Involvement of *miR-3648* mediated *APC2* dysregulation in early onset and breast cancer progression

Mehwish Batool[1], Javeria Qadir[1,2,3],
Misbah Shan[1], Feiya Li[2,3], Faraz A. Malik[1], Hassaan M. Awan[1,]*

[1]Dept. of Biosciences, COMSATS University Islamabad, Park Road, Tarlai Kalan, 45550, Islamabad, Pakistan.
[2]Dept. of Laboratory Medicine and Pathobiology, University of Toronto, ON- Canada.
[3]Sunnybrook Research Institute, Sunnybrook Hospital, ON- Canada

**Corresponding email: hassaan.awan@comsats.edu.pk*

Abstract

Multiple cancers arise due to aberrations in the wingless integrated (Wnt) signaling pathway. Several miRNAs modulate the integral components of the Wnt signaling pathway. *miR-3648* is a human-specific miRNA that is of particular interest due to its minimal off-targeting effect. In this study, we investigated the expression of *miR-3648* and *APC2* in breast cancer patients of Pakistan. Correlations of *miR-3648* and *APC2* expression with clinico-pathological features and breast cancer subtypes were observed in tissue samples by means of quantitative real time PCR. Our results showed that *miR-3648* was relatively downregulated in Luminal A subtype, with corresponding upregulation of *APC2* in these patients. Moreover, the transcript levels of both *miR-3648* and *APC2* were found to be inversely regulated in breast cancer women presented with early disease onset, pre-menopause, low tumor grade, early clinical stage, absence of nodal invasion and metastasis, further suggesting the molecular interplay of these molecules in breast cancer development and progression.

Keywords: *APC2*; breast cancer; luminal A; miR-3648; Wnt signaling

1. Introduction

Breast cancer is one of the most commonly diagnosed cancer worldwide (Sung *et al*, 2021). Various genetic and environmental factors contribute towards breast cancer leading to the accumulation of mutations in essential genes that regulate maintenance, cell growth and tumor microenvironment (Rich *et al*, 2015).

MicroRNAs (miRNAs) are 22-25nt long, single stranded, small non-coding RNAs (Yang *et al*, 2015). They modulate gene expression by targeting 3'UTR of mRNAs, resulting in translation repression or mRNA destabilization, thus, playing an essential role in cellular and biological processes such as differentiation, stress response, apoptosis and cell cycle (Mittal *et al,* 2017; López-Knowles *et al,* 2010). While a specific mRNA could be targeted by multiple miRNAs, a single miRNA can have several mRNA targets as well (Di Vizio *et al*, 2012). miRNAs are virtually

involved in almost every disease including cancers. The regulatory actions of miRNAs on normal expression of oncogenes or tumor suppressor genes lead a cell transit toward cancerous state (Vannini *et al*, 2018). They have great potential to be used as therapeutic target for disease treatment such as in breast cancer (Davey *et al*, 2021; Petrovic *et al*, 2021)

Many cancers arise due to irregularities in the Wnt signaling pathway (Anastas *et al,* 2013). The tumor suppressor Adenomatous polyposis coli (*APC*) plays a role in Wnt signaling pathway, a pathway whose irregularities have been shown to be related with many cancers, and its association has been observed with multiple cancers. Several studies have described alterations in Wnt/APC2 signaling pathway, thus, discerning its role in the process of carcinogenesis (Jafarzadeh & Soltani, 2021; Koni *et al*, 2020; Castagnoli *et al*., 2020). In this regard, there have been quite a number of miRNAs reported to modulate integral components of the Wnt signaling pathway, therefore, contributing to the pathogenesis of various cancers. *miR-3648* is one of the many human specific miRNAs identified, and is of particular interest due to its minimum off-targeting (Hu *et al,* 2012). Previously, a highly sensitive luciferase assay was employed to show that *miR-3648* directly targets APC2 mRNA, harboring three *miR-3648* binding sites (Rashid *et al*, 2017). Following endoplasmic reticulum stress, downregulation of APC2 expression due to elevated *miR-3648* expression results in enhanced cell proliferation (Rashid *et al,* 2017). Its correlation with other cancer types is also reported (Xing, 2019; Emmadi *et al,* 2015). However, its role in breast cancer remains elusive.

