



Evaluation of miRNAs targeted RBPs under hypoxia in cancer

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On a global level, it has been observed that hypoxia significantly reduces total RNA levels and decreases total *de novo* translation. In the presence of hypoxia, wide range of responses have been observed due to RBPs. Some RBPs like HuR, PTBP, TTP, ERBP etc. have been shown to increase or decrease stability of their respective mRNA targets by binding to them. These target mRNAs are typically hypoxia-mediated targets like the master regulator of hypoxia, HIF-1 and its downstream targets involved in processes like cell signaling and angiogenesis (VEGF), metabolism (GLUT1) etc. (Gorospe *et al.* 2011). A stability inducing RBP associates with an mRNA and improves its half-life by preventing binding of degradation inducing RBPs and miRNAs (Zhao *et al.* 2000). Increase or decrease in translation rates of target mRNAs have also been observed as an indirect consequence of hypoxia through RBPs. RBPs can block translation by mobilizing mRNAs into p-bodies (like miRNAs) and RNA granules that form in response to hypoxia. In these structures, the mRNA is stable yet there is interaction with translation machinery (Gottschald *et al.* 2010). RBPs can also activate translation. In the presence of hypoxia, internal ribosome entry sites (IRESs) have been proposed to promoted translation. IRESs are cis acting elements present in some 5' UTRs that mediate ribosome recruitment. Under hypoxia, RBPs are shown to stabilize these IRESs thereby activating translation (Fähling 2009).

ELAV (HuR) class of RNA binding proteins is involved in tandem with miRNAs when it comes to post transcriptional regulation. For example, in response to cellular stress, HuR is dephosphorylated and translocated, which relieves cationic amino acid transporter 1 (CAT1) mRNA from miR-122-mediated repression. HuR is also required for let-7-mediated repression of myc mRNA (Marieke *et al.* 2011). It was also demonstrated that combination of HuR and mir-3134 promotes more stability of HuR targeted transcripts like SOX9, VEGFA and EGFR as compared to stability by HuR alone. The same study also found out that HuR also regulated about 80% of the genes regulated by miR-3134 (Marieke *et al.* 2011). From these studies we can see that there is definitely some amount of interplay between RNA binding proteins involved in

transcriptional regulation and micro RNAs.

Overall, it should be noted that although several *trans*-binding factors (RBPs and miRNAs) have been identified that modulate the expression of hypoxia-response genes in cultured cells, particularly in cancer cells, their interplay and its functional impact under hypoxic conditions in tissues, organs, and organisms remains largely unknown. Our work here identifies miRNAs that may play a major role in hypoxia signaling by regulation of levels of specific RBPs. Since target transcripts of both RBP and miRNAs are several cancer related genes, the perturbations in the RBP-miRNA interactions under hypoxia will most likely affect various hypoxia regulated cellular processes.

The cell lines used were U87MG (Glioblastomamulti-forme) and MCF7 (Breast cancer), which were obtained from NCCS, Pune. These cells were grown in DMEM (Invitrogen) with 10% FBS and 10,000 U/ml PenStrep.

RNA was extracted from the cells using Trizol method with the Fermentas® RNA Purification Kit. The purity and concentration of the RNA extracted was measured using Nanodrop and this RNA was stored at -80°C. cDNA synthesis was performed using VersocDNA synthesis kit from Thermofisher®.

For plasmid transfection, 6 µl of lipofectamin was first added to 200 µl of Opti-MEM media. Side by side, 1.5 µg of plasmid and 200 µl of Opti-MEM media were added in another tube. These two tubes were mixed together in the order that the second mixture is added to the first after 5 min. Then this solution was kept for 20 min at room temperature. After 20 min, this mixture was added carefully in a drop-by-drop manner to the wells, along with DMEM. The cells were subsequently kept for 5 h and after this the media was changed.

