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Impact of MiRNAs on Wnt-related gene activity in breast cancer

Tomasz Sirek[1,2]✉, Agata Sirek[3], Nikola Zmarzły[3], Marcin Opławski[4,5], Katarzyna Król-Jatręga[1,2], Dariusz Boroń[6], Michał Chalcarz[7,8], Piotr Ossowski[3], Konrad Dziobek[4], Damian Strojny[9,10], Joanna Szymańska[3], Julia Gajdeczka[3], Przemysław Borawski[11,13], Kacper Boroń[1] & Beniamin Oskar Grabarek[3,12]

Breast cancer is the most commonly diagnosed cancer in women. The Wnt pathway is involved in the regulation of cell proliferation, differentiation, survival, and migration. Its disruption may promote the induction of breast cancer and its further development. The aim of the study was to identify micro RNA (miRNAs) that could potentially influence the activity of Wnt-related genes in five types of breast cancer in Polish women. Study included patients with five breast cancer subtypes: 130 luminal A, 96 HER2-positive luminal B, 100 HER2-negative luminal B, 36 non-luminal HER2-positive, 43 triple negative breast cancer (TNBC). Tumor tissue was removed during surgery along with a margin of healthy tissue (control group). Expression profile of Wnt-related genes was assessed with mRNA microarrays and reverse transcription quantitative polymerase chain reaction (RT-qPCR). Protein expression was conducted with enzyme-linked immunosorbent assay (ELISA). miRNA profiling was carried out with miRNAs microarrays and the miRDB database. Reduced activity of miR-130a could be related to overexpression of *CCND1* and *GSK3B*. Similarly for miR-199a and *GSK3B*. High activity of miR-2115 could be associated with downregulation of *TCF7L2*. *WNT5 A* overexpression may be linked to low levels of miR-497. In addition, study revealed increased levels of *APC*, *DVL3*, *LEF1* with reduced activity of *FZD4* and *TCF7L1* in all five subtypes of breast cancer.

According to global cancer statistics from 2022, breast cancer is the most commonly diagnosed cancer in women, as well as the leading cause of cancer-related deaths. It has been estimated that nearly 1 in 4 cases is breast cancer, and in terms of deaths, it is 1 in 6 cancer deaths in women worldwide[1]. The National Cancer Registry in Poland revealed that in 2021, 24.2% of women's cancers were breast cancers. They were also the second most common cause of cancer-related deaths. Data regarding young women (20–44 years) are also disturbing, where breast cancer accounts for 28% of diagnoses and 27% of deaths[2]. Treatment for breast cancer depends on its type and includes surgery, chemotherapy, radiotherapy, immunotherapy, hormonal therapy, and targeted therapy[3].

In the classification of breast cancer, the activity of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), as well as Ki67 proliferation index are of great importance[4]. Luminal A is one of the most commonly recognized subtypes and due to its non-aggressive nature, it has a good prognosis. It is characterized by the presence of ER and PR, as well as the absence of HER2 and a low level of Ki-67[5]. In the case of the luminal B subtype, increased proliferation is observed, which results in a worse prognosis compared to luminal A cancer. ER is present on the cell surface, while HER2 is present only in some tumors[6]. Non-luminal HER2-positive subtype shows even more increased proliferation and division. It is characterized by the presence of HER2 and the absence of ER and PR[7]. Triple negative breast cancer (TNBC) is the most aggressive subtype compared to the others, which is associated with a worse prognosis. It lacks ER, PR, and HER2 on the cell surface, which makes its treatment difficult[8].

[1]Department of Plastic Surgery, Faculty of Medicine, Academia of Silesia, Katowice 40-555, Poland. [2]Department of Plastic and Reconstructive Surgery, Hospital for Minimally Invasive and Reconstructive Surgery in Bielsko-Biała, Bielsko-Biala 43-316, Poland. [3]Department of Medical and Health Sciences, Collegium Medicum, WSB University, 41-300 Dabrowa, Górnicza, Poland. [4]Department of Gynecology and Obstetrics with Gynecologic Oncology, Ludwik Rydygier Memorial Specialized Hospital, Kraków 31-826, Poland. [5]Department of Gynecology and Obstetrics, Faculty of Medicine and Health Sciences, Andrzej Frycz Modrzewski University in Kraków, Kraków 30-705, Poland. [6]Uczelnia Medyczna im. Marii Skłodowskiej-Curie, Warszawa 00-136, Poland. [7]Chalcarz Clinic-Aesthetic Surgery, Aesthetic Medicine, Poznan 60-001, Poland. [8]Bieńkowski Medical Center-Plastic Surgery, Bydgoszcz 85-020, Poland. [9]Institute of Health Care, National Academy of Applied Sciences in Przemyśl, Przemyśl 37-700, Poland. [10]New Medical Techniques Specjalist Hospital of St. Family in Rudna Mała, Rzeszów 36-060, Poland. [11]Włocławek, Poland. [12]Department of Molecular, Biology Gyncentrum Fertility Clinic, Katowice 40-055, Poland. [13]Przemyslaw Borawski is an Independent Researcher. ✉email: drtskierka@gmail.com

The Wnt pathway is involved in the regulation of cell proliferation, differentiation, survival, and migration. It has been shown that its disruption may promote the induction of breast cancer and its further development. For this reason, therapies based on its blocking are taken into account[9]. Importantly, the Wnt pathway can function via canonical and non-canonical pathways. The canonical pathway, called the Wnt/β-catenin pathway, mainly controls cell proliferation. It is triggered by the binding of Wnt ligands to the frizzled receptors (FZDs) and low density lipoprotein receptor-related protein 5/6 (LRP5/6) co-receptors. Activation of target genes occurs via transcription factor (TCF) and lymphoid enhancer factor (LEF)[10,11]. The noncanonical pathway is β-catenin-independent and involves the Wnt5a type ligands. It can occur via the Wnt/planar cell polarity (PCP) and Wnt/Ca^{2+}pathways[12,13]. The noncanonical Wnt pathway interacts with many other signaling pathways, making it difficult to find a Wnt pathway-specific target[14]. Another important issue is the balance between the canonical and noncanonical pathways. Studies indicate that Wnt5a, mainly assigned to the noncanonical pathway, can regulate the activity of both pathways, but the mechanisms involved still remain unclear[14]. Roarty et al. reported that loss of transforming growth factor-β (TGF-β) or Wnt5a led to activation of the canonical Wnt pathway in mammary epithelium, redirecting tumor phenotype[15]. On the other hand, Gujral et al. observed overexpression of Wnt5a in metastatic breast cancer cell lines and high-grade tumors[16]. In addition, the Wnt pathway in breast cancer is also associated with immune evasion[17], resistance to apoptosis induced by cisplatin, doxorubicin, paclitaxel[18], induction of cell cycle arrest[19]. The Wnt pathway is therefore complex, and its activity and direction of signaling in breast cancer depend on many factors.

MicroRNAs (miRNAs) are small non-coding RNAs, typically 20–24 nucleotides in length, that play crucial roles in regulating a wide range of biological processes in mammals and other multicellular organisms[20,21]. These miRNAs influence key functions related to cancer, such as cell proliferation, cell cycle regulation, apoptosis, differentiation, migration, and metabolism[22]. While several aspects of miRNA biogenesis and their repressive mechanisms remain unclear, many essential processes have been identified. Most miRNAs are transcribed by RNA polymerase II, either as independent transcription units or embedded within the introns of protein-coding genes. Under normal physiological conditions, miRNAs are integral to feedback loops and contribute to the stability of biological processes by buffering fluctuations in gene expression. This buffering action allows miRNAs to fine-tune protein production, preventing overexpression beyond optimal levels[22–25].

Research on miRNAs and cancer is vast and diverse, encompassing a variety of diseases and experimental approaches. However, studying miRNAs is complicated by their high redundancy. One challenge lies in the fact that a single miRNA often targets multiple mRNAs, meaning that the phenotypic impact of deregulating one miRNA is unlikely to result from a single target. Although many studies focus on individual targets, miRNAs typically exert their full effects through a range of mRNAs, some of which may belong to the same cellular pathway. Additionally, miRNAs often exist in families with similar seed sequences, further complicating experimental interpretation. At the mRNA level, redundancy also occurs, as distinct miRNAs with different seed sequences can co-regulate the same target gene[26–30].

Investigating the role of miRNAs in cancer is further complicated by the genetic heterogeneity of tumors and cancer cell lines. Often, multiple miRNAs are found to be deregulated in the same tumor. Moreover, because individual miRNAs regulate numerous transcripts, their role in cancer can be context dependent[31]. As a result, a particular miRNA might be upregulated in certain cancers, acting as an oncogene, while being downregulated in others, suggesting a tumor suppressor role. For example, miR-29 functions as a tumor suppressor in lung cancer, but exhibits oncogenic properties in breast cancer[32,33]. The aim of the study was to identify miRNAs that could potentially influence the activity of Wnt-related genes in five subtypes of breast cancer in Polish women.

Results

Gene expression profile determined by mRNA microarrays

Among 101 mRNAs corresponding to 46 genes related to the Wnt pathway, one-way ANOVA showed that 18 mRNAs significantly changed their expression in breast cancer compared to control ($p < 0.05$; FC > 2 or <−2). Tukey's post-hoc test revealed that 11 mRNAs significantly changed their expression in luminal A cancer, 13 mRNAs in HER2-positive luminal B cancer, 14 mRNAs in HER2-negative luminal B cancer, 14 mRNAs in non-luminal HER2-positive cancer, 13 mRNAs in TNBC. Venn diagram highlighted differential and common genes (Fig. 1).

