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ORIGINAL CONTRIBUTIONS

Experimental ONCOLOGY

DIFFERENTIAL EXPRESSION PATTERNS OF *AIP*, *UCKL1*, AND *PKN1* GENES IN BREAST CANCER OF DIFFERENT MOLECULAR SUBTYPES

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***Background*: Classification of breast cancer (BC) in the molecular subtypes had the enormous impact on the development of the individualized therapy. Nevertheless, there is a need for additional biomarkers that would help to refine molecular subtypes of BC and propose the therapeutic approach for each patient. *Aim:* To study differential expression patterns of *AIP, UCKL1*, and *PKN1* genes in blood sera and tumor tissue of patients with BC of different molecular subtypes. *Materials and Methods:* The total extracellular RNA was isolated from serum of 26 BC patients. cDNAs was synthesized and quantitative polymerase chain reaction was performed. Also, immunohistochemical studies of UCKL, AIP and PKN1 were performed on deparaffined tissue sections. The study was supplemented by a bioinformatic analysis of the publicly available databases. *Results: AIP* and *UCKL-1* extracellular mRNA levels were 100–1000-fold increased in blood sera of all BC patients, compared to the healthy donors. The highest levels were detected in the luminal A and HER2 (ERRB2) BC subtypes. The highest levels of *PKN1* were detected blood sera of the patients with luminal B and basal subtypes; its expression levels were just 10–100-fold higher in BC samples compared to healthy donors. *Conclusions:* The *UCKL1, AIP, PKN1* genes are overexpressed at the mRNA level in blood sera of BC patients compared to the sera of healthy individuals. Among three genes under study, only for the AIP gene, the pattern of extracellular mRNA expression in sera paralleled to protein expression in BC tissues of each specified molecular subtype.**

***Key Words:* UCKL1, AIP, PKN1, breast cancer, molecular subtypes of breast cancer, expression pattern, bioinformatic analysis.**

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Breast cancer (BC) is a rather aggressive disease that is rapidly "getting younger" and affects more and more women of active working age. BC is one of the first malignancies, which was characterized not only by stage and grade, but also by molecular profiling. BC is subdivided into the subtypes that are managed differently in current clinical practice based on the molecular characteristics of tumor cells [1]. This was achieved by a bioinformatic analysis of the microarray data [2]. Importantly, BC sub-classification allows us to perform personalized diagnostics, create an individualized approach to cure a patient, and to forecast the course of the disease.

All BCs could be divided in three large groups — luminal, basal and HER2 overexpressing tumors [1–3]. To date, there are five molecular subtypes of BC: the luminal A and B, HER2 overexpressing, basal and "normal-like" [4–7]. Sometimes another molecular type of BC is classified, so called "claudin-low" [8]. However, this subtype is poorly characterized and has not been used yet in the clinical practice [9]. In the present work, we would consider only four BC subtypes (Table), molecular profiles of which were extracted from [1, 2, 4].

Classification of BC in the molecular subtypes had the enormous impact on the development of the individualized therapy. Nevertheless, in many cases, cancer cells became resistant to chemotherapeutic drugs, possibly due to the activation of the alternative pathways or loss of receptor expression [10]. Therefore, there is a need for additional biomarkers that would help to refine molecular subtypes of BC and propose the therapeutic approach for each patient. Ideally, oncomarkers should be expressed only by cancer cells; they are often represented by the complex glyco- or lipoproteins (they may be of non-protein origin) [11]. More than 200 molecules are known as tumor markers, but only a few dozen proteins are of diagnostic value. The changes in levels of the certain marker, or in a set of markers, might help to monitor the course of the disease and make prognosis [12–14].

In the present work, we attempted to widen up a range of BC tumor markers. To do so, we have chosen few genes, which were shown to be implicated in cell transformation. We assessed their expression at the mRNA level in blood sera and at a protein level in tumor samples.

Aryl hydrocarbon receptor interacting protein (AIP, also known as ARA9 and XAP-2) (**NP_003968**) [15–17] regulates the expression of many xenobiotic metabolizing enzymes [18], that may play an important role in development of resistance to chemotherapy.

Uridine-cytidine kinase 1 like 1 (UCKL-1) (**NP_060329.2**) [19] was chosen based on its elevated levels and the enhanced activity in damaged tissues, colon tumors [20], hepatocellular carcinomas [21, 22], and under B cell transformation upon Epstein — Barr virus infection [23].

Protein kinase N1 (PKN1, **NP_99872**) [24], the serine-threonine protein kinase, is involved in regulation

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Abbreviations used: AIP – aryl hydrocarbon receptor interacting protein; BC – breast cancer; PKN1 – protein kinase N1; UCKL-1 – uridine-cytidine kinase 1 like 1.

