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Function characterization of miR-4726; a downregulated miRNA in breast cancer

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ABSTRACT: *MicroRNAs play a vital role in carcinogenesis either showing its effect as oncogenic or tumour suppressive manner. MiR-4726 mapped in 17q12 chromosome- a clinically important region, as amplification of this region causes over expression of ERBB2/ HER2 receptor. An earlier study reported that miR-4726 is down regulated in DCIS and IBC compare to benign epithelial cells suggesting a tumour suppressor role. However, the functional role of this miRNA in breast cancer is yet to be identified. To evaluate the functional role, we generated the control (miR-CTRL) and miR-4726 overexpressing sub clones of MCF-7 and MDA MB-231 cells. Proliferation was assessed by manual cell counting, MTS assay and colony forming assay. Cell migration was tested using scratch method. For drug response analysis, microRNA overexpressing cells were exposed to five different chemotherapeutic agents in different concentrations and cell proliferation was assessed by MTS assay at different time intervals. We did not observe any significant differences between the control (miR-CTRL) and miR-4726 expressing cells in regards to proliferation, migration and sensitivity to chemotherapeutic compounds. Taking into account the consistently high expression levels of the miRNA, the lack of a repeatable significant effect indicates that the downregulation of this miRNA in breast cancer is a passenger mutation, and does not exert an effect on fitness of breast cancer cells.*

KEYWORDS:*Breast cancer, MicroRNA, miR-4726, MCF7, Oncogenic, Tumor suppressive*

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I. INTRODUCTION

Breast cancer is one of the most common deadly disease characterized by molecular heterogeneity and considered as the second leading cause of cancer associated death in women worldwide**[1, 2]**. It is broadly categorized as ductal carcinoma in situ (DCIS) that are characterized by the proliferation of malignant ductal epithelial cells within the ducts of breast and invasive breast cancer (IBC) where proliferation of cells occurs in the ducts well as outside of the ducts into the surrounding tissue or another organ**[3]**. Gene expression profiling by multiple research groups have categorised breast cancer into five major molecular subtypes; luminal A, luminal B, HER2/ER+/-, basal-like, and normal breast like**[4].**Several known genetic regulators are altered in breast cancer. Among the regulators, dysregulation of different microRNAs also play a vital role in the carcinogenesis either showing effects as oncogenic or tumor suppressive manner**[5]**. MicroRNAs (miRNAs) are small, non-coding, single stranded 21 nucleotides long RNA transcribed from hairpin like structures in association with diverse enzymes and proteins that negatively regulate the expression of gene post transcriptionally via a sequence specific manner by seeding with the 3′ UTR of cognate mRNAs resulting in the repression of translation and or the degradation**[6-9].**miRBase a central repository for miRNA sequences listed 1881 mature miRNA sequences in their database. Calin*et al* showed that 50% of the annotated miRNAs are associated with different cancers in human**[10]**. For example, mir-125b-1 is mapped on a chromosome 11q24 that is deleted in breast cancer suggesting its role as a tumor suppressor**[10].** miR-155 has been reported to be overexpressed in breast carcinomas suggesting its role as oncomir**[11].** miR-22 is noted as metastamiR in breast cancer**[12].** Alongside, somatic mutation in the DNA is one of the known major cause for the development of cancer where driver mutation is responsible for the proliferation of the cancer cell but the passenger mutation has no effect on cell growth advantage and therefore not contributed to cancer development**[13].** Several non-coding regions of the DNA showed a remarkable mutation frequency but they do not possess driver mutation**[14].**

In human breast cancer, approximately one fourth of the patients show overexpression of HER2/Erbb2**[15]**that ultimately makes the cancer cells to grow in uncontrolled manner. In 2011, Persson *et al* identified some new microRNAs by next-generation sequencing, among them an intronic microRNA miR-4726 mapped in 17q12 chromosome- a clinically important region, as amplification of this region causes

overexpression of HER2/Erbb2 receptor**[16].** Using*in situ* hybridization staining in breast cancer tissue samples, Ingoldsby *et al*, reported thatexpression of miR-4726 is down regulated in ductal carcinoma in situ (DCIS) and invasive breast cancer (IBC) compare to benign epithelial cells,suggesting a tumour suppressor role**[17]**.However, very diminutive information regarding the role of this intronic microRNAs in the development and progression of cancer is available to date so, it is important to know the role of this intronic microRNAs in breast cancer. Since, miR-4726 is a newly identified intronic miRNA hosted in MLLT6 gene we, therefore, aimed to study the functional role of this miRNA in the progression of breast cancer that will shed light to better understanding of this disease.

We investigated the functional properties of miR-4726 in breast cancer. Our result demonstrated that miR-4726 has no role on cell proliferation, migration and drug induced apoptosis in breast cancer and it seems that down regulation of this miRNA is a passenger mutation in breast cancer and does not exert an effect on fitness of breast cancer cells.

II. MATERIALS AND METHODS

2.1 Culturing and treatment of cells:MCF7, MDA MB 231 cells were sourced from ECACC. Cells were grown in Dulbecco's modified medium (DMEM) supplemented with 10 % Fetal bovine serum (FBS), Na-Pyruvate (1mM), L- Glutamine (2 mM), 100U/ ml penicillin and 100 mg/ml streptomycin in a CO2 incubator supplied with 5 % CO2 and at 37®C temperature. MCF7 and MDA MB 231 clones (miR-CTRL & miR-4726) were maintained using the same DMEM along with the aforementioned supplements and puromycin (1µg/ml).