Overexpression of *FZD1* and *FZD2* was characteristic for luminal A cancer. In turn, decreased expression of *WNT6* was characteristic for HER2-negative luminal B cancer, and *FZD3* for both subtypes of luminal B cancers. Overexpression of *FZD6* and silencing of *CTNNB1* were noted in non-luminal HER2-positive cancers and TNBC. Additionally, *FZD7* and *FZD8* were differential genes in all subtypes of breast cancer except luminal A. Moreover, 9 genes significantly changed their expression regardless of the cancer subtype: *APC*, *CCND1*, *DVL3*, *FZD4*, *GSK3B*, *LEF1*, *TCF7L1*, *TCF7L2*, *WNT5 A*. Detailed data for these genes are presented in Table 1.

APC, *CCND1*, *DVL3*, *GSK3B*, *LEF1*, *WNT5 A* showed significant overexpression in breast cancer samples regardless of the subtype, while reduced activity was noted for *FZD4*, *TCF7L1*, *TCF7L2*.

Expression profile of APC, CCND1, DVL3, FZD4, GSK3B, LEF1, TCF7L1, TCF7L2, WNT5 A determined by RT-qPCR and ELISA

In the next step, RT-qPCR was used to determine the expression profile of 9 genes differentiating breast cancer regardless of its subtype (Fig. 2).

The analysis showed that the RT-qPCR results corresponded to those obtained in the microarray experiment. Then, the expression of the studied genes was determined at the protein level (Table 2).

APC, CCND1, DVL3, GSK3B, LEF1 and WNT5 A levels were significantly increased in all types of breast cancer samples compared to the control group. FZD4, TCF7L1, TCF7L2 levels were also decreased, which was

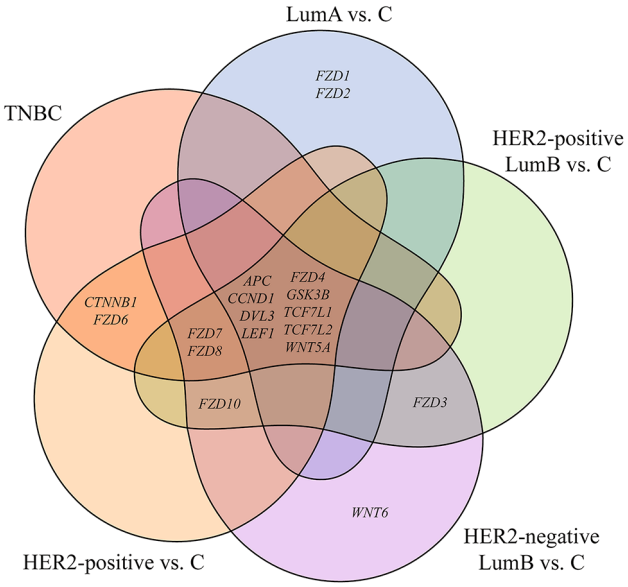

Fig. 1. Venn diagram of genes differentiating breast cancer from the control. LumA, luminal A; LumB, luminal B; HER2, human epidermal growth factor receptor 2; TNBC, triple-negative breast cancer; C, control; APC, adenomatous polyposis coli protein; CCND1; cyclin D; CTNNB1, beta-catenin; DVL3, dishevelled segment polarity protein 3; FZD, frizzled family receptor; GSK3B, glycogen synthase kinase-3 beta; LEF1, lymphoid enhancer binding factor 1; TCF7L1/2, transcription factor 7 like 1/2; WNT5 A/6, Wnt family member 5 A/6.

ID	mRNA	Fold change				
		LumA vs. C	HER2-positive LumB vs. C	HER2-negative LumB vs. C	HER2-positive vs. C	TNBC vs. C
203525_s_at 203526_s_at	*APC*	2.1 2.19	2.13 2.33	2.4 2.27	2.44 2.76	3.12 3.4
208711_s_at	*CCND1*	3.45	4.3	3.8	2.59	4.91
201908_at	*DVL3*	3.09	2.81	3.6	2.72	4.12
218665_at	*FZD4*	−3.24	−3.05	−3.22	−2.46	−3.47
226191_at 209945_s_at	*GSK3B*	2.06 2.28	2.21 3.27	2.29 2.67	2.68 3.96	3.09 4.66
221558_s_at	*LEF1*	3.15	2.2	3.06	2.05	3.72
221016_s_at	*TCF7L1*	−2.7	−3.77	−4.62	−3.47	−4.9
212761_at	*TCF7L2*	−2.8	−4.59	−4.63	−3.65	−5.11
205990_s_at	*WNT5 A*	2.15	2.54	2.78	4.45	5.29

Table 1. List of mRNAs representing genes involved in Wnt signaling differentiating breast cancer from the control regardless of its subtype ($p < 0.05$; FC > 2 or < −2). ID, number of the probe; LumA, luminal A; LumB, luminal B; HER2, human epidermal growth factor receptor 2; TNBC, triple-negative breast cancer; C, control; APC, adenomatous polyposis coli protein; CCND1; cyclin D; DVL3, dishevelled segment polarity protein 3; FZD4, frizzled family receptor 4; GSK3B, glycogen synthase kinase-3 beta; LEF1, lymphoid enhancer binding factor 1; TCF7L1/2, transcription factor 7 like 1/2; WNT5 A, Wnt family member 5 A.

consistent with the analysis at the mRNA level. Furthermore, TCF7L1, TCF7L2 reached levels below detection in all breast cancer subtypes except luminal A.

MiRNA target prediction

The miRNA target prediction analysis has identified several miRNAs that may play crucial roles in regulating Wnt pathway genes in breast cancer. Among these, miR-130a was associated with the overexpression of *CCND1* and *GSK3B*, while miR-199a likely contributed to the downregulation of *FZD4* and the overexpression of *GSK3B*. Additionally, miR-2115 was linked to the reduced expression of *TCF7L2*, and miR-497 appeared to be responsible for the overexpression of *WNT5 A*. No miRNA was found to regulate *APC*, *DVL3*, *LEF1*, or *TCF7L1* under the study's conditions. These findings suggest that miRNAs, particularly miR-130a, miR-199a, miR-2115, and miR-497, play a significant role in modulating the activity of Wnt pathway-related genes, potentially driving dysregulation in breast cancer subtypes. The expression pattern analysis revealed distinct changes in both mRNA and miRNA activity across different breast cancer subtypes. The overexpression of *CCND1* and *GSK3B*

Fig. 2. Expression profile of selected genes determined by RT-qPCR compared with the control ($p < 0.001$). LumA, luminal A; LumB, luminal B; HER2, human epidermal growth factor receptor 2; TNBC, triple-negative breast cancer; C, control; APC, adenomatous polyposis coli protein; CCND1; cyclin D; DVL3, dishevelled segment polarity protein 3; FZD4, frizzled family receptor 4; GSK3B, glycogen synthase kinase-3 beta; LEF1, lymphoid enhancer binding factor 1; TCF7L1/2, transcription factor 7 like 1/2; WNT5 A, Wnt family member 5 A; ACTB, β-actin. Data are presented as mean ± standard deviation. Significant comparisons between groups are marked with symbols on the graph. $, $p < 0.05$ vs. HER2-positive lumB; #, $p < 0.05$ vs. HER2-negative lumB; %, $p < 0.05$ vs. non-luminal HER2; @, $p < 0.05$ vs. TNBC.

Protein [ng/mL]	Control	LumA	HER2-positive LumB	HER2-negative LumB	HER2-positive	TNBC
APC	1.23 ± 0.15	2.51 ± 0.19*	3.06 ± 0.3*	3.18 ± 0.24*	3.4 ± 0.38*	3.98 ± 0.42*
CCND1	1.57 ± 0.2	3.29 ± 0.21*	4.04 ± 0.26*	3.77 ± 0.25*	3.05 ± 0.18*	4.76 ± 0.35*
DVL3	7.94 ± 0.14	19.33 ± 0.23*	18.43 ± 0.31*	22.23 ± 0.25*	18.09 ± 0.18*	31.09 ± 0.28*
FZD4	11.22 ± 0.15	4.63 ± 0.2*	4.98 ± 0.22*	4.5 ± 0.2*	5.26 ± 0.24*	4.25 ± 0.15*
GSK3B	2.26 ± 0.16	3.82 ± 0.21*	3.74 ± 0.19*	4.12 ± 0.24*	4.4 ± 0.26*	5.09 ± 0.18*
LEF1	0.94 ± 0.11	2.8 ± 0.19*	2.47 ± 0.2*	2.66 ± 0.2*	2.34 ± 0.18*	3.16 ± 0.2*
TCF7L1	0.48 ± 0.12	0.2 ± 0.09*	below detection threshold*	below detection threshold*	below detection threshold*	below detection threshold*
TCF7L2	1.68 ± 0.08	0.78 ± 0.1*	below detection threshold*	below detection threshold*	below detection threshold*	below detection threshold*
WNT5 A	1.72 ± 0.15	3.23 ± 0.13*	3.49 ± 0.16*	3.62 ± 0.19*	4.78 ± 0.15*	6.15 ± 0.14*

Table 2. Concentration of APC, CCND1, DVL3, FZD4, GSK3B, LEF1, TCF7L1, TCF7L2, WNT5 A in breast cancer and control group ($p < 0.05$). LumA, luminal A; LumB, luminal B; HER2, human epidermal growth factor receptor 2; TNBC, triple-negative breast cancer; C, control; APC, adenomatous polyposis coli protein; CCND1; cyclin D; DVL3, dishevelled segment polarity protein 3; FZD4, frizzled family receptor 4; GSK3B, glycogen synthase kinase-3 beta; LEF1, lymphoid enhancer binding factor 1; TCF7L1/2, transcription factor 7 like 1/2; WNT5 A, Wnt family member 5 A. * $p < 0.05$ vs. control.