Table. Molecular characteristics of BC subtypes

Subtype/molecular features	Expression of hormone receptors	Specific genes	Transcription factor networks
Luminal A	Estrogen receptor, ESR1, NP_000116 Progesterone receptor, PGR, NP_001189403	High keratin 19, KRT19, NP_002267	ESR1, FOXA1 (NP_004487), GATA3 (NP_001002295)
Luminal B	ESR1 PGR HER2, the oncogene ERB-B2 receptor tyrosine kinase 2, known also as NEU, NP_001005862	High KRT19	ESR1, FOXA1, GATA3, ELF5 (NP_938195), EHF (NP_001364981)
HER2 amplified	HER2 overexpression	High KRT19 Keratin 14, KRT14, NP_000517	ELF5, EHF
Basal	None of above	Keratin 14	TP63 (NP_003713), NFIB (NP_001177666), FOXC1 (NP_001444)

of transcription, the cytoskeleton filament network, cell migration and invasion of tumor cells [25–27].

We show here that these genes are overexpressed at the mRNA level in blood sera of BC patients compared to the sera of healthy individuals, and at protein levels in BC tissue samples.

MATERIALS AND METHODS

A cohort of patients. In the present study, blood sera and tumor tissues were collected from 26 patients with BC, stages I–II, who underwent surgery at the National Cancer Institute of the National Academy of Medical Sciences of Ukraine (Kyiv, Ukraine). All women gave written informed consent to participate in the study, which was approved by the Ethics Committee of RE Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of the National Academy of Sciences of Ukraine. Biopsies were fixed in a neutral buffered 4% formaldehyde solution. After fixation, dehydration, and embedding in paraffin, serial sections were cut at a normal thickness of 5 μm and stained with hematoxylin/eosin for histological diagnosis. BC were graded based on their architectural features, according to the criteria, described in [28], by experienced pathologists. Samples were distributed by a molecular subtype as follows: luminal A — 7 cases, luminal B — 7 cases, Her2 overexpressing — 6 cases, and basal — 6 cases. Sera from 4 healthy individuals (males and females) were used as the control.

RNA isolation, cDNA synthesis and qPCR. The total extracellular RNA was isolated from serum, using the RNeasy Mini Kit (Qiagen Inc, Germany), according to the manufacturer's instructions. The cDNAs were synthesized, using 2 μg of total RNA, M-MLV Reverse Transcriptase, and RNAse inhibitor (Invitrogen, USA), according to the manufacturer's protocol. Quantitative PCR (q-PCR) was performed, using 2 μg cDNA and the HOT FIREPol EvaGreen qPCR Mix (Solis BioDyne, Estonia), on the PCR System 7500 (Applied Biosystem, USA). Primers were the following: for *UCKL-1* (NM_017859) forward 5'-AGCACTATGCGGGCAA GTGCTA-3', reverse 5'-TCTGGATGAGGATGGTGCCGAT-3'; for *AIP* (M_003977) forward 5'-TACTACGAGGTGCTG-GACCACT-3', reverse 5'-GCACTTTGGCAAAG TCAGCCTG-3'; for PKN1 (NM_002741) forward 5'-CTGTTCGCCATC AAGGCTCTGA-3', reverse 5'-CACTGGTCACRGCCGCCAATAT-3'. As an internal control for standardization, a gene encoding TATA-binding protein (*TBP*, NM_003194) was used: forward primer 5'-TTTCTTGCCAGTCTGGAC-3', reverse 5'-CACGAACC ACGGCACTGATT-3'. Relative quantification (comparative Ct (ΔΔCt) method) was used to compare expression levels of the *UCKL-1, AIP* and *PKN1* genes with the internal control. Two or three reactions (each in triplicate) were run for each gene, so the standard deviation might be calculated.

Immunohistochemistry. Immunohistochemical studies of UCKL, AIP and PKN1 were performed on deparaffined tissue sections. Paraffin was dissolved in xylol, and sections were rehydrated with stepwise washing with ethanol in phosphate-buffered saline (99; 90; 70 and 30% EtOH). Sections were then treated with 2% solution of H_2O_2 in methanol at room temperature for 30 min to reduce background. Epitopes were exposed to hot citrate buffer (water bath, 92 °C for 15 min). The rabbit antibodies against these proteins (Cell Signaling, USA) were used for detection, diluted in blocking buffer (2% bovine serum albumin, 0.2% Tween-20, 10% glycerol, 0.05% NaN_3 in phosphate-buffered saline). EnVision system (DakoCytomation, Denmark) was used in 30 min second-step incubation. After washing in phosphate-buffered saline peroxidase activity was assayed using 3,3'-diaminobenzidine. After counterstaining with hematoxylin for 1–2 min, sections were embedded in Canadian balsam and studied by light microscopy.

