GHR, PEX5 AND AXIN2 ARE NEW TARGETS FOR MIR-195 INVOLVING IN LONGEVITY, INCREASING BIOLOGICAL ACTIVITIES AND EMT OF CANCER CELLS FOR HEPATOCELLULAR CARCINOMA CELLS

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Hepatocellular carcinoma causes 500.000 deaths per year (Varnholt *et. al.*, 2008) microRNAs have been discovered as one of the molecular pathways in carcinogenesis (Lee 2006; Lemmer *et. al.,* 2006). They are short (non-coding) RNAs that have vital roles in gene regulation through different approaches such as mRNA degradation or DNA synthesis inhibition (Garzon *et. al.,* 2006; Lee *et. al*,1993; Berezikov *et. al*., 2006; Bereikov *et. al*., 2006; Volinia *et al.*, 2006.) Recent studies showed that miRNA expression signature is having a very significant impact on the prognosis, diagnosis and even classification of different cancer types (Lu *et. al*., 2005; Yanaihara *et. al.*, 2006; Ling *et. al*., 2016; Zhou *et. al.*, 2014; Salido-Guadarrama *et. al.*, 2016). Moreover, the *in vivo* and *in vitro* prediction tools for miRNA targets are very difficult alone so the importance of mixing both *in silico* tools with *in vitro* and *in vivo* techniques could provide much more better results in the last years and different target genes were perfectly predicted using this approach (Amer *et. al.,* 2014) This combination gave more insights for further understanding on how miRNA can regulate gene expression. In addition, it can control even the defense mechanisms in many organisms or control the progression of disease (Melo and Easteller, 2010)

miRNA acts both as tumor suppressor and as oncogene and are aberrantly expressed in a number of cancers (Nana-Sinkam 2011) Hence, the im-

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portance of discovering the role of miRNA in cancer regulation will give insights in understanding this disease and increase the ability to deal with it. miRNA expression profiles are effective for classifying solid and hematologic human cancers.

Since it has been well established that miRNA are involved in tumor initiation and progression, the aim of this study was the identification and analysis of miRNAs could enhance our understanding of the important roles that miRNA play in this complex regulatory network as well as the prediction of miRNA target genes involved in cancer pathways and its validation.

MATERIALS AND METHODS

Cell Culture

The predicted target genes were detected as done before in our previous study (Amer *et al.*, 2014 and El-Hefnawi *et. al.*, 2013) Huh-7.5 (A.T.C.C designation number PTA-8561) was grown in DMEM (Gibco) supplemented with 10% bovine calf serum (Atlanta Biologicals), penicillin and streptomycin (Gibco).

Constructs

The 3UTRs of PEX 5, GHR and AXIN2 were isolated using specific primers for them (Table 1) along with a high-density DNA isolated by DNeasy® Blood & Tissue from Qiagene from Huh7. Then purified, ligated with PmirGLO Dual-Luciferase mina Target Expression Vector (Promega, 9PIE133) and sequenced. The resulted constructs named pmirGLO-PEX5, pmirGLO-GHR and pmirGLO-AXIN2. miRNA-195 precursor synthetic sequence was designed and ordered from GeneScript and inserted into miRNASelect™ pEGP-miR Cloning and Expression Vector (Part No. MIR-EXP-GP).

Cell transfection

pEGP-miR-195 and pmirGLO-PEX5, pmirGLO-GHR and pmirGLO-AXIN2 respectively were co-transected in Huh-7 cell line using HiPerFrct transfection reagent from Qiagen (Cat No. 301704) according to the manufacture protocol. The day before transfection, 5-6 x 105 Huh-7 cells were seeded at 60 mm dish with 4ml DMEM antibiotic free medium. MTT assay (Sigma, M-0408) was done according to manufacture protocol by using 20µ1 of HiPerfect transfection buffer.

The experimental design was three 60 mm dish plates for each co-transfection experiment: five different combinations of vectors used in transfection: the three plates were transfected with as following: 1) [EGP-miR-195 and pmirGLOPEX5, pmirGlo-GHR and pmirGLO-AXIN2 constructs, respectively 2) pEGP-Null expression vector and pmirGLO-PEX5, pmirGLO-HR and pmirGLO-AXIN2 constructs, respectively and 3) non-transfected cells. The expression of miR-195 was detected using GFP 24 h after transfection and pmirGLO-PCMT1 was detected using antibiotic resistance gene.

Stable selection was done with Puromycin and Gentamycin for 72 h.

Luciferase assay system

Luciferase expression was detected 48 h after transfection using luciferase assay system from Promega (Cat. No. E1500). The FlouStar Optima was used to measure firefly luciferase. Each transfection was repeated twice

RESULTS AND DISCUSSION

miR-195 target prediction

The target prediction was done as we described before (Amer *et. al.,* 2014; El-Hefnawi *et. al.,* 2013) using a combination of different software for miR-195 target prediction. Five different programs were used, and minimum free energy was calculated for both the best fitting binding sites and secondary structure hybridization to check the accessibility of a target region (Fig. 1).