Therefore, the aim of this study was to investigate and compare the expression of *miR-3648* and *APC2* in fresh tissue samples of breast cancer patients as well as in the healthy controls in Pakistani cohort. Furthermore, the study also aimed at exploring the potential associations of *miR-3648* and *APC2* with clinico-pathological features and breast cancer subtypes.

2. Materials & Methods

2.1. Tissue Specimen and Data Collection

This research was conducted in agreement with prior approvals from the Ethical Review Board (ERB) of CUI (CUI/Bio/ERB/12-19/03). The study cohort comprised of 80 breast cancer affected women with ages ranging from 27-97 years collected from Holy family Hospital, Rawalpindi in agreement with the ethical guidelines set forth by the collaborating institutes. The mean age was calculated to be 50 years. Freshly excised tumor tissues along with their adjacent healthy tissues (2cm away from tumor area) were collected and stored in RNA*later*® (Invitrogen, California, USA) and maintained at 4^{o}C in an ice bucket during transportation to the laboratory. Demographic data and clinico-pathological characteristics including tumor grade, nodal involvement, stage, and age were obtained from the patients who underwent Modified Radical Mastectomy (MRM).

2.2. RNA isolation and quantification

RNA extraction from tissue samples was carried out using standard TRIzol™ reagent method using an already established protocol (Qadir *et al.,* 2021). Extracted RNA was quantified on a nanodrop (IMPLEN GmbH, Germany). For each sample, 1µl of RNA was loaded onto nanodrop

and absorbance was recorded at 260 and 280nm. A ratio of 260/280 was measured to assess the quality of extracted RNA and samples with a ratio > 1.9 were used for further analysis.

2.3. cDNA Synthesis

To examine the expression of miRNA-*3648* and APC*2*, RNA isolated from each sample was subjected to cDNA synthesis. A cDNA synthesis kit from Thermo Scientific (Cat#K1622) was used to transcribe RNA into cDNA in accordance with the manufacturer's instructions. Oligo dT primers and *miR-3648* stem loop (SL) primer was added to each reaction mixture. The cDNA, thus formed, was confirmed using 2% agarose gel electrophoresis. The sequence of *miR-3648* stem loop primer is given in the (Table 1).

2.4. Quantitative Real Time Polymerase Chain Reaction

Relative expression of *miR-3648* and *APC2* was analyzed using gene specific primers and qPCR SYBR Green Master Mix on quantitative PCR machine (Applied Biosystems). Transcript levels of *miR-3648* and *APC2* were determined based on the threshold cycle (C_t) values. Livak's method was used to analyze the data. GAPDH was used as an internal control for *miR-3648* and *APC2* gene data normalization (Rashid *et al*, 2017). Primers used in this study are given in (Table 1).

2.5. Statistical analysis

Statistical analysis for the results, thus acquired, was performed using nonparametric tests. Mainly Wilcoxon Signed Ranked Test for paired variables while Mann-Whitney and Kruskal Wallis tests were used for unpaired variables. GraphPad Version 5.0 was employed as a tool for statistical analysis.

Table 1. Oligo sequences used in this study.