Bioinformatic data analysis. To analyze expression of genes at the mRNA level, a publicly available data Protein Atlas was used. Human Protein Atlas is available from http://www.proteinatlas.org.

Statistical analysis. GraphPad Prism software (version 8, GraphPad Software, USA) was used to determine the means of the gene expression. The Kruskal — Wallis test for non-parametric criteria for the groups was performed for each gene.

RESULTS AND DISCUSSION

Earlier, we have shown that it is possible to analyze the extracellular mRNA [29] that could be stabilized by a placement in the specific membrane vesicles, such as exosomes (diameter < 150 μm), microvesicles (200–500 μm), oncosomes (1–10 μm), apoptotic bodies, etc. [30, 31]. Of course, such a small number of mRNA molecules is a major problem to perform gene expression studies. On the other hand, such

approach represents the good strategy to develop non-invasive markers that could be analyzed in body fluids, such as blood, urine, saliva, or cerebrospinal fluid [32–35]. Importantly, the quantitative assessment of extracellular mRNA requires normalization with the specific control (in our case, it is *TBP*).

The relative amounts of the extracellular mRNA of the *UCKL1, AIP* and *PKN1* genes were assessed

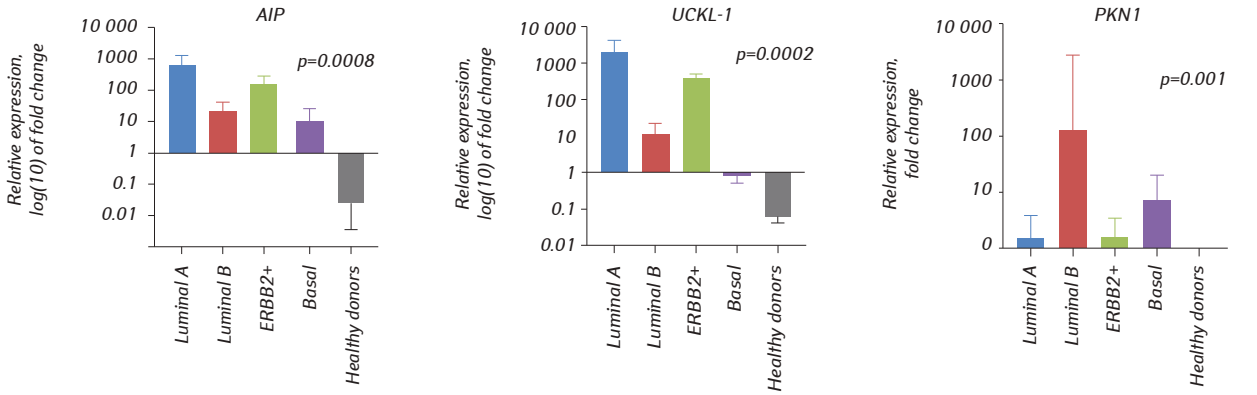

Fig. 1. Expression patterns of the *UCKL1, AIP* and *PKN1* genes at mRNA levels in blood sera assessed by qPCR. Figure was prepared with the help of GrapPrism software; the Kruskal — Wallis test for non-parametric values in groups was applied for each gene. Significant differences are considered when $p \leqslant 0.05$. ERBB2+ stands for the HER2 overexpression subtype

