Involved *microRNAs* in alternative polyadenylation intervene in breast cancer via regulation of cleavage factor “CFIm25”

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Cleavage factor “CFIm25”, as a key repressor at proximal poly (A) site, negatively correlates to cell proliferation and tumorigenicity in various cancers. Hence, understanding CFIm25 mechanism of action in breast cancer would be a great benefit. To this aim four steps were designed. First, potential *miRNAs* that target 3′-UTR of CFIm25 mRNA, retrieved from Targetscan web server. Second, screened *miRNAs* were profiled in 100 breast cancer and 100 normal adjacent samples. Third, *miRNAs* that their expression was inversely correlated to the CFIm25, overexpressed in MDA-MB-231 cell line, and their effect on proliferation and migration monitored via MTT and wound healing assays, respectively. Fourth, interaction of *miRNAs* of interest with 3′-UTR of CFIm25 confirmed via luciferase assay and western blot. Our results indicate that CFIm25 considerably down-regulates in human breast cancer tissue. qRT-PCR assay, luciferase test, and western blotting confirm that CFIm25 itself could be directly regulated by oncomiRs such as *miR-23, -24, -27, -135, -182 and -374*. Besides, according to MTT and wound healing assays of cell lines, CFIm25 knockdown intensifies cell growth, proliferation and migration. Our results also confirm indirect impact of CFIm25 on regulation of mRNA’s 3′–UTR length, which then control corresponding *miRNAs*’ action. *miRNAs* directly control CFIm25 expression level, which then tunes expression of the oncogenes and tumor proliferation. Therefore, regulation of CFIm25 expression level via *miRNAs* is expected to improve treatment responses in breast cancer.

**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| CPSF         | Cleavage and polyadenylation specificity factor |
| CstF         | Cleavage stimulation factor |
| PAS          | Polyadenylation site |
| 3′-UTR       | 3′-Untranslated region |
| UTR-APA      | Untranslated region-alternative polyadenylation |
| miRNA/miR    | MicroRNA |
| CFIm25       | Cleavage factor Im 25 |
| RF           | Random forest classifiers |
| shRNA        | Short hairpin RNA |
| HCC          | Hepatocellular carcinoma |

Living organisms apply complicated mechanisms of gene regulation to generate different cell types, which result in various behaviors from a single genome. Dynamic and highly polymorphic nature of mRNA polyadenylation, as a functional aspect of gene regulation, is one of the most recent discoveries in this field. Cleavage and polyadenylation of nascent mRNA accomplishes via application of two large multimeric complexes, which named as cleavage–polyadenylation specificity factor (CPSF) and cleavage stimulation factor (CstF), respectively. The
miR-182, many cases. That’s why loss of expression correlates with inception or the aggressiveness of tumors24,25.

levels (p < 0.05), while made no change in cells that were transducted with miR-374 MB-231 stable cell lines were generated using miRNA [10]. According to image analysis of western blot gels via Image J software, CFIm25 protein level notably decreased while miR-23 mimetic tumor suppressor roles in several types of cancer such as breast cancer19–23. Besides, miRNAs mimic tumor suppressor roles in many cases. That’s why loss of mirRNA expression correlates with inception or the aggressiveness of tumors34,25.

In this study, we checked if miRNAs are involved with regulation of CFIm25 expression. To examine regulatory impact of miRNAs on endogenously APA master regulator CFIm25, correlation of oncogenic miRNAs with CFIm25 investigated in clinical samples of breast cancer patients. Then, we explored impact of transfected miRNAs in breast cancer cell line, in a set of parallel separate experiments. Identification of numerous oncomiRs that suppress CFIm25, which then induce proximal polyadenylation and mediate in production of shortened oncogenes in breast cancer, confirms involvement of miRNAs in regulation of CFIm25.