The shuffled one hundred random sets for each miRNA-195 were independently hit against its predicted PEX5, GHR and AXIN2 using miRanda software on Linux OS operation system. The prediction score for each hit at the same target was used to calculate the Z-score of the targets according to this equation**: $Z = (\mu - x) / \sigma$ where "x" is the original prediction score, "μ" is the mean, "σ"** is the standard deviation of random sequences scores. P-value was calculated using calculated Z-score to estimate the significance of the predicted targets. Z-score of PEX5, GHR and AXIN2 were 4.71, 4.15 and 4.68, respectively, while P values were 2.51961E-06, 3.25278E-05 and 2.83E-06, respectively, which are highly significant for all the targets.

Gene ontology annotations

The peroxisomal biogenesis factor 5 (PEX5) is having a vital role in peroxisomal protein import as it binds to PTS1-type tripeptide peroxisomal targeting signal (SKL-type) (Dodt *et. al.,* 1995, Wiemer *et. al*., 1995 and Fransen *et. al.,* 1995).

The peroxisome biogenesis disorders (PBD group) are genetically heterogeneous. It is classified to at least 14 distinct genetic groups. Defects in PEX13 and PEX5 are the cause of peroxisome biogenesis disorder complementation group 13 (PBD-CG13) [MIM: 601789]; also known as PBD-CGH. PBD-CG13 is a peroxisomal disorder arising from a failure of protein import into the peroxisomal membrane or matrix (Liu *et. al.,* 1999). Defects in PEX13 and PEX5 are also resulting in adrenoleukodystrophy neonatal (NALD) [MIM: 202370]. NALD is a peroxisome biogenesis disorder (PBD) characterized by the accumulation of very long-chain fatty acids, adrenal insufficiency and mental retardation (Shimozawa *et. al.,* 1999)The cargo handling characteristic of PEX13 and PEX5 are highly interesting in cancer Williams and Distel, 2006)The increased biological activities of cancer cells need to have transportation systems to facilitate their development.

From this point the importance of increasing levels of PEX5 is obviously apparent especially when it is differentially expressed in liver cancers due to miR-195 targeting for it. Growth Hormone Receptor (GHR) encodes a member of the type I cytokine receptor family, which is transmembrane receptor for growth hormone. It is known as growth hormone receptor gene binds to growth hormone and inactivates - and intracellular cascade. GHR is one of the core genes in important pathways like AKT signaling Pathway, regulation of eIF4e and p70 S6 kinase, growth hormone signaling pathway, IGF-1 Receptor and longevity, cytokine receptor interaction and Trefoil factors initiate mucosal healing (Hrington and Carter-Su2001). The Akt/PKB is a pathway in cell signaling. The key activator of this pathway is GHR that activates a long cascade of genes leading to cell survival. The expression level of GHR is high in liver cancers due miR-195 targeting for it. This high expression level leads to activation of this cascade and cell survival of the cancer cells. PI3 Kinase is a lipid kinase that is activated by GHR signals (e.g., GH) transmitted by many transmembrane receptors with protein kinase cytosolic domains. These in turn signal to constitutive membrane threonine kinase called PDK-1 and, simultaneously, to a cytosolic protein kinase called Akt (or alternatively protein kinase B, PKB). Akt indirectly activates (not phosphorylates) mTOR, which lies at the heart of growth regulatory pathways. mTOR promotes cell growth by: activating S6kinase (an activator of translation), activating PKC (so turning on many synthetic and secretory pathways), inhibiting p21 (so releasing cells from G1 arrest) and inhibiting GSK3β (with similar effect, since GSK3β targets cyclin D for proteolysis). Akt is promoting cell survival through phosphorylation of Bad (pro-apoptotic protein). Inhibition of both Akt1 and Akt2 selectively sensitize tumor cells, but not normal cells, to apoptotic stimuli which means in contrast that activation of Akt1 and Akt 2 leads to tumor survival (DeFeo-Jones *et. al.,* 2005 and Song *et. al.,* 2005)

One of the most important pathways that are activated through GHR is regulation of eIF4e and p70 S6 kinase pathway. This pathway is one of the most important pathways in cancers (De Benedetti and Harris, 1999) eIF-4F and p70 Kinase play critical roles in translational regulation . eIF-4F is a complex whose functions include the recognition of the mRNA 5' cap structure (eIF-4), delivery of an RNA helicase to the 5' region (eIF-4A), bridging of the ribosome (eIF-4G) and mRNA, and circularization of mRNA by interaction between the poly (A) binding protein (PABP) and (eIF-4G). Several stimuli, including growth factors like GHR and cytokines, regulate the eIF-4 complex and p70 S6 kinase by initiating a phosphorylation cascade involving the sequential activation of PI3-K, PDK1/2, Akt/PKB, and FRAP/mTOR kinase. FRAP/mTOR, together with an unidentified kinase are responsible for the phosphorylation of 4E-BP. This in turn is leading to its dissociation from MNK1/2.

