



OPEN ER α status of invasive ductal breast carcinoma as a result of regulatory interactions between lysine deacetylases KAT6A and KAT6B

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Breast cancer (BC) is the leading cause of death among cancer patients worldwide. In 2020, almost 12% of all cancers were diagnosed with BC. Therefore, it is important to search for new potential markers of cancer progression that could be helpful in cancer diagnostics and successful anti-cancer therapies. In this study, we investigated the potential role of the lysine acetyltransferases KAT6A and KAT6B in the outcome of patients with invasive breast carcinoma. The expression profiles of KAT6A/B in 495 cases of IDC and 38 cases of mastopathy (FBD) were examined by immunohistochemistry. KAT6A/B expression was also determined in the breast cancer cell lines MCF-7, BT-474, SK-BR-3, T47D, MDA-MB-231, and MDA-MB-231/BO2, as well as in the human epithelial mammary gland cell line hTERT-HME1 – ME16C, both at the mRNA and protein level. Statistical analysis of the results showed that the nuclear expression of KAT6A/B correlates with the estrogen receptor status: KAT6A_{NUC} vs. ER $r = 0.2373$ and KAT6B_{NUC} vs. ER $r = 0.1496$. Statistical analysis clearly showed that KAT6A cytoplasmic and nuclear expression levels were significantly higher in IDC samples than in FBD samples (IRS 5.297 ± 2.884 vs. 2.004 ± 1.072 , $p < 0.0001$; IRS 5.133 ± 4.221 vs. 0.1665 ± 0.4024 , $p < 0.0001$, respectively). Moreover, we noticed strong correlations between ER and PR status and the nuclear expression of KAT6A and KAT6B (*nucKAT6A* vs. ER, $p = 0.0048$; *nucKAT6A* vs. PR $p = 0.0416$; *nucKAT6B* vs. ER $p = 0.0306$; *nucKAT6B* vs. PR $p = 0.0213$). Significantly higher KAT6A and KAT6B expression was found in the ER-positive cell lines T-47D and BT-474, whereas significantly lower expression was observed in the triple-negative cell lines MDA-MB-231 and MDA-MB-231/BO2. The outcomes of small interfering RNA (siRNA)-mediated suppression of KAT6A/B genes revealed that within estrogen receptor (ER) positive and negative cell lines, MCF-7 and MDA-MB-231, attenuation of KAT6A led to concurrent attenuation of KAT6A, whereas suppression of KAT6B resulted in simultaneous attenuation of KAT6A. Furthermore, inhibition of KAT6A/B genes resulted in a reduction in estrogen receptor (ER) mRNA and protein expression levels in MCF-7 and MDA-MB-231 cell lines. Based on our findings, the lysine acetyltransferases KAT6A and KAT6B may be involved in the progression of invasive ductal breast cancer. Further research on other types of cancer may show that KAT6A and KAT6B could serve as diagnostic and prognostic markers for these types of malignancies.

Breast Cancer (BC) is a type of cancer primarily caused by the abnormal and uncontrolled growth of mammary gland cells. Since cancer progression can occur in different types of mammary gland cells, different types of breast cancer can be distinguished¹. Breast cancer has been the most common type of malignancy in women^{1–5}. Recent research shows that one out of eight women in the U.S. will develop Invasive Ductal Breast Carcinoma (IDC) during their lifetime. Nearly 300,000 new cases of IDC are expected to be diagnosed in 2021 in the United States alone^{6,7}. Moreover, for the first time BC has overtaken lung cancer as the most common cancer worldwide (nearly 12% of new cases)^{6,7}. More than 19.3 million new cases of BC were diagnosed in 2020 (which represents almost 11.7% of all cancers), with lung cancer ranking second and colorectal cancer third. There are well-known risk factors responsible for breast cancer development, such as tobacco use, low fruit and vegetable consumption, high BMI, alcohol use, and lack of physical activity^{1,4,5,8–11}. The increase in the world population leads to one more observation: by 2040, approximately 30 million new cases of BC will be diagnosed worldwide. Taking all

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of the above-mentioned statistics into consideration, it is highly justified for scientists to persistently try to find new potential diagnostic and therapeutic markers of breast cancer. Health organizations and state governments are, today more than ever, compelled to focus on and develop modern tools for the successful fight against the rising number of new cancer cases around the world.

Acetylation of histones by lysine acetyltransferases (KATs) is a crucial physiological process in the cell nucleus. This process is a very specific mechanism of epigenetic regulation of gene transcription that interferes with various cellular pathways and processes such as DNA damage repair, metabolism, cellular proliferation, autophagy, mitosis, and signal transduction. ϵ -Lysine acetylation affects protein structure and function via multiple processes, including subcellular localization, enzymatic activity, DNA stability, and protein-DNA and protein-protein interactions^{12–17}. KAT6A (monocytic leukemia zinc finger protein – MOZ, also known as MYST3) and KAT6B (MORF or QKF, also known as MYST4) belong to the MYST family of acetyltransferases, which consists of KAT5–KAT8 proteins. The major function of KAT6 is to acetylate histone H3 at lysine-23 (H3K23) and non-histone chromatin proteins. For this reason, they are crucial in biological mechanisms such as transcription, maintenance, and regulation of hematopoietic and neural stem cells, skeletogenesis meiosis, and cell cycle progression^{18–23}.

Unfortunately, there is still a limited amount of data on the expression levels and possible role of KAT6A/B in cancer progression. Considering their role in some cellular processes, it seems logical to hypothesize that the inhibition of these proteins may provide a therapeutic benefit in cancer. KAT6A/B inhibition leads to cell cycle arrest, which leads to cellular senescence²⁴.

In eukaryotic cells, KAT6A and KAT6B form very stable MOZ and MORF complexes, respectively. Dysregulation of KAT6A and KAT6B function leads to an aggressive form of leukemia leiomyoma, myelodysplastic syndrome, and other blood malignancies and solid tumors. Due to the presence of catalytic subunits, KAT6A and KAT6B can interact with adaptor proteins such as ING5 (inhibitor of growth 5), BRPF1/2/3 (bromodomain PHD proteins 1, 2, or 3), and hEAF6 (homologue of Esa1-associated factor 6)^{22,25,26}. Three of the four subunits of this large catalytic complex (KAT6A/B, ING5, and BRPF1/2/3) contain PTM readers. Additionally, BRPF1/2/3 possesses two PHD fingers, and ING5 possesses one. This crosstalk between KAT6A/B and other complex partners is responsible for maintaining and controlling important pathways in normal signalling and carcinogenesis. It has been shown that KAT6A/B play a significant role in a regulation of ER α expression in breast cancer^{27,28}. Nuclear localization and enzymatic activity are crucial for transcriptional activation of the ESR1 gene, which encodes the ER.