Results

Correlation of CFIm25 expression level with oncogenic miRNAs in breast cancer clinical samples and cell lines. According to bioinformatics analysis, miR-23, miR-24, miR-27, miR-96, miR-135, miR-182, miR-221, miR-222 and miR-374 found to target CFIm25 beside their oncogenic potential. In order to determine whether CFIm25 expression is controlled by miRNAs of interest in breast cancer, we measured the expression levels of potentially involved miRNAs in breast cancer patients (n = 100) and normal adjacent tissues (n = 100) using mir-quantitative qRT–PCR analysis. Results showed considerable elevated level of miRNAs in samples acquired from patients (at least in 6 out of 9 monitored miRNAs), while CFIm25 expression level was diminished significantly (Fig. 1a, b). In the next step, patients categorized as low or high CFIm25-expressing tumors, using the median expression for CFIm25 in the normal sample as a cut-off.

Since CFIm25 level differs in samples acquired from patients, their correspondingly correlated miRNAs might be variable too. By this regard, distribution of the median expression of nine miRNAs of interest evaluated respected to both low and high levels of CFIm25 expression (Fig. 1c).

The median gene expression level of miR-23, miR-24, miR-27, miR-135, miR-182, miR-221 and miR-374 was significantly higher in the patients expressing lower levels of CFIm25, compared to those expressing higher levels (p < 0.05).

Intervention of CFIm25 in length determination of proto-oncogenes’ 3′-UTR. Three out of five proto-oncogenes interact efficiently with CFIm25 and CPSF1 either at proximal or distal of 3′-UTR while the other two proto-oncogenes (EGFR: NM_005228.5, AKT3: NM_005465.5) interact with both proteins merely at distal of the 3′-UTR (Table 1). Therefore, exclusive interaction of CFIm25/CPSF1 complex with distal region of 3′-UTR, which either prevents mRNA translation or induce its degradation34–40. Numerous miRNAs have been shown to be aberrantly expressed, and act as oncogenes in several types of cancer such as breast cancer19–23. Besides, miRNAs mimic tumor suppressor roles in many cases. That’s why loss of mirRNA expression correlates with inception or the aggressiveness of tumors34,25.

In this study, we checked if miRNAs are involved with regulation of CFIm25 expression. To examine regulatory impact of miRNAs on endogenously APA master regulator CFIm25, correlation of oncogenic miRNAs with CFIm25 investigated in clinical samples of breast cancer patients. Then, we explored impact of transfected miRNAs in breast cancer cell line, in a set of parallel separate experiments. Identification of numerous oncomiRs that suppress CFIm25, which then induce proximal polyadenylation and mediate in production of shortened oncogenes in breast cancer, confirms involvement of miRNAs in regulation of CFIm25.

Some of the potentially involved miRNAs in breast cancer, down-regulate CFIm25 in MDA-MB-231 cell line. To confirm the impact of miRNAs on CFIm25 regulation in clinical samples, MDA-MB-231 stable cell lines were generated using miRNA containing lentivirus transduction. We determined that enforced expressions of miR-23, miR-24, miR-27, miR-135, miR-182 and miR-374 significantly down-regulated CFIm25 (p < 0.05), while made no change in cells that were transducted with Ctrl-vector, miR-96, miR-221 and miR-222 (Fig. 2a). Up-regulation of miR-23 and miR-374 down-regulated CFIm25 the most, compared to other miRNAs including miR-24, miR-27, miR-135 and miR-182 (Fig. 2a). According to image analysis of western blot gels via Image J software, CFIm25 protein level notably decreased while miR-23, 27, and 374 were up-regulated (Fig. 2b).

In the next step, we investigated whether 3′-UTR of CFIm25 has functional targets for predicted miRNAs. To this aim, full-length sequence of CFIm25 3′-UTR cloned to psiCHECK-2 vector, which is placed at downstream of Renilla luciferase reporter gene (CFIm25-psiCHECK2). In a parallel experiment, as negative controls, the seed sequence of miR-23, miR-24, miR-27, miR-135, miR-182 and miR-374 mutated intentionally at certain points. The
stem-loop structures that formed following introduction of mutations, cloned to pCDH-TurboGFP vector (Mut-miRNAs) in shRNA format. The HEK293 cells transiently transfected with the CFIm25-pSICHECK2 construct and WT-miRNAs, which led to a significant decrease of reporter activity compared to the control (Fig. 2c). Activity of the reporter construct was unaffected by a simultaneous transfection with Mut-miRNAs carried a mutated seed sequence. Taken together, these data strongly suggest that miR-23, miR-24, miR-27, miR-135, miR-182 and miR-374 directly bind to 3′-UTR of CFIm25’s mRNA.

