Exp Oncol 2014
36, 3, 174–178

Experimental ONCOLOGY

EXPRESSION OF HUMAN BETA-DEFENSINS-1–4 IN THYROID CANCER CELLS AND NEW INSIGHT ON BIOLOGIC ACTIVITY OF hBD-2 *IN VITRO*

*O.V. Zhuravel, O.L. Gerashchenko, M.R. Khetsuriani, M.A. Soldatkina, P.V. Pogrebnoy**
R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of NAS of Ukraine, Kyiv 03022, Ukraine

The study was *aimed* on analysis of human beta-defensin-1–4 (hBDs) mRNA expression in cultured thyroid cancer cells and evaluation of effects of recombinant hBD-2 (rec-hBD-2) on growth patterns, migration properties and expression of E-cadherin and vimentin in these cells. *Methods*: The study was performed on cultured follicular thyroid cancer WRO cells, papillary thyroid cancer TPC1 cells, and anaplastic thyroid cancer KTC-2 cells. For analysis of hBD-1–4 mRNA expression in thyroid cancer cells, semiquantitative RT-PCR was used. Effects of rec-hBD-2 on cell proliferation, viability, and migration were analyzed using direct cell counting, MTT test, and scratch assay respectively. Expression of vimentin and E-cadherin was evaluated by quantitative PCR (qPCR). *Results*: By the data of RT-PCR, all three studied thyroid cancer cell lines express hBD-1 and -4 mRNA, but not hBD-2 mRNA, while hBD-3 expression was detected in WRO and KTC-2 cells. The treatment of TPC-1, WRO, and KTC-2 cells with 100–1000 nM rec-hBD-2 resulted in significant concentration-dependent suppression of cell proliferation, viability, and migratory property. By the data of qPCR, significant up-regulation of vimentin expression was registered in KTC-2 and WRO cells treated with 500 nM rec-hBD-2. Significant down-regulation of E-cadherin expression ($p < 0.05$) was detected only in KTC-2 cells treated with the defensin. Also, it has been shown that TPC-1 cells treated with 500 nM rec-hBD-2 acquired more elongated morphology. *Conclusion*: The data demonstrate that hBD-2 in concentrations higher than 100 nM exerts significant concentration-dependent suppression of thyroid cancer cell growth and migration, and affects vimentin and E-cadherin expression dependent on histologic type of thyroid cancer cells.

***Key Words*: thyroid cancer, human beta-defensin-2, E-cadherin, vimentin, proliferation, viability.**

The incidence of thyroid cancer in Ukraine is constantly rising and in 2012 it comprises 1.8% of all cancers [1]. Thyroid carcinoma is represented by five major histological types — papillary carcinoma (more than 80% cases), follicular, medullary, poorly differentiated, and anaplastic carcinoma. These types of thyroid cancer have different prognosis, the course of the disease and require different treatment. At the same time, fundamental aspects of thyroid tumorigenesis share a number of common underlying molecular mechanisms, among which the process of epithelial-mesenchymal transition (EMT) has been recognized recently among important events which determine metastatic progression [2, 3]. Thyroid cancer cells are often used as model for investigation of EMT mechanisms and elements of EMT machinery.

EMT and reverse event — mesenchymal-epithelial transition (MET) represent recently discovered fundamental processes of epithelial cell plasticity control. During EMT epithelial cells acquire the special properties of mesenchymal cells: cell morphology is changed to a spindle shaped mesenchymal type, migratory and invasive properties of the cells are enhanced, and expression of some markers (in particular, vimentin and E-cadherin) is altered. During EMT, expression of E-cadherin, a cell-to-cell adhesion molecule, is down-regulated, while expression of structural protein vimentin is up-regulated.

It is accepted that EMT could occur in three different biologic settings — embryogenesis/organogenesis (type 1 EMT), tissue regeneration /wound repair (type 2 EMT), and cancer progression/metastasis (type 3 EMT) [4, 5]. It is supposed that all mentioned types of EMT represent different biologic processes but may have a lot in common in regard to underlying molecular events and elements. EMT machinery is under close investigation at present time due to its crucial role in tumor metastasis.