was associated with the downregulation of miR-130a, showing fold changes of − 2.61 in luminal A, − 3.15 in HER2-positive luminal B, − 4.35 in HER2-negative luminal B, − 5.02 in HER2-positive non-luminal, and − 6.24 in TNBC, suggesting miR-130a's reduced regulatory control over these genes. Similarly, miR-199a, which targets *FZD4* and *GSK3B*, was significantly downregulated with fold changes ranging from − 2.07 in luminal A to − 4.69 in TNBC, potentially contributing to the observed dysregulation of these genes. In contrast, miR-2115 showed upregulation, particularly in HER2-positive cancers, with fold changes of 2.01 in luminal A and up to 2.98 in HER2-positive non-luminal cancer, correlating with the reduced expression of *TCF7L2*. Additionally, *WNT5 A* overexpression was linked to the downregulation of miR-497, with fold changes ranging from − 2.39 in luminal A to − 3.38 in TNBC (Table 3).

mRNA	miRNA	Target score	Fold change				
			LumA vs. C	HER2-positive LumB vs. C	HER2-negative LumB vs. C	HER2-positive vs. C	TNBC vs. C
CCND1 *GSK3B*	miR-130a	95 89	−2.61	−3.15	−4.35	−5.02	−6.24
FZD4 *GSK3B*	miR-199a	97 88	−2.07	−2.65	−2.73	−3.18	−4.69
TCF7L2	miR-2115	91	2.01	2.27	2.18	2.98	2.44
WNT5 A	miR-497	98	−2.39	−2.76	−2.62	−2.91	−3.38

Table 3. Expression of MiRNAs potentially involved in the regulation of the studied genes ($p < 0.05$; FC > 2 or < −2). LumA, luminal A; LumB, luminal B; HER2, human epidermal growth factor receptor 2; TNBC, triple-negative breast cancer; C, control; CCND1; cyclin D; FZD4, frizzled family receptor 4; GSK3B, glycogen synthase kinase-3 beta; TCF7L2, transcription factor 7 like 2; WNT5 A, Wnt family member 5 A.

Overall survival (OS) analysis

Overall survival analysis was performed for 9 mRNAs selected in the study: *APC*, *CCND1*, *DVL3*, *FZD4*, *GSK3B*, *LEF1*, *TCF7L1*, *TCF7L2*, *WNT5 A* (Figs. 3, 4, 5, 6 and 7).

In luminal A cancer, poorer survival is associated with low levels of CCND1, LEF1, TCF7L1, WNT5 A and overexpression of GSK3B (Fig. 3).

In HER2-positive luminal B cancer, reduced APC expression and increased TCF7L1 levels negatively impact overall survival (Fig. 4).

In turn, in HER2-negative luminal B cancer, decreased GSK3B and TCF7L2 activity contributes to the worsening of overall survival (Fig. 5).

The analysis also showed a negative impact of APC overexpression and decreased CCND1 and WNT5 A activity on overall survival in non-luminal HER2-positive cancer. (Fig. 6).

In TNBC, high levels of APC worsen overall survival. Among the remaining genes, FZD4 is on the border of statistical significance, and its decreased activity could translate into a worse prognosis (Fig. 7).

Discussion

In this study, we determined the expression level of genes related to the Wnt pathway in 5 subtypes of breast cancer: luminal A, HER2-positive luminal B, HER2-negative luminal B, non-luminal HER2-positive, TNBC. Analysis at the mRNA level showed significant overexpression of *APC*, *CCND1*, *DVL3*, *GSK3B*, *LEF1*, *WNT5 A* with reduced activity of *FZD4*, *TCF7L1*, *TCF7L2*, which was then confirmed at the protein level. The study also predicted which miRNAs differentiating breast cancer tissues from the control may participate in the regulation of the activity of the selected 9 genes.

APC and GSK3B together with axin belong to the catalytic complex, which is part of the Wnt pathway. In the absence of Wnt ligands, a low level of β-catenin is observed, which results from the activity of the complex. It enables the phosphorylation of β-catenin, consequently leading to its ubiquitination and degradation in proteasomes. The appearance of the Wnt ligand and its binding to the FZD receptor activates the Dvl protein, which causes the dissociation of axin from the catalytic complex. As a result, β-catenin is not destroyed, but stabilized and transferred to the nucleus, where transcription of target genes takes place with the help of TCF/LEF transcription factors[34]. *APC* is considered a tumor suppressor due to its involvement in the Wnt pathway and has been described in the context of breast cancer, colorectal cancer, gastric cancer, and prostate cancer. Saelee and Pongtheerat demonstrated reduced *APC*levels in breast cancer resulting from promoter hypermethylation, which was also associated with more aggressive behavior of tumor cells [25–28][35]. Stefanski et al. observed that decreased *APC*activity in breast cancer promotes doxorubicin resistance[36]. *GSK3B* is regulated by the phosphoinositide 3 kinase PI3 K/Akt pathway, which closely interlocks with the Wnt pathway. Quintayo et al. reported that high expression of *GSK3B*promoted the reduction of distant relapse-free survival[37]. Vijay et al. observed that the use of a *GSK3B*inhibitor promoted the reduction of epithelial-mesenchymal transition (EMT) in TNBC. Moreover, higher expression of this gene was correlated with poorer overall patient survival[38]. Gao et al. pointed out that *GSK3B* is present in many cancers and highlighted its involvement in various signaling pathways associated with the avoidance of targeted therapy, chemotherapy, and radiotherapy. They observed a significant increase in *GSK3B*levels in cisplatin-resistant MCF-7/MDR cells[39].

In our study, *APC* was overexpressed in studied breast cancer subtypes, which translated into reduced levels of β-catenin that were particularly significant in non-luminal HER2-positive cancer and TNBC. Interestingly, in these tumors, high levels of *APC* were associated with poorer overall survival. In the case of *GSK3B*, we also noted its overexpression. Our analysis allowed us to identify 2 miRNAs that may potentially participate in the regulation of *GSK3B* activity: miR-130a and miR-199a. We observed a significant decrease in their levels, which may be the reason for excessive activation of *GSK3B*. Chen et al. also reported a decrease in miR-130a activity in breast cancer, which inhibited its invasion and migration[40]. Huang et al. observed that high expression of miR-130a abolished drug resistance in the Doxorubicin-resistant MCF-7/Adr breast cancer cell line[41]. Interestingly, according to our prediction, miR-130a may potentially participate in the regulation of *CCND1*, which was overexpressed in our analysis, indicating increased proliferation[42]. Goel et al. showed that overexpression of this cyclin in breast cancer is very important in HER2 therapy resistance[43]. Jeffreys et al. also observed that *CCND1*overexpression was associated with poor prognosis, especially in ER positive breast cancer[44]. In the case of miR-199a, Kim et al. showed a significant decrease in its activity in the breast cancer lines MDA-MB-231,

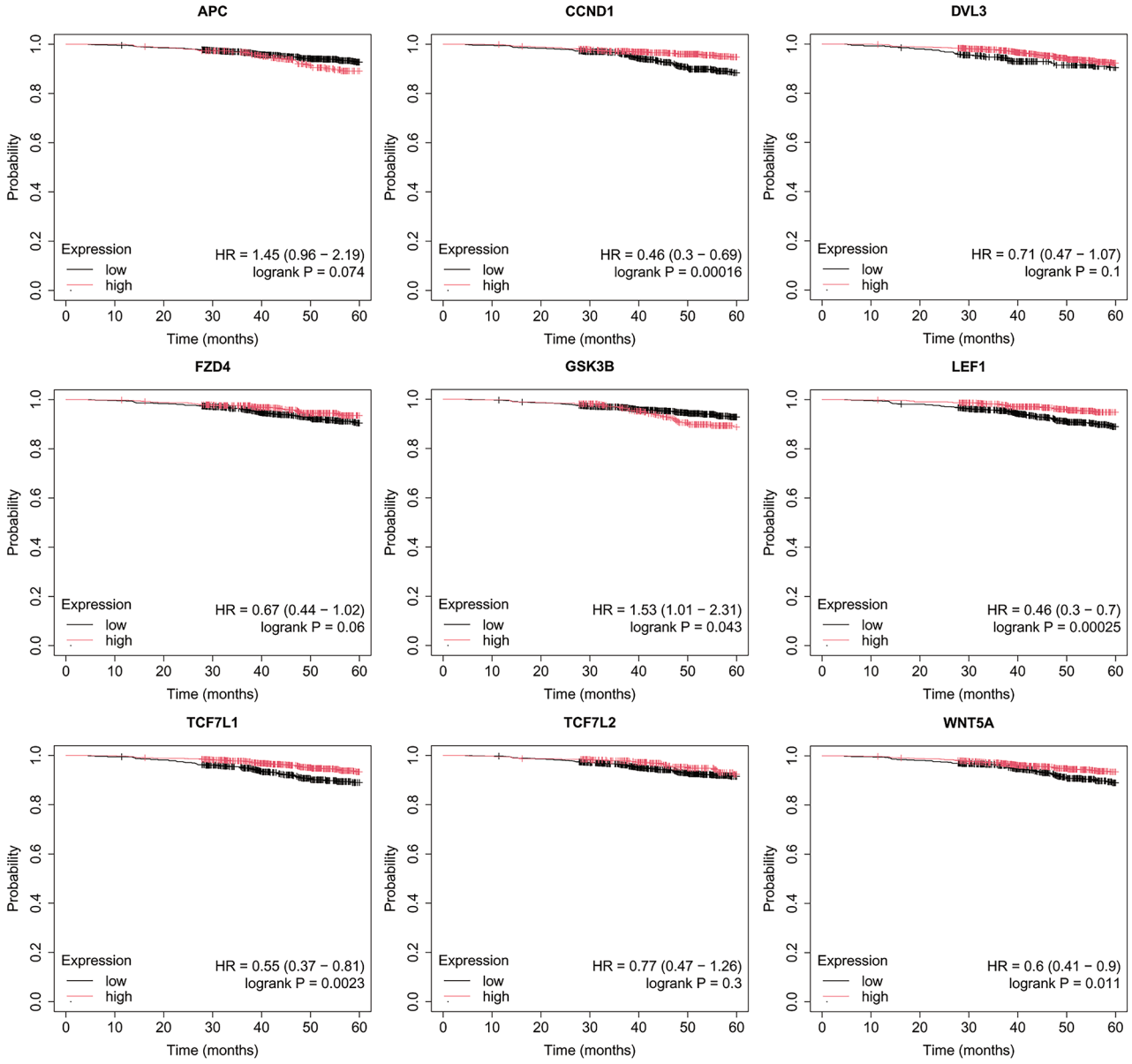

Fig. 3. Overall survival analysis in luminal A cancer. APC, adenomatous polyposis coli protein; CCND1; cyclin D; DVL3, dishevelled segment polarity protein 3; FZD4, frizzled family receptor 4; GSK3B, glycogen synthase kinase-3 beta; LEF1, lymphoid enhancer binding factor 1; TCF7L1/2, transcription factor 7 like 1/2; WNT5 A, Wnt family member 5 A.

