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Let-7 microRNA: tumor suppression activity in breast cancer

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Abstract

Breast cancer is the leading cause of cancer death among women and characterized by deregulated expression of several tumor suppressor or oncogenic miRNAs. Mutations in these miRNAs resulted in abnormalities in cell-cycle exit as well as in the execution of a terminal differentiation program, preventing cells from reaching their fully differentiated state. The objective of this study was the identification and characterization of miR-let-7c as a potential tumor suppressor in breast cancer. Levels of expression of miR-let-7c were examined in breast cancer cell lines and tissues analysis using qRT-PCR. The results from this study show that the level of let-7c was significantly decreased in tumor compared to their matched normal breast tissues, and further, decreased in metastatic breast tumor. Furthermore, down-regulation of let-7c increased cell proliferation of breast cancer cells line. Reconstitution of Let-7c by lentiviral-mediated intratumoral delivery significantly reduced tumor burden in xenografts of breast cancer cells. Thus, profiling miRNA-Let-7 is in need to clarify intercellular signaling and discover a novel breast tumor marker and therapeutic targets.

Keywords: Breast cancer; Let-7; mRNA; Xenograft; qRT-PCR

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Introduction

Breast cancer (BC) is most frequently diagnosed cancer and remains one of leading cause of cancer-related death in women worldwide. Altered signaling pathways [1], mutation in genes [8], activation of oncogenic pathways [2], DNA damage and non-targeted effects of chemotherapeutic agents [3], significantly contributes in cancer progression. Therapeutic strategies including chemotherapy, application of toxins obtained from pathogen [4] have shown limited clinical efficacy against cancer [5].

During past one and half decade, enormous growth in the field of microRNAs (miRNAs) biology have been witnessed and it has been suggested that targeting these small molecules holds potential therapeutic efficacy for cancer [6]. miRNAs are evolutionary conserved, single-stranded and contains approximately 22 nucleotides RNA molecules that alter the expression of gene at the post-transcriptional level.

In nucleus miRNAs are transcribed by RNA polymerase II as pri-miRNAs and subsequently cleaved by ribonuclease III, Drosha, to form a ~70 nucleotide long pre-miRNA. Thereafter, the pre-miRNA is transported to the cytoplasm and processed by the RNase III protein, Dicer, to yield 18–25 nucleotide long miRNA duplex [7].

After unwinding, one of the strands incorporated into the RNA-induced silencing complex, that subsequently interacts with complementary sequences in the 3′ untranslated regions (3′ UTRs) of the target mRNA transcripts. A single miRNA is capable of regulating multiple mRNAs of various functions [8].

Further, dysregulation of miRNAs abrogates the normal functioning of the cellular system that promotes several pathological conditions such as cancer [9]. Abnormal expression of miRNAs such as let-7 has been reported in several malignancies including BC. In year 2000 Reinhart et al. demonstrated that let-7 miRNA alters the phenotype of nematode and regulates the development of Caenorhabditis elegans [10].

In human, 10 members of the let-7 family have been identified, including let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, let-7i, miR-98 and miR-202. In normal physiological conditions let-7 is primarily involved in gene regulation, cell adhesion and muscle formation. Accumulating evidence suggests that let-7 is downregulated in numerous types of cancer, including gastric tumors [11], colon cancer [13], lung cancer [13], Burkitt's lymphoma and BC [14].

Material and Patients

A total of 29 primary breast cancer specimens obtained from Japanese female patients who underwent surgical treatment from 2003 to 2012 in [1]Division of Biochemistry, Loma Linda University, Mortensen, USA were available for examination in this study. The age of the subjects ranged from 30 to 88 years (median: 57 years), and 21 cases (41–86 years: median 58.6 years) were selected for PCR array analysis among these 26 cases. Tissues were fixed with 10% formalin for 24–48 h at room temperature and were embedded in paraffin. Research protocol for this study was approved by the ethics committee of [1]Division of Biochemistry, Loma Linda University, Mortensen, USA.

Immunohistochemistry

of LIN28/LIN28B Immunohistochemical staining was performed using a biotinstreptavidin method with Histofine kit (Nichirei Co. Ltd., Tokyo, Japan). The slides were deparaffinized in xylene,followed by hydration through a series of alcohol. Hydrogen peroxidase was used to block intrinsic peroxidase activity. Following the application of primary antibodies, the reacted sections were incubated at 4 ◦C for 24 h. The primary antibodies, LIN28 (55CT58.12.1: mouse monoclonal antibody), and LIN28B (rabbit polyclonal antibody) were commercially obtained from Abcam Ltd. (Cambridge, UK). Human testis tissue was used as a positive control of immunostaining [15]. The following primary antibodies were used for other immunohistochemical analysis: monoclonal antibodies for estrogen receptor (ER; ER1D5;