KAT6B is a close homologue of KAT6A, sharing approximately 90% identity with the acetyl-CoA binding site. First discovered in 1996, KAT6A is associated with gene rearrangement in leukemia, with KAT6B being shown to play a similar role^{29,30}. KAT6A was also found to play a role in acute myeloid leukemia, where it binds to the CREB protein due to gene translocation t(8;16)(p11;p13)³¹.

According to the latest scientific reports, KAT6A and KAT6B can be responsible for the formation of malignancies such as liver, breast, urogenital, gastrointestinal tract, endometrium and lung tumours^{12,24,27,32–40}. Unfortunately, as in the case of the MOZ protein, there is no confirmed direct influence of the MORF protein on cancer formation. It is presumed that inhibiting these proteins could revolutionize the fight against cancer. For this reason, many studies have taken into account the detailed assessment of not only the action and function of this protein but also a safe method for its inhibition, along with the assessment of the remission or even elimination of cancer cells from the human body.

KAT6A/B has been shown to be responsible for an imbalance in acetylation processes that influences the expression patterns of tumor suppressors and proto-oncogenes^{28,41}. The hyperacetylation of some proto-oncogenes increases their expression and converts them into oncogenes. In contrast, hypoacetylation of tumor suppressor genes silences them and/or decreases their expression levels.

KAT6A/B not only influences the expression pattern of other transcripts but is also dependent on epigenetic modulations, such as the regulation of the methylation of the promoter region, miRNAs, and lncRNAs. It has recently been discovered that the *KAT6B* promoter region is mediated by EZH2 and hypermethylated by H3K27 in congenital scoliosis (CS)⁴². This methylation leads to an increase in the proliferation ability of chondrocytes in CS, which may be related to the RUNX2/Wnt/ β -catenin signalling pathway. High amplification levels of the *KAT6A* and *KAT6B* genes have been discovered in different malignancies, including ovarian and breast cancer, colon and rectal adenocarcinomas, lung adenocarcinoma, and uterine cervical cancer^{30,43–45}. Moreover, KAT6A/B was co-expressed with Nuclear Receptor Coactivators 1 and 3 (NCOA1 and NCOA3)⁴⁶. This co-expression was found in breast, endometrial, pancreatic, and prostate cancers, where it promotes tumor growth, invasion, metastasis, and chemoresistance^{47,48}.

KAT6A was discovered to be both a target and repressor of miR-223 gene expression feedback during macrophage differentiation⁴⁵. Additionally, miR-224 has been proved to be downregulated in the HT29 colon cancer cell line when KAT6A is overexpressed⁴⁹. In contrast, KAT6B is regulated by miR-22 and miR-4513 in gastric cancer, resulting in cancer cell proliferation, invasion, and promotion of epithelial-to-mesenchymal transition³⁵.

KAT6A and KAT6B proteins stimulate cancer cell growth by influencing the cell cycle. For this reason, inhibiting their action can stop malignancy and put it into a completely dormant state without destroying its cells. However, before such a drug is developed, a number of studies should be conducted to assess the expression of MYST proteins (especially MOZ and MORF) in cancer, so that future drugs are as effective as possible^{12,32,33,50–53}.

Materials and methods

Patients and clinical samples

The experiments were carried out using archival paraffin blocks of invasive ductal breast carcinomas ($n = 495$) and fibrocystic breast disease (FBD) – mastopathy ($n = 38$). Samples were obtained during surgical resection

between 2010 and 2016 at the Polish Mother Memorial Hospital Research Institute in Lodz. Paraffin sections of the IDC and FBD samples were stained with hematoxylin and eosin (H&E) to verify the appropriateness of immunohistochemical (IHC) analyses. Clinical data were derived from hospital archives and are summarized in Table 1.

Histopathological analysis of IHC reactions

KAT6A and KAT6B immunocytochemical expression was established using the ImmunoReactive Score (IRS) according to Remmele and Stenger⁵⁴. This scale considers both the intensity of the color (staining) of the reaction and the percentage of positively stained cells. The final score, which represents the product (multiplication) of these two parameters, ranged from 0 to 12. Statistical analysis was performed using the Mann-Whitney U-test, ANOVA Kruskal-Wallis test, and Spearman test. Differences were considered statistically significant at $p < 0.05$.

Cell lines

For our studies, the breast carcinoma cell lines MCF-7, T-47D, and MDA-MB-231 (obtained from American Type Culture Collection ATCC, Manassas, VA, USA), SK-BR-3, BT-474 (from the Cell Lines Collection of the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy of the Polish Academy of Science, Wrocław, Poland) and MDA-MB-231/BO2 (courtesy of Dr. Philippe Clezardin, INSERM U664, France), as well as immortalized normal breast cells (hTERT-HME1 – ME16C, from ATCC) were used. The breast cancer cell lines were cultured in α -MEM medium supplemented with 2 mM L-glutamine (Lonza, Basel, Switzerland), 10% fetal calf serum (FCS; Invitrogen Carlsbad, CA, USA), and antibiotics. The ME16C cell line was cultured in the MEGM Bulletkit medium (Lonza, Basel, Switzerland). All media were supplemented with FBS (Sigma) to a final concentration of 10%. The cells were cultured at 37 °C in a 5% CO₂.

Immunohistochemistry (IHC)

For the IHC reactions, we used IDC and FBD samples fixed in 10% buffered formalin and embedded in paraffin. To determine the expression of KAT6A and KAT6B, murine monoclonal mouse antibodies directed

Characteristic	No. (%) of patients (n = 495)
<i>Tumour size</i>	
T1 (< 2 cm)	190 (38.0)
T2 (2–5 cm)	129 (26.0)
T3 (> 5 cm)	130 (26.0)
T4	46 (9.0)
<i>Tumour stage - pT</i>	
1	313 (63.23)
2	152 (30.71)
3	19 (3.84)
4	11 (2.22)
<i>Grading</i>	
I	92 (18.59)
II	312 (63.03)
III	91 (18.38)
<i>Nodal status pN</i>	
N0	298 (60.20)
N1	167 (33.74)
N2	18 (3.64)
N3	12 (2.42)
<i>ER status</i>	
Positive	324 (65.45)
Negative	171 (34.55)
<i>PR status</i>	
Positive	312 (63.03)
Negative	183 (36.97)
<i>HER2 status</i>	
Positive	311 (62.83)
Negative	184 (37.17)
<i>Triple negative status</i>	
Positive	40 (8.08)
Negative	455 (91.92)

Table 1. Clinicopathological data of the studied cases of invasive ductal breast carcinoma.

against KAT6A (1:200; PA5-66742, Thermo Fisher Scientific, Waltham, MA, USA) and KAT6B (1:200; PA5-52251, Thermo Fisher Scientific) were used. IHC was performed using Autostainer Link 48 (DakoCytomation, Glostrup, Denmark) to provide reliable and repeatable conditions.