S. No	Oligo	Sequence
1	miR-3648 (SL)	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCC
2.	miR-3648 (F)	CACAGCCGCGGGGAT
3.	miR-3648 (R)	CCAGTGCAGGGTCCGAGGTA
4.	APC2 (F)	CGCTGCAGGAGCTGAAGAT
5.	APC2 (R)	GGCTGGAGTTGTCCCTTAGC
6.	GAPDH (F)	CAAGGTCATCCATGACAACTTT
7.	GAPDH (R)	GTCCACCACCCTGTTGACAACTTG
8.	β-catenin (F)	TGCAGCTTCTGGGTTCCGATGATA
9.	β-catenin (R)	AGATGGCAGGCTCAGTGATGTCTT

3. Results

The obtained patient data was distributed according to clinico-pathological parameters including age, menopausal status, tumor size, tumor grade, tumor stage (I-IV), nodal involvement and metastasis. To establish the clinical relevance of *miR-3648* and *APC2* in breast cancer patients, their change in expression levels was shown as depicted (Table 2).

Table 2. Expressional Association of *miR-3648* and *APC2* with Clinico-pathological Parameters and Intrinsic Molecular Subtypes in Breast Cancer

Variable(s)	Categories	Relative Expression of *miR-3648* (Mean±SEM)	Relative Expression of *APC2* (Mean±SEM)	*p*-Value
Tumor Tissues	80 Samples	1.095± 0.3759	0.3325± 0.1461	**0.0002*** **
Age	<50 Yrs	1.190± 0.4357	0.2389± 0.1259	0.0002***
	>50 Yrs	0.5850± 0.2519	170.9± 167.1	0.1993
Grade	I	0.5169± 0.5131	0.02738± 0.02695	**0.0234***
	II	0.8850± 0.3773	29.56± 29.13	**0.0406***
	III	1.226±0.4782	0.4335±0.2248	0.111
Stage	I	0.8568±0.5737	0.03199±0.02661	**0.0234***
	II	1.350±0.7832	0.4172±0.2494	0.3564
	III	0.9887±0.5012	14.45±14.05	0.0503
	IV	1.071±0.9495	1.070±1.001	0.8960
Menopausal Status	Pre-Menopause	0.6632±0.3308	0.1056±0.05867	**0.009** **
	Post-Menopause	1.996±0.9377	21.59±20.43	0.0572
Tumor Size	<2cm	0.8620± 0.6105	2.043± 1.453	0.1099
	2-5cm	2.095± 1.192	0.3092± 0.1645	**0.0028** **
	>5cm	0.6952± 0.2859	10.71± 10.38	0.0754
Nodal Involvement	Positive (N1)	1.157± 0.4279	8.998± 8.482	0.0877
	Negative (N0)	0.7187± 0.3745	0.1332± 0.09612	**0.0017** **
Metastasis	M0	1.100±0.4097	5.305±5.065	**0.0009*** **
	M1	1.071±0.9495	1.070±1.001	0.896
Intrinsic Molecular Subtypes	Luminal A	0.02584± 0.01604	1.167± 1.061	**0.0245***
	Luminal B	2.350± 1.177	0.8384± 0.4512	0.1276
	HER2 Positive	0.04797± 0.04303	0.006327± 0.003353	0.0510
	TNBC	7.258± 7.219	3.631± 1.726	0.4688

3.1. Expression profile of *miR-3648* and *APC2* in tumor tissue samples

Expression profiling of *miR-3648* and *APC2* in breast cancer patients revealed that transcript levels of both molecules were negatively correlated, i.e., *miR-3648* showed slight upregulation in tumor samples whereas *APC2* was found to be downregulated, suggesting that the loss of *APC2* function in breast cancer patients of Pakistan may partially be due to the presence of *miR-3648* (Figure 1A). Interestingly, the difference in expression levels of these two entities was statistically significant with $p<0.001$.

3.2. Association of *miR-3648* and *APC2* expression with Clinico-pathological Parameters

APC2 mRNA expression was observed to be significantly upregulated in patients with moderately differentiated tumors, whereby, *miR-3648* was relatively downregulated in these patients. This expressional variation between the *miR-3648* and *APC2* was found to be statistically significant (Figure 1B). In pre-menopausal breast cancer patients, there was a slight upregulation of *miR-3648* but downregulation of *APC2* (Figure 1C). This implied the potential role of *APC2* being a tumor suppressor with regulatory roles in premenopausal patients. Regarding different age groups, *APC2* mRNA expression was observed to be downregulated in younger patients with ages <50 years whereas *miR-3648* was relatively upregulated in these patients. This variation between these two molecular entities was found to be statistically significant (Figure 1D).