SYBR® Green dye used to detect the quantity of cDNA (obtained from the miRNA or mRNA) in a given sample. This dye detected only when it is bound to double stranded DNA (dsDNA). As the amplification of the cDNA takes place, more of the dsDNA formed and consequently more SYBR® Green was detected. The number of cycles (C_t) required by a sample to reach a threshold value for detection of the dye is recorded. These were normalized against a housekeeping gene/miRNA to determine the fold change of the miRNA in the sample. This method was used to

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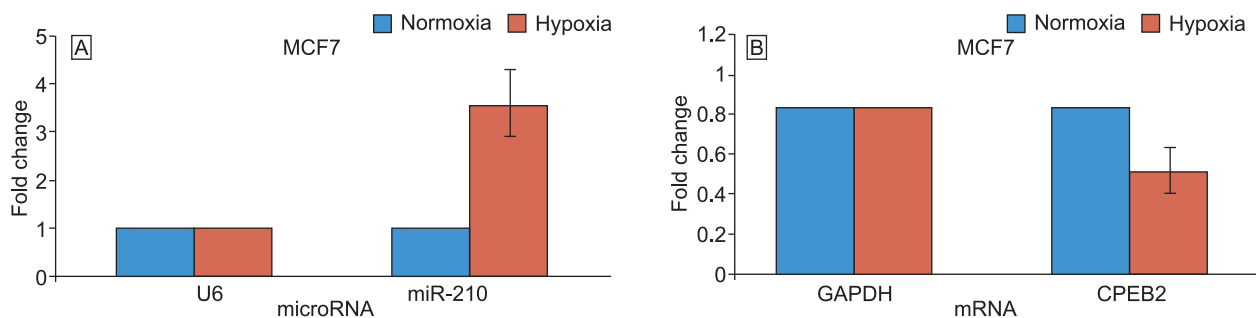


Fig. 1. Level of miR-210 (A) and CPEB2 (B) in response to hypoxia treatment (48 h) in MCF7 cell line. RNU6B (U6) and GAPDH were used for the normalization of the data in respective experiments.

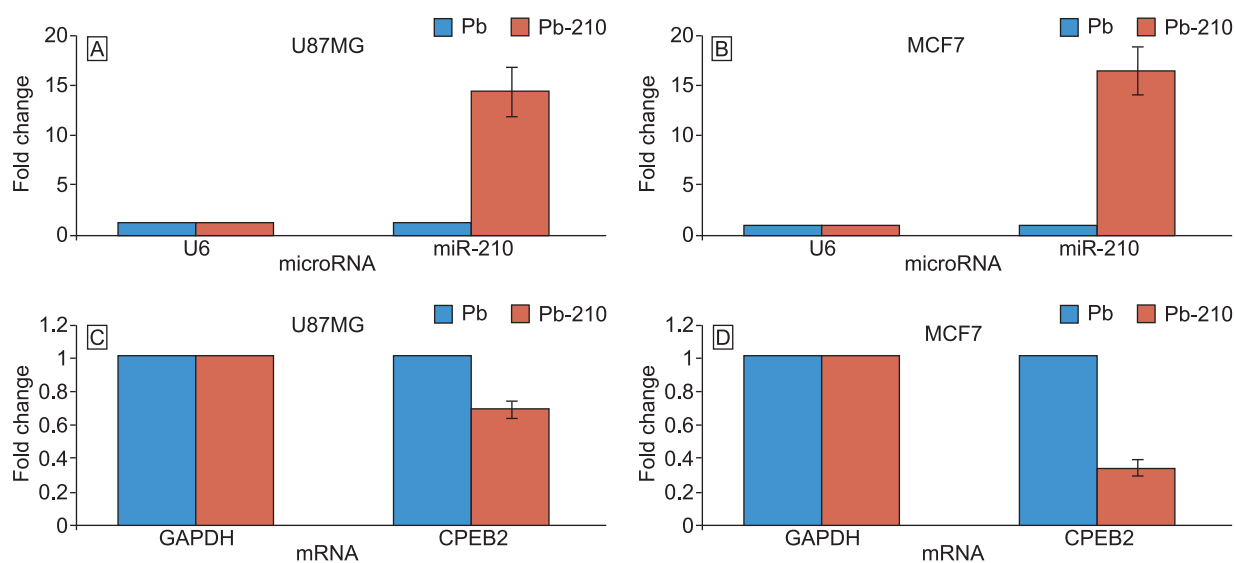


Fig. 2. Level of miR-210 and CPEB2 in response to hypoxia treatment (48 h) in U87MG and MCF7 cell line. U6 and GAPDH were used for the normalization of the data.

quantify the miRNAs in the control and infected samples.

MCF7 cells were grown separately under normoxia (21% O₂) and hypoxia (0.2% O₂) for 48 h and the levels of miR-210 and CPEB2 were determined using stem-loop qRT-PCR and qRT-PCR respectively (Fig. 1). We observed that miR-210 is upregulated and correspondingly CPEB2 is downregulated in hypoxia.

miR-210 overexpression construct (pB-210) and empty vector (pB) were transfected in MCF7 and U87MG and the levels of miR-210 and CPEB2 were determined using stem loop qRT-PCR or qRT-PCR respectively (Fig. 2). CPEB2 was downregulated in both U87MG and MCF7, on overexpression of miR-22.