According to our findings that suggested miR-23 and miR-347 had something to do with CFIm25 down-regulation, we used miR-Off-23 and miR-Off-374 to silence miR-23 and miR-374. Now, by using miR-Off-23 and miR-Off-374, we can confirm if silencing of miR-23 and miR-374 directly controls overexpression of CFIm25 in breast cancer’s cell line or not. To this aim, miR-Off-23 and miR-Off-374 transduced to MDA-MB-231 cell line and efficiency of transduction monitored using qRT–PCR analysis. Transduction of miR-Off-23 and miR-Off-374 resulted in significant overexpression of CFIm25 as demonstrated in Fig. 2d (by 3.3 and 2.89 fold increase in case of miR-Off-23 and miR-Off -374, respectively).

Selected miRNAs can regulate cell proliferation in breast cancer cell line partly by modulating CFIm25 mRNA. To determine importance of CFIm25 in regulation of MDA-MB-231 proliferation, we intentionally performed MTT and wound healing assays in miRNAs transduced MDA-MB-231 cells. Result of the MTT assay confirmed increment of “cell proliferation index” in transduced cells as compared with that of the control groups (p<0.05; Fig. 3a).

To clarify direct impact of miRNAs on cell proliferation through regulation of 3’-UTR of CFIm25 mRNA, MDA-MB-231 cells once transduced with miR-Off-23/miR-Off-374 and assessed by MTT assay. In the second approach, transduced cells transfected by shCFIm25 vector and evaluated by MTT assay. When miR-Off-23 and...
miR-Off-374 transduced to cells and transiently co-transfected with shCFIm25 vector (Fig. 3b, c), CFIm25 expression level diminished and cell proliferation significantly increased (Fig. 3d). Collectively, these results suggest that miR-Off-23/-374 vectors up-regulated CFIm25 protein level, which eventually suppressed proliferation of cancer cells.

Wound healing assay exemplifies CFIm25 balance with miR-23, -374. The wound healing assay designed to evaluate role of miR-Off-23/miR-Off-374 in MDA-MB-231 in cell migration. As what is shown in Table 1...

Table 1. Proto-oncogenes that CFIm25 and CPSF1 target their 3′UTR mRNA. The cases that are highlighted in dark grey represent proto-oncogenes with positive interaction probability. The RF (Random Forest) classifier is a probability index, which falls between 0 (the lowest probability) to 1 (the highest probability).