RNA extraction, cDNA synthesis and real-time PCR reactions

Total RNA from the studied cell lines was isolated using an RNeasy Mini Kit (Qiagen, Hilden, Germany). Next, the total RNA was transcribed to cDNA using the High Capacity Reverse Transcriptase kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. RT-qPCR was carried out in 20 μ L volumes using the iTaq Universal Probes Supermix (Bio-Rad Laboratories, Hercules, CA, USA) on a 7500 Real-time PCR System. The TaqMan-specific probes used in the experiment (Hs01063026_m1 for KAT6A, Hs00202463_m1 for KAT6B, Hs_01046816_m1 for *ESR1*, and Hs 99999903_m1 for *ACTB* as a reference gene) were also obtained from Applied Biosystems. All the above-mentioned reactions were performed in triplicate under the following conditions: activation of polymerase at 50 °C for 2 min, initial denaturation at 94 °C for 10 min followed by 40 cycles of denaturation at 94 °C for 15 s, and annealing and elongation at 60 °C for 1 min. The relative mRNA expression of the studied markers was calculated using the $\Delta\Delta C_t$ method.

SDS-PAGE and western blotting

Whole cell lysates from the cell lines were obtained using Cell Lysis Buffer (Thermo Fisher Scientific) with the addition of a cocktail of inhibitors (Sigma, St. Louis MO, USA), 250 units of Benzonase[®] (Merck Millipore, Bedford, MA, USA), and 2 mM phenylmethanesulfonyl fluoride (PMSF). The lysates were mixed with 4X SDS-PAGE gel loading buffer (200 mM Tris-HCl [pH 6.8], 400 mM DTT, 8% SDS, 0.4% bromophenol blue, and 40% glycerol), loaded on a 10% acrylamide gel, separated by SDS-PAGE under reducing conditions, and then transferred onto a PVDF membrane in the XCell SureLock[™] Mini Gel Electrophoresis System (Thermo Fisher Scientific). After protein transfer, the membrane was incubated in a blocking solution (4% BSA in TBST buffer) for 1 h at RT, followed by overnight incubation at 4 °C with anti-KAT6A (1:500; PA5-66742, Thermo Fisher Scientific), anti-KAT6B (1:500; PA5-52251, Thermo Fisher Scientific), and anti-ER α (1:750, MA5-14501, Thermo Fisher Scientific). Subsequently, the membrane was rinsed with TBST buffer and incubated for 1 h at RT with secondary donkey anti-mouse antibody conjugated with HRP, diluted 1:3000 (709-035-149; Jackson ImmunoResearch, Mill Valley, CA, USA), and then rinsed and treated with the Immuno-Star HRP Chemiluminescent kit (Bio-Rad). Rabbit anti-human β -actin monoclonal antibody (#4970; Cell Signaling Technology, Danvers, MA, USA), diluted 1:1000, was used as the internal control. Western blotting results were analyzed using the ChemiDoc MP System (Bio-Rad).

siRNA transfection

Ambion predesigned siRNAs, including *GAPDH* siRNA as a positive control and scrambled sequence siRNA as a negative control, were used in the experiments. Specific siRNAs used were as follows: s15534 for KAT6A (*MYST-3*, Chr.8.) and s108335 for KAT6B (*MORF*, Chr.10.). MCF-7, an ER α -positive cell line, and MDA-MB-231, an ER α -negative cell line, were grown in 6-well plates as previously described. The concentration of siRNA and quantity of transfection reagent were determined experimentally. Cells were trypsinized, centrifuged at 1000 rpm for 5 min at 4 °C, and resuspended in fresh media before transfection. Ambion's siPORT NeoFX (6 μ l/well) lipid-based transfection reagent and siRNAs (50 nM final concentration) were individually diluted in OptiMEM and mixed. After 10 min, the transfection complexes were overlaid with 2×10^5 cells/well. Verification of KAT6A and KAT6B silencing and their impact on the ER α expression profile in an estrogen receptor (ER)-positive cell line was conducted.

Statistical analysis

The Shapiro-Wilk test was used to evaluate the normality assumption of the groups examined. The Wilcoxon signed-rank test was used to compare the differences between the LSCC and NMLT groups. Additionally, Spearman's correlation test was performed to analyze the existing correlations. All statistical analyses were performed using Prism 8.1.0 (GraphPad Software, La Jolla, CA, USA). The results were considered statistically significant at $p < 0.05$.

Results

KAT6A/B are associated with breast tumours features

Clinicopathological features of 495 patients with invasive ductal breast carcinoma were analyzed. KAT6A/B expression was measured using immunohistochemistry. The correlations between KAT6A/B expression, clinicopathological features, and prognosis were assessed. Using IHC, we determined the cellular localization and expression levels of KAT6A/B in 38 cases of FBD and 495 cases of IDC (Fig. 1). Nuclear expression of KAT6A and KAT6B was observed in 396 (80.0%) and 475 (96.0%) IDC samples, respectively, compared with 4 (10.5%) and 35 (92.0%) samples, respectively. Additionally, cytoplasmic expression of KAT6A and KAT6B was observed in 463 (93.5%) and 27 (5.45%) IDC samples, respectively, compared with 27 (71.0%) and 0 (0.0%) samples, respectively. Statistical analysis showed that KAT6A cytoplasmic and nuclear expression levels were significantly higher in IDC samples than in FBD (IRS 5.297 ± 2.884 vs. 2.004 ± 1.072 , $p < 0.0001$, Mann-Whitney test), and (IRS 5.133 ± 4.221 vs. 0.1665 ± 0.4024 , $p < 0.0001$, Mann-Whitney test) (Fig. 1E), respectively. In addition, a significantly higher cytoplasmic expression level of KAT6B was observed in IDC samples than in FBD samples (IRS 5.296 ± 4.2144 vs. 0.145 ± 0.079 , $p < 0.0001$, Mann-Whitney test) (Fig. 1F). The cytoplasmic expression profiles of KAT6A were significantly higher in grade 1 cases than in grade 3 cases ($p = 0.0109$, Mann-Whitney test), as well as in grade 2 cases than in grade 3 cases ($p = 0.0003$, Mann-Whitney test). Additionally, nuclear expression levels of KAT6A were elevated in grade 2 cases compared to grade 1 cases ($p = 0.0001$, Mann-