In the present study we have investigated a possible effect of human beta-defensin-2 (hBD-2) on expression of special EMT markers in thyroid cancer cells. hBD-2 is an inducible cationic peptide, a member of hBD family of antimicrobial peptides, involved in antimicrobial protection of human body and in wound healing processes [6–9]. In a number of studies it has been demonstrated that some hBDs, especially hBD-2, play an important role in wound repair affecting keratinocyte migration and proliferation and accelerating wound healing. Hypothetically, these antimicrobials could be involved in II type EMT, however, this hypothesis has not been analyzed yet. From the other hand, in *in vitro* setting it has been shown that hBD-2 and hBD-4 may regulate the growth of cultured human cancer cells via cell cycle control and affect their migratory properties and malignant potential in a concentration-dependent manner [10–12]. Also, deregulation of expression of some defensins in human tumors of different histogenesis has been shown, and possible implication of these antimicrobials in tumor development has been stated

Received: May 14, 2014.
*Correspondence: E-mail: pogrebnoy@onconet.kiev.ua
Abbreviations used: EMT – epithelial-mesenchymal transition; hBD – human beta-defensin; MET – mesenchymal-epithelial transition; qPCR – quantitative PCR; rec-hBD-2 – recombinant human beta-defensin-2; RT-PCR – reverse transcription polymerase chain reaction.

[13, 14]. Moreover, the latest studies demonstrated an association between expression of hBD-1 and in oral squamous cell carcinoma and migratory and invasive properties of OSCC cells, and suggested that hBD-1 expression in OSCC may be considered as prognostic marker in this type of cancer [15]. However, in regard to thyroid cancer, no data on expression and biological effects of hBDs have been reported so far.

Therefore, in the present study we have analyzed the profile of hBD-1–4 mRNA expression in three human thyroid cancer cell lines originating from papillary carcinoma (TPC-1 cell line), follicular carcinoma (WRO cell line), and anaplastic cancer (KTC-2 cell line). Also, we have studied an influence of recombinant hBD-2 (rec-hBD-2) on proliferation and viability of these cells, their morphology and migratory properties, and expression of important EMT markers — E-cadherin and vimentin. Taken together, our data demonstrate that hBD-2 not only controls the growth and migratory properties of human thyroid cancer cells but also significantly affects expression levels of EMT markers and alters cell morphology toward spindle-shaped phenotype.

MATERIALS AND METHODS

Cell lines. In the study, follicular thyroid cancer WRO cell line, papillary thyroid cancer TPC1 cell line, and anaplastic thyroid cancer KTC-2 cell line were used. The cells were cultured in DMEM culture medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin G sodium, 100 μg/mL streptomycin sulfate in humidified 5% CO_2 atmosphere at 37 °C.

Preparation of rec-hBD-2. To study the effect of exogenous defensin on cell growth, we used purified rec-hBD-2 [16]. In brief, *E.coli BL21(DE3)* cells transformed with GST-hBD-2-recombinant plasmid were induced with 1 mM IPTG for 6 h, pelleted by centrifugation, resuspended in lysis buffer (50 mM Tris-HCl, pH 7.6; 250 mM NaCl; 1% Triton X-100 and cocktail of protease and phosphatase inhibitors), and disrupted using ultrasound disintegrator (UD-11 Automatic, Poland). Then cell lysate was applied to affinity chromatography on glutathione-agarose column (GE Healthcare, Sweden) with following cleavage of the defensin from fusion protein by thrombin digestion. hBD-2 peptide was further purified by reverse phase chromatography on Sep-Pack C18 cartridge (Waters, USA), vacuum-dried, and re-dissolved in water. Protein concentration was determined by UV absorbance at 280 nm using spectrophotometer Nanodrop-1000 (USA).

Direct cell counting. To study the effect of rec-hBD-2 on cell proliferation, KTC-2, TPC-1, and WRO cells were routinely cultured in 24-well plates ($5 \cdot 10^4$ cells per well) to nearly 50% confluence, then culture medium was replaced with fresh DMEM supplemented with 2.5% FBS. Rec-hBD-2 was added into the medium in concentrations of 0.1; 1; 10; 100; 500; 1000 nM, followed by culturing for 48 h. After the treatment, cells were washed with PBS, detached with trypsin, and counted in hemocytometer. The percentage of dead cells was analyzed using trypan blue staining.