| 3′ UTR of the mRNA | Possible CFIm interaction site (TGTA) | Proximal (P) | Distal (D) | Poly–adenylation signal (PAS) | ATTAAA | AATAAA | RF classifier for CFIm25 | RF classifier for CPSF1 | RF classifier for CFIm25/CPSF1 |
|---------------------|--------------------------------------|--------------|------------|-----------------------------|--------|--------|------------------------|------------------------|-----------------------------|
| EGFR: NM_005228.5   | 1482–1487                            | P            | 208        | 1695–1700                   | 0.45   | 0.55   | 0.7                    |                        |                             |
|                     | 1482–1487                            | D            | 208        | 1695–1700                   | 0.45   | 0.55   | 0.7                    |                        |                             |
|                     | 1482–1487                            |              | 208        | 1695–1700                   | 0.45   | 0.55   | 0.7                    |                        |                             |
|                     | 1482–1487                            |              | 208        | 1695–1700                   | 0.45   | 0.55   | 0.7                    |                        |                             |
|                     | 1482–1487                            |              | 208        | 1695–1700                   | 0.45   | 0.55   | 0.7                    |                        |                             |
|                     | 1482–1487                            |              | 208        | 1695–1700                   | 0.45   | 0.55   | 0.7                    |                        |                             |
| MYC: NM_002467.6    | 263–266                              | P            | 11         | 276–282                     | 0.5    | 0.5    | 0.5                    | 0.5                    | 0.6                         |
|                     | 263–266                              | D            | 19         | 2406–2411                   | 0.45   | 0.75   | 0.7                    |                        |                             |
|                     | 263–266                              |              | 19         | 2406–2411                   | 0.45   | 0.75   | 0.7                    |                        |                             |
|                     | 263–266                              |              | 19         | 2406–2411                   | 0.45   | 0.75   | 0.7                    |                        |                             |
|                     | 263–266                              |              | 19         | 2406–2411                   | 0.45   | 0.75   | 0.7                    |                        |                             |
|                     | 263–266                              |              | 19         | 2406–2411                   | 0.45   | 0.75   | 0.7                    |                        |                             |
|                     | 263–266                              |              | 19         | 2406–2411                   | 0.45   | 0.75   | 0.7                    |                        |                             |
| PTEN: AF067844.1    | 200–203                              | P            | 11         | 276–282                     | 0.5    | 0.5    | 0.5                    | 0.5                    | 0.6                         |
|                     | 200–203                              | D            | 19         | 2406–2411                   | 0.45   | 0.75   | 0.7                    |                        |                             |
|                     | 200–203                              |              | 19         | 2406–2411                   | 0.45   | 0.75   | 0.7                    |                        |                             |
|                     | 200–203                              |              | 19         | 2406–2411                   | 0.45   | 0.75   | 0.7                    |                        |                             |
|                     | 200–203                              |              | 19         | 2406–2411                   | 0.45   | 0.75   | 0.7                    |                        |                             |
|                     | 200–203                              |              | 19         | 2406–2411                   | 0.45   | 0.75   | 0.7                    |                        |                             |
|                     | 200–203                              |              | 19         | 2406–2411                   | 0.45   | 0.75   | 0.7                    |                        |                             |
| AKT2: NM_001626.6   | 1504–1507                            | P            | 11         | 276–282                     | 0.5    | 0.5    | 0.5                    | 0.5                    | 0.6                         |
|                     | 1504–1507                            | D            | 19         | 2406–2411                   | 0.45   | 0.75   | 0.7                    |                        |                             |
|                     | 1504–1507                            |              | 19         | 2406–2411                   | 0.45   | 0.75   | 0.7                    |                        |                             |
|                     | 1504–1507                            |              | 19         | 2406–2411                   | 0.45   | 0.75   | 0.7                    |                        |                             |
|                     | 1504–1507                            |              | 19         | 2406–2411                   | 0.45   | 0.75   | 0.7                    |                        |                             |
|                     | 1504–1507                            |              | 19         | 2406–2411                   | 0.45   | 0.75   | 0.7                    |                        |                             |
| AKT3: NM_005465.5   | 1560–1563                            | P            | 11         | 276–282                     | 0.5    | 0.5    | 0.5                    | 0.5                    | 0.6                         |
|                     | 1560–1563                            | D            | 19         | 2406–2411                   | 0.45   | 0.75   | 0.7                    |                        |                             |
|                     | 1560–1563                            |              | 19         | 2406–2411                   | 0.45   | 0.75   | 0.7                    |                        |                             |
|                     | 1560–1563                            |              | 19         | 2406–2411                   | 0.45   | 0.75   | 0.7                    |                        |                             |
|                     | 1560–1563                            |              | 19         | 2406–2411                   | 0.45   | 0.75   | 0.7                    |                        |                             |
|                     | 1560–1563                            |              | 19         | 2406–2411                   | 0.45   | 0.75   | 0.7                    |                        |                             |
|                     | 1560–1563                            |              | 19         | 2406–2411                   | 0.45   | 0.75   | 0.7                    |                        |                             |

Wound healing assay exemplifies CFIm25 balance with miR-23, -374. The wound healing assay designed to evaluate role of miR-Off-23/miR-Off-374 in MDA-MB-231 in cell migration. As what is shown in...
Fig. 4, down-regulation of miR-23 and miR-374 (by miR-off-23, -374 transfection) significantly decreased migration ability of transduced cells. However, shCFIm25 vector improved migration. Results of migration assay showed that number of migrated cells in the miR-Off-23/miR-Off-374 transduced group, was less than that in the shCFIm25 group. This last finding confirmed that the migration ability of MDA-MB-231 cells was inhibited by miR-Off-23/miR-Off-374, which had been found previously to be under influence of CFIm25 expression level (Fig. 5).