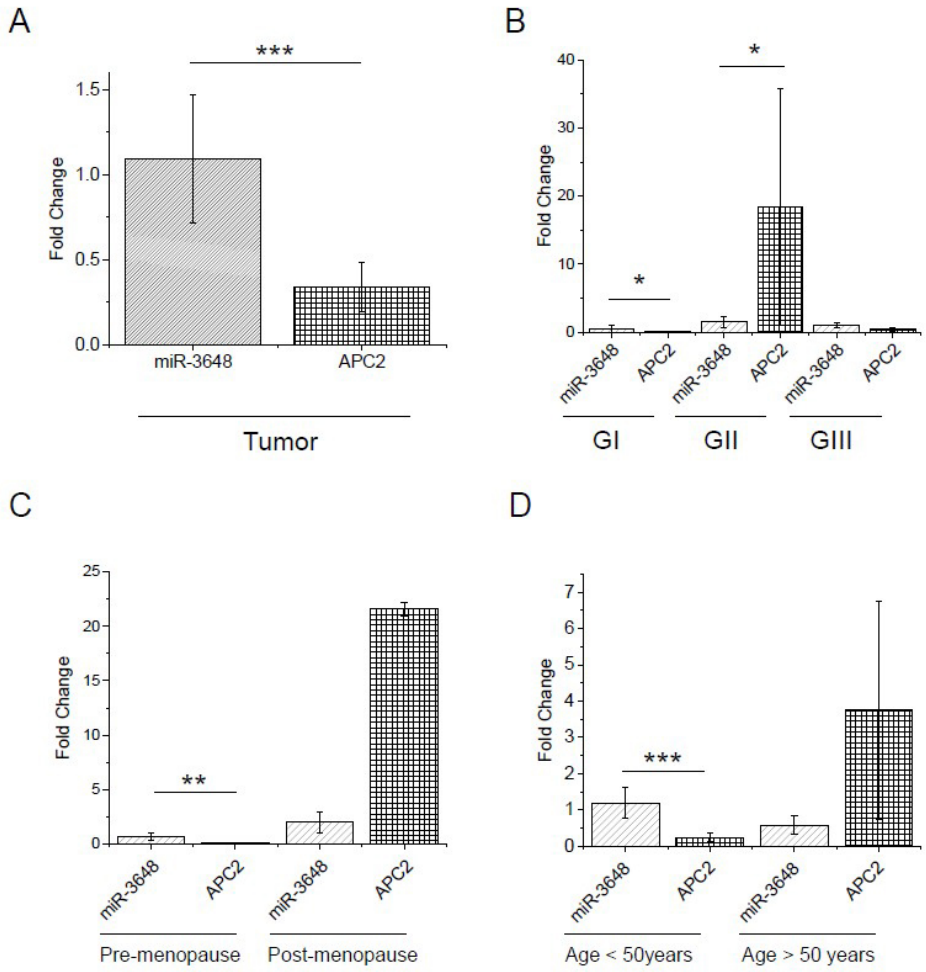

Fig. 1. Expression of *miR-3648* and *APC2* in breast tumor samples and its association with clinico-pathological parameters. (A) Significant downregulation of *APC2* expression was observed in breast tumors with miR-3848 upregulation. (B) In moderately differentiated tumors, *APC2* gene expression was significantly upregulated with *miR-3648* downregulation unlike well differentiated and poorly differentiated tumors. Similarly (C, D) shows significant downregulation of *APC2* in early disease onset and pre-menopausal women. $p<0.05$ was considered as statistically significant.

