4.1: Status of miR-101 in sporadic breast cancer tissues and validation of its new targets

The expression profile of miR-101 was assessed in 56 tissue samples (28 tumor and 28 adjoining normal tissues), with approximately 70% decrease in all tumors as compared to paired normal tissues (pValue = 6.49 x 10^{-6}). The decreasing trend was consistent with the increasing stages of cancer (pValue = 0.028) (Figure 4.1). The 3’UTR region of UBE2N and SMARCA4, the two genes involved in DDR pathway, were predicted as novel targets for hsa-miR-101 by all the target prediction software tools used.

The two novel targets identified by computational analysis were verified by in vitro Dual-Luciferase Reporter Assays and the expression of luciferase measured from Luc gene in presence of 3’UTR binding site of UBE2N and SMARCA4 independently and in presence of miR-101. The reporter gene expression decreased significantly in MCF7 (p = 0.001 & 0.005) and HepG2 (p = 0.00019 & 0.000142) cells when compared to the control experiments where miR-101 was absent (Figure 4.2).

4.2: Effect of miR-101 over-expression

Transfection of pEP-miR-101 in MCF7 cells was carried out in a manner to induce more than 2-Fold increase in expression of miR-101 to understand the biological outcome after its over-expression. It was clear that this microRNA has a role not only in mTOR or EZH2 pathways (Cao et al 2010, Wang et al 2008) but also assumingly in DNA damage response (DDR) pathway due to the involvement of UBE2N and SMARCA4 as its new targets. Over-expression of miR-101, lead to a 16-Fold increase in expression of ATM and 3.5-Fold of P53 in Real time expression studies. Whereas, the expression of P21 decreased as compared to the untransfected cells (Figure 4.3).

The cellular viability monitored by MTT Assay after transfection with pEP-miR-101 decreased to 86 % (Figure 4.4). To ascertain that the decrease in cellular viability was primarily due to an increase in DNA damage, because of the involvement of the novel targets as ascertained in this study, a Halo Assay was performed. It was observed that in
presence of miR-101 there was a 2.9-Fold (1.53 to 4.4) increase in NDF (Nuclear Diffusion Factor) values as compared to control cells (Figure 4.5).

![Figure 4.1](image_url)

(a)

(b)

Figure 4.1: Fold change in expression of miR-101 in total 28 Tumor samples as compared to 29 Normal samples, p Value = 6.49x10^-6 (a) and in Stage-2, Stage-3, Stage-4 of breast cancer patients with respect to Stage-1, p Value = 0.028 (b).

4.3: Expediting the role of miR-101 in induced DNA damage conditions

Since the two novel targets identified in this study (UBE2N & SMARCA4) are known to play a critical role in DDR pathway, we designed an assay where DNA damage was either induced independently or together with miR-101 over-expression and the three different concentrations of etoposide (1µM, 5µM & 10µM), a well-established chemical to cause a generalized DNA damage. As compared to untreated cells the expression of miR-101 increased 10-Fold at 1µM and 2.2-Fold at 5µM whereas the expression
decreased at 10µM. When miR-101 was over-expressed in Etoposide treated cells, a similar expression profile was obtained, but with a marginal increase. The fold change in expression of miR-101 increased to 11-Folds at 1µM, 7-Fold at 5µM and 3-Fold at 10µM concentrations (Figure 4.6). The requirement of miR-101 for activating cellular death via DNA damage augmented the effect of miR-101 production by DNA damaged cells.