Discussion

It is confirmed that most of the human genes have multiple polyadenylation signals, which dynamically regulate their variations in 3′-UTR length26. The CFIm25, as a newly discovered repressor of proximal poly (A) site usage, has a significant role in UTR-APA site regulation. The CFIm25, as a subunit of cleavage factor complex, recruits CPSF6 and/or CPSF7 along with CPSF5, and activates 3′-mRNA cleavage site and polyadenylation processing machinery3,11. Down-regulation of CFIm25, significantly diminishes “distal poly-A signal” length, and enhances tumorigenic properties and size of the tumor cells. However, CFIm25 overexpression reduces aforementioned properties and inhibits tumor growth. An explanation for this is that by choosing a proximal PAS, the 3′-UTR length of the mRNAs shortens, which then eliminates miRNAs binding sites and revokes regulation through miRNAs11,27–29. Here, we approved CFIm25 is indirectly involved in regulation of many genes through changing...
′-UTR length, which then eliminates binding sites of their corresponding miRNAs. Nonetheless, more studies are still needed to clarify comprehensive regulatory mechanism of CFIm25 gene by miRNAs.

Although breast cancer is the most common cancer diagnosed in women, survival rates of breast cancer have improved recently, which is mostly due to factors such as a new personalized approach to cancer treatment and a better understanding of the molecular mechanism of the disease. In this study, we used human breast cancer specimens to analyze their CFIm25 expression profile. We confirmed that CFIm25 is significantly down-regulated in breast cancer samples, compared to the controls. This phenomenon is consistent with the previous study that introduced CFIm25 as APA regulator that was also down-regulated in glioblastoma and came up with cancer proliferation and tumorigenicity. Besides, it was reported that expression level of CFIm25 in hepatocellular carcinoma (HCC) was negatively correlated to the metastatic potential of HCC cell line through increasing E-cadherin level, while glutaminase mRNA isoforms, which contain distinct 3′-UTR (KGA and GAC), showed a complex interplay between RNA processing and microRNA repression in controlling glutamine metabolism in cancer cells.

In the next step of the current study, we selected nine oncomiRs using bioinformatics analysis. They all confirmed to have recognition sites in the 3′-UTR of the CFIm25 (miR-23: 2 sites, miR-24: 2 sites, miR-27: 3 sites, miR-96: 1 site, miR-135: 2 sites, miR-182: 1 site, miR-221: 2 sites, miR-222: 2 sites and miR-374: 4 sites). So far, several studies investigated role of oncomiRs in different cancers including breast cancer. However, no experimental work has performed to clarify effects of these miRNAs on CFIm25 expression level and, as a result, in regulation of UTR-APA site. When clinical samples divided into two groups, whose CFIm25 expression was more or lower than the median expression of control samples, a negative correlation between level of miR-23, miR-24, miR-27, miR-96, and miR-374 and CFIm25’s expression was found. This negative correlation in turn suggests that CFIm25 is being regulated by aforementioned miRNAs. Besides, in vitro data from transduced MDA-MB-231 cells confirmed that high levels of miR-23, miR-24, miR-27, miR-135, miR-182 and miR-374 was negatively correlated with either CFIm25 mRNA or its protein content.