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Immunotech, Marseille, France), progesterone receptor (PR; MAB429; Chemicon International Inc., CA, US), and Ki-67 (MIB-1; DakoCytomation Co. Ltd., Kyoto, Japan), and rabbit polyclonal antibody for HER2/neu (AO485; DakoCytomation).

miRNA PCR array

Human Cancer RT2 Profiler PCR Array system (QIAGEN, Mannheim, Germany) was used as demonstrated in previous reports in order to measure expression of let-7 family in a quantitative fashion in isolated breast carcinoma cells [16]. Laser capture microdissection was carried out to isolate carcinoma cells from 8 mm formalin fixed and paraffin embedded tissue sections (FFPE) using MMI CellCut (Molecular Machines and Industries, Flughofstrasse, Glattbrug, Switzerland). Approximately 5000 carcinoma cells were obtained from individual tissue sections. These isolated carcinoma cells were deparaffinized at 60 ◦C for 3 h. Extraction of miRNA was performed as instructed by Pure Link miRNA Isolation Kit (Invitrogen, Carlsbad, CA), followed by cDNA synthesis using RT2 miRNA First Strand Kit (QIAGEN). qRT-PCR was employed with ABI7500 real-time PCR system (Applied Biosystems, Forster city, CA, USA) to determine expression levels of let-7 miRNAs using RT2 profiler PCR Array Data Analysis for statistical analysis.

Statistical analysis

Stat View 5.0J Software (SAS Institute Inc., NC, USA) was used for analytical findings of clinicopathological features in the cases examined. The values for patient's age, ER LI, ER LI, and PRLI were represented as the mean ± SD. For hierarchical clustering analysis, LIN28 expression of each sample was categorized based upon its immunoreactivity, compared with results of microarray analysis of let-7 family. The tree structures were formulated using TreeView programs according to the degree of similarity. We compared the results of PCR array with those of immunohistochemistry using Bonferroni/Dunn and Mann–Whitney U test. The significance of each value was determined when P value was less than 0.05. The statistical differences between LNI28/LIN28B and clinicopathological parameters were determined using Chi-square analysis; 95% confidence interval calculated for confirmation of statistical significance.

Results

LIN28 and LIN28B in breast cancer tissues an association between LIN28 and clinicopathological parameters was summarized in Table 1. There was a significantly positive correlation between the LIN28 and ER expression (P = 0.007 for LI, P = 0.002), PR (P = 0.034) and Ki-67 (P = 0.049), whereas the status of HER2 was inversely associated with LIN28 status (P = 0.036) of the cases examined. No significant correlation was detected between ER and LIN28. In addition, LIN28 and LIN28B (P = 0.009) were positively correlated in all the cases evaluated. An association between LIN28B and clinicopathological features was summarized in Table 2. There was a significantly positive association between LIN28B and Ki-67 status (P = 0.049), but no other significant associations were detected.

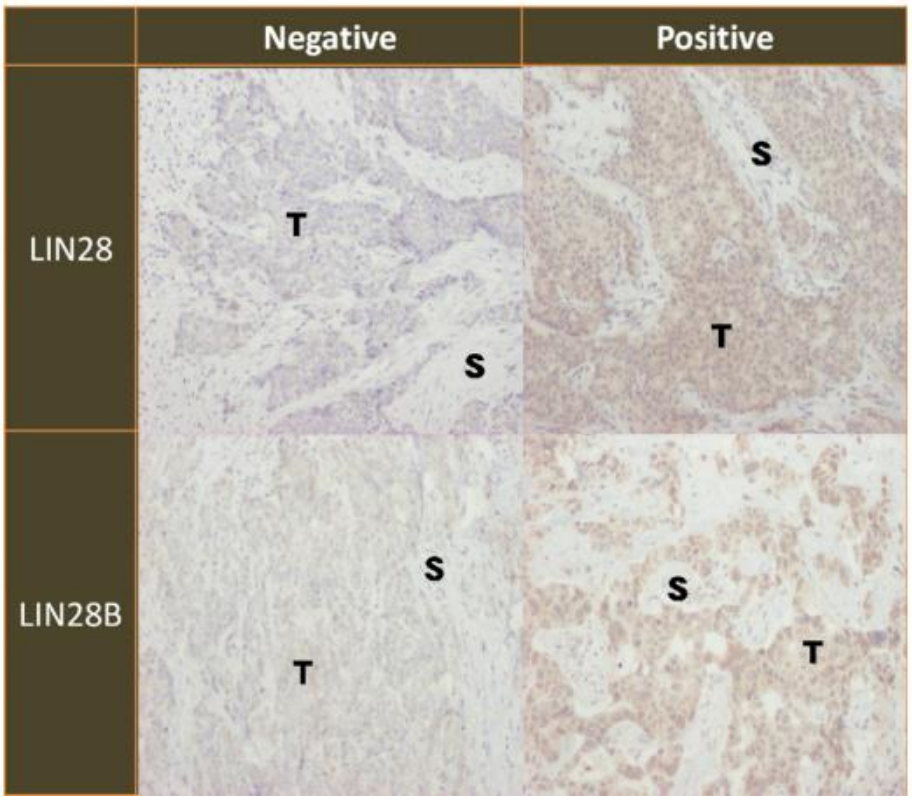

Figure 1.

