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DETERMINATION OF SURVIVIN AND PROTEIN KINASE AKT IN PAPILLARY THYROID CARCINOMA AND METASTASIS TISSUES*

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Survivin is an evolutionarily conserved eukaryotic protein, a member of Inhibitors of Apoptosis proteins (IAP) family, that is essential for cell division and can inhibit cell death. Normally it is expressed only in actively proliferating cells, and is upregulated in most cancers; consequently, it has received significant attention as a potential oncotherapeutic target [1].

Unlike other IAPs, survivin possesses a unique structure, a single BIR domain and an α-helical folding of the C-terminal region [2]. In addition to the full length wild-type transcript of survivin, twenty variants originating from alternative splicing have been described [3]. Survivin is overexpressed in a large number of cancers, including melanoma, glioma, squamous cell carcinoma, ovarian, breast, thyroid, lung, prostate, gastric and hematological cancers. Survivin overexpression has been observed in several types of endocrine cancers, including differentiated and medullary thyroid cancer [2, 4–7].

Akt is a central signalling molecule of the PI3K/Akt signalling pathway. It is a serine/threonine-specific protein kinase and involved in apoptosis, proliferation, transcription and cells migration [8]. Aberrant activation of this pathway has been identified in a wide range of thyroid cancers. Several studies have revealed that Akt actively engages with the migratory process in motile cells, including metastatic cancer cells [9].

Survivin is generally modulated by cancer pro-survival cell signaling pathways, such as PI3K/Akt, in several cancer contexts. Experimental evidences report that some Akt inhibitors (trastuzumab) not only modulate expression of this signaling pathway but also downstream survivin expression [2].

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The **aim** of the study was to compare the levels of survivin expression and protein kinase Akt activation in tissue samples of goiter, papillary thyroid carcinoma (PTC), metastases, and conditionally normal tissue.

MATERIALS AND METHODS

Postoperative samples of tumor tissue, metastasis, and conditionally normal (non-tumor, histologically unchanged) tissue, obtained from the surgical department of the Institute's clinic, were used for the study. The research protocol was approved by the Ethics Committee of the Institute (protocol #26-KE, April 10, 2019). All patients signed informed consent for further diagnostic and scientific research on their biomaterials.

Quantitative PCR (qPCR) [10].

Instruments, Reagents and Analysis. Reactions were carried out on the following instruments: thermocycler 2720 (Applied Biosystems, USA) and qTower 3 84 G (Analytik Jena, Germany). Data were analysed using instrument software, Microsoft Excel and GraphPad PRISM 10.

RNA extraction. All assays were carried out using RNA extracted from anonymised patient samples. RNA was isolated with TRIzol reagent [11]. Total RNA samples were washed with 75% ethanol, dried and resuspended in 20–50 μL of RNase-free water.

All oligonucleotides were resuspended in sterile RNase-free water at 100 μM and stored in aliquots at −20°C.

RT-qPCR Reactions. RNA was reverse transcribed using component RevertAid RT Kit (ThermoScientific, #K1691). A mixture of 2 μg RNA and 1 μL 100 μM Oligo (dT)18 primer was incubated at 65°C for 5 min, then chilled on ice. Te following components in the indicated order were added, 4 μL 5x Reaction buffer, 1 μL RiboLock RNAse Inhibitor (20 U/μL), 2 μL 10mM dNTP Mix, 1 μL RevertAid RT (200 U/μL). The tubes were transferred to a thermocycler (Applied Biosystem 2720, USA) with the heated lid set to 112°C and incubated using the following protocol: 42°C for 1 h, 70°C for 5 min. 1 μL cDNA aliquots were used for further qPCR analysis.

qPCR was done using SYBR Green master mix (Thermo Scientific, #K0251). Gene expressions were normalized to ß-actin, and fold differences were calculated using the comparative CT method: $2^{-(\Delta\Delta CT)}$, where ΔΔCT refers to (normalized tumor/metastasis sample) — (normalized control (norm) sample). The cDNA (1 μL) was added to reaction mix, containing 12.5 μL Maxima SYBR Green qPCR Master Mix (2X), primers — 0.3 μM of each and nuclease-free water to 25 μL. Each of reaction mixtures were pipetted into 3 wells of qPCR plate. Plate was spun for 5 min at 2000 rpm. qPCR reactions were carried out for 40 cycles with 15 s denaturation at 95°C and 1 min polymerisation at 60°C.