![Figure 4.2: Graphical representation of Luciferase assay for invitro analysis of miR-101 targeting 3’UTR of UBE2N in MCF7 cells, p value = 0.001 (a)& in HeLa cells, pValue = 0.00019 (b), and also 3’UTR of SMARCA4 in MCF7 cells, p Value = 0.005 (c) & in HeLa cells, p Value = 0.000142 (d).](image)

In order to monitor the extent of induced DNA damage in a time dependent manner, the gene expression profile in etoposide treated (1µM, 5µM & 10µM) cells for 12hrs showed decreased expression of ATM with the increasing concentration when compared to untreated cells. Both P21 and P53 expressions also increased. When the expression was compared with 5µM etoposide exposure it was observed that P21 and P53 increased from 1µM to 5µM, and decreased at 10µM concentrations. The etoposide

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treated cells when transfected with pEP-miR-101 for over-expression of miR-101, the ATM and P53 expression remained same between treated and untreated cells, although there was a significant increase in expression, which was approximately 10-Fold and 6 fold, respectively, in presence of miR-101. The expression of P21 increased gradually with increase in concentration of etoposide in presence of miR-101 (Figure 4.3).

![Figure 4.3: Fold change in expression of ATM, P53 and P21 in presence and absence of miR-101 and in combination with different intensities of DNA damage induced via etoposide treatment.](image)

The cellular viability of the cells treated with 1µM, 5µM & 10µM, Etoposide, decreased to 90%, 86% & 75%, respectively. Similarly when MCF7 cells were subjected to a combination treatment of microRNA and 1µM, 5µM & 10µM etoposide, it was observed that the percentage of viable cells decreased to 71%, 61% & 50%, respectively, as compared to independently overexpressed microRNA or etoposide treated experiments (Figure 4.4).

5µM concentration of etoposide when selected as optimum for treatment, it was observed that there was a significant increase in NDF values of cells treated with both etoposide and over-expressing miR-101 as compared to independent treatments (pValue = 2.314 x 10^-19). The NDF values obtained in cells exposed to 5µM Etoposide and/or over-expressing miR-101 for 12hrs, were 4.46, 4.97, and 6.22, respectively (Figure 4.5).
Figure 4.4: The decrease in percentage of cellular viability of MCF7 cells in presence of miR-101 and combination treatment with different concentrations of etoposide.

Figure 4.5: Fold change in relative NDF values determined by Halo Assay on MCF7 cells treated with miR-101 and etoposide. (p Value = 2.314 x 10^-19).
Figure 4.6: Graphical representation of fold change in expression of miR-101 when pEP-miR-101 is transfected in MCF7 cells, when MCF7 cells are treated for 12hrs with etoposide, alone and in combination.

Figure 4.7: Senescence associated β-galactosidase staining in MCF7 cells.

4.4: Direct involvement of miR-101 in senescence

The results obtained suggested the involvement of miR-101 in senescence. For better understanding and establishing a direct role, cells were transfected with pEP-miR-101 and were stained with β-galactosidase 12hrs after transfection. On comparing the
staining between miR-101 transfected and control cells it was observed that presence of miR-101 resulted in senescence. However, mock transfection also resulted in senescence but the amount of such cells was far less as compared to that obtained in miR-101 transfected cells. The quantity of senescent cells matched that of 1µM etoposide treated cells; taken as positive control for this particular assay (Figure 4.7).

4.5: Discussion

In 2005, three studies published in NATURE established a close correlation between altered expression of specific miRNA and tumorigenesis(He et al 2005, Iorio et al 2005, Michael et al 2003, O'Donnell et al 2005). Since then, most miRNAs have been shown to express at lower levels in tumors than normal tissues, assuming the role of tumour suppressors. miR-101 has been suggested to act as a tumor suppressor in breast cancer, however, its mode of regulation is not clearly understood. Despite the information available on several of the proposed targets of miR-101, including ATM, where breast tumors could be sensitized for radiation [6], the exact mechanism remains unresolved.

Previously we have reported (Gochhait et al 2009, Pal et al) that with increasing tumor stages the expression of DDR genes is altered, suggesting the perturbed status of the DDR pathway at later stages to facilitate tumor progression. UBE2N plays a vital role in activating the repair pathway by ubiquitinating the phosphorylated H2AX and MDC1 complex. Formation of this complex occurs by virtue of ATM (Bonner et al 2008). This ubiquitinilated complex has the property to activate two vital cell survival pathways, the homologous recombination and repair by binding to RAP80, BARD1, BRCA1.
(Sobhian et al 2007) and the chromatin remodeling and transcriptional activation by forming a complex with RAP80, BRCA1, SMARCA4, CCNDC98 (Bochar et al 2000). Over-expression of miR-101 effectively down regulates the novel targets: UBE2N & SMARCA4; suppressing the DNA Repair, homologous recombination, chromatin remodeling and transcriptional activation of a cell (Figure 4.10). Thus, it was pertinent in this study to validate further the role of miR-101.