2.2 Plasmid construct and generation of stable cell lines:miRExpress TM pEZX-MR03 plasmids (miR-CTRL and miR-4726) were sourced from GeneCopeia to produce lentiviral vectors for the expression of miR-CTRL and miR-4726 separately. The mature MicroRNA sequence of miR-4726 that was included in transfection plasmids is- ACCCAGGUUCCCUCUGGCCGCA. The lentivector is designed for co-expression of GFP along with miRNA. We generated stable subclones of miR-4726 by transfecting MCF7 and MDA MB 231 cells with lentivirus made using control and miR-4726 expression vector pEZX-MR03 along with packaging plasmids in HEK 293T cells by using jetPEI transfection reagent (Polypus transfection, VWR International Ltd, Dublin, Ireland) and puromycin selection (1µg/ml) for 7 days according to the manufacturer's instruction.

2.3 Extraction of RNA, RT-PCR, qRT-PCR: Total RNA was isolated by harvesting the cells with Trizol (Life Technologies) according to the manufacturer's guidelines. RT (Reverse transcription) was done with 4 µg of total RNA and random hexamers (Promega) using ImProm-II TM Reverse Transcription system (Promega). Real time PCR (qRT-PCR) for genes was carried out using cDNA products mixed with 2X TaqMan master mix and 20X TaqMan gene expression assays (IDT). Relative expression was calculated from CT values obtained after 40 cycle of PCR reaction in AB5000 instrument (Applied Biosystems) using ΔΔCT method. Real time PCR for analysing miRNA expression was carried out by synthesizing cDNA using ImProm-II™ Reverse Transcription System and miRNA specific stem-loop primers (Applied Biosystems) according to the user manual of the kit. Primers for miR-4726: RT primer: 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACT GGATACGACCTGGGG; qRT-PCR assay: F5′ - TTCCTCATGCTGACCTCCCTCCTG;R 5′ – ATCCTGCCTCTCCTTCCTCCAC.

2.4 Cell proliferation assay

2.4.1 Manual cell counting assay: Cells (MCF-7 and MDA MB-231 clones expressing miR-CTRL and miR-4726) were plated in 6-well plate (50,000 cells/well) and were then trypsinized and counted manually by using haemocytometer at different time intervals (Day 0, 1,3,5,7). Fold change was calculated with relative to the cell number at Day 0.

2.4.2MTS cell proliferation assay: Cells (MCF-7 and MDA MB-231 clones expressing miR-CTRL and miR-4726) were plated in 96-well plates (2,000 cells/well) with 20 wells per cell lines. Cell proliferation was assessed by MTS assay at different time intervals (Day 0, 1, 3, 5, 7). MTS assay was carried out by adding 20 µl MTS/PMS in each well followed by 4 hours incubation in CO2 incubator. Absorbance was recorded using microplate reader at 490 nm and fold change was calculated with relative to the absorbance at Day 0.

2.4.3Colony forming assay: Cells (MCF-7 and MDA MB-231 clones expressing miR-CTRL and miR-4726) were plated in 6-well plate (5,000 cells/well). After 7 days of plating cells were observed to have formed colonies under microscope. Media was discarded from the plate and cells were washed with HBSS. To stain the colonies 1 ml Coomassiebrilliant blue was added to the wells and incubated on a shaker for 10 minutes.

Colonies of the cell were then washed with water for three times. Finally stained colonies were observed visually and compared.

2.5Cell migration assay: Scratch assay: Scratch was done in a confluent monolayer of MCF-7 and MDA MB-231 clones (miR-CTRL and miR-4726) by P-200 tip and migration of cells into the wound was observed at 12 hours interval using GFP enabled fluorescence microscope (Evos FL Life Technologies) and photographed accordingly. These images were compared to examine differences in cell migration.

2.6Sensitivity assay to therapeutics: Stable sub-clones of MCF7 and MDA-MB-231 (miR-CTRL and miR-4726) cells were seeded in 20 wells for each cell line in 96-well plates (5,000 cells/well). After incubation for 24hrs, medium was replaced with different chemotherapeutic agents namely doxorubicin, Tunicamycin, 5-Fluorouracil (5-FU), Bortezomib, docetaxelcontaining medium in four ascending concentrations. Cells were then incubated again for 48hrs and MTS assay was carried out at 490 nm to determine effect of miR-4726 in mediating drug induced apoptosis.

2.7Survival analysis for miR-4726: The prognostic value of the miR-4726 in breast cancer was analyzed using Kaplan-Meier Plotter (http://kmplot.com/analysis/) and determined overall survival using TCGA dataset. The patient samples are divided into two groups. The two patient groups (higher and lower expression levels) were compared using a Kaplan-Meier survival plot. The hazard ratio with 95% confidence intervals and log rank p value was calculated.

2.8Statistical Analysis: Statistical analysis in differential expression of miR-4726 in TMA sections of different breast cancers was carried out using R-statistical software (V2.12.0), SPSS (v 20), Minitab 16 and Graph Pad software. The data presented for cell proliferation, drug response analysis and qRTPCR are expressed as mean ± SD for three independent experiments. Differences between the treatment groups were assessed using Two-tailed paired student's t-tests. The values with a $p<0.05$ were considered statistically significant.