Western Blotting was conducted in accordance with recommendations [12]. Snap-frozen tissue specimens were homogenized with using tissue grinder kit in lysis buffer (25 mM Tris-HCl pH 7.4, 1% Triton X100, 150 mM NaCl, 0.1% SDS, 1 mM EDTA, 1% Sodium deoxycholate) with 50 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF and Protease Inhibitor Cocktail Complete Mini (Roche). Lysate aliquots were mixed with Laemmli buffer (30% glycerol, 3% SDS, 125 mM Tris-HCl, pH 6.8), resolved by 12% SDS-PAGE, and transferred to nitrocellulose membrane, stained with Ponceau S, blocked with TBST (150 mM NaCl, 50 mM Tris-HCl, Tween 20, pH 7.4) containing 5% BSA or 5% non-fat dried milk and incubated with primary antibodies diluted in 5% BSA in TBST. After washing and incubation with horseradish peroxidase-coupled secondary antibodies, detection was performed with Western Blotting Luminol Reagent (Santa Cruz, sc-2048).

Table 1

Primers used

β-actin	Forward: 5′-GAA-ATCGTG-CGTGACATTAA-3′; Revers: 5′-CCA-GAC-AGC-ACT-GTG-TTG-G-3′
Survivin	Forward: 5′-CTTTCTCAAGGACCACCGCATC-3′, Revers: 5′-CAATCCATGGCAGCCAGCTGC-3′

Protein bands detected by Western blotting were scanned in the Gel-Pro analyzer program 4.0 by IOD indicator — integrated optical density of each track. Unit of measurement — conventional optical unit.

Antibodies. The following primary antibodies were used: rabbit monoclonal antibody against phospho-Akt (Ser473) (Cell signaling, #4060S); mouse monoclonal antibody conjugated to horseradish peroxidase (HRP) (Abcam, ab20272). All primary antibodies were used at dilution 1:1000. Secondary goat anti-rabbit antibodies conjugated to HRP (Cell signaling, #7074S) were used at dilution 1:3500.

Statistical analysis and data presentation were performed using Origin 2019b software. The results of the study are presented as M ± SE. Student's *t*-test was used to compare data groups. Values of $p \leq 0.05$ were considered significant.

RESULTS AND THEIR DISCUSSION

Housekeeping proteins are essential proteins involved in basic, fundamental cellular functions, like metabolism and cell structure, and are used as internal controls for Western blot analysis to normalize data and account for variations in sample loading. The ß-actin was used as housekeeping protein in this study.

Melt curve analysis is a crucial quality control step in real-time PCR (qPCR) to ensure the amplification of a single, specific product, which is essential for accurate gene expression quantification. Melting curves for ß-actin and survivin show a single peak, indicating that the primers are specific and only one PCR product

