (stage)	Expression of *hBD–3* gene	
			T	N
1	57	$T_1 N_0 M_0$ (2)	–	+
2	55	$T_2 N_0 M_0$ (2)	+++	+
3	60	(4a)	++	–
4	62	$T_2 N_0 M_0$ (2)	+++	+
5	64	$T_3 N_2 M_0$ (4a)	+++	–
6	56	$T_3 N_1 M_1$ (4b)	+	++
7	69	$T_2 N_0 M_0$ (2)	++	+
8	50	$T_2 N_0 M_0$ (2)	++	+
9	47	$T_2 N_0 M_0$ (2)	++	–
10	45	$T_1 N_0 M_0$ (2)	+	+
11	52	$T_1 N_0 M_0$ (1)	++	–
12	64	$T_2 N_0 M_0$ (2)	++	+
13	67	$T_2 N_0 M_0$ (2)	++	+
14	48	$T_2 N_0 M_0$ (2)	+++	–
15	55	$T_2 N_1 M_0$ (2)	++	–
16	56	$T_3 N_2 M_0$ (4b)	–	+
17	60	$T_2 N_0 M_0$ (2)	+++	–
18	39	$T_1 N_0 M_0$ (1)	+	+
19	55	$T_2 N_0 M_0$ (2)	++	–
20	64	$T_3 N_0 M_0$ (2)	+++	+

Notes: T — tumor tissue, N — normal tissue.

Total RNA from A431 cells and tissue samples was isolated according to the method of Chomczynski, Sacchi [7] with modifications as described earlier [1] and RT–PCR with hBD–3 specific primers has been carried out as described below. The reaction of reverse transcription was conducted in RT buffer (Amplisense, Russia) with 1–2 μg of RNA, 200 units of M–MuLV–reverse transcriptase (Amplisense, Russia) and hBD–3 specific primers in total volume of 25 μl. RT–PCR with G3PDH–specific primers: 5′–TGCACCACCAACTGCTTAGC forward and 5′–GGCATGGACTGTGGTCATGAG reverse was used as a positive control of re–

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Abbreviations used: EGF — epidermal growth factor; hBD–3 — human beta–defensin 3; RT–PCR — reverse transcription polymerase chain reaction.

verse transcription reaction (Sigma, USA). HBD–3 specific polymerase chain reaction (PCR) primers were designed according to nucleotide database NCBI with the use of Oligo Program (Free Software) and had further sequences: 5′–CAGCGTGGGGTGAAGCCTAGCA forward and 5′– TTTCTTCGGCAGCATTTTCGGC reverse with the annealing temperature of 60 °C.

For polymerase chain reaction, 2 μl of reverse transcription mixture has been used. The PCR was conducted in the total volume of 25 μl with the use of Taq–polymerase (Amplisense, Russia). 35 cycles of Perkin Elmer Cetus ver. 2,2 amplificator at the temperature mode: 15 s — 95 °C, 15 s — 60 °C and 15 s — 72 °C has been chosen for hBD–3 cDNA amplification.

PCR results were analyzed by electrophoresis in 3% agarose gel. For futher verification hBD–3 cDNA was precipitated from the PCR mixture, dissolved in deionized water and then digested by specific restriction enzymes. The sites for restriction endonucleases on hBD–3 cDNA have been found with the use of DNAMAN ver. 1.0 program (Lynnon, Canada).

In total, for the hBD–3 mRNA expression 20 samples of human vulval tumor tissues in comparison with adjacent normal tissues has been analyzed (see Table). Our data showed the significant increase in the expression of *hBD–3* gene and its induction in vulval tumor samples in 7 and 8 cases respectively (Table, Fig.1, *c*). Two cases were characterized by equal levels of hBD–3 mRNA in tumors and normal tissues, 1 case — by increased hBD–3 mRNA level in normal tissue in comparison with the tumor, and 2 cases — by the absence of hBD–3 expression in malignant epithelium. So, in 75% of cases the expression of *hBD–3* gene was increased in vulval tumors in comparison with adjacent normal tissues.