III. RESULTS

3.1 Aberrant expression pattern of miR-4726in breast cancer

Expression of miR-4726-3p/5p and its host gene MLLT6 was evaluated in a panel of breast cancer cell lines (MCF7, MDA MB 231, BT474, T47D, SKBR3). MLLT6 expression was predominantly higher in all breast cancer cell line compare to MCF10A and miR-4726-3p/5p expression did not follow the same pattern of expression as its host gene. Apparently, the expression of miR-4726 was relatively low in MCF7, MDA MB231 and SKBR3 **(Fig.1A, 1B).** It suggests that there is no correlation between the expression levels of host gene MLLT6 and miR-4726.

3.2 Generation and characterization of the stable clones expressing miR-4726

To evaluate the functional properties of miR-4726 in breast cancer, control and miR-4726 overexpressing stable sub clones ofMCF7 (MCF7-miR-CTRL & MCF7-miR-4726) and MDA MB231 (MDA MB 231-miR-CTRL & MDA MB 231-miR-4726) were generated. For this purpose, parental MCF7 and MDA MB 231 cells were transduced with lentivirus prepared using miR-CTRL and miR-4726 expression plasmid having a backbone of pEZX-MR03 vector followed by selection in puromycin containing medium for 7 days that co-express GFP along with the transferred miRNA (either control or miR-4726) as the plasmids comprise a GFP tag adjacent to the miRNA sequence**(Fig.2A).**Expression of GFP in the stable sub clones of MCF7 and MDA MB 231 cells was observed under fluorescence microscope indicating plasmids co-expressing GFP and miRNA sequence have been transduced successfully and actively expressed in the clones**(Fig.2B)**. The relative expression of miR-4726 in the sub-clones was measured using ΔΔCT method from the ct values obtained from Taqman qRT-PCR and it was observed a remarkable higher expression of miR-4726 in MCF7-miR-4726 and MDA MB 231 clones. When compared to control clones, the generated miR-4726 clones significantly overexpressed the miR-4726 **(Fig.2C, 2D)**. Therefore, MCF7-miR-CTRL; MCF7-miR-4726 and MDA MB 231-miR-CTRL; MDA MB 231-miR-4726 clones were used in subsequent experiments to characterize the functional property of miR-4726 in breast cancer cells.

3.3 Effect of miR-4726*on*the growth of breast cancer cells

Next the role of miR-4726 on the proliferation of breast cancer cells was determined. Three commonly used techniques named manual cell counting, colony forming assay and MTS cell proliferation assay were employed to assess the growth potentiality of miR-4726. miR-CTRL and miR-4726 overexpressing clones (MCF7 and MDA MB 231) were plated in a six-well plate followed by trypsinization and manual counting on alternative days using a haemocytometer over the course of seven days. Manual counting of cells exhibit no

difference in the growth of breast cancer cells**(Fig. 3A, 3B)**. MTS cell proliferation assay a widely used colorimetric method for determining the number of viable cells during proliferation. This assay was carried out for the clones of MCF7 and MDA MB 231 by seeding cells (2000 cells /well) in 96 well plate. Absorbance was recorded at 490 nm on alterative days followed by the addition of MTS for 4 hours incubation over a period of one week. No difference was observed in the fold change of cell growth for miR-4726 overexpressing clones in compare to miR-CTRL clones of both cell lines**(Fig.3C, 3D)**. The capability of a single cell to form colony is a commonly used technique for assessing the growth of cancer cells. Exactly same number of MCF7 and MDA MB 231 clones (miR-CTRL and miR-4726 overexpressing) were seeded in six-well plates followed by incubation in CO_2 incubator until the formation of colonies. Visual inspection of the Coomassie Brilliant Blue staining of the colonies revealed no difference in the number of stained colonies for miR-CTRL and miR-4726 overexpressing clones of both cell lines**(Fig.3E, 3F).**Takendata together from three different experiments indicate that miR-4726 has no role on the growth of breast cancer cell lines.

3.4 Role of miR-4726 *on*the migration of breast cancer cells

Migration potentiality is one the important hallmark among the hallmarks of cancer. This phenotype was tested using *in vitro* scratch assay in the clones (miR-CTRL and miR-4726 overexpressing) of MCF7 and MDA MB 231 cells. Scratch wounds were created by scratching confluent cell monolayers with a P200 pipette tip followed by observing the migration of cells into the wound at 12 hours interval for a duration of 36 hours using fluorescence microscope and the healing of the wound was photographed accordingly. We did not notice any difference in miR-CTRL and miR-4726 overexpressing cells in the healing of wounds for both the cell lines**(Fig.4A, 4B)**. So, it is evident that miR-4726 has no role in the migration of breast cancer cells.

3.5 Effect of miR-4726 expression in the sensitivity of chemotherapeutics

The role of different miRNAs in the effectiveness of chemotherapeutic agents is now under investigation as resistance to the therapeutic compounds becoming a challenge in the treatment of different cancer including breast cancer. To identify the effect of miR-4726 in the sensitivity towards different chemotherapeutic compounds,MCF7 and MDA MB 231 clones (miR-CTRL, miR-4726 overexpressing) were exposed to five different compounds in four ascending concentrations for 48 hours commonly named as 5-F-uracil, doxorubicin, bortezomib, docetaxel, tunicamycin. These agents were chosen to expose cells to several different mechanisms of toxicity may also elucidate the target pathways of the miRNAs. But, miR-4726 did not show any difference in sensitivity towards these five compounds compare to control in both cell lines**(Fig.5A-5J)**. Thereby, it is evident that miR-4726 has no effect on sensitivity of breast cancer cells to chemotherapeutic agents.