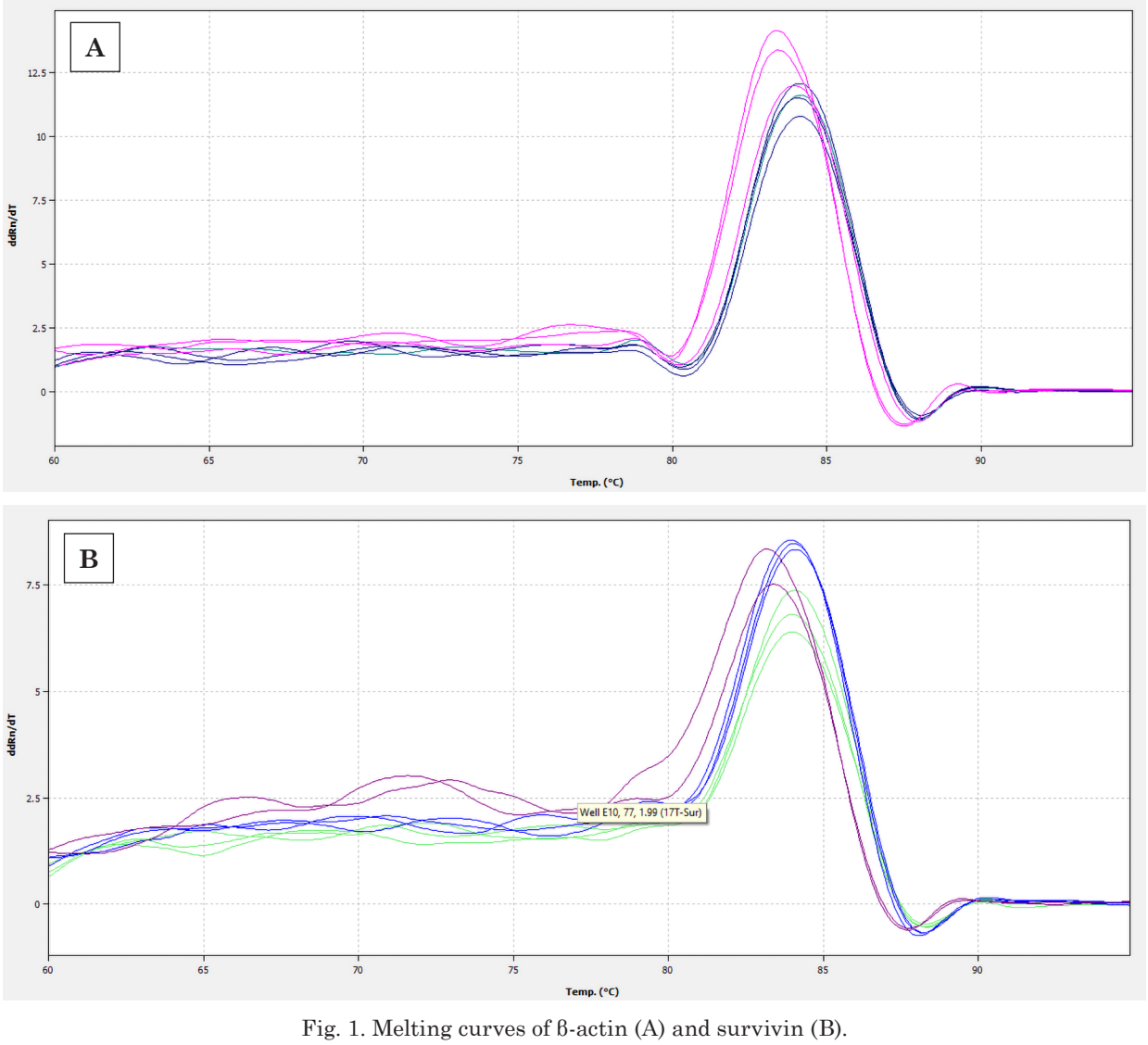

Fig. 1. Melting curves of ß-actin (A) and survivin (B).

is being amplified. The melting temperature of ß-actin amplification product lies between 83 and 84°C (Fig. 1A) and survivin melting temperature lies around 84°C (Fig. 1B).

The study included patients with PTC without metastasis, PTC with metastasis and goiters. Group 1 included patients with goiter (G), group 2 — with multinodular goiter (MNG), group 3 — PTC without metastasis, group 4 — PTC with metastasis, group, 5 — metastasis tissue (Fig. 2).

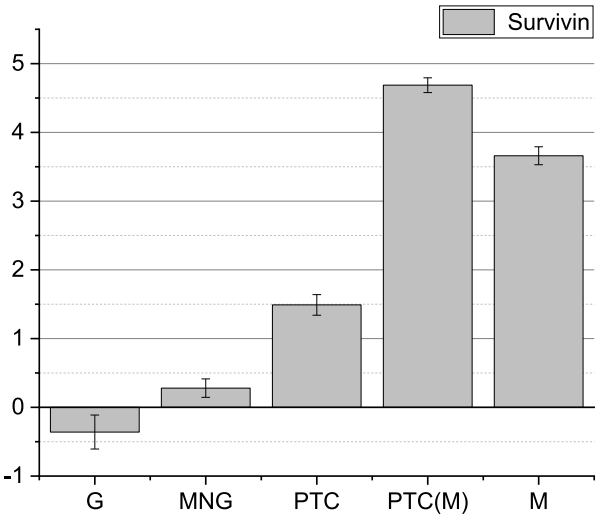

Fig. 2. Quantitative PCR results presenting mRNA expression levels of survivin.
Note: G — goiter, MNG — multinodular goiter, PTC — carcinoma without metastasis, PTC (M) — carcinoma with metastasis, M — metastasis. Y axis — fold change (log_2) compared with conditionally normal tissue. The expression values of survivin for all groups significantly differ from each other.