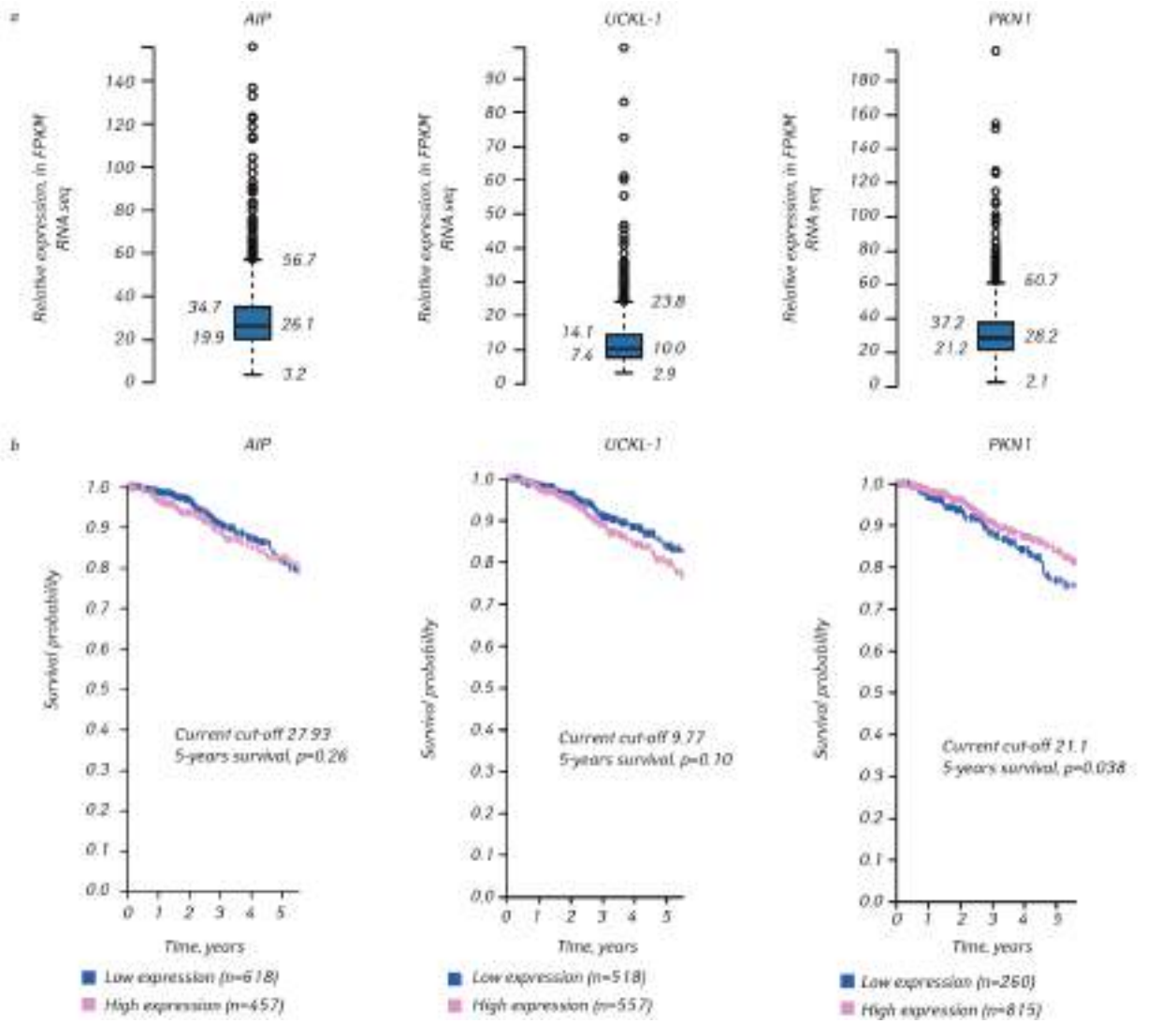

Fig. 2. Relative expression of the *UCKL1, AIP* and *PKN1* genes and a survival analysis according to the Protein Atlas portal: *a* — elative expression, based on RNA seq, according to the Protein Atlas portal. Expression is shown in the fragments per kilobase million units, providing a digital measure of the abundance of transcripts; *b* — the *UCKL1* and *AIP* are not predictive markers, while *PKN1* might be one (adapted from: https://www.proteinatlas.org/ENSG00000110711-AIP/pathology/breast+cancer;https://www.proteinatlas.org/ENSG00000198276-UCKL1/pathology/breast+cancer; https://www.proteinatlas.org/ENSG00000123143-PKN1/pathology/breast+cancer)