3.3. Association of *miR-3648* and *APC2* expression with TNM and Molecular Breast Cancer Subtypes

As shown in (Figure 2A), breast tumor samples with size between 2-5cm showed increased transcript levels of *miR-3648* while *APC2* was relatively decreased in these patients. Similar pattern of expression for these two was observed in patients who exhibited no lymph node involvement (Figure 2B). Moreover, an upregulation of *APC2* gene accompanied with a downregulation of *miR-3648* in breast cancer patients with no distant metastasis was observed, and was statistically significant, as shown in (Figure 2C).

Interestingly, both *miR-3648* and *APC2* were observed to be significantly associated at molecular level, explicitly in Luminal B breast cancer subtype in Pakistani women. Similar pattern of *miR-3648* and *APC2* expression was also observed in triple negative breast cancer (TNBC) breast tumors subtype, however, the findings were not statistically significant. It may be inferred that *miR-3648* and *APC2* may have role in estrogen-receptor (ER) and progesterone-receptor (PR) regulation in breast cancer patients (Figure 2D).

APC2 interacts with *β-catenin* and negatively regulates the *β-catenin* signaling pathway (Pai *et al.,* 2017). Downregulation of *APC2* should influence the downstream expression of *β-catenin*, therefore, the expression of *β-catenin* was measured and as expected, the overall expression of *β-catenin* mRNA was significantly upregulated (Figure 2E). Furthermore, its expression showed inverse relationship with the expression of *APC2* in pre-metastatic tumors of patients having pre-menopausal status with age < 50 (Figure 2F). These findings further corroborated with our findings that *miR-3648* mediated repression of *APC2* influence the early onset of breast cancer.