The expression of survivin mRNA in PTC tissue with metastasis significantly higher than its expression in conditionally normal (more than 4.5 times) and goiter tissues (see Fig. 2). The expression of survivin mRNA in PTC without metastasis more than three times lower compared to PTC with metastasis. In metastasis themselves the level of survivin expression is lower than in the primary tumor with metastasis but substantially higher than in tissue of PTC without metastasis (see Fig. 2) and other tissues.

Thus, survivin levels in the primary tumour may be useful for predicting metastasis development. Survivin expression may also serve as a marker for differentiating between malignant and benign neoplasms.

The study of Akt activation included patients with PTC without metastasis, PTC with metastasis and metastasis. Group 1 included patients with PTC without metastasis, group 2 — PTC with metastasis, group 3 — metastasis tissue.

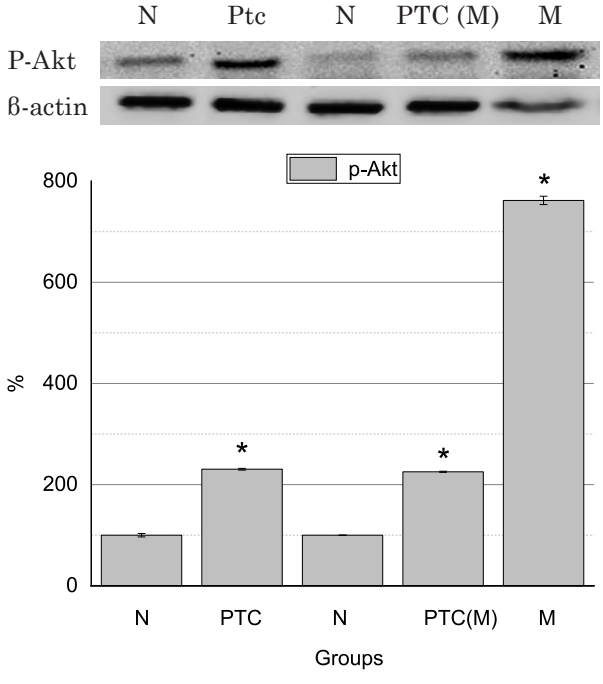

Fig. 3. Expression levels of p-Akt in thyroid samples.
Note: The image above is the result of a Western blot; the figure below is a quantification of a Western blot in %. Mol. weight of ß-actin — 42 kDa; p-Akt ~ 60 kDa. N — conditionally normal tissue (100 %), PTC — carcinoma without metastasis, PTC (M) — carcinoma with metastasis, M — metastasis. * — the differences from the previous group are significant, $P \leq 0.05$.

We presented the obtained data in % to calculate the amount of Akt in tumor tissue relative to the corresponding conditionally normal tissue.

The expression pattern of the phosphorylated (activated) form of Akt is somewhat different from the expression of survivin (see Figs. 2 and 3). Although the level of active kinase in the primary tumor is higher than in the conditionally normal tissue, there is no difference between the tumor without metastases and the tumor with metastases. At the same time, unlike survivin, the level of Akt in metastases is almost 4 times higher than in the primary tumor and almost 8 times higher than in the conditionally normal tissue (see Fig. 3).

Survivin is mainly expressed in cancer tumors. Expression in normal tissues is low, making it a valuable diagnostic and prognostic marker and target for antitumor therapy [13, 14]. The potential prognostic value of survivin is variably reported depending on the lung cancer stage [15].