The three key observations made by our group i.e. (i) decrease in expression of miR-101, with increasing stages of breast cancer, when compared to normal cells, (ii) the identification of 2 DDR genes as direct targets and (iii) the altered expression of DDR genes observed previously in cancer patients (Gochhait et al 2009, Pal et al) were indicative of the association of miR-101 regulation with DNA damage (Figure 4.8). The bio-informatics as well experimental target prediction of SMARCA4 and UBE2N supported the same (Figure 4.9). Till date, to the best of our knowledge no microRNA has been implicated indirectly regulating the DDR pathway.

![Figure 4.9: Pictorial representation of has-miR-101 targeting the 3’UTR of UBE2N and SMARCA4.](image)

A large body of evidence has established that DNA damage is not only required for maintenance of senescence (d’Adda di Fagagna 2008) but also triggers apoptosis via senescence (Chen et al 2007). The increase in expression of ATM/P53 has been reported in re-oxygenated hematopoietic progenitor cells to cause senescence by cell cycle arrest before leading to apoptosis (Zhang et al 2005). Out of many studied proteins showing an association between damage and senescence, the up-regulated expression of KDM2B in mouse embryonic fibroblast was shown to help in bypassing senescence. It was also shown by the same group that the expression of miR-101 was
reduced in presence of KDM2B (Tzatsos et al). However the precise role of miR-101 in DNA damage and senescence, if any, has remained elusive.

In order to establish the suggestive role, an assay system was developed where the behavior of a cell under differentially induced generalized DNA damage with etoposide (Montecucco et al 2001) was studied in presence and absence of the induced over-expression of miR-101. This was followed by Halo Assay. The extent of damage caused by 12hrs etoposide exposure (1µM, 5µM & 10µM) varied from mild, moderate and severe, respectively; whereas the 24hrs of exposure to cells resulted in more or less similar damage at all the three concentrations. The average nuclear diffusion factor (NDF) values (Figure 4.11 and 4.12) supported the observations made on cell survival as well. The time dependent uniform outcome on long exposures suggested a probable phenomenon of senescence leading to apoptosis, irrespective of the dose of etoposide (the DNA damage inducing drug) used. Keeping in view the different intensities of damage at different time intervals, capable of modulating DDR pathway without causing complete death to cells, 12hrs exposure time was considered for further experimentation.

Well established markers of DNA damage, ATM (Zhang et al 2012), P21 (Mauro et al 2012) and P53 (Krejci et al 2008), were studied to assess the status of activation of DDR pathway to substantiate further the observation of induced DNA damage assessed in Halo assay. The increase in expression of P21 and P53, on exposure to etoposide, suggested a gradual up-regulation of DDR pathway in response to damage induced. Whereas, the decrease in expression of ATM suggested that the up-regulated DDR pathway decreased the accumulation of damaged DNA. The relative decrease in expression of ATM, P21 and P53 at 10µM etoposide exposed cells supported the non-functionality of DDR pathway at a higher dose, resulting in the increased damage and severe cell death, as was evident from Halo and MTT Assays. Under these exposures no specific signature for senescence could be inferred. Interestingly, the expression of miR-101 was maximum at 1µM which decreased gradually from 5µM to 10µM etoposide exposed cells. Since the cells under study were cancerous in nature, the maximum increase in expression of miR-101 at 1µM with gradual decrease later could possibly be to regulate the DDR or could be hypothesized as a shift occurring from senescence to apoptosis.
In presence of miR-101, an increase in DNA damage and decrease in cellular survival was observed. Also the expression profile of miR-101, i.e decrease in expression with increasing damage, in over-expressing and etoposide exposed cells were same as that of only etoposide exposed cells. This profile obtained in cancerous cells suggested that in presence of damage a specific concentration of miR-101 was required for regulating the DNA damage, in order to decide the cellular fate towards death. Presence of miR-101 increased the expression of ATM and P53, whereas the expression of P21 decreased. These findings were similar to the change in expression of ATM/P53 in presence of miR-101 and etoposide exposure observed by us. Apart from MTT and Halo Assay, the over-expression of Apoptotic marker: CASP3 in presence of miR-101 also supported the activation of Apoptotic pathway (Figure 4.13). The results obtained both in dose and time dependent experiments were indicative of initiating senescence via DNA damage by miR-101.
Graph A: Change in NDF values of MCF7 cells treated with 1uM, 5uM and 10uM concentration of etoposide at (A) 12hrs and (B) 24hrs with a p Value of 5.0578 X 10^{-39} and 1.36 X 10^{-20}, respectively.