CAL120, and HCC1395, which are characterized by high metastatic potential[39]. Li et al. demonstrated that miR-199a could inhibit the migration and invasion of breast cancer cells[45,46]. Our study also indicated the association of this miRNA with *FZD4*, the expression of which was significantly reduced regardless of the breast cancer subtype. Gupta et al. showed that Wnt ligand binding to *FZD4*induces EMT via the canonical pathway in prostate cancer[47]. In contrast, Zougros et al. reported a significant decrease in *FZD4*expression in 80% of the breast cancer samples, while in the remaining 20% the expression was unchanged compared to the control[48].

In our study, we found overexpression of *DVL3*, which is probably not related to the regulation at the miRNA level. Zou et al. also reported high levels of *DVL3* in breast cancer, which was associated with increased activity of *CCND1*and β-catenin, thereby activating the canonical Wnt pathway and promoting tumor cell growth[49]. Transcription factors *LEF1* and *TCF*, including *TCF7L1* and *TCFL2*, play an important role in the canonical Wnt pathway. In the presence of β-catenin, they function as co-activators of Wnt pathway target genes[50]. Previous studies indicate overexpression of *LEF1*in breast cancer and its association with less favorable outcomes[51,52]. Lima et al. also reported a significant increase in *TCF7L1* and decrease in *TCF7L2*in breast cancer[51], which is partially consistent with our study. We observed overexpression of *LEF1* with decreased activity of both TCF transcription factors. In addition, we predicted a link between low expression of *TCF7L2*and high activity of miR-2115. This miRNA molecule is poorly characterized so far. Singh et al. demonstrated miR-2115 overexpression

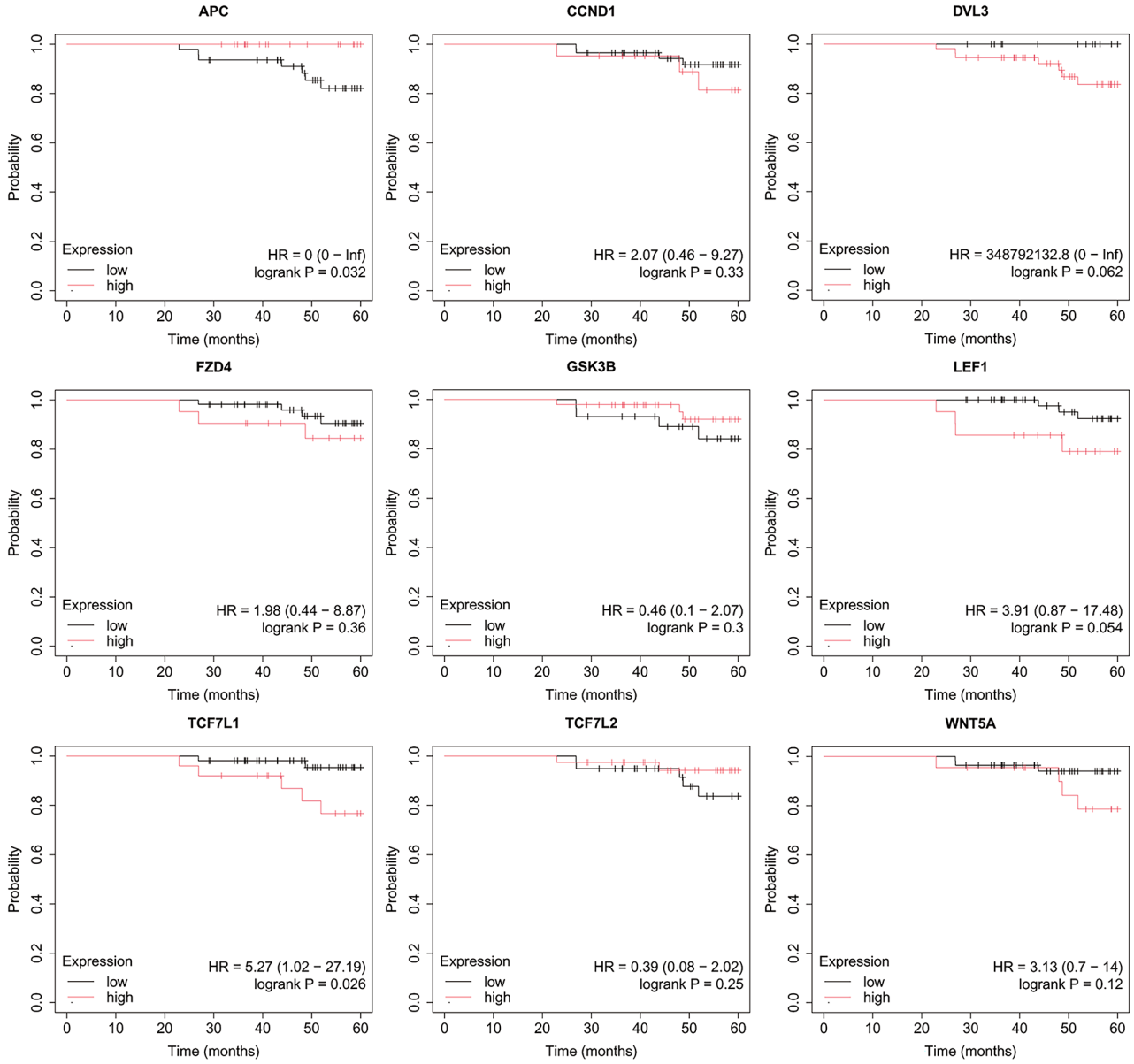

Fig. 4. Overall survival analysis in triple-negative breast cancer. APC, adenomatous polyposis coli protein; CCND1; cyclin D; DVL3, dishevelled segment polarity protein 3; FZD4, frizzled family receptor 4; GSK3B, glycogen synthase kinase-3 beta; LEF1, lymphoid enhancer binding factor 1; TCF7L1/2, transcription factor 7 like 1/2; WNT5 A, Wnt family member 5 A.

in squamous cell carcinoma[53]. However, Shen et al. suggested its potential involvement in clear cell renal cell carcinoma[54].

In our study, we found significant overexpression of *WNT5 A*, which may be related to reduced miR-497 levels. Wnt5a is considered a representative ligand of the noncanonical Wnt pathway, which involves multiple signaling cascades that often overlap[55]. *WNT5 A*is considered an oncogene in various cancers, including gastric cancer, where its overexpression promoted cell migration and invasion, correlating with tumor aggressiveness[56]. Pukrop et al. demonstrated the involvement of *WNT5 A*in promoting breast cancer metastasis[57].

Interestingly, MacMillan et al. pointed out that some studies indicate that WNT5 A can antagonize the canonical Wnt pathway. They suggested that WNT5 A may act as both an oncogene and a suppressor, depending on the cellular context[58]. Zhu et al. also emphasized that the promotion of metastasis by Wnt5a is microenvironment-dependent, as its overexpression was also observed in tumor-associated macrophages. This indicates multiple factors that determine the fate of Wnt5a in cancer cells[55]. In the case of miR-497, its downregulation is present in many cancers, including breast cancer[59]. Increasing its activity allows for inhibition of proliferation and invasion of breast cancer cells[60].

The study allowed us to determine the expression profile of genes associated with the Wnt pathway in five subtypes of breast cancer. We also made predictions about which miRNAs may participate in the regulation of