3.6 Association of miR-4726 in overall survival of breast cancer patients

The prognostic value of miR-4726 was determined in breast cancer patients using TCGA data set. Survival analysis of breast cancer data set using KM plotter revealed that in breast cancer patient's lower expression of miR-4726 was associated with better overall survival (**Fig. 6)**

IV. DISCUSSION

Small noncoding RNAs- microRNAs are now considering as prospective diagnostic markers and therapeutic targetsin different cancers**[18].**Oncogenic microRNAs are getting overexpressed and tumor suppressive microRNAs are downregulated in different cancers suggesting a potential role of miRNAs in the carcinogenesis**[11, 19]**. This study demonstrates that the expression of miR-4726 is downregulated in ductal carcinoma in situ and invasive breast cancer in comparison to benign epithelial tissue while in situ hybridization was utilized to study the expression of this miRNA suggesting a tumor suppressive role for miR-4726.

It is evident that miRNAs facilitate or inhibit cell proliferation, migration, invasion of different cancer. Schmitt et al have reported that ErbB2 intronic miRNA-4728 inhibits breast cancer cell proliferation, migration and invasion along with enhanced apoptosis while oncogenic miR-21 is associated with breast cancer cell proliferation, colony formation, migration and invasion**[20, 21].** We have assessed cell proliferation and migration for miR-4726 in MCF7 and MDA231 clones that are expressing this microRNA suggests that miR-4726 has no effect on the growth and migration of breast cancer cells.

Resistance to chemotherapy is still a challenge in different cancers including breast cancer. Along with diverse mechanisms of drug resistance to chemotherapeutic compounds, miRNAs are identified as one of the majorcontroller of important genes that are associated with resistanceto chemotherapy**[22].**It is speculated that due to enhanced chemo-resistance of different drugs in cancers, miRNA based treatment alone or in combination with current drugs may be an alternative option for the effective treatment of cancer. A growing

number of studies demonstrated that down regulation of miR-489 is associated with resistance to doxorubicin**[23],** and miRNA-34a is associated with docetaxel resistance in breast cancer **[24]**. In our study, we have treated miR-4726 expressing clones of MCF7 and MDA MB 231 cells by five different compounds namely-ddoxorubicin; a chemotherapeutic drug which functions by intercalating DNA- causing lethal damage, Tunicamycin; used to induce the unfolded protein response (UPR) through blocking of N-linked glycosylation, Fluorouracil (5-FU); an antimetabolite which inhibits synthesis and repair of DNA, Bortezomib; a therapeutic proteasome inhibitor, docetaxel; which inhibits mitotic cell division between metaphase and anaphase. We did not observe any enhanced sensitivity or resistance towards any of the compound for miR-4726 expressing clones of breast cancer cells suggesting that this miRNA have no role in the sensitivity of chemotherapeutic agents in the treatment of breast cancer.

Studies suggests that miRNA is a prognostic biomarker in several human cancers including breast cancer**[25].**For example- higher expression of miRNAs grouped in miR-17-92 cluster have been shown to be associated with a good prognosis in the luminal A breast cancer subtype, whereas higher miRNAs in the same cluster have been observed with a poor prognosis in the HER2-enriched and TNBC breast cancer subtypes**[26].**Our KM plotter analyzed data showed that lower expression of miR-4726 is associated with good overall survival in breast cancer. To the best of our knowledge, this is the first study on miR-4726 to evaluate the prognostic value of this miRNA in breast cancer.

V. CONCLUSIONS

Taken together, it can be concluded that miR-4726 has no effect on breast cancer cell proliferation, migration or enhanced sensitivity or resistance towards chemotherapeutic compounds. Since, this miR-4726 is found to be downregulated in human breast cancer it seems down regulation might be due to passenger mutation of this miRNA which further supports the findings that passenger mutation does not contribute to the development of cancer**.**

VI. DECLARATION FOR CONFLICT OF INTEREST

The author is declaring no conflict of interest for this work.

VII. ETHICAL STATEMENT

The entire work was carried out in *in-vitro* cell based models. There was no involvement of human or animal in this work.

VIII. FUNDING

This work was not supported by any specific grant from any funding agencies in the public, commercial, or not-for-profit sectors.

IX. AUTHOR CONTRIBUTION

Muhammad Mosaraf Hossain: Conceptualization, methodology, investigation, analysis of data, visualization, writing/reviewing and Editing of manuscript.

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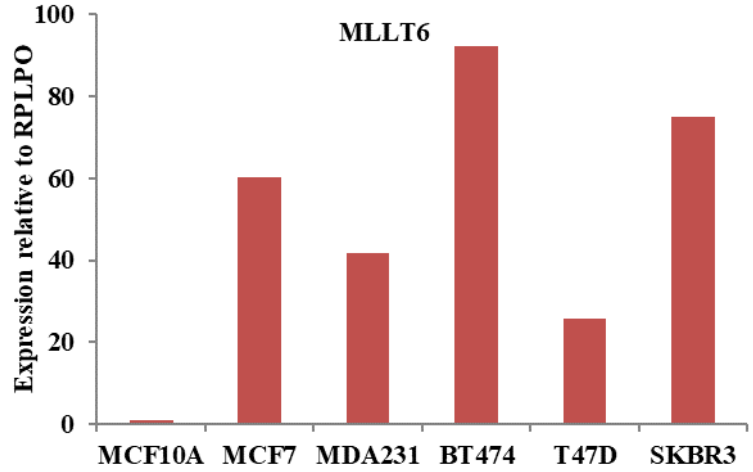
(A)