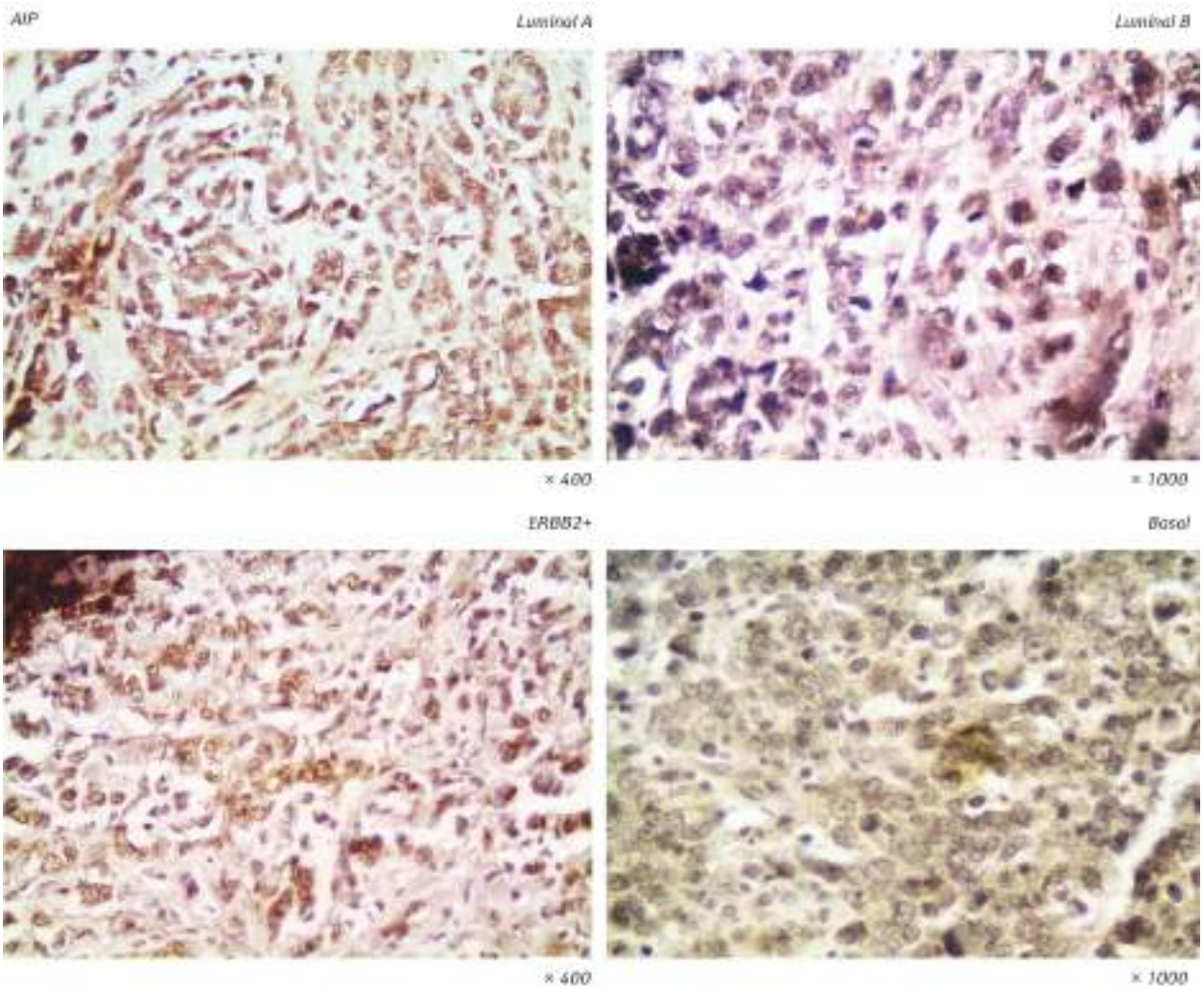

Fig. 3. Expression of the AIP protein in BC tissues. Notice the highest AIP signal in BC tissue of the luminal A and HER2 (ERRB2) overexpression subtypes

by qPCR in blood sera of 26 BC patients and of 4 healthy individuals. As shown in Fig. 1, expression levels of these genes in blood sera are much higher in BC patients, compared with healthy individuals. For *AIP* and *UCKL-1*, the difference makes hundreds and thousands fold.

Concerning differences between the BC subtypes, *AIP* and *UCKl-1* showed similar patterns – they were expressed at the highest levels in luminal A BC cases, and at the lowest – in basal BC cases.

The levels of extracellular *PKN1* mRNA were much lower, compared to the *AIP* and *UCKl-1*; difference was approximately one magnitude (Fig. 1). The expression pattern was also different — the highest levels were detected in the cases of the luminal B and basal BC subtypes.

To compare the obtained results with the expression pattern of the above studied genes in tumor samples, the Protein Atlas portal was analyzed [36, 37]. According to the RNAseq data, *AIP* and *PKN1* were expressed at the similar levels, while the median value for the *UCKL-1* expression was two-three folds lower (Fig. 2, *a*). We have to mention that the RNA seq gives the special values — the ratio of the number of reads on the total number of transcripts in a created library. Hence, it can not be compared directly to expression of genes at the mRNA levels, obtained by q-PCR. Moreover, no subclassification was performed for BC tumors.

Using the RNAseq data, a Protein Atlas team calculated the survival probability of BC patients with gene expression above the median value (high expression) and below the median value (low expression). No significant differences were found for *AIP* and *UCKL-1*, even if *UCKL-1* showed a trend to be higher in patients with a poorer 5-year survival rate (Fig. 2, *b*). *PKN-1* can be considered a prognostic marker for BC, the higher levels of PKN-1 are favorable for the 5-year survival rate (Fig. 2, *b*). It was shown before that PKN1 levels were altered in tumor cell lines [26, 27].