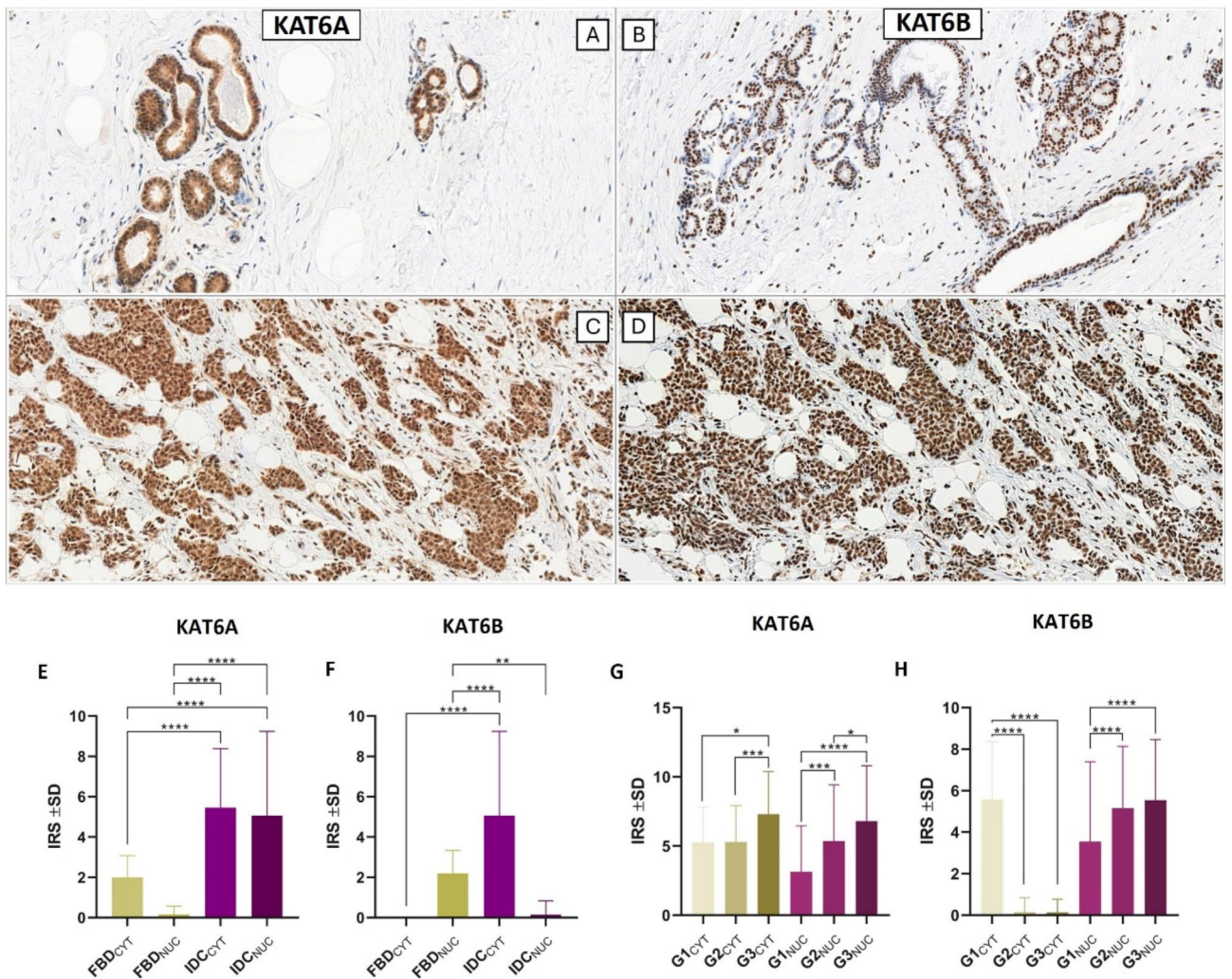


Fig. 1. Differentiated nuclear-cytoplasmic expression of KAT6A in FBD (A) and IDC (C), and nuclear expression of KAT6B in FBD (B) and IDC (D) cases. Original magnification, $\times 400$. KAT6A and KAT6B IHC reactions statistical analysis in FBD ($n = 38$) and IDC ($n = 495$) cases. Analysis of cytoplasmic and nuclear KAT6A expression levels (E) ($p < 0.0001$, Mann-Whitney test). KAT6B's significantly higher expression level between IDC and FBD samples ($p < 0.0001$, Mann-Whitney test) – (F). KAT6A's and KAT6B's expression in IDC with regard to malignancy Grade (G and H), respectively. * $p < 0.1$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Whitney test), grade 3 cases compared to grade 1 cases ($p < 0.0001$, Mann-Whitney test), and grade 3 cases compared to grade 2 cases ($p = 0.0265$, Mann-Whitney test) (Fig. 1G). Remarkably, there was a statistically significant decrease in cytoplasmic expression levels of KAT6B in grade 2 and grade 3 cases compared to grade 1 cases ($p < 0.0001$, Mann-Whitney test) (Fig. 1H). Similarly, nuclear expression levels of KAT6B were elevated in grade 2 cases compared to grade 1 cases and in grade 3 cases compared to grade 1 cases ($p < 0.0001$, Mann-Whitney test) (Fig. 1H).

As shown in Fig. 2, the Spearman correlation test revealed a strong positive correlation between KAT6A nuclear and cytoplasmic expression ($r = 0.3053$, $p < 0.0001$), KAT6A and KAT6B nuclear expression ($r = 0.3023$, $p < 0.0001$), and between KAT6A cytoplasmic expression and KAT6B nuclear expression ($r = 0.3212$, $p < 0.0001$). Additionally, statistical analysis showed correlations between the status of the ER and the expression of KAT6A and KAT6B (KAT6A_{CYT} vs. ER $r = 0.3421$, KAT6A_{NUC} vs. ER $r = 0.4373$, KAT6B_{NUC} vs. ER $r = 0.2496$).

We used Kaplan-Meier analysis to analyze the effect of KAT6A and KAT6B on the overall survival of patients with IDC. In patients with grade 3 tumors, a higher cytoplasmic KAT6A expression had a negative impact on the overall survival time compared to the group with lower KAT6A cytoplasmic expression ($p = 0.0427$) (Fig. 3A). According to the TNM staging, stage IIA patients with higher nuclear KAT6A expression levels had shorter overall survival times than those with lower expression levels ($p = 0.0387$) (Fig. 3B). Finally, the survival time of



Fig. 2. KAT6A_{CYT}, KAT6A_{NUC}, KAT6B_{CYT}, KAT6B_{NUC}, ER, PR, and HER2 Spearman rank correlation matrix among 495 breast cancer samples (IDC). Red colour indicates a negative correlation and blue indicates a positive correlations. The correlation coefficients are reflected in the circle's size and colour intensity. The correlation coefficients and matching colours are displayed in the colour bar on the right side of the graphic.

stage IA patients was significantly lower in groups with a higher expression of nuclear KAT6B compared to those with a lower expression ($p=0.0173$) (Fig. 3C).

KAT6A/B mRNAs and protein expression levels in BC cell lines

Real-time PCR gene expression analysis revealed that all studied breast cancer cell lines and the normal breast cell line ME16C showed significant KAT6A and KAT6B gene expression levels. A significantly higher expression level of KAT6A was found in the SK-BR-3 ($p<0.0001$) and T-47D ($p<0.01$) cell lines. In contrast, a significantly higher expression level of KAT6B was observed in the MCF-7 ($p<0.001$), SK-BR-3 ($p<0.0001$), and MDA-MB-231/BO2 ($p<0.001$) cell lines. Moreover, a significantly lower expression level of KAT6A was observed in the MCF-7 ($p<0.1$) and MDA-MB-231 ($p<0.01$) cell lines, and a significantly lower expression level of KAT6B was observed in the MDA-MB-231 ($p<0.0001$) cell line (Fig. 4).