As it was shown earlier, the expression of hBD–2 may be induced *in vitro* upon the action of EGF/TGF–alpha [2]. According to the literature data, vulval tumors are generally characterized by hyperexpression of growth factors [8], leading to activation of MAPK cascade and AP–1 transcription factor [9]. It has been shown, that hBD–3 gene contains putative AP–1 binding site at its 5′–promoter region [10]. To study whether the expression of *hBD–3* gene could be induced by EGF, we have analyzed hBD–3 mRNA expression in A431 cells upon action of EGF by RT–PCR. The results have demonstrated the presence of hBD–3 mRNA in EGF–stimulated cells and its absence in non–stimulated control (Fig. 2, *a*). The *hBD–3* gene fragment was verified by restriction analysis as indicated above (Fig. 2, *b*).

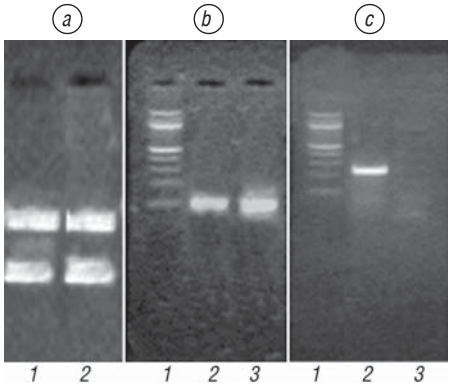

Fig. 1. Expression of hBD–3 mRNA in surgical specimens of vulval tumors in comparison with conventionally normal tissues. Electrophoretic analysis of total RNA from vulval tumor tissues (*a*, line *1*) and normal epithelium (*a*, line *2*). Expression of *G3PDH* gene 99 bases fragment in tumor (*b*, line *2*) and normal tissue (*b*, line *3*). Expression of *hBD–3* gene in tumor (*c*, line *2*) and normal tissue (*c*, line *3*). Lines *b*, *1*, *c, 1* correspond to 100 b ladder molecular weight standards (Biolabs, USA).

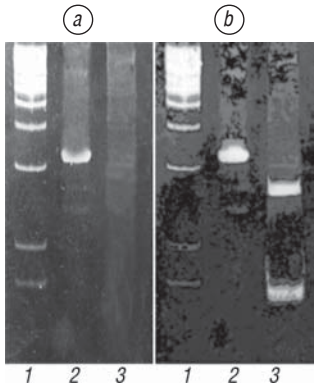

Fig. 2. The expression of *hBD–3* gene in A431 cell line (*a*). Lines *2* and *3* — expression of *hBD–3* gene in EGF–stimulated and non–stimulated A431 cell line respectively; line *1* — 200 b ladder molecular weight standards with MassRuler DNA ladder mix (Fermentas, Lietuva). Rectriction analysis of amplified hBD–3 cDNA fragment (*b*): line *1* — 200 b ladder molecular weight standards with MassRuler DNA ladder mix (Fermentas, Litva); line *2* — full–size *hBD–3* gene; line *3* — 70 bp and 150 bp fragments of *hBD–3* gene after restriction with PvuII.

One may conclude that the induction of hBD–3 mRNA expression in tumor tissues may be possibly caused by action of growth factors. From the other hand, malignant transformation of epithelial tissues is frequently accompanied by infection or inflammation processes [11], and the expression of hBD–3 mRNA may be induced by LPS or IL–1 [4]. Further studies on peculiarities of hBDs expression in human tumor tissues may shed a light on the involvement of antimicrobial peptides in oncogenesis.

REFERENCES

1. **Lisovskiy IL, Soldatkina MA, Lytvyn DI, Markeeva NV, Turchak OV, Nespryad'ko SV, Vinnitskaya AB, Nosach LN, Povnitsa OU, Pogrebnoy PV.** Pattern of beta-defensin 2 and EGFR mRNAs expression in cervical and vulvae cancer cells. Exp Oncol 2001; **23**: 248–52.