Hence, next, we examined expression of the selected genes at the protein levels, using immunohistochemistry.

The AIP protein signal was the highest in the luminal A and HER2 (ERRB2) overexpression BC subtypes (Fig. 3) showing the similar pattern with expression of the extracellular *AIP* mRNA in patient sera (see Fig. 1). The AIP protein is involved in cell transformation,

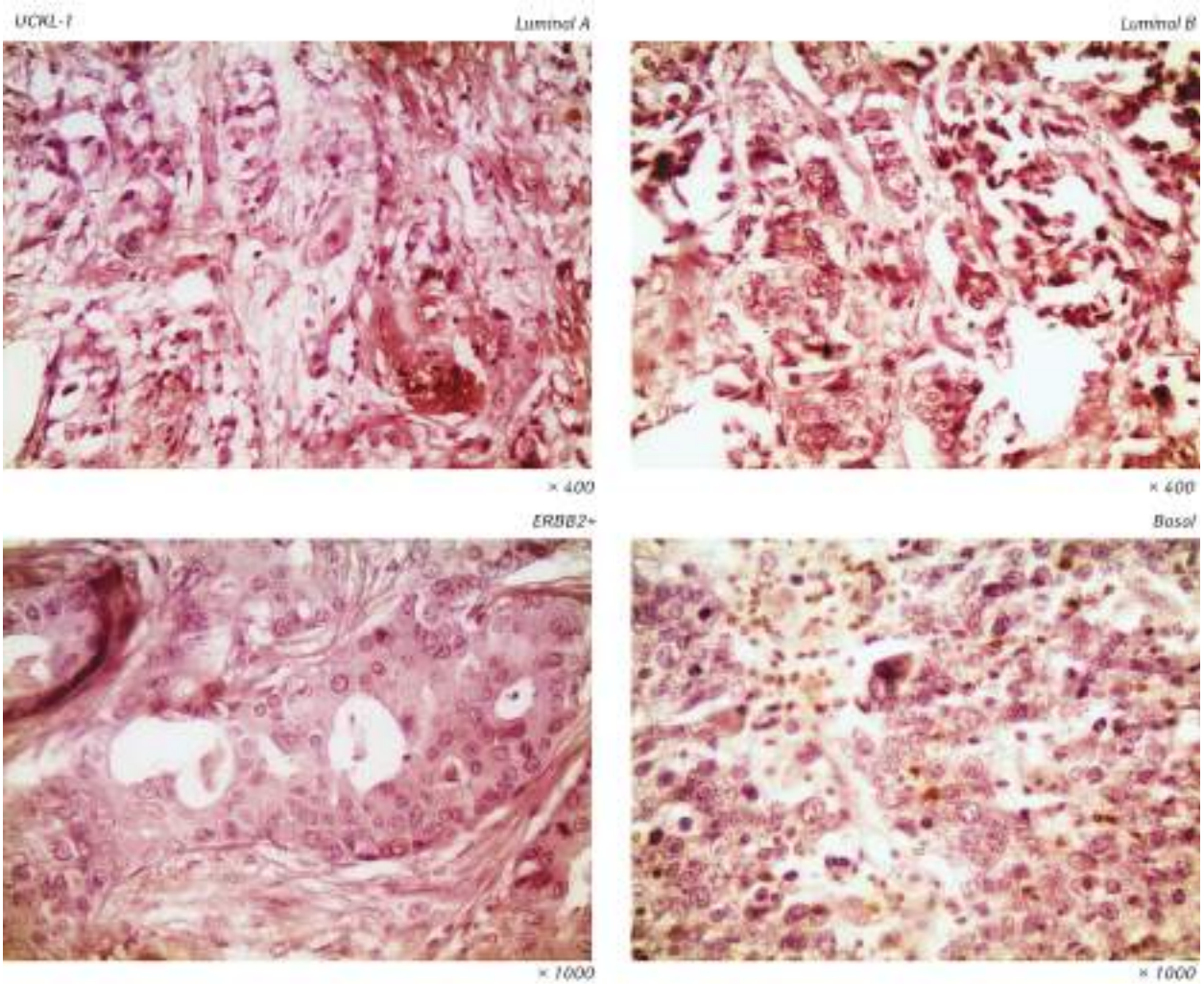

Fig. 4. Expression of the UCKI-1 protein in BC tissues. Notice the weak signal in all subtypes of BC tissues

induced by Epstein-Barr virus [38] and the hepatitis B virus [17]. The AIP protein binds to several nuclear receptors regulating transactivation of enzymes involved in metabolism of xenobiotics [18]. Importantly, mutations in the *AIP* gene can cause pituitary adenomas [39, 40].