MTT assay. To evaluate the effect of rec-hBD-2 on cell viability, MTT-test has been applied [17]. Thyroid cancer cells were seeded into 96-well plates ($7 \cdot 10^3$ cells per well) and incubated with rec-hBD-2 at the concentration range from 100 pM to 1 μM in DMEM supplemented with 2.5% FBS for 48 h. Then cells were routinely treated with MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) according to standard protocol, and colorimetric reaction was evaluated with the use of ELISA reader (Awareness Technology Inc, USA) at $\lambda = 545$.

Scratch wound healing assay. An effect of rec-hBD-2 on mobility of cultured human thyroid cancer cells was performed by scratch wound healing assay [18]. The cells were seeded in 6-well plates ($1.5 \cdot 10^5$ cells per well) and routinely grown till 80% confluence, than a scratch was performed using a tip, and the cells were grown for next 24 h in the presence of 500 or 1000 nM rec-hBD-2 in serum free medium. Then the cell migration into wound area was examined using inverted microscope Axioplan (Zeiss, Germany).

RT-PCR analysis of hBD-1–4 mRNA expression in thyroid cancer cells. To evaluate the hBD-1–4 mRNA expression in thyroid cancer cells, total RNA was extracted from KTC-2, WRO and TPC-1 cell lysates using Trizol RNA Isolation Kit (Neogene, Russian Federation) according to manufacturer's protocol. For detection of hBD-1–4 mRNA expression, semiquantitative RT-PCR analysis was performed using specific primers (Table). The expression level of beta-actin (the house-keeping gene) served as a loading control. The products of RT-PCR were routinely analyzed by electrophoresis in agarose gel.

Table. Primers for the genes of interest

Gene	Primers
DEFB1 (hBD-1)	F: TGTTGCCTGCCAGTCGCCATGAG
	R: TCACTTGCAGCACTTGGCCTTCCC
DEFB4 (hBD-2)	F: 5′-GAAGCTCCCAGCCATCAGCC
	R: 5′-GTCGCACGTCTCTGATGAGGGA
DEFB103 (hBD-3)	F: CCTGTTTTTGGTGCCTGTTCC
	R: CTTTCTTCGGCAGCATTTTCG
DEFB104 (hBD-4)	F: GAAGCTCCCAGCCATCAGCC
	R: GTCGCACGTCTCTGATGAGGGA
Beta-actin	F: CTGGAACGGTGAAGGTGACA
	R: AAGGGACTTCTTGTAACAATGCA

Quantitative PCR analysis of vimentin and E-cadherin expression in thyroid cancer cells. Expression of vimentin and E-cadherin in KTC-2, WRO and TPC-1 cells treated with 500 nM rec-hBD-2 for 24 h, was analyzed using qPCR. Total RNA was extracted from cell lysates using Trizol RNA Isolation Kit (Neogene, Russian Federation) according to manufacturer's protocol. 2 μg of RNA were reverse transcribed using an M-MLV Reverse Transcriptase, Ribonuclease Inhibitor Ribolock (Thermo Scientific Inc., USA) and oligo(dT)18 primer. Expression levels of vimentin and E-cadherin genes were analyzed by qPCR on ABI 7500 Fast real-time PCR system (Applied Biosystems, USA) using 0.2 μM of each primer and SYBR Green master mix (Thermo Scientific Inc., USA) and 1 μl cDNA. The following conditions of reaction were used: 10 min 95 °C initial denaturation; 40 cycles15 s 95 °C denaturation, 60 s 60 °C primer annealing/elongation. The fluorescence was recorded