We checked for the reported expression levels of miR-210 and CPEB2 in various cancers to look for inverse correlation of expression using StarBase software that integrates data from TCGA database. We did find an inverse correlation in some cancers, i.e. miR-210 was up-regulated while CPEB2 was down-regulated (Fig. 3).

MCF7 cells and U87MG were grown separately under normoxia (21% O₂) and hypoxia (0.2% O₂) for 48 h and the levels of miR-193b and PTBP1 were determined using

stem-loop qRT-PCR and qRT-PCR respectively (Fig. 4). We observed that miR-193b is upregulated in both the cell lines while PTBP1 was downregulated in U87MG. Expression of PTBP1 in MCF7 could not be ascertained owing to RNA contamination.

miR-193b overexpression construct (pC-193b) or empty vector (pC) were transfected in MCF7 and U87MG and the levels of miR-193b were determined (Fig. 5). We observed that no overexpression of the miRNA in either U87MG or MCF7 cell lines took place and thus, we did not proceed to check for the levels of PTBP1.

We checked for the expression levels of miR-193b and PTBP1 in various cancers to look for inverse correlation of expression using StarBase software that integrates data from TCGA database. We found that while miR-193b was overexpressed in some and underexpressed in other cancers, PTBP1 was uniformly over-expressed (Fig. 6).

While several trans-binding factors (RBPs and miRNAs) have been recognized to change the expression of hypoxia-responsive genes in cultured cells, predominantly in cancer cells, their interaction and its functional impact under hypoxic conditions leftovers. Our work here is an attempt

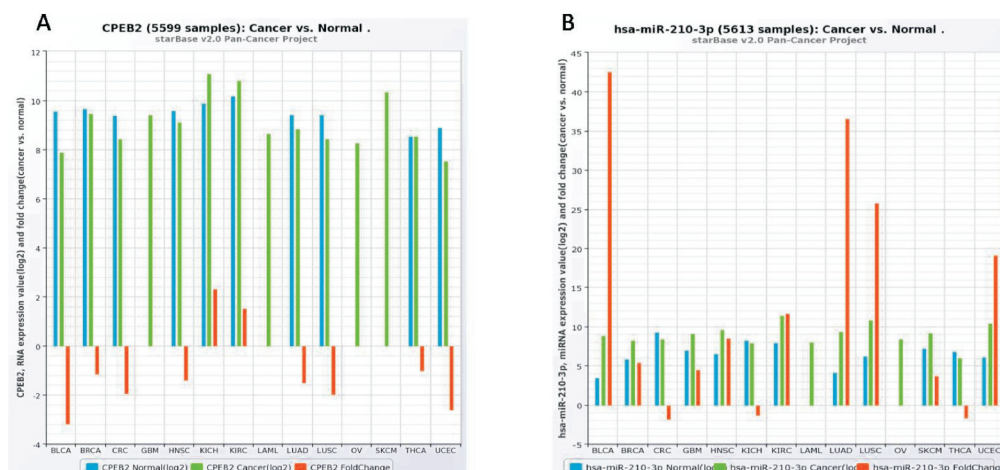


Fig. 3. Expression change of miR-210 (B) and CPEB2 (A) in normal vs cancer cells for 14 different cancer types.

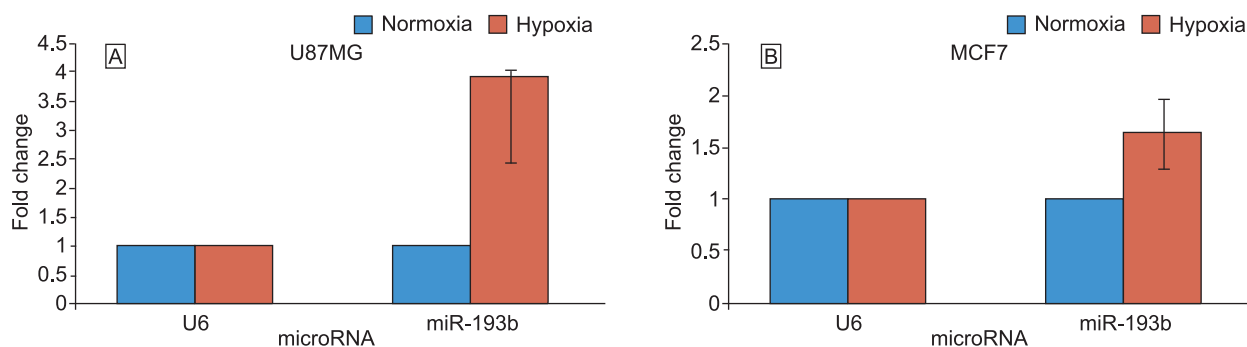


Fig. 4. Level of miR-193b in response to hypoxia treatment (48 h) in U87MG and MCF7 cell line. RNU6B (U6) was used for the normalization of the data.