Survivin expression in normal thyroid follicles is low or moderate. It increases with the degree of dedifferentiation [14]. Most PTCs show moderate to high expression of survivin. Higher expression is observed in tumors with lymph node metastasis and distant metastases [16, 17], with a tendency towards extrathyroidal extension and advanced stage [16, 18]. Survivin expression intensity in metastatic tissue is the same as in primary tumors [19]. Up-regulation of expression contributes to tumor progression to an aggressive and poorly differentiated phenotype [5, 14]. There is a strong association between concomitant high expression of survivin with vascular endothelial growth factor C (VEGF-C) and the metastatic status of lymph nodes in PTC patients, which suggests their cooperation in the metastatic process [19]. The association of high levels of co-expression of VEGF-C, survivin, and matrix metallopeptidase 9 with lymphatic metastases and local invasiveness of PTC suggests their potential utility as prognostic biomarkers of aggressive carcinomas [19–21].

Survivin, a member of the apoptosis inhibitor family, is involved in the regulation of cell cycle and proliferation [21, 22]. It was shown a strong positive correlation between the expression of survivin and proliferating cell nuclear antigen (PCNA) and thyroid lesions. The expression of survivin and PCNA in malignant thyroid tumors was significantly higher compared to benign lesions [14, 22]. Survivin expression has high specificity and sensitivity for distinguishing malignant and benign types of thyroid tumors. It can be considered as a biological marker of thyroid malignancy and used in clinical practice [22]. It may also be useful to test the level of survivin in serum as a biomarker that can be determined before surgery. Both tumor expression and high serum levels of survivin may be associated with poor prognosis in patients with thyroid cancer [23].

There are frequent alterations of the PI3K-Akt pathway in various types of human cancers. Amplification of the genes encoding PI3K/Akt lead to the constitutive activation of this pathway. Recent evidence has suggested that Akt plays an important role in cancer cell migration and invasion [9].

The positive expression rates of PI3K, Akt and survivin were significantly higher in the gastric cancer tissues. Expression levels of PI3K, Akt and survivin were significantly correlated with TNM stage, differentiation grade, lymph node and distant metastases. Cooperative relationships were identified between PI3K and Akt, PI3K and survivin, suggesting the involvement of the PI3K/Akt/survivin signaling pathway in the tumorigenesis of gastric cancer. Thus, protein expression of Akt and survivin were significantly associated with the development, progression and metastasis of gastric cancer and may have value as diagnostic and prognostic markers [24].

High melanocyte survivin expression significantly correlated with thickness of primary melanoma. A significant positive correlation was found between melanocyte survivin and phospho-Akt and VEGF expression. Survivin was significantly associated with the presence of metastasis. Thus, survivin, via Akt and VEGF, seems to play an important role in melanoma and could represent an important prognostic marker of melanoma progression [25].

Akt could also activate NF-κB and up-regulate the transcription of pro-survival genes. Active NF-κB enters the nucleus and up-regulates the transcription of Bcl-2, Bcl-xL, XIAP, survivin and Akt, which finally to up-regulate a set of EMT transcription factors including Snai1, Slug, Zeb1, Zeb2, and Twist, which regulate the expression of epithelial and mesenchymal markers at a transcriptional level. The mesenchylmal stem-like cells displayed the aggressive phenotypes, including invasion, metastasis, anti-apoptosis, drug resistance and stemness [8].

For nearly two decades, survivin has been proposed as a potential anticancer target. Several survivin inhibition strategies seem to compromise tumor development. Direct inhibitors of survivin have already been developed and work in this direction continues [2, 26].

CONCLUSIONS

Thus, survivin is overexpressed in a large number of cancers including differentiated and medullary thyroid cancer. Increased survivin mRNA expression in papillary thyroid carcinoma may be a marker of metastasis formation. It can also be used to differentiate between benign and malignant thyroid lesions. The level of the phosphorylated (activated) form of Akt, which could interplay with survivin, is especially high in metastasis.

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DETERMINATION OF SURVIVIN AND PROTEIN KINASE AKT IN PAPILLARY THYROID CARCINOMA AND METASTASIS TISSUES

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Background. Survivin is a member of Inhibitors of Apoptosis proteins (IAP) family. It is expressed in actively proliferating cells, and is upregulated in most cancers; consequently, it has received significant attention as a potential oncotherapeutic target. Akt is a central signalling molecule of the phosphoinositide 3-kinase (PI3K)/Akt signalling pathway. It is a serine/threonine-specific protein kinase and involved in apoptosis, prolif-

eration, transcription and migration. Aberrant activation of this pathway has been identified in a wide range of cancers and thyroid as well.