The analysis of KAT6A and KAT6B protein levels using western blotting revealed decreased expression levels of KAT6A (250 kDa) in all studied cell lines: MCF-7 ($p<0.0001$), BT-474 ($p<0.0001$), SK-BR-3 ($p<0.0001$) and T-47D ($p<0.001$), MDA-MB-231 ($p<0.001$) and MDA-MB-231/BO2 ($p<0.001$) cell lines, as well as an increased expression level of KAT6B (215 kDa) in MCF-7 ($p<0.001$), BT-474 ($p<0.0001$), SK-BR-3 ($p<0.0001$), T-47D ($p<0.01$), and MDA-MB-231/BO2 ($p<0.01$) cell lines (Fig. 5).

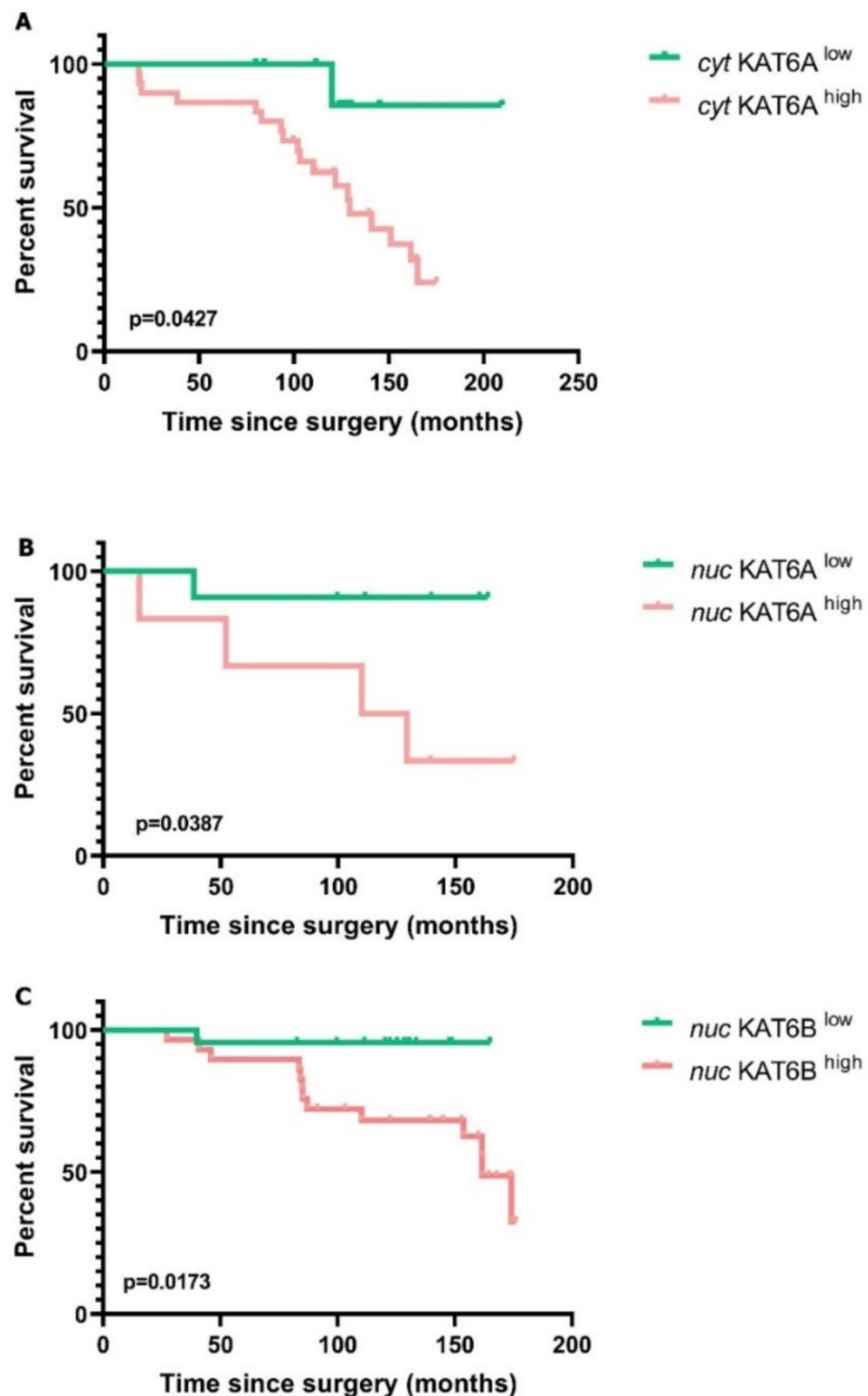


Fig. 3. Survival curve established by data obtained from KM-plot. **(A)** – the survival time of IDC patients with high expression of KAT6A_{CYT} with regard to TNM IIA stage is lower than that of patients with low expression ($p=0.0427$). **(B)** – among the patients with high KAT6A_{NUC} expression in the Grade 3 group, the survival time is lower than in patients with low expression ($p=0.0387$). **(C)** – TNM stage IIA patients with higher KAT6B_{NUC} expression show a shorter survival time compared to those with low KAT6B_{NUC} expressions ($p=0.0173$).