in this direction and suggests subsistence of interactions amid these two key classes of post-transcriptional regulators under normal or hypoxic conditions.

The combination of miRNA:RBP pairs used in this study yielded some interesting results. We studied two RBP:miRNA pairs, viz. CPEB2 – miR-210, PTBP1 – miR-193b. Interestingly, we found that CPEB family members (CPEB1 and CPEB2) are regulated by two hypoxia-regulated miRNAs. An inverse correlation of expression was also seen in miR-210-CPEB2 pair in various cancer patients with miR-210 overexpression and CPEB2 downregulation in preponderance of cancers. In our results, we also get a comparable result. On overexpressing miR-210, downregulation of CPEB2 was observed. An interesting facet into this study would be to look at the downstream targets of these RNA binding proteins. CPEB, for instance, regulates the myc mRNA which is associated with the cell cycle and apoptosis (Gorospe *et al.* 2011). Uncontrolled translation of the myc mRNA may cause the cell to bypass senescence and potentially enter a carcinogenic phase (Burns and Richter 2008). CPEB is also recognized to target p53, a tumour suppressor protein. It was reported that knockdown of CPEB resulted in ~50% decrease in p53 protein levels (Groisman 2002). Therefore, by regulating the CPEB mRNA through over expressing

the miRNAs conferred in this study, we can foresee a means to manage CPEB and thereby may have power over the levels of myc and p53 mRNAs which could potentially influence the conversion of normal cell into a cancerous cell or succession of cancer. Subsequently, the discovery of first two miRNAs, *lin-4* and *let-7*, perceptive of miRNAs in standard physiology and diseased circumstances, such as cancer (Orellana and Kasinski 2015) has highly developed. Progression in the field have been likely due to state-of-the-art technologies such as high throughput screening and deep sequencing, but chiefly due to the improvement of understanding appropriate *in vivo* model systems (Steinkraus *et al.* 2016). Therefore, various model systems that have been instrumental in elucidating the roles of miRNAs in cancers and the technologies that have been extensively applied to generate this animal model systems. Briefly, the utility of *in vivo* models in evaluating the potential of miRNAs as therapeutic agents or targets for treatment of various cancers will depend upon proper animal model.

SUMMARY

Micro RNAs (miRNAs) are miniature, non-coding RNA molecules which are implicated in post-transcriptional regulation of genes. RNA binding proteins (RBPs) are