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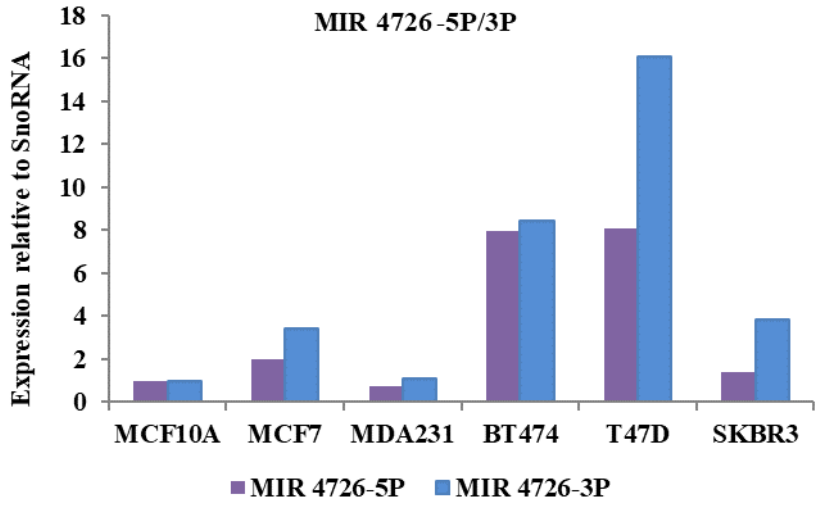

Figure 1. Endogenous expression of miR-4726 in panel of breast cancer cell line (A, B).A panel of breast cancer cell lines (MCF7, MDA MB 231, BT474, T47D, SKBR3) were harvested for RNA isolation using Trizol. The relative expression level of host gene MLLT6 for miR-4726 and miR-4726 itself were quantified by qRT-PCR normalizing against RPLPO for host gene and snoRNA for miR-4726.