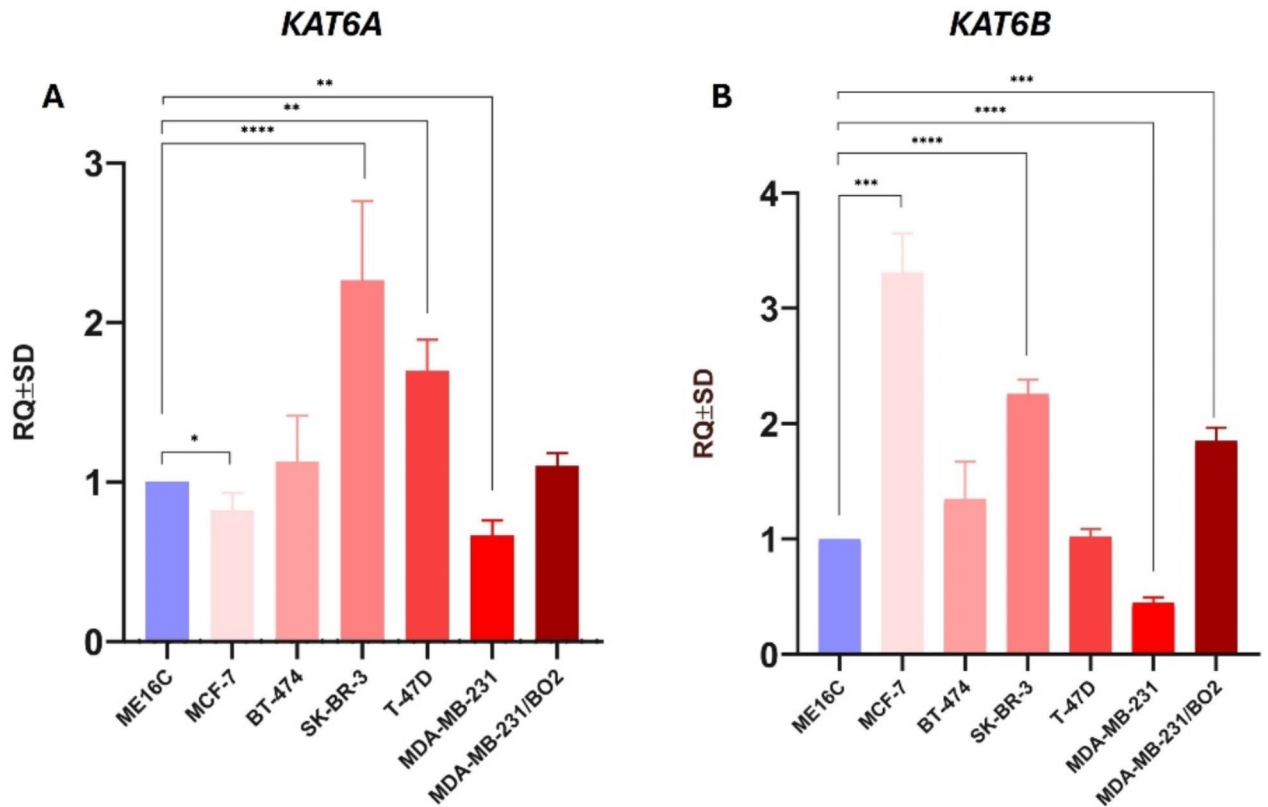


Fig. 4. Characteristics of breast cancer cell lines according to KAT6A (A) and KAT6B (B) expressions. RT-qPCR was used to analyse KAT6A and KAT6B mRNAs and *ACTB* was used as a reference gene. Analysis performed using unpaired t-test. * $p < 0.1$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

siRNA-related knock-down revealed the interactions between KAT6A and KAT6B proteins that influence the ER α levels in breast cancer cells

To assess the impact of siRNA-induced gene silencing on KAT6A/B expression, specific siRNAs were introduced into MCF-7 (ER α -positive) and MDA-MB-231 (ER α -negative) breast cancer cell lines for 24 and 48 h. The effectiveness of transfection was evaluated using RT-qPCR and Western blot analyses. As depicted in Fig. 6, a statistically significant decrease in the expression levels of the targeted genes was observed at both the 24 h and 48 h time points. In MCF-7 cells, downregulation of KAT6A led to concurrent downregulation of KAT6B after 24 and 48 h ($p < 0.0001$ and $p < 0.0001$, respectively), along with decreased expression of ESR1 after 48 h ($p < 0.0001$) compared to control cells. Simultaneously, in MDA-MB-231 cells, downregulation of KAT6B was evident after 24 h and 48 h ($p < 0.0001$ and $p < 0.0001$, respectively). Furthermore, in MCF-7 cells, downregulation of KAT6B resulted in the suppression of KAT6A and ESR1 after 24 and 48 h ($p < 0.0001$ and $p < 0.0001$, respectively). Similarly, in MDA-MB-231 cells, knockdown of the KAT6B gene led to inhibition of KAT6A mRNA after 24 h and 48 h ($p < 0.0001$). Western blot analysis results corroborated the gene expression inhibition findings. Specifically, we observed a significant decrease in both the mRNA and protein levels of Estrogen Receptor Alpha (ER α) following KAT6A knockdown at 48 h ($p < 0.0001$) and KAT6B knockdown at both 24 h ($p < 0.0001$) and 48 h ($p < 0.0001$) via siRNA-mediated silencing (Fig. 6A and C). Notably, 24 h post-KAT6A silencing, there was a significant increase in the ER α transcript ($p < 0.01$) and protein levels ($p < 0.0001$). Furthermore, in both ER α -positive and ER α -negative cell lines, significant downregulation of KAT6B was observed following KAT6A silencing, and vice versa.

Discussion

ϵ -Amino lysine acetylation is one of the most important post-transcriptional modifications (PTM). It has an impact not only at the protein level but also at the metabolome level. Lysine residues were first discovered as regulators of histone-dependent gene transcription; therefore, enzymes catalyzing lysine (K) acetylation were named histone acetyltransferases (HATs). Most HATs are multiprotein complexes with the ability to bind to diverse catalytic subunits that define their enzymatic activity. Because of HATs function, it should not come as a surprise that scientists have started to observe the amplification of their genes in some malignancies. Yu et al. showed that *KAT6A* is amplified by 11% and/or overexpressed in 15% of breast tumors⁵⁵. This increase in *KAT6A* gene abundance in the cell genome results in worse clinical outcomes in estrogen receptor-positive (ER+) breast cancers.