Figure 4.11: Change in NDF values of MCF7 cells treated with 1uM, 5uM and 10uM concentration of etoposide at (A) 12hrs and (B) 24hrs with a p Value of 5.0578 X 10^{-39} and 1.36 X 10^{-20}, respectively.

Keeping in mind the suggestive roles of miR-101 defined by our study we tried to understand whether such a role could be utilized for therapeutic purposes. It has been observed that the drug resistance property of cancer cells results in therapy failure (Komarova and Wodarz 2005). Senescence has been observed to play a major role in drug sensitivity, specifically via DNA damage (Berns 2002). Even senescence has been
looked at as an answer to drug resistance property and enhancing cancer treatment indirectly (Gordon and Nelson).

Figure 4. 12: Halo Assay performed in MCF7 cells defining the extent of DNA damage in untreated / untransfected cells (a), miR-101 over expressing (b), 5uM Etoposide treatment (c) and combination effect of both (d).

Understanding the DNA damage conditions in presence of miR-101 and all the exposure concentrations of etoposide, the optimum expression of miR-101, ATM, P53 and P21 under moderate DNA damage conditions at 5µM etoposide appeared as the point for switch between arrest and apoptosis. We could observe that the percentage of DNA damage induced by miR-101 was similar to what exposure at 5µM of etoposide could achieve. When microRNA and moderate damage by etoposide were given invitro to cancer cells, the context of DNA damage increased considerably. To reconfirm the potential effect of miR-101, the expression of microRNA was increased through invitro
transfection by pEP-miR-101 and subjected to prolonged expression. Due to the presence of miR-101 and the unique role identified by us, we were unable to establish miR-101 over-expressing stable cell lines thus validating the hypothesis made by us. The results defined a particular combination of miR-101 and 5µM etoposide as most favorable for treatment purposes, with the expectation to minimize the side effects otherwise inflicted with high doses of DNA damage inducing drugs.

![Graph showing expression status of CASP3](image)

**Figure 4.13: Expression status of CASP3 in pEP-miR-101 transfected and etoposide treated MCF7 cells**

Our findings suggest that miR-101 possibly plays a very important role in helping cell decide its fate in response to DNA damage and senescence. The expression of miR-101 and other important genes in three different conditions of damage i.e mild, moderate and severe clearly support that each situation has a different requirement for miR-101. The apparent role of miR-101 from our study seems to switching of gears from survival to death with a simultaneous potential to cause DNA damage as and when required for supporting cellular senescence.
4.6: Conclusion

miR-101 was identified not only as a tumor suppressor but also as a regulatory mediator molecule used by the cell to help decide its fate under DNA damage conditions. Apart from this miR-101 can also be used as a therapeutic agent for treatment of breast cancer affected patients. The rate of cell death induced alone by miR-101 and in combination with low doses of Etoposide (with a decrease in usage of upto 200%) is expected to minimize the hazardous side effects of the drug and help increase life expectancy. This emergent role of microRNA opens an opportunity to evolve combinations of microRNAs and anticancer drugs in low doses for treatment purposes.
4.7: References