Immnunolocalization of LIN28 and LIN28B in breast carcinoma tissues. A total of 26 specimens were classified according to the degree of relative immunointensity; negative.

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Expression of let-7 family and its correlation with LIN28 and LIN28B Histopathological examination revealed an important interaction between the status of LIN28 and hormone receptors in breast carcinoma cells as described above. We therefore focused upon the effects of LIN28 towards let-7 miRNA family in human breast carcinoma cells.

Hierarchical clustering analysis of PCR array demonstrated that the status of let-7 miRNA expression tended to be correlated with that of LIN28 immunoreactivity in human breast carcinoma cells (Fig. 2A). LIN28 positive group tended to be associated with reduced expression of let-7, whereas cases with negative LIN28 status tended to be associated with increased expression of the great majority of let-7 miRNAs. Fig. 2B–I represented miRNA concentration according to LIN28 expression of the cases.

Of eight let-7 miRNA family members, the level of let-7d ($P = 0.026$, 0.184-fold) and let-7f ($P = 0.037$, 0.341-fold) expression in LIN28 positive group was lower than that in LIN28 negative group of the patients. No significant associations were, however, detected between LIN28 immunoreactivity and other let-7 family members in breast carcinoma cells. There were also no significant correlations between LIN28B immunoreactivity and the expression levels of let-7 family in breast carcinoma cells.

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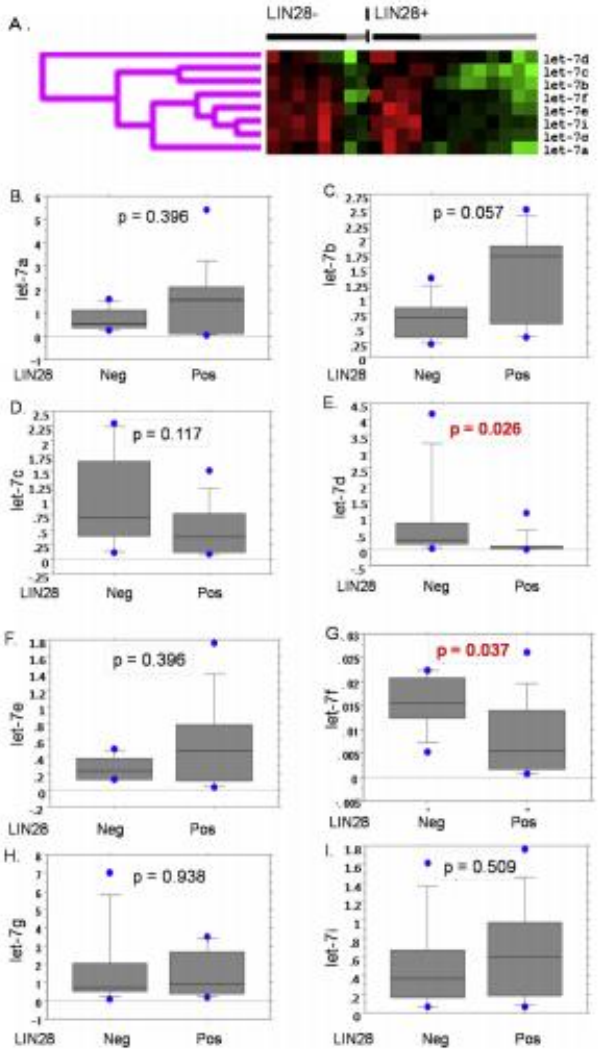

Figure. 2.

Expression of let-7 miRNA and LIN28. (A) Hierarchical cluster analysis demonstrating expression of let-7 miRNA family in 21 breast carcinoma cases. The cases were classified into two groups; LIN28 (−) and LIN28 (+). The black bars represent high expression of let-7 miRNAs, and the grey bars represent low expression of let-7 miRNAs. Red, high miRNA expression; green, low miRNA expression. (B)–(I) The box plot representing relative mature miRNA expression in y-axis, and LIN28 expression in x-axis. A horizontal line in the box plot illustrates the median value. The upper and lower bars indicate the 90th and 10th percentiles, respectively. The statistical analysis was performed using a Mann–Whitney U test. P 0.05 is indicated in bold.