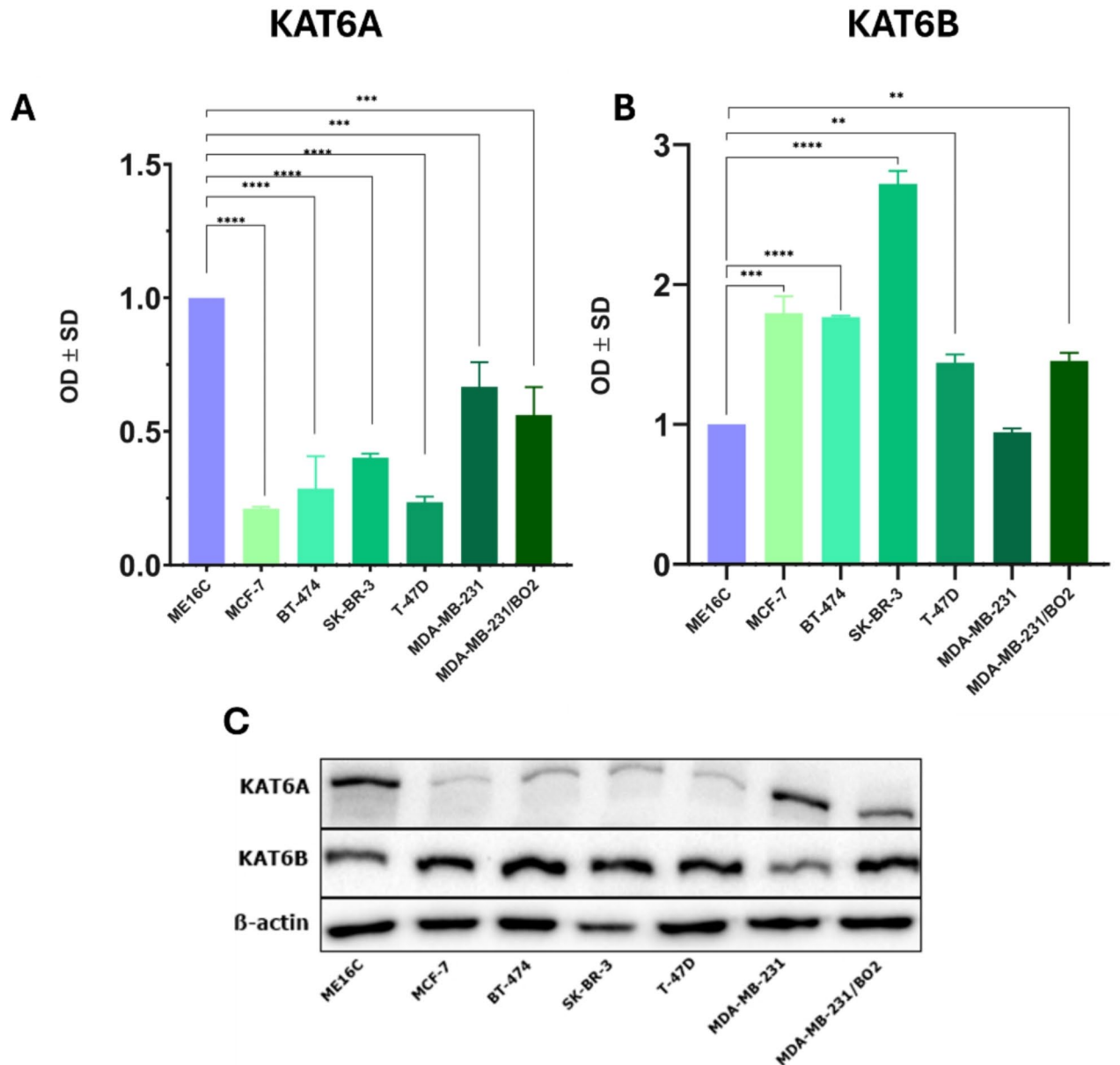


Fig. 5. Densitometric analysis of KAT6A (A) and KAT6B (B) bands of the selected breast cancer cell lines (western blot). Different expressions of the KAT proteins studied were noticed in all breast cancer cell lines and the control cell line (ME16C). Estrogen receptor (ER) positive cell lines (MCF-7, BT-474 and T-47D) show relatively higher expression levels of KAT6A and KAT6B proteins compared to ER-negative cell lines (ME16C, SK-BR-3, MDA-MB-231 and MDA-MB-231/BO2). Analysis performed using unpaired t-test. * $p < 0.1$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. (C) β -actin was used as an internal control. The data show the average standard deviation of three independent experiments. ***, $p < 0.001$; **** $p < 0.0001$. The results were identical after three repetitions of this experiment. It was repeated with similar results in two independent experiments. Source data are provided as a Supplementary S1.

Recent studies in mice clearly show that KAT6A/B acetyltransferases are involved in senescence processes, where their function is coupled with the tumor suppressor p16^{24,53,55-57}. Moreover, KAT6A/B enzymes exhibit a double-sided feature. They can both activate and suppress senescence via the acetylation of histone and non-histone substrates. PML, MOZ, and p53 interact with each other, increasing MOZ-mediated p53 acetylation and enhancing p53 activity. At the same time, MOZ is an important factor that stimulates *INK4A/ARF* suppressor locus. Therefore, MOZ suppresses *INK4A/ARF*-dependent senescence. MORF/MOZ proteins show very high evolutionary conservation among different organisms such as yeasts, flies, and mammals. This suggests that they play a crucial role in cell biology, especially in the regulation of the cell cycle and the maintenance of stem cells. A fluctuation in the physiological balance of KAT6A and KAT6B leads to different pathological states, such as microcephaly, global developmental delay, Noonan syndrome, Say-Barber-Biesecker-Young-Simpson syndrome,

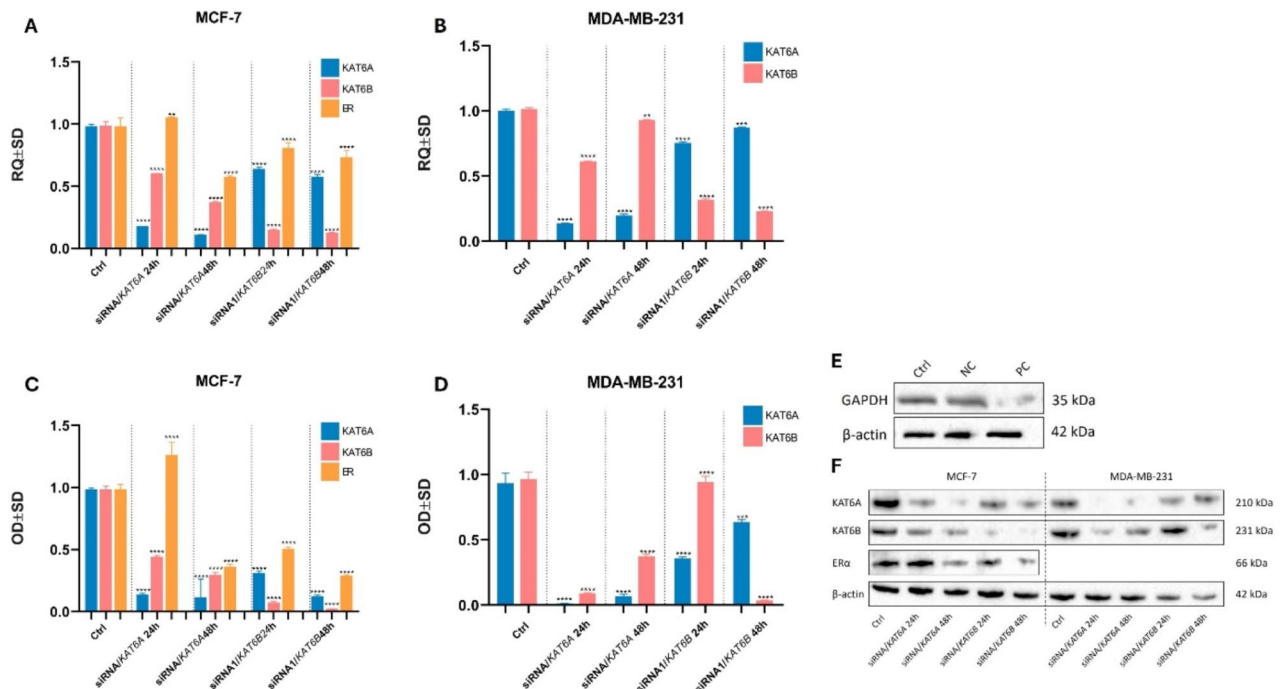


Fig. 6. Downregulation of KAT6A/B mRNAs and protein expression by siRNA-induced knock-down in breast cancer cell lines MCF-7 and MDA-MB-231. Quantitative polymerase chain reaction was used to analyze KAT6A, KAT6B and ER α mRNA expression in MCF-7 cell line (A) and KAT6A and KAT6B in MDA-MB-231 cell line (B). Western blot analysis (F) measured the effects of siRNA-mediated knockdown of KAT6A, KAT6B and ER in MCF-7 (C) and KAT6A/B in MDA-MB-231 (D) cell lines. GAPDH was used as an internal control (E). Data represent the mean and standard deviation of three independent experiments. Comparisons between groups were done using Student's t-test: * $p < 0.1$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Source data are provided as a Supplementary S1.