during the annealing/elongation step in each cycle. A melting curve analysis was performed at the end of each PCR by gradually increasing the temperature from 60 to 95 °C while recording the fluorescence. A single peak at the melting temperature of the PCR-product confirmed primer specificity. The β_2-microglobulin was used as a reference gene to standardize the level of other transcripts. Following primers were used for vimentin: F — 5′-GTGAATACCAAGACCTGCTCAA-3′, R — 5`-AGGGAGGAAAAGTTTGGAAGAG-3′; for E-cadherin: F — 5′- CTGGTTCAGATCAAATCCAACA-3′, R — 5′-CTTCAGCCATCCTGTTTCTCTT-3′ and β_2-microglobulin: F — 5′-CCGTGTGAACCATGTGACTTTGTC-3′, R — 5′-TGCGGCATCTTCAAACCTCCATGATG-3′. The relative amounts of the transcripts were first normalized to the reference gene and then normalized to the gene expression level in the untreated samples according to the $2^{-\Delta\Delta Ct}$ method [19], statistical analysis was performed using statistical application of Microsoft Excel software based on *t*-test with $p < 0.05$ representing significance.

Statistical analysis. Data on direct cell counting and MTT data were reported as the mean ± SD of values obtained from three independent experiments and analyzed by Student's *t*-test to assess the statistical significance of the differences between the groups. A statistically significant difference was considered at $p < 0.05$.

RESULTS AND DISCUSSION

Expression of hBD-1–4 mRNA in human thyroid cancer cells. Semiquantitative RT-PCR analysis of expression of hBD-1–4 mRNA in human papillary carcinoma TPC-1 cells, follicular carcinoma WRO cells, and anaplastic carcinoma KTC-2 cells has revealed the presence of mRNA for hBD-1 and -4 and no detectable hBD-2 expression in all cell lines. hBD-3 expression was registered in 2 from 3 cell lines (WRO and KTC-2 cells). WRO cells were characterized by high expression of hBD-3 while TPC1 — by high expression of hBD-4 (Fig. 1). So, thyroid cancer cells of different histological type express beta-defensins-1,-3 and -4, but not hBD-2. To our knowledge, it is the first report on hBDs expression in thyroid cancer cells. Up-to-date, just a single study reported on low-level expression of hBD-4 mRNA in normal thyroid gland [20].

Effect of rec-hBD-2 on proliferation and viability of human thyroid cancer cells in vitro. We have analyzed an effect of rec-hBD-2 on proliferation and viability of TPC-1, WRO, and KTC-2 cells. As it has been shown using direct cell counting technique (Fig. 2), in concentrations higher than 100 nM rec-hBD-2 exerted concentration-dependent growth suppression of the thyroid cancer cells, especially KTC-2 cells, and in concentrations lower than 100 nM exerts no significant influence on cell counts (see Fig. 2).

As it has been shown by MTT assay, viability of cultured TPC-1, WRO, and KTC-2 cells is affected by rec-hBD-2 in a similar concentration-dependent manner (Fig. 3). Our data have shown that in concentrations lower than 100 nM rec-hBD-2 had no significant effect on viability of TPC-1 and WRO cells and stimulated viability of KTC-2 cells at 100 pM concentration (see Fig. 3). In all three cell lines significant suppression of cell viability in the presence of rec-hBD-2 in concentrations higher than 100 nM has been registered. No viable cells have been revealed after treatment of the cells with 1 μM rec-hBD-2.

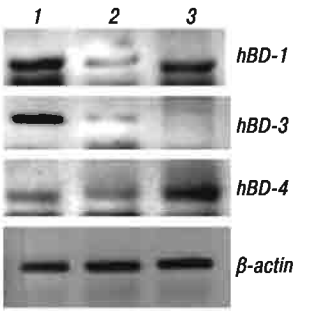

Fig. 1. RT-PCR analysis of hBDs mRNA expression in thyroid cancer cells: 1 — WRO; 2 — KTC-2; 3 — TPC1. *β-actin* is used as a house-keeping gene