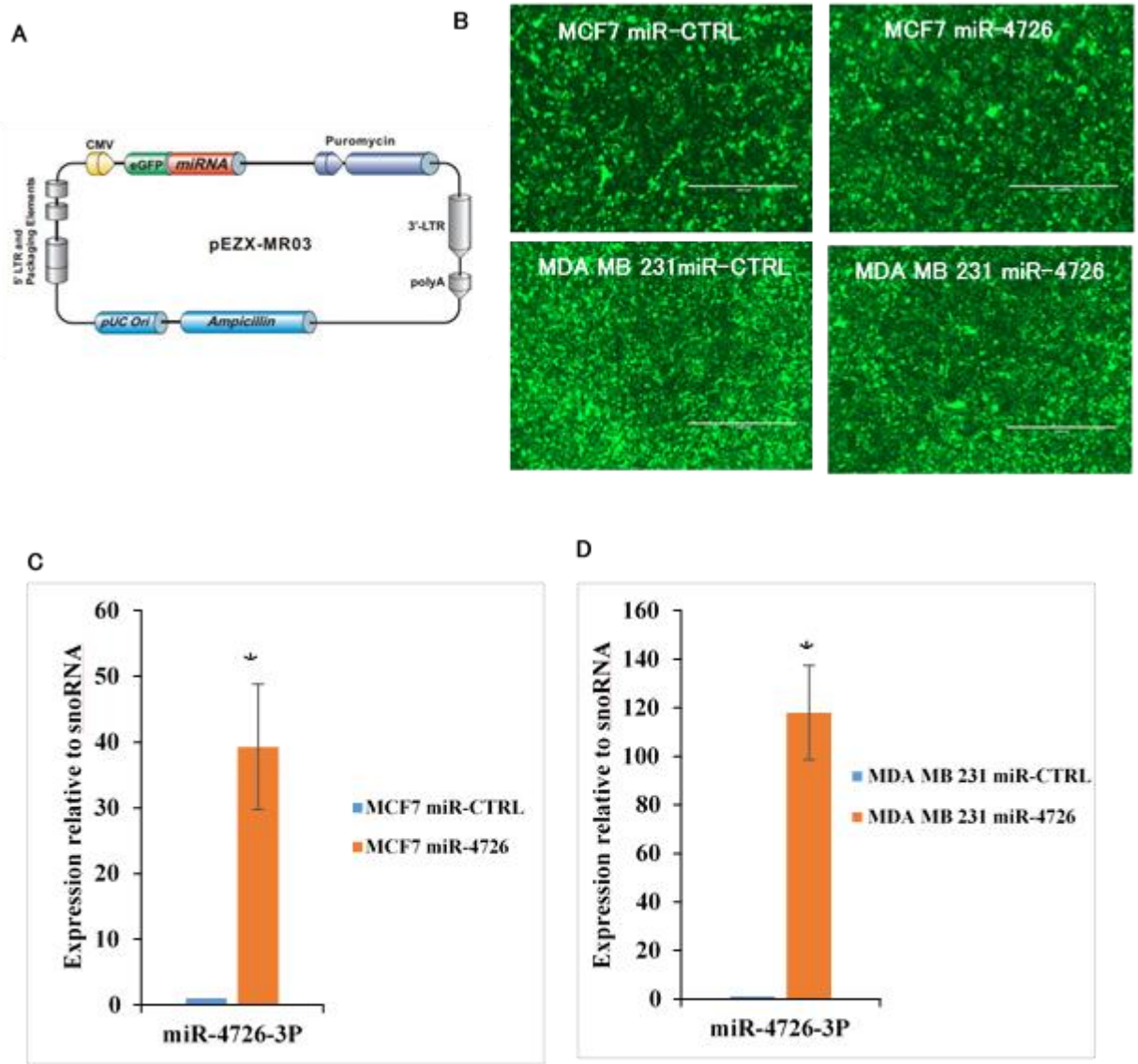

Figure 2: Generation and characterization of the stable clones expressing miR-4726.(A) Map of lentiviral vector used to generate miR-CTRL and miR-4726 expressing cells. **(B)** Expression of miR-4726 or control was confirmed by GFP expression as GFP is co-expressed with the miRNA expression in sub clones of MCF7 and MDA MB 231 cells. **(C)** miR-4726 and miR-CTRL expressing MCF7 clones were harvested for RNA isolation using Trizol and expression levels of miR-4726 was confirmed by Taqman qRT-PCR, normalizing against snoRNA. **(D)** RNA was also isolated from MDA MB 231 miR-CTRL and miR-4726 clones and the expression of miR-4726 was confirmed by Taqman qRT-PCR, normalizing against snoRNA.