The aim of the study was to compare the expression of survivin and activation of protein kinase Akt in the tissues of patients with goiter, multinodular goiter (MNG), papillary thyroid carcinoma (PTC) with and without metastasis (Mts) to the lymph nodes.

Materials and methods. Postoperative specimens of tumor tissue, metastasis (Mts), benign neoplasms (goiters) and conditionally normal tissue were used for the studies. The expression of survivin mRNA was determined using real-time polymerase chain reaction (qPCR). The expression of Akt was determined using Western-blotting.

Results. The obtained data indicate that the expression of the survivin mRNA in PTC tissue and Mts significantly exceeds the level of its expression in conditionally normal (more than 4.5 times) and goiter tissues. The expression of survivin in PTC without metastasis more than three times lower compared to PTC with metastasis. In metastasis the level of IAP mRNA expression is lower than in the primary tumor. The expression of the phosphorylated (activated) form of Akt in PTC tissue and Mts significantly higher than level of its expression in conditionally normal tissue. The level of Akt in Mts is almost 4 times higher than in the primary tumor and almost 8 times higher than in the conditionally normal tissue.

Conclusions. Thus, an increased survivin mRNA expression in papillary thyroid carcinoma may be marker of metastasis formation. It can also be used to differentiate between benign and malignant thyroid lesions. The level of the phosphorylated (activated) form of Akt is especially high in metastasis.

Keywords: papillary thyroid carcinoma, metastasis, survivin, protein kinase Akt.

ВИЗНАЧЕННЯ СУРВІВІНУ ТА ПРОТЕЇНКІНІЗИ AKT У ТКАНИНІ ПАПІЛЯРНОЇ КАРЦИНОМИ ЩИТОПОДІБНОЇ ЗАЛОЗИ ТА МЕТАСТАЗІВ

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Актуальність. Сурвівін належить до родини білків інгібіторів апоптозу (IAP). Він експресується в проліферуючих клітинах і має підвищену активність в більшості видів раку, а, отже, отримав значну увагу як потенційна онкотерапевтична мішень. Akt – центральна молекула сигнального шляху PI3K/Akt. Це серин/треонін-специфічна протеїнкіназа, яка задіяна в апоптозі, проліферації, транскрипції та міграції. Аберантна активація цього шляху була виявлена в широкому спектрі видів раку поряд з новоутвореннями щитоподібної залози.

Метою дослідження було порівняння експресії сурвівіну та активації протеїнкінази Akt у тканинах пацієнтів із зобом, багатовузловим зобом (БВЗ), папілярною карциномою щитоподібної залози (ПКЩЗ) з метастазами (Мтс) у лімфатичні вузли та без них.

Матеріали та методи. Для досліджень використовували післяопераційні зразки пухлинної тканини, Мтс, доброякісних новоутворень (зобів) та умовно нормальної тканини. Експресію мРНК сурвівіну визначали за допомогою полімеразної ланцюгової реакції в реальному часі (кількісної ПЛР). Експресію Akt визначали за допомогою вестерн-блоттингу.

Результати. Отримані дані свідчать про те, що експресія мРНК сурвівіну в тканині ПКЩЗ та Мтс значно перевищує рівень його експресії в умовно нормальних тканинах (у понад 4,5 рази) та тканинах зобу. Експресія сурвівіну в ПКЩЗ без метастазів утричі нижча порівняно з ПКЩЗ з метастазами. У метастазах рівень експресії мРНК IAP нижчий відносно первинної пухлини. Експресія фосфорильованої (активованої) форми Akt у тканині ПКЩЗ та Мтс значно вища, ніж рівень її експресії в умовно нормальній тканині. Рівень Akt у метастазах майже в 4 рази вищий, ніж у первинній пухлині, та майже у 8 разів вищий, ніж в умовно нормальній тканині.

Висновки. Таким чином, підвищення експресії мРНК сурвівіну в папілярній карциномі щитоподібної залози може бути маркером утворення метастазів. Його також можна використовувати для диференціації доброякісних та злоякісних уражень щитоподібної залози. Рівень фосфорильованої (активованої) форми Akt особливо високий у тканині метастазів.

Ключові слова: папілярна карцинома щитоподібної залози, метастазування, сурвівін, протеїнкіназа Akt.