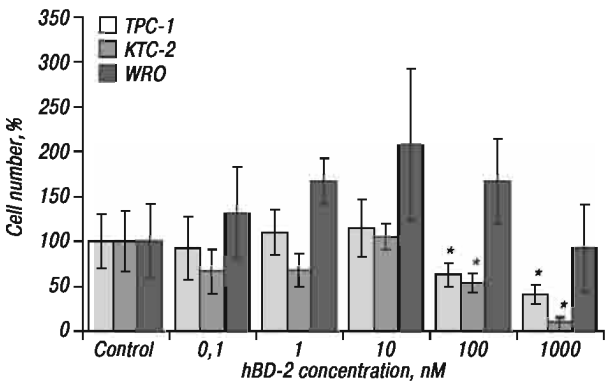

Fig. 2. A concentration-dependent effect of rec-hBD-2 on the number of viable cultured cells of TPC-1, KTC-2, and WRO lines. The number of attached cells was evaluated by direct cell counting. The data of three independent experiments are presented as the mean ± SD. *The difference is significant as compared to appropriate control ($p < 0.05$)

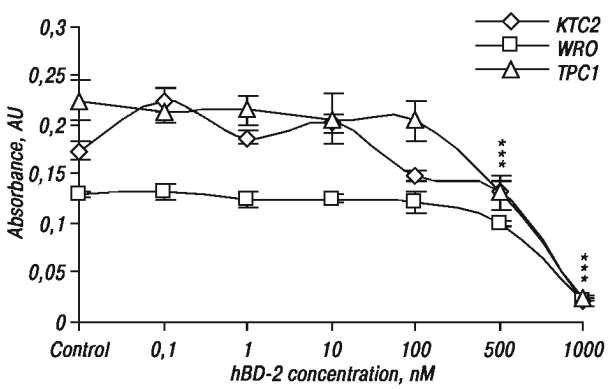

Fig. 3. A concentration-dependent effect of rec-hBD-2 on viability of TPC-1, WRO, and KTC-2 cells (MTT analysis). The data of three independent experiments are presented as the mean ± SD.*$p < 0.05$

The data on the influence of rec-hBD-2 on proliferation and viability of thyroid cancer cells are in accordance with the reported earlier results on a concentration-dependent growth suppressive effects of this defensin toward cancer cells of other histological types (such as carcinoma cells of A431 and A549 lines, cultured human melanoma cells [10, 12]). As it was reported, growth suppression caused by hBD-2 *in vi-*

tro is exerted via cell cycle arrest at G1/S checkpoint, down-regulation of cyclin D1 expression and activation of pRB [10, 12]. In the present research we did not study cell cycle distribution of thyroid cancer cells treated with rec-hBD-2; we suppose that the mechanism of hBD-2 dependent growth suppression in thyroid cancer cells may be similarly exerted via blockage of cell cycle. Here, we have focused our attention on other effects of rec-hBD-2 in growth-inhibitory concentrations toward cultured thyroid cancer cells, in particular, its effects on morphology and migration ability of these cells, and on expression of common EMT markers — vimentin and E-cadherin.

Effect of rec-hBD-2 on thyroid cancer cell migration and morphology. To analyze a possible effect of rec-hBD-2 on cancer cell migration, wound healing assay has been applied. It was revealed that 100 nM and 500 nM of rec-hBD-2 significantly suppressed migration of TPC-1 cells (Fig. 4) as well as WRO and KTC-2 (data not shown). Also, we have noted an altered morphology of TPC-1 cells after 48 h treatment with 500 nM or 1000 nM rec-hBD-2 (Fig. 5). TPC-1 cells treated with rec-hBD-2 acquire more elongated fibroblast-like shape compared to the untreated control cells. No notable morphological changes in WRO and KTC-2 cells treated with 500 nM or 1000 nM rec-hBD-2 were observed (data not shown).

From the reports of other authors it is known that some human defensins may affect cancer cell migration, in particular, hBD-1 suppresses migration and invasion of oral squamous cell carcinoma cells [15], hBD-3 suppresses head and neck cancer cell migration [21], hBD-4 may stimulate or inhibit migration of A431 and TPC-1 cells in scratch assay dependent on its concentration [11]. However, little is known yet about possible impact of defensins as peptides affecting cell migratory capability *in vivo* as well as the mechanisms of defensin-dependent suppression of cell migratory properties. In our study, we detected not only hBD-2 dependent suppression of thyroid cancer cell migration, but also alterations in TPC-1 cell morphology toward more elongated mesenchymal phenotype. Therefore, in the last part of our study we have studied the effects of rec-hBD-2 on expression levels of EMT markers in thyroid cancer cells *in vitro*.