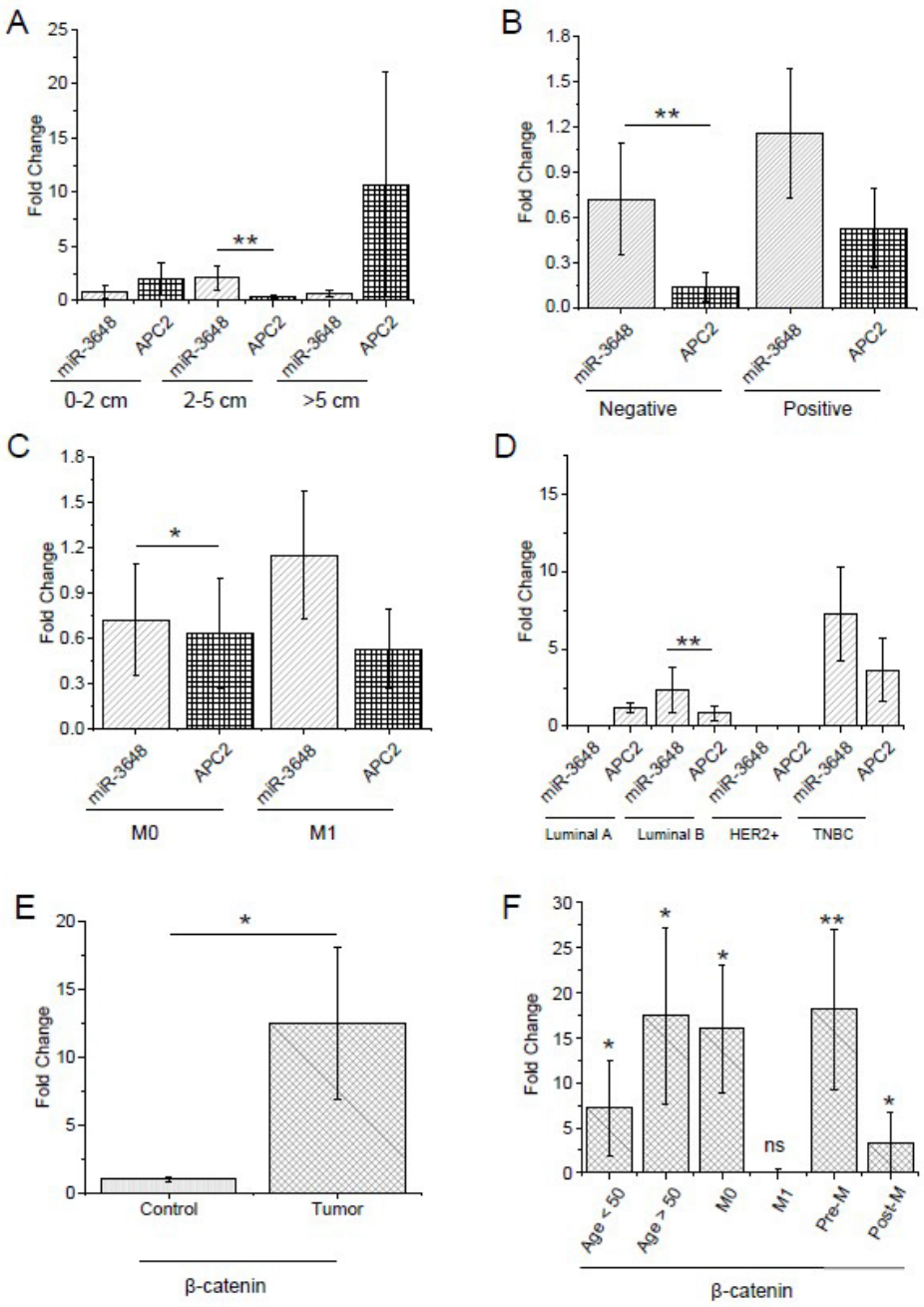

Fig. 2. Expressional association of *miR-3648* and *APC2* with TNM and molecular subtypes in breast cancer. (A) Increased expression of *miR-3648* and corresponding downregulation of *APC2* was observed in moderate sized tumors. (B) Decreased expression of *APC2* in tumors devoid of lymph node involvement and in (C) non-metastatic tumors. (D) Strong association of *miR-3648* higher expression and corresponding low expression of *APC2* in Luminal B subtype hints at ER & PR regulation in breast cancer. (E) Higher *β-catenin* expression in breast tumors and in (F) pre-metastatic tumors of patients of age <50 and pre-menopausal status. M0, no metastasis. M1, metastasis. TNBC, triple negative breast cancer. ER, Estrogen receptor. PR, progesterone receptor. $p<0.05$ was considered as statistically significant.

4. Discussion

Aberrations in the Wnt signaling pathway have been shown to be associated with multiple cancers (Anastas & Moon, 2013). Several studies have described that the alterations in Wnt/APC2 signaling pathway play roles in the process of carcinogenesis (Wen *et al.,* 2020). There have been quite a number of miRNAs reported to modulate integral components of the Wnt signaling pathway, therefore, contributing to the pathogenesis of various cancers (Tabnak *et al.,* 2021). Of these, *miR-3648* is one of the many human specific miRNAs identified and is of particular interest due to its minimum off-targeting (Hu *et al*, 2012). Previously, the dysregulation of *miR-3648* and *APC2* genes have been reported in different cancers, with *miR-3648* to be upregulated in bladder cancer, neuroblastoma, and prostate cancer (Xing, 2019; Saeki *et al*, 2018; Sun *et al,*2019). However, there is limited or no data regarding *miR-3648* and *APC2* altered gene expression in breast cancer. Hence, the current study was the first to explore the expression levels of *miR-3648* and *APC2* and their correlation and clinical relevance in breast cancer patients.

Expression profiling of *miR-3648* and *APC2* in breast cancer patients revealed the roles of these molecules in the process of carcinogenesis. Specifically, the transcript levels of both molecules were inversely regulated i.e., *miR-3648* showed slight downregulation in luminal type A whereas *APC2* was found to be upregulated, exhibiting enhanced tumor suppressor activity of *APC2* in the absence of *miR-3648* in patients with this intrinsic breast cancer subtype.