According to previous reports, low level of CFIm25 leads to cancer proliferation and migration, which is in agreement with our MTT and wound healing assay results. Importantly, functional regulatory role of miR-23 and miR-374 on CFIm25 mRNA, approved via MTT assay. This assay confirmed increased growth of MDA-MB-231 cells, which were co-transduced with miR-Off-23/miR-Off-374 and CFIm25 knock-down vectors. CFIm25 is known to be crucial in controlling invasion and metastasis of HCC. It is also confirmed that

Figure 3. Viability test of MDA-MB-231 cells that were transduced with different cloned miRNAs. (a) MTT assay of MDA-MB-231 cells that are transduced with miRNAs of interest. MTT assay of transducted MDA-MB-231 cells by (b) miR-Off-23 and (c) miR-Off-374 that transfected by shCFIm25 vector. Each time point was expressed as total absorbance at 570 nm after background subtraction (Y axis). Points are mean of three experiments and error bars represent standard deviation. (d) CFIm25 expression level in shCFIm25 transfected cells. The star above the bar, represents the significant difference respect to the corresponding control reference.
activation of APA sites can affect the migratory capacity of cancer cells as well as fibroblasts. According to the results of wound healing assay, regulation of CFIm25 expression by miR-23 and 374 directly controls breast cancer cells' migration and invasion. Since invasion and metastasis are the underlying causes of poor long-term survival of breast cancer patients, regulation of CFIm25 via miRs could be considered as a promising strategy for inhibition of metastasis in future clinical studies.

Parental genes of mRNAs with shorter 3'-UTRs, are more prone to up-regulation during tumorigenesis due to increased escaping from miRNA repression. It is determined that almost 67% genes with shorter 3'-UTRs in tumors, have lost at least one predicted miRNA-binding site. In some cases, overexpression of oncogenic proteins comes up without regulatory intervenience of the proto-oncogenes. One example of this phenomenon is cancer cell lines (such as MDA-MB-231) that express substantial amounts of mRNA isoforms with shortened 3'-UTRs. CFIm25 is a regulatory protein that controls key factors such as EGFR and MYC that are involved in NF-κB and MAPK/ERK pathways. Our finding suggests that regulation of proto-oncogenes by CFIm25, could be mediated via APA and shortening of 3'-UTR of their corresponding mRNAs, which eventually result in progression of breast cancer. The MYC, PTEN and AKT2 proto-oncogenes are more responsive to CFIm25 than EGFR and AKT3, since they have several potential interaction sites either at proximal or distal of their mRNAs 3'-UTRs (Table 1). Tuning impact of CFIm25 on MYC, PTEN and AKT2 will then control downstream phenomena such as glutamine metabolism in cancer cells, which could be applied as a strategy for targeted therapy. Besides, 3'-UTR of EGFR's mRNA contain multiple microRNA target sites, which are associated with cell cycle arrest and cell death. As long as CFIm25 interacts with distal region of EGFR's 3'-UTR mRNA (Table 1),

Figure 4. Wound healing assay of transducted MDA-MB-231 cells. Migration of transducted MDA-MB-231 cells toward the scratched area (rectangle) is monitored by 12-h intervals.
suppression of breast cancer tumorigenicity by miRNAs is highly plausible. In a similar way, interaction of CFIm25 with distal region of AKT3 3′-UTR mRNA, facilitates down-regulation of AKT3, which then regulates migration and metastasis in breast cancer cells. These two examples can clarify how CFIm25 interaction with distal region of EGFR and AKT3 3′-UTR mRNAs, can facilitates suppression of breast cancer tumorigenicity and metastasis, respectively.

Conclusion

In summary, CFIm25 is down-regulated in human breast cancer tissues compared to the adjacent non-cancerous breast tissues. Besides, CFIm25 mRNA is also regulated by several miRNAs including miR-23, miR-24, miR-27, miR-135, miR-182 and miR-374. Since CFIm25 is a key factor in cancer proliferation, identification of its modulators, such as miRNAs, facilitates development of molecular-targeted therapeutics for various cancers including breast cancer. Our in-vitro results confirm functionality of certain miRNAs toward regulation of 3′-UTR via CFIm25 in breast cancer cells, which then introduce it as an emerging field of study in pre-clinical studies of breast cancer.