Expression of vimentin and E-cadherin in thyroid cancer cells treated with rec-hBD-2. The study of expression of two common EMT markers — vimentin and E-cadherin — has been carried out using qPCR analysis in TPC-1, KTC-2 and WRO cells treated with 500 nM rec-hBD-2 for 24 h. It has been found out that in KTC-2 cells treated with the defensin, expression level of vimentin was significantly higher, and E-cadherin — notably lower than in untreated control cells by 3.39 ± 0.19 and 0.59 ± 0.034 times, respectively ($p < 0.05$) (Fig. 6). In WRO cells, treatment with defensin led to significant up-regulation of vimentin expression by 3.07 ± 0.13 times, and had a tendency for down-regulation of E-cadherin expression level (Fig. 6). In TPC-1 cells vimentin level was unaffected by rec-hBD-2 treatment while E-cadherin expression is undetectable in this cell line (Fig. 6).

To our knowledge, here we present the first evidence on effects of hBD-2 on vimentin and E-cadherin expression in human cancer cells. Up-to-date, a recent study has reported on effects of synthetic human alpha-defensin-5 (HD-5), antimicrobial peptide produced by the Paneth cells on expression of E-cadherin in squamous cell line Het-1A *in vitro* [22]. Using immunocytochemistry, flow cytometry, and Western blotting, the authors showed that the E-cadherin expression was down-regulated in HD-5 treated cells and suppose that HD-5 produced by metaplastic Paneth cells may be involved in the development of Barrett's esophagus due to its ability to decrease E-cadherin expression [22].

In conclusion, the data of present research demonstrate that hBD-2 in concentrations higher than

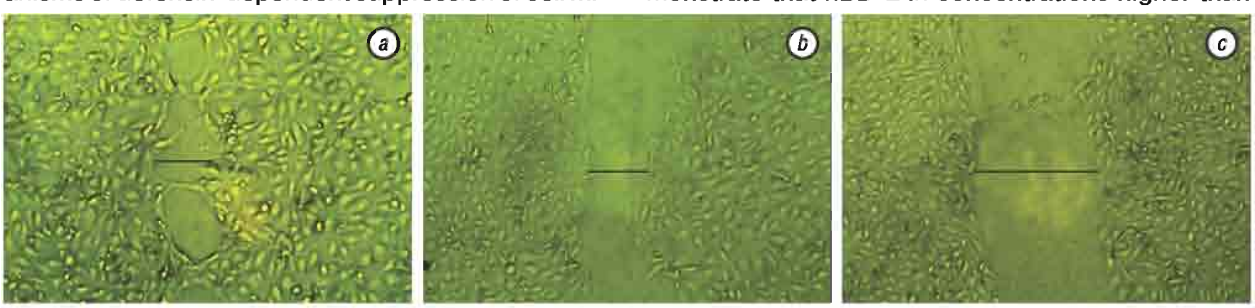

Fig. 4. Wound healing assay performed in TPC-1 cells treated with 100 nM (*b*) or 500 nM (*c*) rec-hBD-2 for 48 h. *a* — control untreated cells. The results of a typical experiment are presented. Magnification ×400