blepharophimosis-ptosis-epicanthus inversus syndrome, and genitopatellar syndrome^{58–64}. Moreover, there is increasing evidence for the role of KAT6A/B in malignancies such as leukemia, medulloblastoma, breast cancer, endometrial serous carcinoma, ovarian cancer, and colorectal cancer^{26,34,51,65–67}. However, to date, p53 has been identified as the only non-histone target of KAT6A. New findings suggest that HAT proteins may also be involved in β -catenin regulation, promoting proliferation and invasion, as well as cancer cell metastasis. Overactivation of the Wnt/ β -catenin signalling pathway results in the progression of different types of malignancies, including breast, lung, hepatocellular, gastric, prostate, colorectal, bone, and ovarian cancer. Therefore, it is highly possible that further investigation of KAT6A/B expression patterns and their possible mechanisms among cancers will help to better understand cancer physiology. Based on the fact that KAT6A/B are responsible for the drug resistance of cancer cells, targeting those acetyltransferases and their effector substrates may help in BC clinical treatment. It has already been proven and tested that KAT6B inhibition increases the response to treatment in SCLC patients treated with irinotecan³³.

The results of molecular biology techniques showed varied expression patterns of KAT6A and KAT6B among the studied cell lines, both at the mRNA and protein levels. These findings correlate with the results obtained for the BC patient samples. The results presented in this study correspond to the findings of another study that showed that ER-positive cell lines are characterized by increased KAT6A/B gene expression levels compared to the normal epithelial breast cell line MEF16C and triple-negative cell lines MDA-MB-231 and MDA-MB-231/BO2. The SK-BR-3 cell line is ER-negative, but recent studies suggest that it weakly expresses the estrogen receptor. Therefore, increased expression of KAT6A and KAT6B in this cell line may also be involved in the KAT6A/B/ER axis. Moreover, successful knockdown of the KAT6A gene results in a profound reduction in ER α expression. The results of this study support these findings. Our observations indicate that nuclear expression levels of KAT6A and KAT6B positively correlate with estrogen receptor (ER) status in invasive ductal carcinoma (IDC) cases. This finding suggests their functional role in the transcription of the estrogen receptor gene and other associated transcripts due to their nuclear localization. We observed an interesting paradox where higher expression of KAT6A and KAT6B is associated with estrogen receptor (ER) positivity, yet patients with elevated levels of these proteins exhibit reduced survival times. This could be attributed to the dual role of KAT6A and KAT6B. While these proteins may promote the growth of ER-positive breast cancers, which are typically more responsive to hormone therapies and initially have a more favorable prognosis, they may simultaneously activate oncogenic pathways that drive more aggressive tumor behavior. Consequently, the higher expression of KAT6A and KAT6B may not only support ER-mediated tumor growth but also contribute to enhanced tumor

progression, increased metastatic potential, and resistance to therapy. Therefore, despite the typically favorable prognosis associated with ER-positive status, the overexpression of KAT6A and KAT6B appears to counteract this advantage, leading to poorer clinical outcomes for patients.

Consequently, KAT6A and KAT6B may serve as potential therapeutic targets in ER-positive breast cancer. Furthermore, siRNA-mediated knockdown of KAT6A/B genes demonstrated their involvement in the regulation of ESR1 transcription, suggesting that the status of KAT6A/B may correlate with the receptor status of invasive ductal carcinoma (IDC) cells. Interestingly, knockdown of KAT6A resulted in the downregulation of KAT6B, suggesting a potential regulatory interaction between these two genes. KAT6A and KAT6B are members of the MYST family of histone acetyltransferases and their structural similarity often results in functional redundancy. This could occur in one of the following scenarios: (1) *functional redundancy*: KAT6A and KAT6B may have overlapping cellular functions. Knocking down KAT6A could disrupt cellular processes that depend on histone acetylation, leading to compensatory downregulation of KAT6B to mitigate the effects of KAT6A loss; (2) *feedback mechanisms*: KAT6B expression could be regulated by feedback mechanisms in response to changes in histone acetylation levels caused by KAT6A knockdown. If KAT6A knockdown leads to alterations in histone acetylation patterns, the cell may respond by downregulating KAT6B to maintain homeostasis. (3) *regulatory interactions*: KAT6A and KAT6B may directly or indirectly regulate each other's expression through transcriptional or post-transcriptional mechanisms. Knocking down KAT6A disrupts these regulatory interactions, resulting in the downregulation of KAT6B; (4) *compensatory responses*: Cells often employ compensatory mechanisms to maintain essential cellular functions in response to genetic perturbations. Downregulation of KAT6B could be part of a compensatory response aimed at restoring cellular balance in the absence of KAT6A; (5) *functional crosstalk*: KAT6A and KAT6B may participate in common signaling pathways or biological processes. Knocking down KAT6A may affect these shared functions, indirectly influencing KAT6B expression. Therefore, we believe that this regulation is particularly important in ER-positive breast cancer, where KAT6A/B-mediated acetylation enhances ER expression and activity, thereby promoting tumor growth and proliferation. Consequently, KAT6A and KAT6B are not only biomarkers for ER status but also potential therapeutic targets for treating ER-positive breast cancer.

Overall, the downregulation of KAT6B upon KAT6A knockdown suggested a complex interplay between these two genes in cellular regulation, likely involving multiple molecular mechanisms. Further investigation is needed to fully elucidate the underlying regulatory mechanisms and the functional consequences of this relationship.

In conclusion, the data presented in this study clearly showed an increase in KAT6A and KAT6B expression levels in IDC compared to those in mastopathy cases. This suggests that these two HAT proteins may be involved in cancer biology. Therefore, it is important to further study the possible mechanisms by which KAT6A/B is involved. Pharmacological targeting of KAT6A/B via inhibitors could be a novel strategy for antitumor treatment.

Data availability

All data associated with this study are present in the manuscript. Materials used in this study are available from the corresponding author upon reasonable request.

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Author contributions

Conceptualization, M.O.; data curation, M.O., B.S., and H.R.; formal analysis, M.O.; funding acquisition, M.O.; investigation, M.O., M.M., A.R., and A.P.; methodology, M.O.; resources, M.O.; supervision, M.O.; visualization, M.O.; writing—original draft, M.O.; writing—review and editing, M.O., A.P., and M.M. All authors have read and agreed to the published version of the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Institutional Review Board Statement

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Bioethics Committee of Wrocław Medical University (No. 116/2024 N).

Informed consent

for histopathological examination and further research using the collected tumor material was obtained from all subjects involved in this study.

Additional information

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