Methods

Cell lines and patient sample collection. Hundred fresh frozen samples of human breast cancer tumors and their normal adjacent tissue obtained from the Iran national tumor bank of Cancer Institute (Imam Khomeini hospital, Tehran University of medical sciences, Tehran, Iran). Sample collection performed according to the international tumor bank SOP protocol and written informed consent obtained from patients. This study was approved by the cancer institute of Imam Khomeini Hospital, Tehran, Iran. SOP protocol and consent designed in accordance with ethical standards of Tehran University of medical sciences and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Human embryonic kidney cell line HEK293T (RRID:CVCL_0063) and human breast cancer cell line MDA-MB-231 (RRID:CVCL_0062) were obtained from Pasteur Institute of Iran (IPI) and Iranian biological resource center, respectively. Aforementioned institutes check the authenticity of human cell lines via DNA profiling annually. The obtained cell lines cultured in Dulbecco’s modified Eagle’s Medium (DMEM; Gibco), supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin–streptomycin (Gibco) at 37 °C with 5% CO2 incubation.

Bioinformatic analysis. The miRNAs that were susceptible to target CFIm25 mRNA 3′-UTR acquired using prediction web servers such as TargetScan, miRWalk (version 3.0) and DianaTool. The standard cutoff for screening predicted miRNAs was 8 mer, at the most, while the percentage of context ++ score (CS) did not exceed 95%. Predicted miRNAs with the high score were investigated in the miRCancer database. Finally, miRNAs with the oncogenic roles were selected for the rest of the research, including miR-23, miR-24, miR-27, miR-96, miR-135, miR-182, miR-221, miR-222 and miR-374.

In order to find out if CFIm25 efficiently targets 3′-UTR of proto-oncogenes’ mRNAs, three steps were followed. First, 3′-UTR mRNA of twenty proto-oncogenes that known to be involved in breast cancer, retrieved from UTR database. Second, twenty retrieved proto-oncogenes including: NM_053056.2, NM_001759.4, NM_000077.4, JX391994.1, S67388.1, NM_005163.2, NM_001626.6, NM_005465.5, BC040540.1, AF067844.1,
chased from ABM Company and packaged into lentiviruses particles. For luciferase assay, full length of
pMDG plasmid (containing packaging plasmid), and pPAX2 supernatants were harvested for two or three times, every 12 h, concentrated by ultracentrifuge at 47,000×
Focusing Flow Cytometer, ABI, FlowJo software)48. Furukawa et al. method47.

Table 2. Empty vector without any cloned sequence (cloning were listed in supplementary
miRNAs and at downstream of the sequences, cloned into the mammalian expression vector pCDH.TurboGFP
minor modifications20,46. Primer sequences are listed in supplementary Table 1 and 2.

Plasmids, viral vectors construction and luciferase assay. For the construction of miRNA-expressing
vectors, genomic fragment consists of the stem-loop structure of each miRNA and theirs flanking genomic
sequences, cloned into the mammalian expression vector pLEX.jred and pCDH.TurboGFP at downstream of the
cytomegalovirus (CMV) promoter. The PCR primers used for miRNAs cloning were listed in supplementary
Table 2. Empty vector without any cloned sequence (pLEX-Ctrl/ pCDH-Ctrl) was used as control in accordance
with Furukawa et al. method47.

HEK293T cells were transiently co-transfected by miRNA-expressing lentivectors (or empty backbones),
pPA2X plasmid (packaging plasmid), and pMDG plasmid (containing rev-G) using calcium phosphate. Lentivirus
supernatants were harvested for two or three times, every 12 h, concentrated by ultracentrifuge at 47.000×g for
2 h at 4 °C. Lentivirus titer examined by flow cytometry analysis of reporter positive 293 T cells (Attune Acoustic
Focusing Flow Cytometer, ABI, FlowJo software)48.