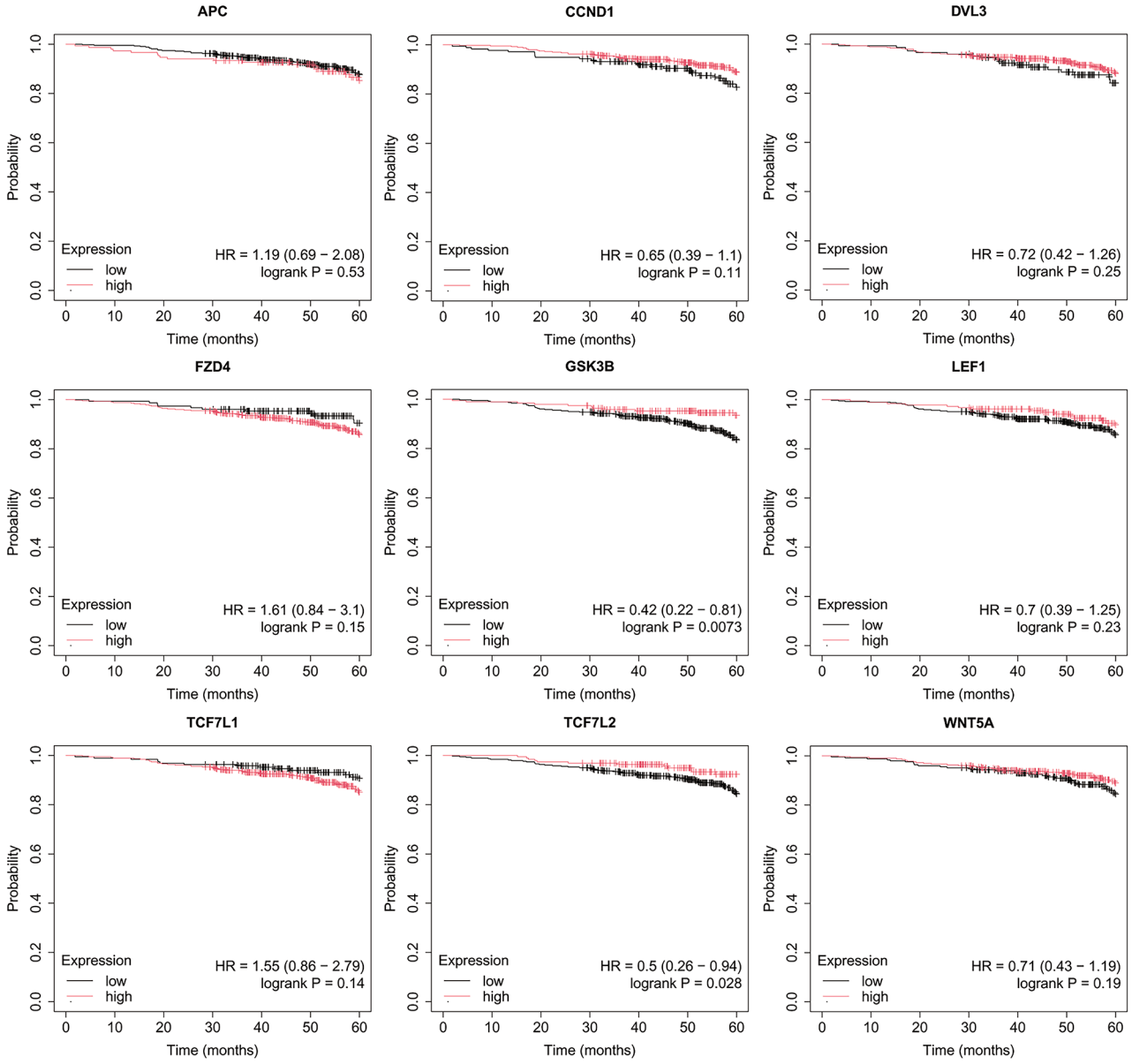

Fig. 5. Overall survival analysis in HER2-positive luminal B cancer. APC, adenomatous polyposis coli protein; CCND1; cyclin D; DVL3, dishevelled segment polarity protein 3; FZD4, frizzled family receptor 4; GSK3B, glycogen synthase kinase-3 beta; LEF1, lymphoid enhancer binding factor 1; TCF7L1/2, transcription factor 7 like 1/2; WNT5 A, Wnt family member 5 A.

their activity. Considering the overexpression of *APC*, *GSK3B*, with reduced levels of β-catenin, *FZD4*, *TCF7L1* and *TCFL2*, this may suggest suppression of the canonical Wnt pathway. Overexpression of *CCND1* and *LEF1* may indicate their participation in other, β-catenin-independent pathways. The overexpression of *WNT5 A* is also important, as it may suggest a shift in the balance between Wnt pathways in favor of the noncanonical pathway. This mechanism may also involve miRNAs, including miR-130a, miR-199a, miR-479, whose reduced activity allows for uncontrolled expression of genes associated with the Wnt pathway.

The interplay between miRNAs and the Wnt signaling pathway in breast cancer is complex and crucial for understanding tumor progression and subtype-specific behavior. miRNAs act as post-transcriptional regulators of Wnt pathway-related genes, influencing key cellular processes such as proliferation, migration, and survival. In this study, miR-130a and miR-199a were identified as negative regulators of *GSK3B*, a key kinase in the Wnt/β-catenin pathway, and their downregulation led to *GSK3B* overexpression, potentially enhancing Wnt signaling activity. Similarly, the reduced expression of *FZD4* was associated with the suppression of miR-199a, further disrupting Wnt receptor function. On the other hand, miR-497 downregulation correlated with *WNT5 A* overexpression, indicating a shift toward non-canonical Wnt signaling, which is often linked to increased metastasis and tumor aggressiveness. The upregulation of miR-2115, linked to reduced *TCF7L2* expression, points to the modulation of canonical Wnt signaling by miRNAs in specific subtypes. This intricate regulatory

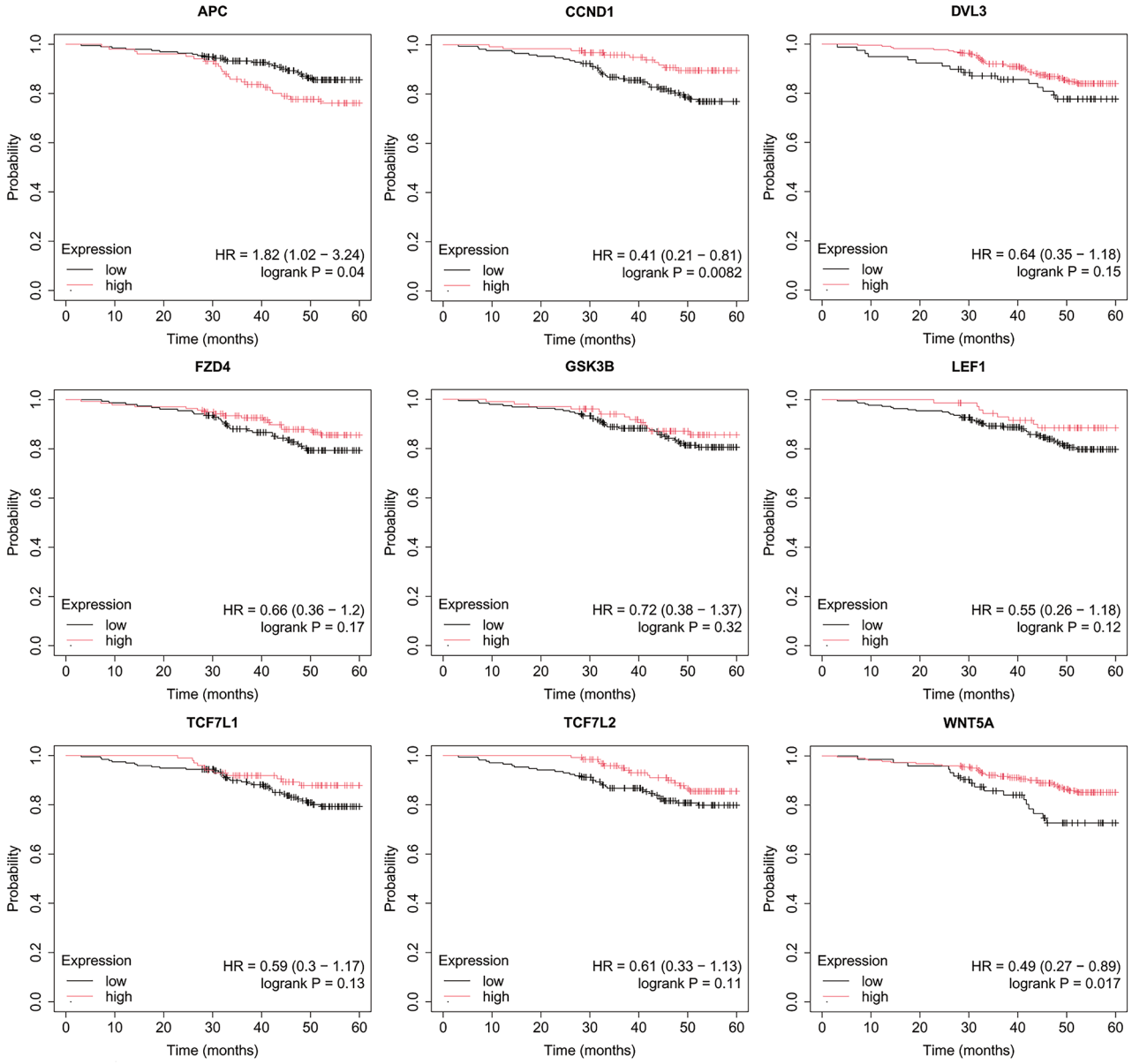

Fig. 6. Overall survival analysis in HER2-negative luminal B cancer. APC, adenomatous polyposis coli protein; CCND1; cyclin D; DVL3, dishevelled segment polarity protein 3; FZD4, frizzled family receptor 4; GSK3B, glycogen synthase kinase-3 beta; LEF1, lymphoid enhancer binding factor 1; TCF7L1/2, transcription factor 7 like 1/2; WNT5 A, Wnt family member 5 A.

network between miRNAs and the Wnt pathway underscores the potential for miRNA-based therapeutic strategies targeting Wnt-driven oncogenesis in breast cancer.

Interestingly, although our cohort consisted exclusively of patients with non-metastatic breast cancer (T1 N0M0), the observed dysregulation of Wnt-related genes and miRNAs may have early prognostic significance. The consistent overexpression of *WNT5 A*, a key component of the non-canonical Wnt pathway associated with metastasis and tumor aggressiveness, alongside the downregulation of its regulatory miR-497, suggests a potential shift in signaling balance even at early disease stages. This is further supported by decreased *FZD4* expression, which may indicate disruption of canonical Wnt signaling and increased invasive potential. These findings highlight that Wnt pathway alterations could precede overt metastasis and serve as early indicators of tumor progression or therapeutic resistance.