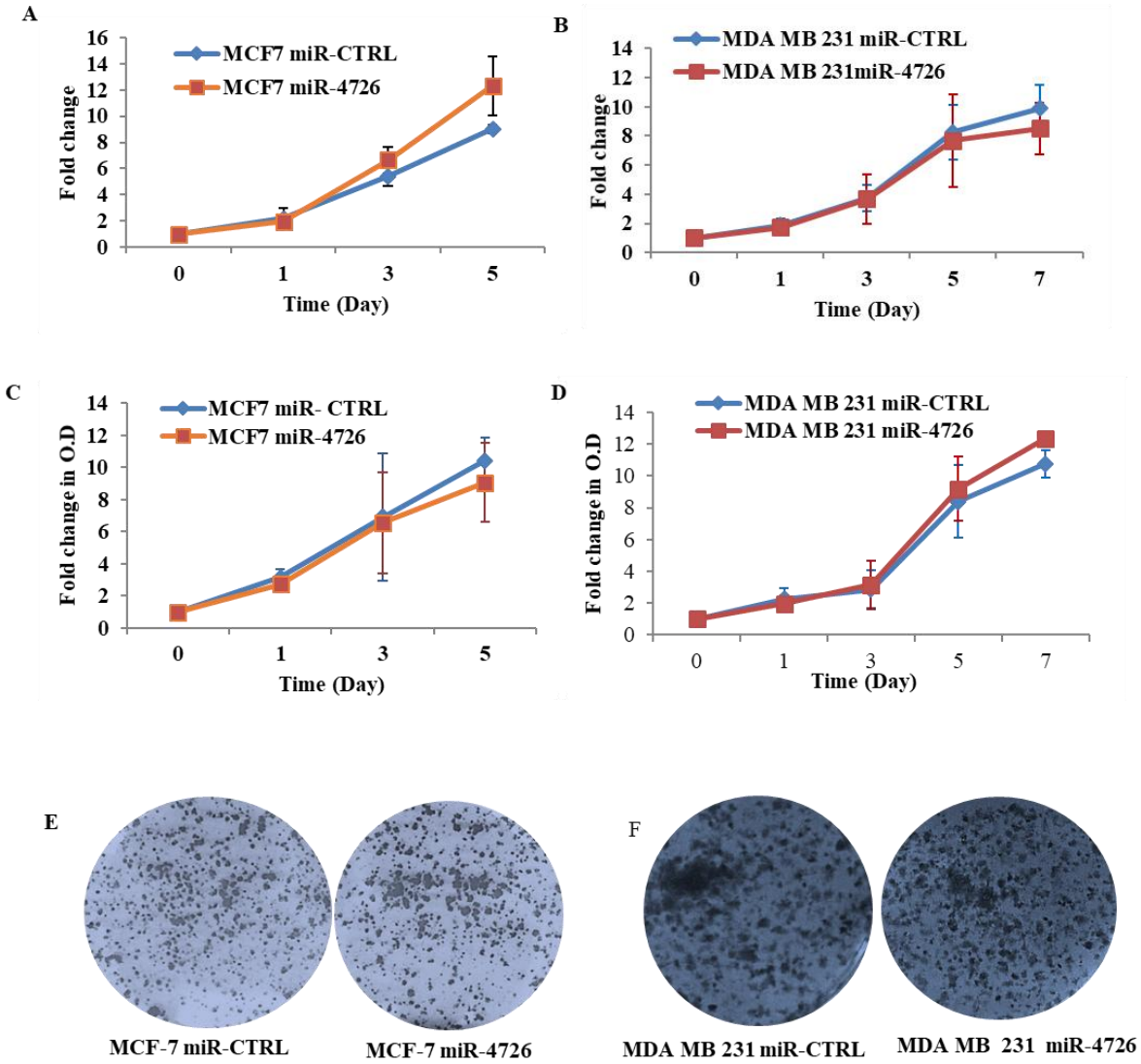

Figure 3. Effect of miR-4726onthe proliferation of breast cancer cell. (A, B) miR-4726 expressing MCF7 and MDA MB 231 clones (50000 cells/well) were seeded in each well of 6-well plate and counted the cells manually on 24 hours interval using haemocytometer and fold change was calculated with relative to cell counting at Day 0. Data represents here as mean ± SD from three independent experiments. **(C, D)** miR-4726 expressing MCF-7 and MDA MB-231 clones along with the respective control clones were plated in 96-well plate (2,000 cells/well). Cell proliferation was assessed by MTS assay at different time intervals (Day 0, 1,3,5,7). Absorbance was recorded using microplate reader at 490 nm and fold change was calculated with relative to the absorbance at Day 0. **(E, F)** miR-4726 expressing clones (5000 cells/well) (MCF7 and MDA Mb 231) were plated in each well of 6-well plate and let them to grow till it is observed to have formed colonies under microscope. Colonies are then stained with Coomassie brilliant blue. Finally stained colonies were observed visually and compared.

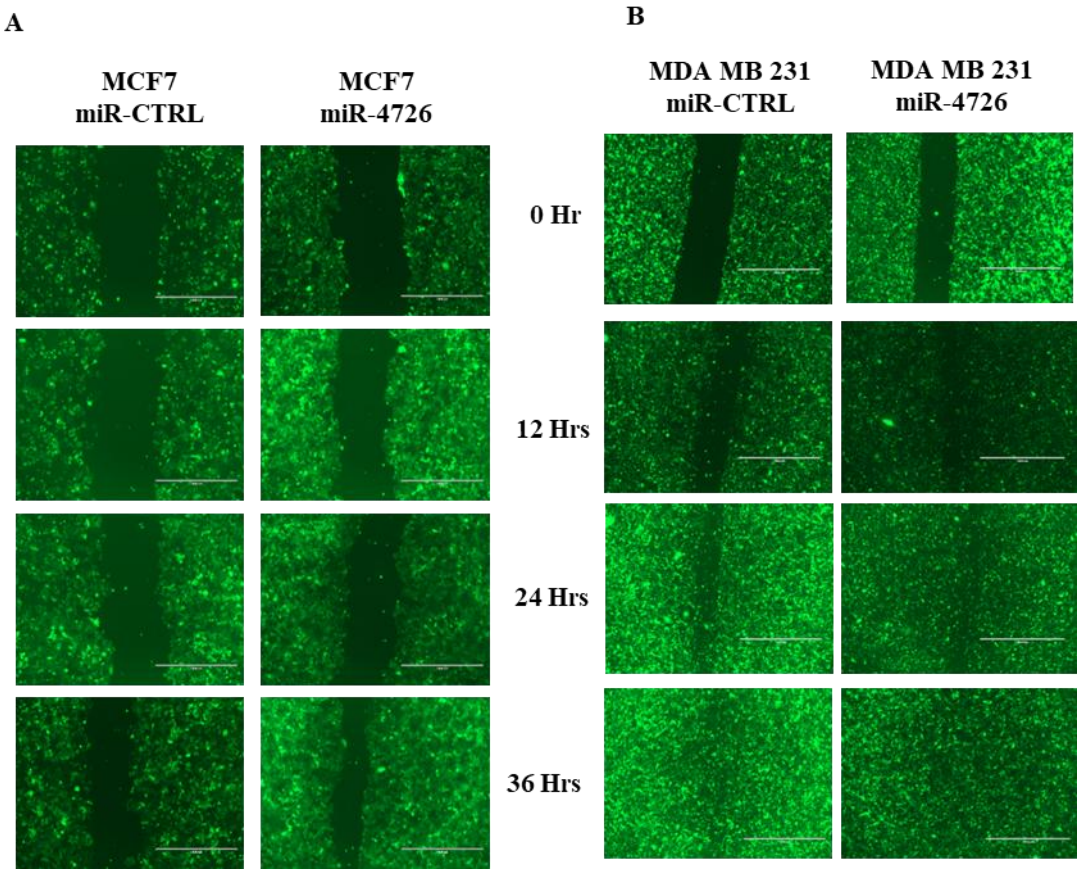

Figure 4. Role of miR-4726 onthe migration of breast cancer cell. (A) Scratch was done in a confluent monolayer of MCF-7 clones (miR-CTRL and miR-4726) by P200 tip and migration of cells into the wound was observed at 12 hours interval using GFP enabled fluorescence microscope. **(B)** Similar experiment was done for MDA MB 231 clones and observed the migration of the cells to heal the wounds using fluorescence microscope.