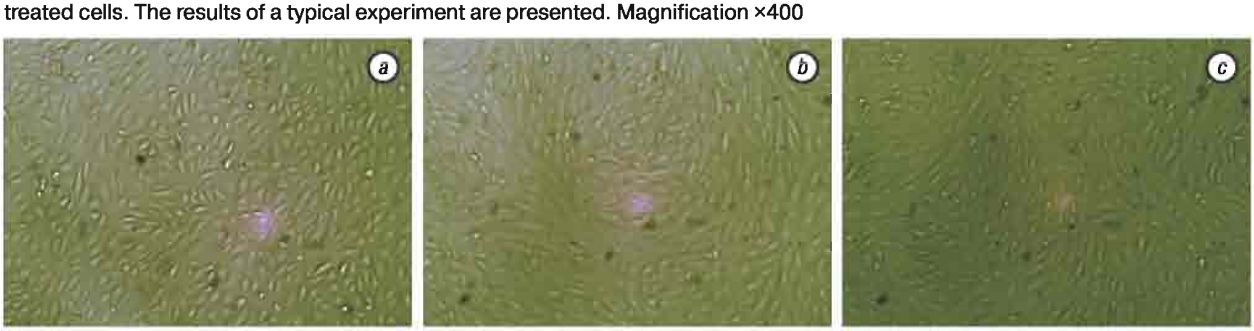

Fig. 5. Morphology of TPC1 cells treated with 500 nM rec-hBD2 (*b*) or 1000 nM rec-hBD2 (*c*) for 48 h. *a* — control cells. Magnification ×400

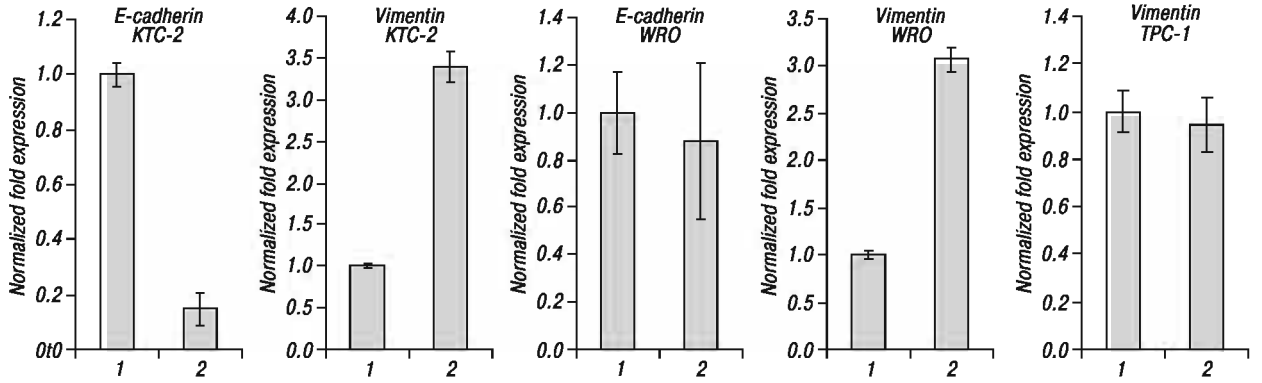

Fig. 6. Relative expression of vimentin and E-cadherin mRNA in KTC-2, WRO, TPC-1 cells treated with 500 nM rec-hBD-2 for 24 h (2) (qPCR data) compared to untreated cells (1). The β_2-microglobulin was used as a reference gene to standardize the level of other transcripts. The relative amounts of the transcripts were first normalized to the reference gene and then normalized to the gene expression level in the un-treated samples according to the $2^{-\Delta\Delta Ct}$ method

100 nM exerts an inhibitory effect on cell growth and migration of three thyroid cancer cell lines. At the same time effects of hBD-2 on expression of vimentin and E-cadherin strongly depended on thyroid cancer cell type. The most prominent up-regulation of vimentin expression and down-regulation of E-cadherin expression were observed in anaplastic thyroid cancer cells, and at lesser extent in follicular thyroid cancer cells while in papillary thyroid cancer cells vimentin expression seems to be unaffected by hBD-2 treatment, and acquired elongated morphology. Taken together, these data point on effects of hBD-2 toward some important EMT markers and gain new insight on the biological activity of this defensin toward cancer cells.

ACKNOWLEDGEMENT

This work was in part supported with grant 0110U005758 of National Academy of Sciences of Ukraine "Fundamental Basis of Molecular and Cellular Biotechnologies".

REFERENCES

1. Cancer in Ukraine. Bull Nat Cancer Register of Ukraine. ZP Fedorenko et al., eds. Kyiv 2013: 62–3.

2. **Hombach-Klonisch S, Natarajan S, Thanasupawat T, *et al.*** Mechanisms of therapeutic resistance in cancer (stem) cells with emphasis on thyroid cancer cells. Front Endocrinol (Lausanne) 2014; **5**: 37.