According to an already published report, almost 10% from total reported breast cancer cases are categorized as luminal B subtype (Parise & Caggiano, 2014). Interestingly, another more recent study reported higher prevalence of luminal B subtype in Pakistani population (Qadir *et al,* 2020). TNBC, being the most aggressive cancer is not easy to treat. In the current study cohort, however, no significant association of altered *miR-3648* and *APC2* expression could be established in TNBC subtype. According to previous studies, the change observed specifically in triple negative and luminal breast cancer subtypes may be due to imbalance of estrogen and progesterone hormones (Radojicic *et al,* 2011). It may possibly be attributed to the cross-talk between *APC2*, *miR-3648* and estrogen receptor alpha (ERα), progesterone receptor (*PR)* and growth factor receptor (*HER2)* in breast cancer (Daly *et al,* 2017). Consistent to these studies, *miR-3648* may be playing a very important role in regulating the expression of these receptors by regulating *APC2* activity but this aspect needs to be explored further.

APC2 gene is reported as a tumor suppressor gene. Literature review suggests the involvement of *APC2* gene in the *Wnt* Signaling, a pathway whose dysregulation leads to breast cancer (Daly *et al,* 2017). In ovarian homeostasis, *APC2* regulates the *Wnt* signaling control and acts as a tumor suppressor gene (Mohamed *et al,* 2019). Although, *APC2* gene dysregulation is well studied in breast cancer but using mouse mammary epithelium (Daly *et al,* 2017). Particularly, the expression profile of *APC2* gene in human breast cancer has yet to be examined in the context of its regulation by miRNA-3648. Hence, this study was the first to report the regulatory interplay between these two entities in breast cancer patients of Pakistan.

To examine the clinical relevance of *miR-3648* and *APC2* in breast cancer patients, parameters including age, tumor size, grade, stage, metastasis, nodal involvement and menopausal status were observed and compared. Among different age groups, *APC2* mRNA expression was significantly downregulated in younger patients with ages <50 years but accompanied with a slightly upregulated level of *miR-3648*. These findings endorsed the loss of *APC2* function in patients exhibiting early disease onset i.e., breast cancer, in a *miR-3648* dependent manner. Comparably, patients with age older than 50 years, showed no significant association between *APC2* and *miR-3648*. Furthermore, relatively higher expression of *β-catenin* due to downregulation of *APC2* was also observed in samples corresponding to early disease onset (Figure 2E and 2F). While 3'UTR of *β-catenin* mRNA is devoid of *miR-3648* binding sites, the observed dysregulation of *APC2* expression may in part be due to dysregulation of *miR-3648* and higher expression of *β-catenin* that was *APC2* dependent. These findings explain the role of *APC2* dysregulation mediated by *miR-3648* levels in breast cancer initiation, development and progression in Pakistani women.

5. Conclusion and Perspective

The current study concluded that *miR-3648* is relatively downregulated in Luminal A breast cancer subtype, with corresponding upregulation of *APC2* in these patients. Moreover, the transcript levels of both *miR-3648* and *APC2* are inversely regulated in breast cancer affected women who presented with early disease onset, pre-menopause, low tumor grade, early clinical stage, absence of nodal invasion and metastasis. This study affirmed the molecular interplay of these molecules in breast cancer development and progression.

Knowing that miRNAs follow an exclusive mechanism of directly targeting the transcripts and the proteins, thus, modulating their functions, an in-depth analysis of miRNA function is still required. The present study will add to the existing pool of information published for miRNAs that specifically regulate Wnt signaling pathway in cancer. Pertinently, extensive *in vitro* and *in vivo* research studies are required to establish the translational significance of *miR-3648* in conjunction with WNT signaling pathway in breast tumorigenesis. Such findings, if adequately furnished, can elucidate the usability of miRNAs as tools for prognosis as well as therapy for cancer patients in the future.

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Conflict of Interest

The authors of the study have no conflict of interest to declare.

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