This study presents several limitations. Firstly, the relatively small sample size, particularly for certain subtypes such as HER2 + and TNBC, may limit the broader applicability of the findings. A larger cohort would be required to strengthen the statistical validity and enhance the generalizability of the results. Secondly, the focus on a homogeneous Polish female population restricts the diversity of the sample, which may affect the extent to which these findings can be applied to other demographic groups. Thirdly, although gene expression was assessed using microarray analysis and qRT-PCR, these techniques have inherent limitations and may not fully capture the

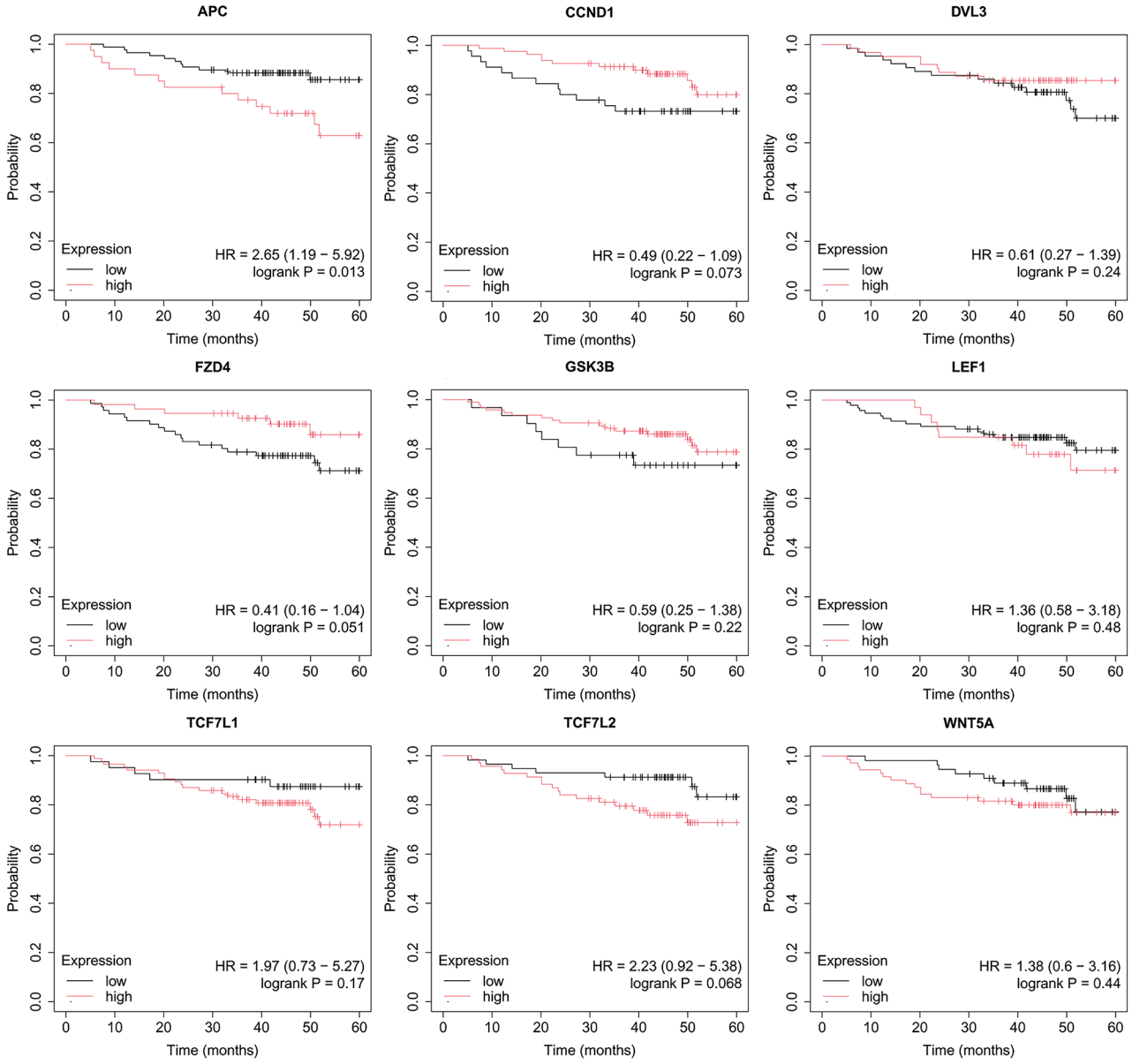

Fig. 7. Overall survival analysis in non-luminal HER2-positive cancer. APC, adenomatous polyposis coli protein; CCND1; cyclin D; DVL3, dishevelled segment polarity protein 3; FZD4, frizzled family receptor 4; GSK3B, glycogen synthase kinase-3 beta; LEF1, lymphoid enhancer binding factor 1; TCF7L1/2, transcription factor 7 like 1/2; WNT5 A, Wnt family member 5 A.

complexities of gene regulation. More advanced methodologies, such as RNA sequencing, would provide a more comprehensive and nuanced understanding of gene expression patterns. Additionally, protein concentration was measured solely using ELISA, and incorporating other techniques, such as immunohistochemistry and western blotting, would provide a more detailed and robust analysis of protein expression.

However, despite these limitations, the study also has several strengths. The integrative approach, combining gene expression profiling, miRNA analysis, and protein quantification via ELISA, enabled a thorough investigation into the role of histaminergic pathways in breast cancer. By including multiple breast cancer subtypes, the findings offer relevance across different patient groups, enhancing the potential impact of the study. The rigorous application of standardized protocols for both gene expression and miRNA analyses ensured high levels of accuracy, reliability, and reproducibility. Furthermore, the use of validated databases for miRNA target prediction allowed for an in-depth exploration of the regulatory mechanisms driving histamine-related changes in gene expression.

Summarizing, our study identified potential relationships between Wnt-related genes and miRNAs in various subtypes of breast cancer, which may be useful in further studies. Analysis methods were used at several stages of genetic information flow, including mRNA, miRNA, and proteins.

Molecular subtype	Grade			Age		BMI [kg/m^2]
	G1	G2	G3	< 50 years	> 50 years	
Luminal A	23 (18%)	48 (37%)	59 (45%)	43 (33%)	87 (67%)	30.78 ± 2.76
HER2-positive luminal B	23 (24%)	57 (59%)	16 (17%)	19 (20%)	77 (80%)	32.09 ± 6.19
HER2-negative luminal B	31 (31%)	57 (57%)	12 (12%)	32 (32%)	68 (68%)	30.18 ± 4.56
Non-luminal HER2-positive	9 (25%)	12 (33%)	15 (42%)	9 (25%)	27 (75%)	33.18 ± 5.67
TNBC	14 (32%)	21 (49%)	8 (19%)	10 (23%)	33 (77%)	34.67 ± 2.98

Table 4. Characteristics of patients. HER2, human epidermal growth factor receptor 2; TNBC, triple-negative breast cancer; BMI, body mass index.

Inclusion criteria	Exclusion criteria
Expressing informed, voluntary consent to participate in the study	Failure to express informed, voluntary consent to participate in the study
Patients diagnosed with one of the five breast cancer subtypes (luminal A, luminal B HER2-, luminal B HER2+, non-luminal HER2+, and TNBC)	Patients with a history of other malignancies
Patients who underwent surgical removal of the tumor along with a margin of healthy tissue	Patients who received neoadjuvant chemotherapy or radiotherapy prior to surgery.
Patients aged between 18 and 75 years	Patients aged below 18 and over 75 years
Patients classified as T1 N0M0 according to TNM classification	Patients with metastatic disease (stages II-IV)
Patients who did not receive any treatment prior to surgery (e.g., chemotherapy, hormone therapy, or radiotherapy	Patients who received any treatment prior to surgery (e.g., chemotherapy, hormone therapy, or radiotherapy

Table 5. Inclusion and exclusion criteria.

Methods

Patients

The study included 405 patients with different breast cancer subtypes: 130 with luminal A, 96 with HER2-positive luminal B, 100 with HER2-negative luminal B, 36 with non-luminal HER2-positive, and 43 with triple-negative breast cancer (TNBC). During surgery, the tumor tissue was removed along with a margin of healthy tissue. Pathological evaluation was used to differentiate between tumor tissue (study groups) and tumor-free tissue (control group). All patients were classified as T1 N0M0. Table 4 presents the characteristics of the patients, whereas the Table 5 presents inclusion and exclusion criteria for study and control groups.

This study was conducted in accordance with the, 2013 Helsinki Declaration and was approved by the Bioethical Committee of the Regional Medical Chamber in Krakow (March 10, 2023; 81/KBL/OIL/2023). Informed consent was obtained from all patients.

Preparation of samples to molecular analysis

The tumor tissue, along with a margin of healthy tissue, was removed and placed in RNAlater solution (ThermoFisher Scientific, Waltham, MA, USA). A homogenizer (T18 Digital Ultra-Turrax, IKA Poland Sp. z o.o., Warsaw, Poland) was used to thoroughly homogenize the samples, which were then placed horizontally on ice and gently agitated on a rocking plate for 1 h. Afterward, the samples were centrifuged (12,000 × g, 4 °C, 15 min), and the supernatant from each sample was collected and stored at − 80 °C until further analysis.

Extraction of total ribonucleic acid (RNA)

Next, total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA; cat. no. 15596026). Extracts were purified using the RNeasy mini kit (QIAGEN, Hilden, Germany; cat. no. 74104) and DNase I (Fermentas International Inc., Burlington, ON, Canada; cat. no. 18047019). Qualitative and quantitative evaluation of the extracts was performed by 1% agarose gel electrophoresis and absorbance measurement.

mRNA microarray analysis

The expression profile was determined with HG-U133 A 2_0 oligonucleotide microarrays (Affymetrix, Santa Clara, CA, USA) and the GeneChip™ 3′IVT PLUS kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA; cat. no. 902416).