3. **Hardin H, Montemayor C, Lloyd RV.** Thyrod cancer stem-like cells and epithelial-mesenchymal transition in thyroid cancers. Hum Pathol 2013; **44**: 1707–13.

4. **Kalluri R, Weinberg RA.** The basics of epithelial-mesenchymal transition. J Clin Invest 2009; **119**: 1420–28.

5. **Talbot LJ, Bhattacharya SD, Kuo PC.** Epithelial-mesenchymal transition, the tumor microenvironment, and metastatic behavior of epithelial malignancies. Int J Mol Biol 2012; **3**: 117–36.

6. **Niyonsaba F, Ushio H, Nakano N, *et al.*** Antimicrobial peptides human beta-defensins stimulate epidermal keratinocyte migration, proliferation and production of proinflammatory cytokines and chemokines. J Invest Dermatol 2007; **127**: 594–604.

7. **Dressel S, Harder J, Cordes J, *et al.*** Differential expression of antimicrobial peptides in margins of chronic wounds. Exp Dermatol 2010; **19**: 628–32.

8. **Hirsch T, Spielmann M, Zuhaili B, *et al.*** Human beta-defensin-3 promotes wound healing in infected diabetic wounds. Gene Med 2009; **11**: 220–8.

9. **Kesting MR, Stoeckelhuber M, Hölzle F, *et al.*** Expression of antimicrobial peptides in cutaneous infections after skin surgery. Br J Dermatol 2010; **163**: 121–7.

10. **Zhuravel E, Shestakova T, Efanova O, *et al.*** Human beta-defensin-2 controls cell cycle in malignant epithelial cells: in vitro study. Exp Oncol 2011; **33**: 114–21.

11. **Gerashchenko OL, Zhuravel EV, Skachkova OV, *et al.*** Biologic activities of recombinant human-beta-defensin-4 toward cultured human cancer cells. Exp Oncol 2013; **35**: 84–91.

12. **Gerashchenko O, Zhuravel E, Skachkova O, *et al.*** Involvement of human beta-defensin-2 in regulation of malignant potential of cultured human melanoma cells. Exp Oncol 2014; **36**: 17–23.

13. **Droin N, Hendrab J-B, Ducoroyb P, *et al.*** Human defensins as cancer biomarkers and antitumour molecules. J Proteomics 2009; **72**: 918–27.

14. **Prado-Montes de Oca E.** Human beta-defensin 1: a restless warrior against allergies, infections and cancer. Int J Biochem Cell Biol 2010; **42**: 800–4.

15. **Han Q, Wang R, Sun C, *et al.*** Human beta-defensin-1 suppresses tumor migration and invasion and is an independent predictor for survival of oral squamous cell carcinoma patients. PLoS One 2014; **9**: e91867.

16. **Lisovskiy IL, Markeeva NV, Shnitsar VM, *et al.*** Production of recombinant of hBD-2 — human antimicrobial peptide expressed in cervical and vulval cancer. Exp Oncol 2003; **25**: 36–9.

17. **Mosman T.** Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Immunol Methods 1983; **65**: 55–63.

18. **Lampugnani MG.** Cell migration into a wounded area *in vitro*. Methods Mol Biol 1999; **96**: 177–82.

19. **Pfaffl MW.** A new mathematical model for relative quantification in real-time RT–PCR. Nucl Acids Res Volume 2001; **29**: e45.

20. **García JR, Krause A, Schulz S, *et al.*** Human beta-defensin 4: a novel inducible peptide with a specific salt-sensitive spectrum of antimicrobial activity. FASEB J 2001; **15**: 1819–21.

21. **Wang K, Wang JH, Baskaran H, *et al.*** Effect of human beta-defensin-3 on head and neck cancer cell migration using micro-fabricated cell islands. Head Neck Oncol 2012; **4**: 41.

22. **Nomura Y, Tanabe H, Moriichi K, *et al.*** Reduction of E-cadherin by human defensin-5 in esophageal squamous cells. Biochem Biophys Res Commun 2013; **439**: 71–7.

Copyright © Experimental Oncology, 2014