2. **Lisovskiy IL, Soldatkina MA, Lytvyn DI, Markeeva NV, Turchak OV, Nespryad'ko SV, Vinnitskaya AB, Pogrebnoy PV.** Expression of defensin genes in A431 and M-Hella cell lines and in tumors of human vulval and cervical carcinoma. Exp Oncol 2001; **23**: 119–22.

3. **Harder J, Bartels J, Christophers E, Schroder J.** Isolation and characterization of human β-defensin-3, a novel human inducible peptide antibiotic. J Biol Chem 2001; **276**: 5707–13.

4. **Lehrer RI, Ganz T.** Defensins of vertebrate animals. Curr Opin Immunol 2002; **14**: 96–102.

5. **Ganz T.** Defensins: antimicrobial peptides of vertebrates. C R Biol 2004; **327**: 539–49.

6. **Moon SK, Lee HY, Li JD, Nagura M, Kang SH, Chun YM, Linthicum FH, Ganz T, Andalibi A, Lim DJ.** Activation of a Src-dependent Raf-MEK1/2-ERK signa-

ling pathway is required for IL-1alpha-induced upregulation of beta-defensin 2 in human middle ear epithelial cells. Biochim Biophys Acta 2002; **1590**: 41–51.

7. **Chomczynski P, Sacchi N.** Single-step method of RNA isolation by acid guanidinium thiocianate-phenol-chloroform extraction. Anal Biochem 1987; **162**: 156–9.

8. **Johnson G, Mannel R, Khalifa M, Walker JM, Wren M, Min KW, Benbrook DM.** Epidermal growth factor receptor in vulval malignancies and its relationship to metastasis and patient survival. Gynecol Oncol 1997; **65**: 425–9.

9. **Karin M.** The regulation of AP-1 activity by mitogen-activated protein kinases. J Biol Chem 1995; **270**: 16483–6.

10. **Harder J, Meyer-Hoffert U, Wehkamp K, Schwichtenberg L, Schroder JM.** Differential gene induction of human beta-defensins (hBD-1, -2, -3, and -4) in keratinocytes is inhibited by retinoic acid. J Invest Dermatol 2004; **123**: 522–9.

11. **Couzin N.** New light on inflammation-tumor link. ScienceNOW 2004: **806**: 1–10.

ЭКСПРЕССИЯ ГЕНА БЕТА–ДЕФЕНСИНА 3 В ЛИНИИ КЛЕТОК А431 И В ЗЛОКАЧЕСТВЕННЫХ НОВООБРАЗОВАНИЯХ ВУЛЬВЫ ЧЕЛОВЕКА

Целью **данной работы является изучение экспрессии гена бета-дефенсина 3 в клеточной линии А431 и в злокачественных новообразованиях вульвы человека.** ***Материалы и методы:*** **была проанализирована экспрессия гена бета-дефенсина 3 методом полуколичественного ОТ-ПЦР в клетках линии А431, а также в 20 образцах опухолевой ткани вульвы человека в сравнении с условно-нормальными тканями вульвы.** ***Результаты:*** **было показано, что в клетках линии А431 экспрессия гена бета-дефенсина 3 индуцируется под воздействием эпидермального фактора роста. При анализе образцов тканей вульвы человека в 8 случаях экспрессия гена бета-дефенсина 3 была отмечена только в опухолевой ткани при ее отсутствии в соответствующих контролях, в 7 случаях отмечено значительное повышение экспрессии данного гена в опухоли в сравнении с нормальной тканью. В остальных случаях уровень экспрессии бета-дефенсина 3 был либо на одинаковом уровне в нормальной и опухолевой ткани, либо незначительно повышен в нетрансформированной ткани. В 2 случаях экспрессия гена бета-дефенсина 3 была отмечена лишь в образцах нормальной ткани.** ***Выводы:*** **в клетках линии А431 индуктором экспрессии гена бета-дефенсина 3 может выступать эпидермальный фактор роста. Злокачественные новообразования вульвы человека характеризуются повышенной экспрессией мРНК бета-дефенсина 3.**

Ключевые слова**: бета-дефенсин 3 человека (hBD-3), новообразования вульвы человека, злокачественная трансформация, эпидермальный фактор роста, линия клеток А431, ОТ-ПЦР.**