The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database was utilized to identify genes associated with the Wnt signaling pathway. Specifically, the KEGG pathway map for the Wnt signaling pathway (entry hsa04310, ID N00056) was referenced to compile a list of 46 genes known to be involved in this signaling cascade. These genes were then matched to their corresponding probes on the microarray platform used in the study, resulting in a total of 101 distinct mRNA transcripts being identified and included in the subsequent gene expression analyses.

mRNA	RT-qPCR Amplification primers (5′–3′)
APC	Forward: AGGCTGCATGAGAGCACTTGTG Reverse: CACACTTCCAACTTCTCGCAACG
CCND1	Forward: TCTACACCGACAACTCCATCCG Reverse: TCTGGCATTTTGGAGAGGAAGTG
DVL3	Forward: GTGACCGCATGTGGCTCAAGAT Reverse: CGTGAAGCCTTCCACATTGTGG
FZD4	Forward: TTCACACCGCTCATCCAGTACG Reverse: ACGGGTTCACAGCGTCTCTTGA
GSK3B	Forward: CCGACTAACACCACTGGAAGCT Reverse: AGGATGGTAGCCAGAGGTGGAT
LEF1	Forward: CTACCCATCCTCACTGTCAGTC Reverse: GGATGTTCCTGTTTGACCTGAGG
TCF7L1	Forward: TCAAGGACACGAGGTCACCATC Reverse: GGAGAAGTGGTCATTGCTGTAGG
TCF7L2	Forward: GAATCGTCCCAGAGTGATGTCG Reverse: TGCACTCAGCTACGACCTTTGC
WNT5 A	Forward: ATTAATTCTGGCTCCACTTG Reverse: GGTTATTCATACCTAGCGAC
ACTB	Forward: TCACCCACACTGTGCCCATCTACGA Reverse: CAGCGGAACCGCTCATTGCCAATGG

Table 6. RT-qPCR primers. APC, adenomatous polyposis coli protein; CCND1; cyclin D; DVL3, dishevelled segment polarity protein 3; FZD4, frizzled family receptor 4; GSK3B, glycogen synthase kinase-3 beta; LEF1, lymphoid enhancer binding factor 1; TCF7L1/2, transcription factor 7 like 1/2; WNT5 A, Wnt family member 5 A; ACTB, β-actin.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

To verify the results of the microarray experiment for 9 genes differentiating breast cancer regardless of its subtype, RT-qPCR was performed using the SensiFast SYBR No-ROX One-Step Kit (Bioline, London, UK). The expression profile was calculated using the $2^{-\Delta\Delta Ct}$ method. β-actin (ACTB) was used as an endogenous control. Primer sequences are provided in Table 6.

MiRNA profiling of Wnt-related MiRNAs and their potential impact on gene expression

To identify miRNAs significantly changing their activity in different breast cancer subtypes compared to controls, miRNA microarrays 2.0 (Affymetrix, Santa Clara, California, USA), FlashTag Biotin HSR RNA Labeling Kit (Affymetrix, Santa Clara, California, USA) and Hybridization Wash and Stain Kit (Affymetrix, Santa Clara, California, USA) were used. Then, predictions were made, which of these miRNAs may participate in the regulation of activity of 9 selected genes: adenomatous polyposis coli protein (*APC*), cyclin D (*CCND1*), dishevelled segment polarity protein 3 (*DVL3*), frizzled family receptor 4 (*FZD4*), glycogen synthase kinase-3 beta (*GSK3B*), lymphoid enhancer binding factor 1 (*LEF1*), transcription factor 7 like 1 (*TCF7L1*), transcription factor 7 like 2 (*TCF7L2*), Wnt family member 5 A (*WNT5 A*). For this purpose, mirDB tool (http://mirdb.org) was used and a target score ≥ 80 was assumed to increase the credibility of prediction[61].

Enzyme-Linked immunosorbent assay (ELISA)

The protein levels were determined by ELISA (Abbexa, Cambridge, UK) using ELISA kits: Human Adenomatosis Polyposis Coli Protein (APC) Kit (MyBioSource, Inc., San Diego, CA, USA; cat. no. MBS2020448), Human Cyclin D (CCND1) Kit (MyBioSource, Inc., San Diego, CA, USA; cat. no. MBS723526), Human dishevelled homolog 3 (DVL3) Kit (MyBioSource, Inc., San Diego, CA, USA; cat. no. MBS9330816), Human Frizzled Homolog 4 (FZD4) Kit (MyBioSource, Inc., San Diego, CA, USA; cat. no. MBS164473), Human Glycogen synthase kinase-3 beta (GSK3B) Kit (MyBioSource, Inc., San Diego, CA, USA; cat. no. MBS2883314), Human Lymphoid enhancer-binding factor 1 (LEF1) Kit (MyBioSource, Inc., San Diego, CA, USA; cat. no. MBS2890483), Human transcription factor 7 like 1 (TCF7L1) Kit (MyBioSource, Inc., San Diego, CA, USA; cat. no. MBS9329473), Human transcription factor 7 like 2 (TCF7L2) Kit (MyBioSource, Inc., San Diego, CA, USA; cat. no. MBS2088212), Human wingless-type MMTV integration site family, member 5 A (WNT5 A) Kit (MyBioSource, Inc., San Diego, CA, USA; cat. no. MBS901538),

Statistical analysis

Analysis of microarray experiment results was performed in Transcriptome Analysis Console (Thermo Fisher Scientific, Waltham, MA, USA). One-way analysis of variance (ANOVA) and Tukey's post hoc test were performed ($p < 0.05$; FC > 2 or FC < − 2). RT-qPCR and ELISA results were analyzed using Statistica 13.3 (StatSoft, Krakow, Poland). The Shapiro-Wilk test was used to assess normality of distribution. Its absence allowed further analysis using Kruskal-Wallis and Dunn's tests.

A sampling calculator was used to estimate the group size[62]. Considering approximately 19,620 women diagnosed with breast cancer in Poland in 2019[63], and assuming a 95% confidence level and a 5% margin of error, the recommended number of study participants was 377.

The overall survival (OS) status in each breast cancer subtype was presented using the Kaplan-Meier plot (http://kmplot.com/; accessed: June 25, 2024)[64,65]. The follow up threshold has been set to 60 months.

Data availability

The data that support the findings of this study are available from the corresponding author, [T.S.], upon reasonable request.

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References

1. Bray, F. et al. Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* **74** (3), 229–263 (2024).
2. 0_krn-2023-book. -2024-01-22.pdf [Internet]. [cited 2024 Aug 11]. Available from: https://onkologia.org.pl/sites/default/files/publications/2024-01/0_krn-2023-book-2024-01-22.pdf
3. Wang, J. & Wu, S. G. Breast cancer: an overview of current therapeutic strategies, challenge, and perspectives. *Breast Cancer (Dove Med. Press)*. **15**, 721–730 (2023).
4. Yang, X. et al. A primary luminal/HER2 negative breast cancer patient with mismatch repair deficiency. *Cell. Death Discov.* **9** (1), 365 (2023).
5. Łukasiewicz, S. et al. Breast Cancer-Epidemiology, risk factors, classification, prognostic markers, and current treatment Strategies-An updated review. *Cancers (Basel)*. **13** (17), 4287 (2021).
6. Yang, Z. J. et al. The regrouping of luminal B (HER2 negative), a better discriminator of outcome and recurrence score. *Cancer Med.* **12** (3), 2493–2504 (2023).
7. Falato, C., Schettini, F., Pascual, T., Brasó-Maristany, F. & Prat, A. Clinical implications of the intrinsic molecular subtypes in hormone receptor-positive and HER2-negative metastatic breast cancer. *Cancer Treat. Rev.* **112**, 102496 (2023).
8. Thomas, A., Reis-Filho, J. S., Geyer, C. E. & Wen, H. Y. Rare subtypes of triple negative breast cancer: current Understanding and future directions. *NPJ Breast Cancer*. **9** (1), 55 (2023).
9. Pohl, S. G. et al. Wnt signaling in triple-negative breast cancer. *Oncogenesis* **6** (4), e310–e310 (2017).
10. Liu, J. et al. Wnt/β-catenin signalling: function, biological mechanisms, and therapeutic opportunities. *Sig Transduct. Target. Ther.* **7** (1), 1–23 (2022).
11. Merikhian, P., Eisavand, M. R. & Farahmand, L. Triple-negative breast cancer: Understanding Wnt signaling in drug resistance. *Cancer Cell. Int.* **21**, 419 (2021).
12. Chae, W. J. & Bothwell, A. L. M. Canonical and Non-Canonical Wnt signaling in immune cells. *Trends Immunol.* **39** (10), 830–847 (2018).
13. Qin, K. et al. Canonical and noncanonical Wnt signaling: multilayered mediators, signaling mechanisms and major signaling crosstalk. *Genes Dis.* **11** (1), 103–134 (2024).
14. Xu, X., Zhang, M., Xu, F. & Jiang, S. Wnt signaling in breast cancer: biological mechanisms, challenges and opportunities. *Mol. Cancer*. **19** (1), 165 (2020).
15. Roarty, K., Baxley, S. E., Crowley, M. R., Frost, A. R. & Serra, R. Loss of TGF-beta or Wnt5a results in an increase in Wnt/beta-catenin activity and redirects mammary tumour phenotype. *Breast Cancer Res.* **11** (2), R19 (2009).
16. Gujral, T. S. et al. A noncanonical Frizzled2 pathway regulates Epithelial-Mesenchymal transition and metastasis. *Cell* **159** (4), 844–856 (2014).
17. Castagnoli, L. et al. WNT signaling modulates PD-L1 expression in the stem cell compartment of triple-negative breast cancer. *Oncogene* **38** (21), 4047–4060 (2019).
18. VanKlompenberg, M. K., Bedalov, C. O., Soto, K. F. & Prosperi, J. R. APC selectively mediates response to chemotherapeutic agents in breast cancer. *BMC Cancer.* **15**, 457 (2015).
19. Gavilán, E. et al. Breast cancer cell line MCF7 escapes from G1/S arrest induced by proteasome Inhibition through a GSK-3β dependent mechanism. *Sci. Rep.* **5**, 10027 (2015).
20. Van Rooij, E. The Art of MicroRNA research. *Circul. Res.* **108** (2), 219–234 (2011).
21. Lu, T. X., Rothenberg, M. E. & MicroRNA *J. Allergy Clin. Immunol.* ;**141**(4):1202–1207. (2018).
22. Rani, V. & Sengar, R. S. Biogenesis and mechanisms of microRNA-mediated gene regulation. *Biotech. Bioeng.* **119** (3), 685–692 (2022).
23. Komatsu, S., Kitai, H. & Suzuki, H. I. Network regulation of MicroRNA biogenesis and target interaction. *Cells* **12** (2), 306 (2023).
24. Shang, R., Lee, S., Senavirathne, G. & Lai, E. C. MicroRNAs in action: biogenesis, function and regulation. *Nat. Rev. Genet.* **24** (12), 816–833 (2023).
25. Bofill-De Ros, X. & Vang Ørom, U. A. Recent progress in MiRNA biogenesis and decay. *RNA Biol.* **21** (1), 1–8 (2024).
26. Inoue, J. & Inazawa, J. Cancer-associated MiRNAs and their therapeutic potential. *J. Hum. Genet.* **66** (9), 937–945 (2021).
27. Correia de Sousa, M., Gjorgjieva, M., Dolicka, D., Sobolewski, C. & Foti, M. Deciphering MiRNAs' action through MiRNA editing. *Int. J. Mol. Sci.* **20** (24), 6249 (2019).
28. O'Brien, J., Hayder, H., Zayed, Y. & Peng, C. Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. Frontiers in Endocrinology [Internet]. 2018 [cited 2022 Sep 21];9. Available from: https://www.frontiersin.org/articles/https://doi.org/10.3389/fendo.2018.00402
29. Sirek, T. et al. MiRNAs in signal transduction of SMAD proteins in breast Cancer. *Int. J. Mol. Sci.* **25** (18), 10088 (2024).
30. Staszkiewicz, R. et al. Variances in the expression profile of circadian Clock-Related genes in astrocytic brain tumors. *Cancers* **16** (13), 2335 (2024).
31. Tahiri, A. et al. Deregulation of cancer-related MiRNAs is a common event in both benign and malignant human breast tumors. *Carcinogenesis* **35** (1), 76–85 (2014).
32. MicroRNA-29 family. reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B | PNAS [Internet]. [cited 2022 May 19]. Available from: https://www.pnas.org/doi/full/https://doi.org/10.1073/pnas.0707628104
33. Gebeshuber, C. A., Zatloukal, K. & Martinez, J. miR-29a suppresses tristetraprolin, which is a regulator of epithelial Polarity and metastasis. *EMBO Rep.* **10** (4), 400–405 (2009).
34. Zhan, T., Rindtorff, N. & Boutros, M. Wnt signaling in cancer. *Oncogene* **36** (11), 1461–1473 (2017).
35. Saelee, P. & Pongtheerat, T. APC promoter hypermethylation as a prognostic marker in breast Cancer patients. *Asian Pac. J. Cancer Prev.* **21** (12), 3627–3632 (2020).
36. Stefanski, C. D., Keffler, K., McClintock, S., Milac, L. & Prosperi, J. R. APC loss affects DNA damage repair causing doxorubicin resistance in breast cancer cells. *Neoplasia* **21** (12), 1143–1150 (2019).
37. Quintayo, M. A. et al. GSK3β and Cyclin D1 expression predicts outcome in early breast cancer patients. *Breast Cancer Res. Treat.* **136** (1), 161–168 (2012).