To down-regulate miR-23 and miR-374 expression, pLenti-III-miR-Off constructs containing GFP marker purchased
from ABM Company and packaged into lentiviruses particles. For luciferase assay, full length of CFlm25
3′-UTR amplified by PCR using the forward 5′-GCAAGCTTAGCTTCTTCTGCCC-3′ and reverse 5′-GCA
CTAGTGTACAAATATTATAA-3′, respectively. The CFlm25 3′-UTR was then cloned to downstream of the
luciferase gene in the pSICHECK2 vector (Promega). Mutant stem-loop forms of each miRNA, harboring two mutations in seed sequence, cloned in the lentiviral vector and named Mut-miR, which was considered as negative control. The HEK293 cells were transiently transfected with wild type or mutant miRNA-expressing vector and CFIm25-pSICHECK2 vector using lipofectamine 2000 (Invitrogen)48.

Luciferase assay performed after 48 h of post-transfection, using the dual-luciferase reporter assay system
(Promega). Renilla luciferase signal normalized against firefly luciferase activity to monitor transfection effi-
ciency. Data are the means of experiments performed in triplicate.

Western blot. The MDA-MB-231 cells were lysed using cell lysis buffer (50 Mm Tris, pH = 8.0, 150 mM
NaCl,1% NP-40, 0.5% sodium deoxycholat, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM EDTA).
The polypeptide component were resolved by electrophoresis at 200 V using 12% SDS–polyacrylamide gel electrophoresis (PAGE) transferred into poly vinyl den fluoride membrane and immersed in 5% non-fat milk powder over one hour at room temperature. Upon completion of the transfer, membranes were incubated with CFlm25 and β-actin primary antibodies (Abcam). After washing, membranes probed with horseradish peroxi-
dase-conjugated secondary antibodies (1:1,000, Abcam) and developed for detection by chemiluminescence on Kodak X-film. In order to quantify bands in western blot gel, acquired images of CFlm25 and beta actin bands with quality of 75 out of 100 compared as a matter of expression level, using Image J software. Higher number of averaged white pixels considered as higher amount of expression.

Short hairpin RNA (shRNA) design and experiments. For knockdown endogenous CFlm25, target
sequence cloned as shRNA format in the pCDH-turboGFP. The shCFIm25 target sequence is 5′-GCCCTCA
TTCTTATTTCAAGAT-3′. An empty shRNA vector (shCtrl) used as a negative control. The MDA-MB-231cells
seeded into a six-well plate and transfected by pCDH-shCFIm25 or pCDH-shCtrl and lipofectamine 3,000 (Invitrogen) according to the manufacturer instruction.

Cell viability assay. For investigation of cell proliferation, experiments performed into three groups. In
the first group, MDA-MB-231 cells transduced with miR-Off-23/miR-Off-374 expressing vectors. In the second

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group, MDA-MB-231 cells that were co-transduced with miR-Off-23/miR-Off-374 expressing vectors were transfected by pCDH-shCFIm25. In the third group, MDA-MB-231 cells transfected merely by pCDH-shCFIm25. At 48, 72 and 96 h after transduction/ transfection, 10 μL of MTT reagent added into wells and incubated for next three hours. Next, 100 μL DMSO was added and the resulting absorbance measured at 550 nm using a multi-well spectrophotometer (Bio-Tek).

**Wound healing assay.** Wound healing assay performed to evaluate MDA-MB-231 cell migration. The MDA-MB-231 cells seeded in 24-well tissue culture plate until their growth rate reached a confluence of ~ 80%. The monolayer scratched gently with a 20-μL pipette tip across the center of the well. After scratching, wells washed twice with medium to remove the detached cells. Cell migration to the scratch area monitored for additional 48 h and photographed every 12 h. Number of cells moved from the border of scratch toward the empty area considered as the “migration index”. Related calculations performed by ImageJ software.

Received: 21 September 2019; Accepted: 24 June 2020
Published online: 14 July 2020

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F.K. and S.M.A.H. designed the study, M.T., G.S., performed the experiments. I.R. analyzed the data, provided intellectual support and expertise toward interpretation of results. A.E. collaborated in cell line preparation and culture set up. All authors contributed to the edition and the critical review of the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-68406-3.

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