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.
[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, 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% CO2 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, TPC-2, TPC-1, and WRO cells were routinely cultured in 24-well plates (5 • 104 cells per well) to nearly 50% confluence, then 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).

**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 • 103 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 λ = 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 • 105 cells per well) and routinely grown till 80% confluence, then 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).

**Q-PCR analysis of hBD-1–4 mRNA expression in thyroid cancer cells.** To evaluate the effect of exogenous recombinant human defensins on cell proliferation, KTC-2, WRO and TPC-1 cell lines were used. The cells were grown to nearly 50% confluence, then treated 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 to 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).

**RT-PCR analysis of hBD-1–4 mRNA expression in thyroid cancer cells.** To evaluate the effect of exogenous recombinant human defensins on cell proliferation, KTC-2, WRO and TPC-1 cell lines were used. The cells were grown to nearly 50% confluence, then treated 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 to 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).

**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 cell lines was studied. The cells were grown to nearly 50% confluence, then treated 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 to 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).

**Quantitative PCR analysis of the genes of interest**

- **DEFB1 (hBD-1):** F: TCGTGGCCGGCCATTGCACGAG R: TACCTCGCAACATGTCGCCATCCC
- **DEFB4 (hBD-2):** F: 5´-GAAGCTCCAGCCACAGCCAGGCA R: 5´-GTCGCACGTCTCTGATGAGGGA
- **DEFB103 (hBD-3):** F: CTTTITTTGGTGGCCTTGTC R: CGTACCACGTCTGATAGGGA
- **DEFB104 (hBD-4):** F: GAATGCCAGCCACAGCCACAGGCA R: GTCGACACGTCTGATAGGGA
- **Beta-actin:** F: CTGGGACGGTGAAGGTGGACA R: AAGGGACCTCTGTGAAACTGGA

**Table.** Primes for the genes of interest

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEFB1 (hBD-1)</td>
<td>F: TCGTGGCCGGCCATTGCACGAG R: TACCTCGCAACATGTCGCCATCCC</td>
</tr>
<tr>
<td>DEFB4 (hBD-2)</td>
<td>F: 5´-GAAGCTCCAGCCACAGCCAGGCA R: 5´-GTCGCACGTCTCTGATGAGGGA</td>
</tr>
<tr>
<td>DEFB103 (hBD-3)</td>
<td>F: CTTTITTTGGTGGCCTTGTC R: CGTACCACGTCTGATAGGGA</td>
</tr>
<tr>
<td>DEFB104 (hBD-4)</td>
<td>F: GAATGCCAGCCACAGCCACAGGCA R: GTCGACACGTCTGATAGGGA</td>
</tr>
<tr>
<td>Beta-actin</td>
<td>F: CTGGGACGGTGAAGGTGGACA R: AAGGGACCTCTGTGAAACTGGA</td>
</tr>
</tbody>
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significant difference was considered at
of the differences between the groups. A statistically
by Student’s
obtained from three independent experiments and analyzed
MTT data were reported as the mean ± SD of values ob-
0.05 representing significance.

Statistical analysis. Data on direct cell counting and
MTT data were reported as the mean ± SD of values ob-
tained 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 thy-
roid 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 detec-
table hBD-2 expression in all cell lines. hBD-3 expres-
sion was registered in 2 from 3 cell lines (WRO and
KTC-2 cells), WRO cells were characterized by high
expression of hBD-3 while TPC-1 — 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 expres-
sion 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 sig-
nificant effect on viability of TPC-1 and WRO cells and
stimulated viability of KTC-2 cells at 100 pM concen-
tration (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 vi-
ability 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
trosis 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

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**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
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 depend 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.

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