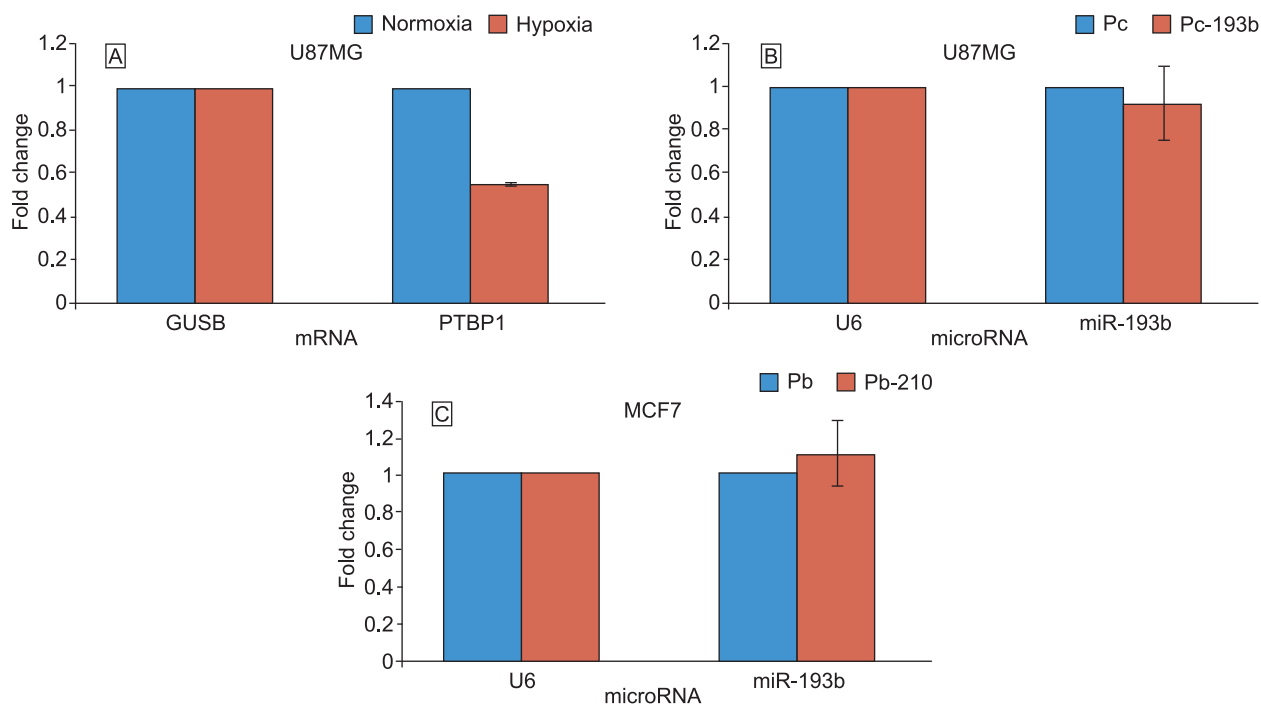


Fig. 5. Level of PTBP1 (A), miR-193b (B) and miR-193b (C) in response to hypoxia treatment (48 h) in U87MG and MCF7 cell line. B Glucuronidase (GUSB and U6) were used for the normalization of the data.

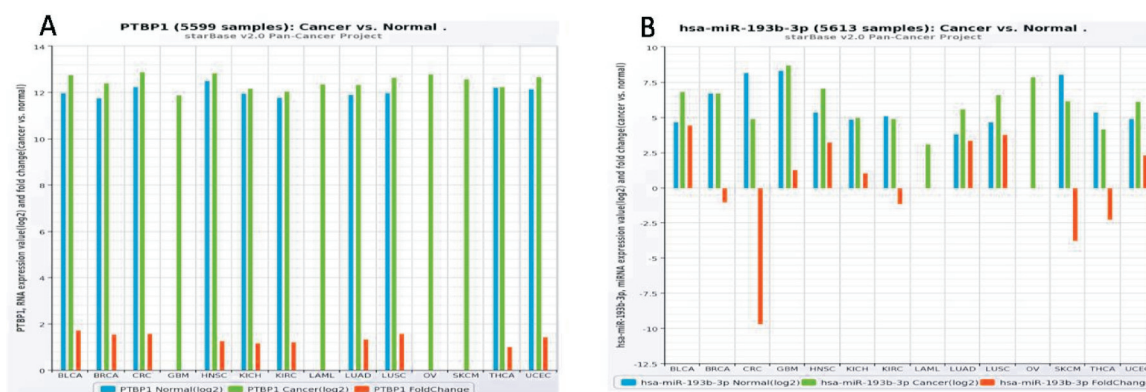


Fig. 6. Expression change of miR-193b (B), PTBP1 (A) in normal vs. cancer cells for 14 different cancers.

involved in a diversity of functions including post-transcriptional regulation of genes. They have also been exposed to influence the messenger RNA stability by binding to their identified sequences at the 3' untranslated region (UTR). Specific RBPs and miRNAs uncovered to be key participant in hypoxia signaling which is present in cancer cells, have been shown to involve in the outcome of the cell and drive towards tumor aggressiveness. Since both RBPs and miRNAs have been shown to be overstated by hypoxia and additionally significant since the site of battle of both players are the same (3'UTR of mRNA), we suggests that they might have role in tandem or be occupied in each other's regulation. Stem loop qRT-PCR was used to check the expression levels of these target miRNAs and the corresponding RBPs in cancer cell lines, namely U87MG (Glioblastoma) and MCF7 (Breast cancer) under hypoxic and normoxic conditions. Transient over expression of the aforesaid miRNAs in the two cancer cell lines was

examined using qRT-PCR to see the regulatory effects of these microRNAs on the corresponding target RBPs. Taken together, our results advocate that there could possibly be communication between the miRNA-RBP pairs that we had chosen and assessing the possibility as therapeutic agents for treatment of various cancers will depend upon suitable animal model.

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