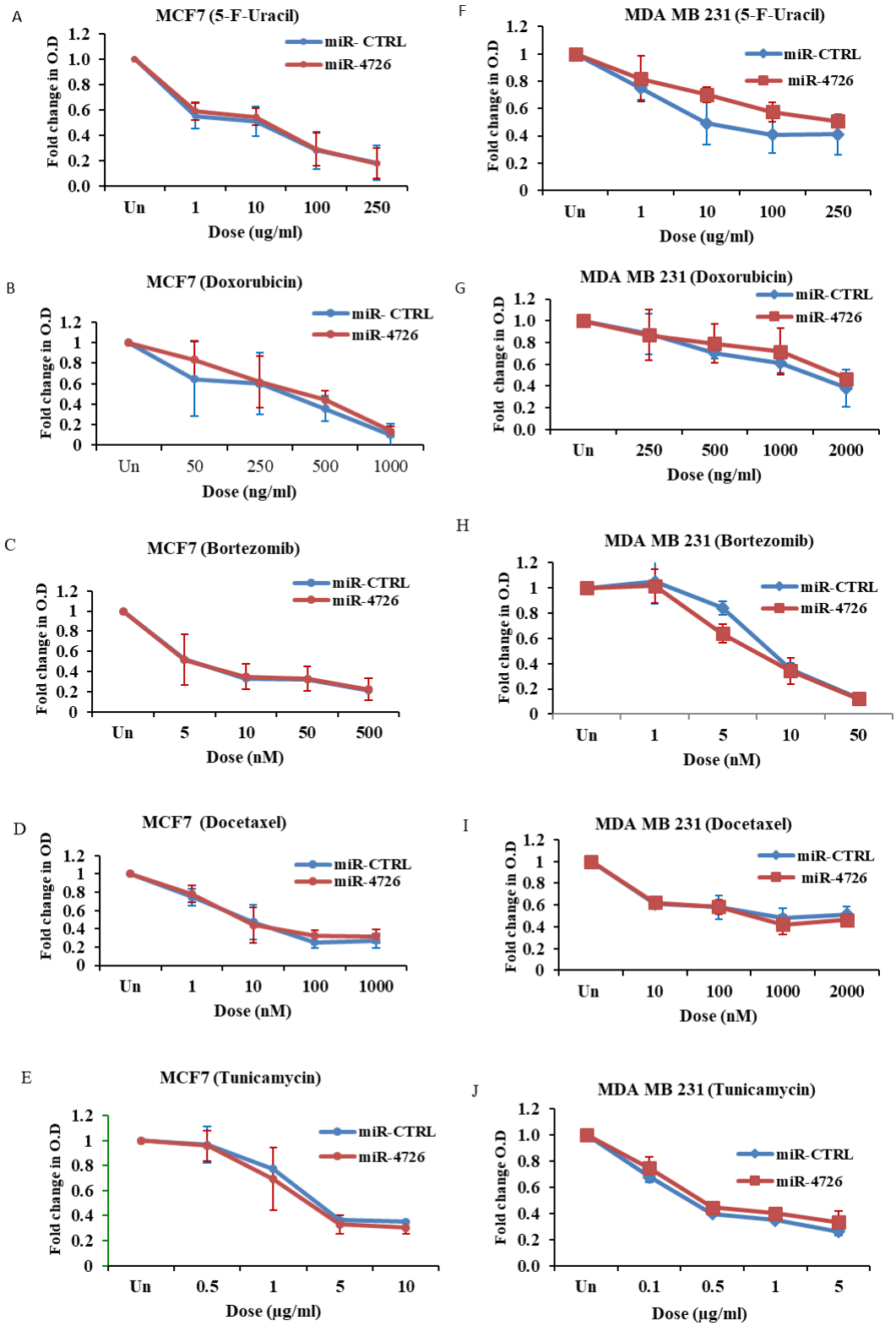

Figure 5. Effect of miR-4726 onthe drug sensitivity towards breast cancer cells. (A-E) MCF-7 (miR-CTRL and miR-4726) clones were plated (5000 cells/well) in 96 well plate and on following day treated with five different chemotherapeutic agents (5-F-Uracil, Doxorubicin, Tunicamycin, Bortezomib, Docetaxel) in four ascending concentrations and let the cells to grow for 48 hours after treatment. Proliferation of the cells were assessed by MTS assay and fold change was calculated with relative to the absorbance at Day 0. Data represents here as mean ± SD from three independent experiments. **(F-J)** MDA MB 231 (miR-CTRL and miR-4726) clones were plated (5000 cells/well) in 96 well plate and on following day treated with five different

chemotherapeutic agents (5-F-Uracil, Doxorubicin, Tunicamycin, Bortezomib, Docetaxel) in four ascending concentrations and let the cells to grow for 48 hours after treatment. Proliferation of the cells were assessed by MTS assay and fold change was calculated with relative to the absorbance at Day 0. Data represents here as mean ± SD from three independent experiments.

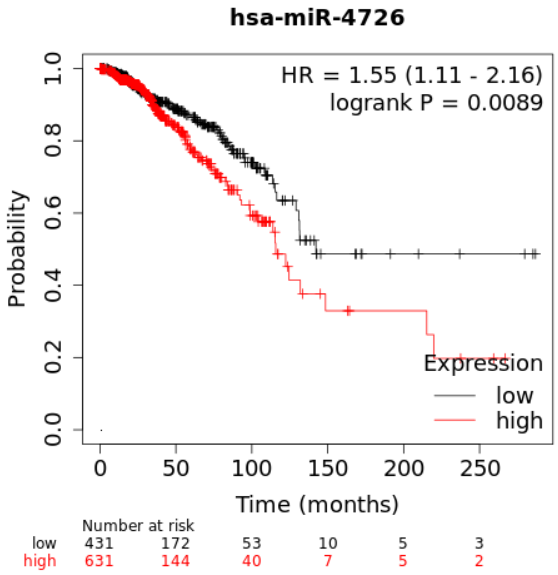

Figure 6. Role of miR-4726 onthe survival of breast cancer patients. Overall survival analysis was carried out by KM plotter (a computational tool) using TCGA dataset to determine the association of miR-4726 expression with overall survival of breast cancer patients

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