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Discussion

Metastasis is a process in which cancer cell break away from the original site and travels the blood or lymphatic system to the other parts of body and form new tumors. Chemotaxis is believed to be a fundamental cause of metastasis in which external signals orient and attract tumor cells [18-26].

Overexpression of certain receptors facilitates BC cells to get attracted and move to another site. CXCR7 is one of the receptor that is found to be overexpressed in BC and participate in metastasis [27]. CCR7 is activated by binding chemokines CCL21 and CCL19 [28-33]. T cells utilize CCL21 to enter lymphoid tissues from circulation. As for as CCL19 is concerned, it is expressed by mature dendritic cells which activates T cells [34].

However, tumor cells exploit the conditions by expressing CCR7 that helps them to localize in lymph node after receiving the chemotactic signals from CCL19 or CCL21. miRNAs have been suggested to suppress the expression of numerous cancer-related genes that subsequently reduces tumorigenesis and metastasis in BC and several other cancers [35]. A study examined the roles of CCR7 and miRNA in breast cancer metastasis suggested that *let-7* family binds participate in the process of metastasis [36].

Let-7a was found to influence the CCR7 down expression by targeting 3′UTR of CCR7, thereby downregulating BC cell invasion and migration. Similar results were confirmed in study performed using zebrafish embryo models. *Let-7a*, a member of family *let-7* act as a tumor suppressor by regulating the expression of RAS and HMGA2 oncogenes [37]. Further, decreased levels of *let-7a* was found to associate with elevated RAS expression in lung squamous carcinoma [38].

Unexpectedly, *let-7* can also have detrimental effects. Even though *let-7* has been demonstrated to have tumor suppressive effects in various cancer types, emerging data suggest that, counterintuitively, in some cases *let-7* may act as an oncogene. Several groups have demonstrated that the *let-7a3* locus is highly methylated in normal tissues, but hypomethylated in lung and ovarian tumors, with *higher* expression of mature *let-7a* in cancers [40].

Over expression of *let-7a3* in lung cancer cells results in increased aggressiveness of cells, assessed via anchorage independent assay and increase in gene expression associated with cell proliferation, as well as down-regulation of genes associated with adhesion, relevant to tumor progression and metastasis [41]. Higher *let-7a3*, *let-7b*, and *let-7c* levels in ovarian and hepatic cancers are correlated with poor prognosis and decreased overall survival [7].

let-7e is increased in and positively affects migration and invasion of esophageal squamous cell carcinoma cells, possibly via targeting ARID3a [42]. Since ARID3a negatively correlates with pluripotency, decreasing it could contribute to stemness [4]. *Let-7f* and *let-7e* have been shown to be upregulated in tongue squamous carcinoma, and *let-7c*, *let-7d*, and *let-7f* are upregulated in aggressive relative to non-aggressive tumors [7]. Mir-98 has been shown to

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increase chemoresistance via indirect repression of mir-152 by targeting Dicer1. Mir-152 controls RAD51 expression, contributing to the poor prognosis of EOC patients with increased levels of mir-98 [100]. In certain in vitro conditions such as starvation, *let-7* paradoxically induces expression of HMGA2 [42].

All of these indicate the complexity of the relationship between *let-7* and cancer cell aggressiveness, and illustrate the fact that the actions of any miRNA are context dependent. The set of genes expressed in a particular cell determines the available *let-7* targets. Thus, it is important that *let-7* overexpression treatment strategies be tailored towards individualized clinical scenarios based on specific miRNA expression profiles, as opposed to overarching treatment schemas spanning across multiple malignancy types [43].

Tumor microenvironment and stroma are also important to consider when developing new Demonstrated that increased *let-7* expression in tumor associated macrophages (TAMs) results in conversion into the M2 phenotype. While tumor infiltration by TAMs with M1 phenotype have pro-inflammatory activity and better prognosis, the M2 phenotype is associated with increased angiogenesis and increased tumor burden [44].

Let-7 delivery as a therapeutic regimen therefore has to be specific to cancer cells due to its oncogenic functions in tumor immune cells. Even though a few studies demonstrated *let-7* as having oncogenic functions and correlating with poor prognosis, the vast majority of evidence suggests otherwise. Therefore, *let-7* remains a potential therapeutic target [34].

A number of studies have shown that the inhibition of let7 miRNA precursor resulted in promotion of various oncogenic activity [45]. Results of our present study also demonstrated that the status of LIN28 may influence the prognosis of patients with estrogen-dependent breast carcinomas. By summing up previous data and our study, it is possible that LIN28 facilitates downregulation of let-7 miRNA in breast malignancy, particularly let-7f precursor [5]. From this, we can reasonably hypothesize a blockage of let-7 maturation process by LIN28 at post-transcriptional level will increase intratumoral estrogen synthesis via aromatase reactivation in human breast cancer tissues.

Competing interests

The authors declare that they have no competing interests.

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