Unexpectedly, the UCKL-1 protein signal was very weak in all BC subtypes (Fig. 4), in contrast to high levels of extracellular mRNA of this gene (see Fig. 1). Actually, there could be two explanations, at least. Probably, the anti-UCKI-1 antibody was not binding to antigen well. On the other hand, the UCKI-1 protein levels could be low, if this protein is degraded fast. There are no data yet on this subject. Anyway, the *UCKL-1* gene could be a candidate for the prognostic marker, due to the huge difference in the levels of its extracellular mRNA in blood sera of BC patients in comparison with healthy individuals. The further studies are needed, on the larger cohort of BC patients at the different stages of tumor progression.

Expression pattern of the PKN1 protein in the BC tissues was rather high (Fig. 5). The highest levels were observed in the HER2 overexpressing phenotype, contrary to the extracellular mRNA pattern for the *PKN-1* gene (see Fig. 1). Of course, levels of the protein say not much about its activity. Probably, a function of PKN1 as the serine-threonine protein kinase is inactivated in breast tumors. However, this should be yet evaluated.

From the three chosen genes, only one, *AIP*, exerted similar patterns of expression as the extracellular mRNA in patient sera and the protein in BC tissues.

AIP is also known as a homologue of immunophilin ARA9 and as X-associated protein 2 of hepatitis B virus [16]. The AIP protein belongs to the FKBP family of proteins that have prolyl isomerase activity and are linked functionally to cyclophilins and immunophilins. FKBP proteins function as chaperones, binding to proline-rich proteins. The AIP protein is usually present in the cytoplasm as part of a multiprotein complex with different nuclear receptors but is transported to the nucleus upon ligand-receptor binding. The AIP protein functions in the aryl-hydrocarbon receptor-mediated signaling that is responsible for metabolism of heavy organic compounds [38].

Due to involvement of AIP in metabolism of xenobiotics, the next question might be whether extracellular mRNA of AIP can be a prognostic marker for response of BC patients to chemotherapeutic agents. This