38. Vijay, G. V. et al. GSK3β regulates epithelial-mesenchymal transition and cancer stem cell properties in triple-negative breast cancer. *Breast Cancer Res.* **21** (1), 37 (2019).
39. Gao, C. et al. Regulation of AKT phosphorylation by GSK3β and PTEN to control chemoresistance in breast cancer. *Breast Cancer Res. Treat.* **176** (2), 291–301 (2019).
40. Chen, X. et al. microRNA-130a suppresses breast cancer cell migration and invasion by targeting FOSL1 and upregulating ZO-1. *J. Cell. Biochem.* **119** (6), 4945–4956 (2018).
41. Huang, J. et al. MicroRNA-130a reduces drug resistance in breast cancer. *Int. J. Clin. Exp. Pathol.* **12** (7), 2699–2705 (2019).
42. Alam, S., Zunic, A., Venkat, S., Feigin, M. E. & Atanassov, B. S. Regulation of Cyclin D1 degradation by ubiquitin specific protease 27X is critical for Cancer cell proliferation and tumor growth. *Mol. Cancer Res.* **20** (12), 1751–1762 (2022).
43. Goel, S. et al. Overcoming therapeutic resistance in HER2-Positive breast cancers with CDK4/6 inhibitors. *Cancer Cell.* **29** (3), 255–269 (2016).
44. Jeffreys, S. A. et al. Prognostic and predictive value of CCND1/Cyclin D1 amplification in breast Cancer with a focus on postmenopausal patients: A systematic review and Meta-Analysis. *Front. Endocrinol. (Lausanne).* **13**, 895729 (2022).
45. Li, S. Q., Wang, Z. H., Mi, X. G., Liu, L. & Tan, Y. MiR-199a/b-3p suppresses migration and invasion of breast cancer cells by downregulating PAK4/MEK/ERK signaling pathway. *IUBMB Life.* **67** (10), 768–777 (2015).
46. Li, W. et al. miR-199a-5p regulates B1 integrin through Ets-1 to suppress invasion in breast cancer. *Cancer Sci.* **107** (7), 916–923 (2016).
47. Gupta, S. et al. FZD4 as a mediator of ERG oncogene-induced WNT signaling and epithelial-to-mesenchymal transition in human prostate cancer cells. *Cancer Res.* **70** (17), 6735–6745 (2010).
48. Zougros, A. et al. mRNA coexpression patterns of Wnt pathway components and their clinicopathological associations in breast and colorectal cancer. *Pathol. - Res. Pract.* **227**, 153649 (2021).
49. Zou, Y. F., Xie, C. W., Yang, S. X. & Xiong, J. P. AMPK activators suppress breast cancer cell growth by inhibiting DVL3-facilitated Wnt/β-catenin signaling pathway activity. *Mol. Med. Rep.* **15** (2), 899–907 (2017).
50. Bem, J. et al. Wnt/β-catenin signaling in brain development and mental disorders: keeping TCF7L2 in Mind. *FEBS Lett.* **593** (13), 1654–1674 (2019).
51. Lima, B. M. et al. Biomarker potential of the LEF1/TCF family members in breast cancer: bioinformatic investigation on expression and clinical significance. *Genet. Mol. Biol.* **46**(4):e20220346 .
52. Bucan, V. et al. LEF-1 regulates proliferation and MMP-7 transcription in breast cancer cells. *Genes Cells*. **17** (7), 559–567 (2012).
53. Singh, A. et al. Differential diagnosis of non-small cell lung carcinoma by Circulating MicroRNA. *J. Cancer Res. Ther.* **16** (1), 127–131 (2020).
54. Shen, T., Song, Y., Wang, X. & Wang, H. Characterizing the molecular heterogeneity of clear cell renal cell carcinoma subgroups classified by MiRNA expression profile. *Front. Mol. Biosci.* **9**, 967934 (2022).
55. Zhu, N. et al. Challenging role of Wnt5a and its signaling pathway in cancer metastasis (Review). *Experimental Therapeutic Med.* **8** (1), 3–8 (2014).
56. Hanaki, H. et al. An anti-Wnt5a antibody suppresses metastasis of gastric cancer cells in vivo by inhibiting receptor-mediated endocytosis. *Mol. Cancer Ther.* **11** (2), 298–307 (2012).
57. Pukrop, T. et al. Wnt 5a signaling is critical for macrophage-induced invasion of breast cancer cell lines. *Proc. Natl. Acad. Sci. U S A.* **103** (14), 5454–5459 (2006).
58. MacMillan, C. D. et al. Stage of breast Cancer progression influences cellular response to activation of the WNT/Planar cell Polarity pathway. *Sci. Rep.* **4**, 6315 (2014).
59. Luo, G. et al. Regulation of microRNA–497 expression in human cancer (Review). *Oncol. Lett.* **21** (1), 1–1 (2021).
60. Li, Y., Hua, K., Jin, J. & Fang, L. miR-497 inhibits proliferation and invasion in triple-negative breast cancer cells via YAP1. *Oncol. Lett.* **22** (2), 580 (2021).
61. Chen, Y. & Wang, X. MiRDB: an online database for prediction of functional MicroRNA targets. *Nucleic Acids Res.* **48** (D1), D127–D131 (2020).
62. Kalkulator doboru próby [Internet]. [cited 2022 Apr 19]. Available from: https://www.naukowiec.org/dobor.html
63. Krajowy Rejestr Nowotworów. Nowotwór piersi – 2019. [Internet]. Available from: https://onkologia.org.pl/sites/default/files/Pier%C5%9B.pdf
64. Győrffy, B. Integrated analysis of public datasets for the discovery and validation of survival-associated genes in solid tumors. *Innov. (Camb).* **5** (3), 100625 (2024).
65. Győrffy, B. Transcriptome-level discovery of survival-associated biomarkers and therapy targets in non-small-cell lung cancer. *Br. J. Pharmacol.* **181** (3), 362–374 (2024).

Author contributions

Conceptualization, T.S., A.S., and B.O.G.; methodology, D.B.; T.S.; M.O.; software, B.O.G. and N.Z; formal analysis, K.D. and K.B.; investigation, T.S.; resources, P.O.; J.G.; K.K.-J.; J.S. and D.S.; data curation, B.O.G. and N. Z.; writing—original draft preparation, D.S.; K.D.; T.S.; M.C.; A.S.; D.B.; P.O.; writing—review and editing, M.O.; T.S.; B.O.G.; visualization, P.O.; supervision, T.S.; B.O.G.; project administration, B.O.G. All authors read and approved the final manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Institutional review board statement

This study adhered to the 2013 Declaration of Helsinki guidelines for human experimentation. This study was approved by the Bioethical Committee of the Regional Medical Chamber in Krakow (No. 81/KBL/OIL/2023, dated 10 March 2023).

Informed consent statement

Written **i**nformed consent was obtained from all patients.

Additional information

Correspondence and requests for materials should be addressed to T.S.

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