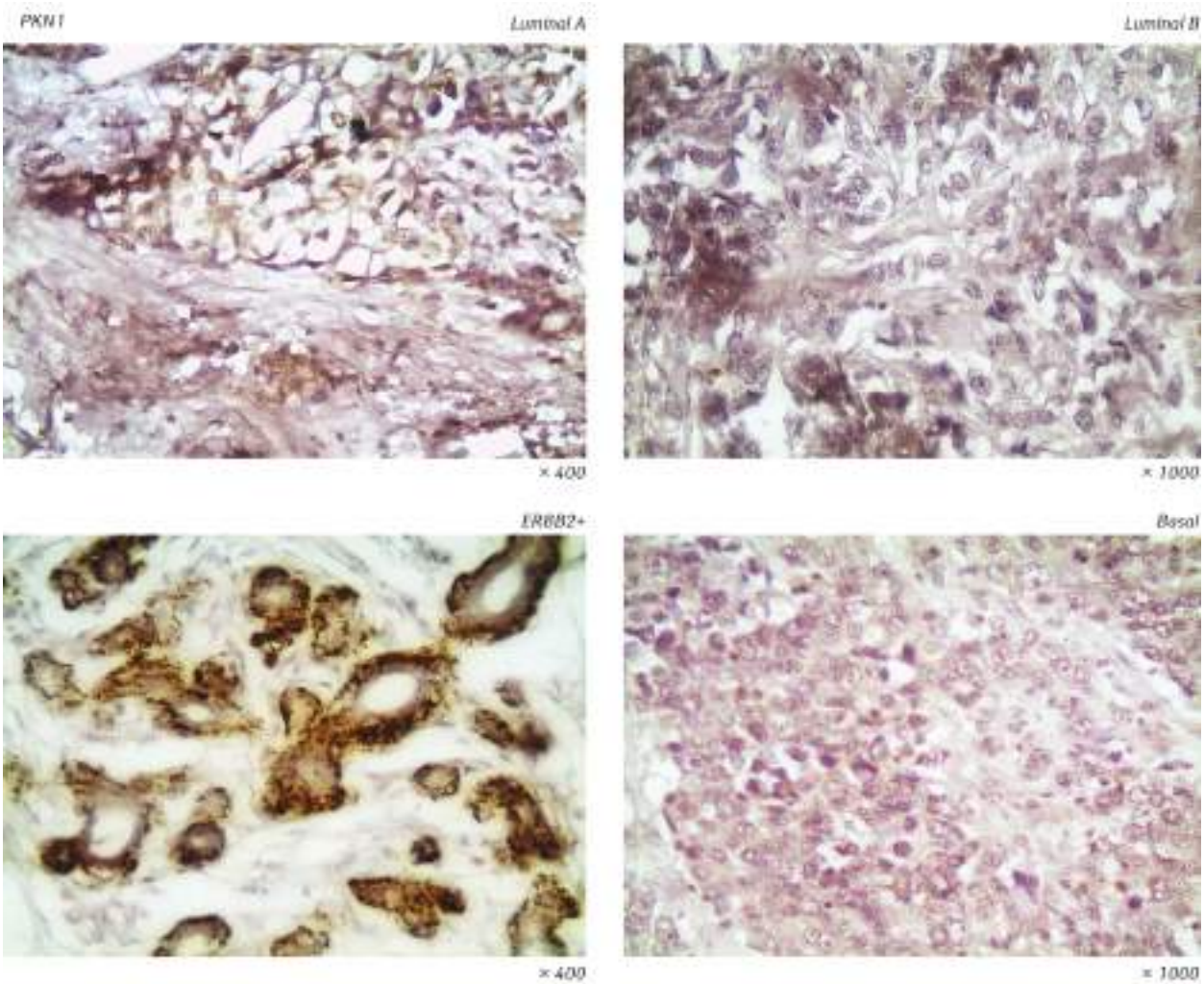

Fig. 5. Expression of the PKN1 protein in BC tissues. Notice the highest AIP signal in the HER2 (ERRB2) overexpressing subtype

should be further investigated, on the larger and wider cohort of BC patients.

To sum up, among three genes under study, only for the *AIP* gene, the pattern of extracellular mRNA expression in sera paralleled to AIP protein expression in BC tissues of each specified molecular subtype. *AIP* was up to 1000-fold increased in blood sera of all BC patients compared to the healthy donors. The highest levels were detected in the luminal A and HER2 (ERRB2) overexpressing subtypes. To assess whether the expression levels of the *AIP* gene in the BC patient sera may be used as an additional criterion for differential diagnostic of BC subtypes, further studies on a larger cohort of BC patients are required.

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COMPETING INTEREST

The author(s) declare that they have no competing interests.

AUTHORS CONTRIBUTION

KA, TZ and LK carried out the immunofluorescence staining. KA and LK performed qPCR. EK and LK performed a bioinformatic and statistical analysis. NL and TZ collected BC tissue and serum samples. NL, VC, LK conceived and designed the study, and together with EK participated in its coordination. LK and EK drafted the manuscript. All authors read, edited and approved the final manuscript.

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ДИФЕРЕНЦІЙНИЙ ПАТЕРН ЕКСПРЕСІЇ ГЕНІВ *AIP*, *UCKL1*, І PKN1 У ЗРАЗКАХ РАКУ МОЛОЧНОЇ ЗАЛОЗИ В ЗАЛЕЖНОСТІ ВІД МОЛЕКУЛЯРНОГО ПІДТИПУ ПУХЛИНИ

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Мета: Визначити диференційні патерни експресії генів *AIP, UCKL1* та *PKN1* у сироватці крові та зразках пухлинної тканини хворих на різні молекулярні підтипи раку молочної залози (РМЗ). ***Матеріали та методи:*** Позаклітинну РНК виділяли із сироватки крові 26 хворих на РМЗ. Синтезували кДНК та проводили кількісний аналіз методом полімеразної ланцюгової реакції. Імуногістохімічні дослідження AIP, UCKL1 та PKN1 проводили на депарафінованих зрізах пухлинної тканини. Проводили також біоінформаційний аналіз загальнодоступних баз даних. ***Результати:*** Рівні позаклітинної мРНК для генів *AIP* і *UCKL-1* були збільшені в 100–1000 разів у всіх зразках РМЗ у порівнянні з умовно здоровими донорами. Найвищі рівні були виявлені в наступних підтипах РМЗ: люмінальний А та з надекспресією HER2 (ERRB2). Найвищі рівні *PKN1* були виявлені у зразках люмінального В і базального підтипів, проте різниця в експресії цього гена між зразками РМЗ та умовно здоровими донорами становила тільки 10–100 разів. ***Висновки.*** Лише ген *AIP* показав однаковий патерн експресії, як для позаклітинної мРНК у сироватці крові пацієнтів, так і протеїну в пухлинній тканині хворих на РМЗ, причому пухлини було згруповано за молекулярними підтипами. Дослідження рівнів експресії гена *AIP* у сироватці крові пацієнтів з РМЗ може бути використано як додатковий критерій для характеристики молекулярного підтипу РМЗ та прогресування пухлини.

Ключові слова: UCKL1, AIP, PKN1, рак молочної залози, молекулярні підтипи раку молочної залози, модель експресії, біоінформаційний аналіз.