Moreover, ERK and p38 MAPK, phosphorylates and activates eIF-4E. Both processes promoting the association of eIF-4E and eIF-4G to form the active eIF-4F complex which is a necessary component of the 48s initiation complex. Phosphorylation of ribosomal protein 56 by p70 S6 kinase stimulates the translation of mRNAs with a 5' oligopyrimidine tract which typically encode components of protein synthesis (Graff *et. al*., 2008). Therefore, the high expression levels of GHR will in turn stimulate the expression level of eIF-4 which is obviously highly expressed in cancers.

IGF-1 belongs to the insulin family of peptides and acts as growth factor in many tissues and tumors. The restriction of caloric intake is one of the means to increase lifespan in a wide range of organisms. Reducing the consumption of calories increases the lifespan of many different organisms, including mice (Holzenberger *et. al*., 2003).

Moreover, short-term changes in physiological measures like insulin responsiveness have been experimentally observed due to caloric restriction (Bluher *et. al*., 2003) while no experimental indications to increase lifespan.

Caloric restriction not only increases lifespan, but decreases age-related deterioration of systems and physiological responses, reducing age related diseases like cancer and neurodegenerative disease. Caloric restriction in animals reduces the levels of insulin and plasma glucose. In addition, it reduces inflammatory responses. In some cases, it may reduce oxidative stress through reduced oxidative metabolism, further contributing to the health benefits of reduced calorie intake. The reduction in inflammation may be related to reduced plasma glucose and in humans could reduce an inflammation connection to cancer, heart disease, and Alzheimer's disease. GHR is activator for IGF-1 that in turn activates the cascade of longevity. This means that the higher expression level of GHR leads to activation of this cascade. The cancer cells need this cascade to stay longer with high efficiency which is one of the cancer hallmarks.

AXIN2 is thought to work mainly as a tumor suppressor gene through negative regulation of Wnt signaling pathway (Hugang *et al*., 2009, Klaus *et al.,* 2008 and Waaler *et. al.,* 2012) However, rather than functioning as tumor suppressor, is demonstrated that Axin2 acts as a potent promoter of carcinoma behavior by up-regulating the activity of the transcriptional repressor, Snai11, inducing a functional epithelial-mesenchymal transition (EMT) program and driving metastatic activity. Silencing Axin2 expression decreases Snai11 activity. In turn this inhibits CRC invasion and metastatic activities in concert with global effects on the Wnt-regulated cancer cell transcriptome. In addition to reversing EMT. The further identification of Axin2 and nuclear Snai11 proteins at the invasive front of human CRCs supports a revised model wherein Axin2 acts as potent tumor promoter *in vivo* (Waaler *et. al.,* 2012; Wu ZQ *et. al.,* 2012) This finding is major result for us

because AXIN2 is a target for miR-195 which is lowly expressed in cancer, therefore the expression level of AXIN2 is supposed to be high, while the main role or mechanism that AXIN2 contribute in cancer is still unknown if it works as a tumor suppressor only or as a tumor activator also though an alternative mechanisms as we also assume.

Cell transfection and luciferase assay system

Assessment of expression level of PEX 5, GHR and AXIN2 in the presence of miR-195 was necessary to validate the prediction results *in vitro* as we did *in silico* using the shuffling method as described before. PmirGLO-3'UTR-PEX5, pmirGLO-3'UTR-GHR and pmirGLO-3'UTR-AXIN2 constructs were co-transfected with miRNASelectTMpEP-mir-195 in Huh-7, respectively. In addition to transfected cells with both Null-pRGP-miRNA-195 and pmirGLO-3'UTR-PEX5, pmiGLO-3'UTR-GHR and pmirGLO-3'UTR-Axin2 constructs as a negative control. The luciferase assay was measured for each combination for the targets in comparison with the co-transfection with the Null-pRGP-miRNA-195. MTT assay was tested with 20μ1 HiPerfect and the cell viability was reported as 90%. The measurements for GHR, PEX5, AXIN2 were highly significant with P-values 0.00029, 0.0005, 0.0004, respectively. In summation, it is evident from the aforementioned results that miRNA could be used as a dependable biomarker for early detection of liver cancer.

However, this calls for further studies to substantiate these findings.

SUMMARY

Recently, micro RNAs have been shown to regulate gene expression of genes in many organisms. They bind to target mRNA transcripts. In a sequence specific manner, including mRNA degradation, translational repression or endonucleolytic cleavage. Some studies showed that miRNA expression correlates with various cancers. Combination of multiple properties e.g. free-energy, sequence pattern, hairpin shaped precursor rather than miRNAs complementarity to their targets provide a more desirable accuracy in miRNA target prediction. Different programs were used to predict novel targets for differentially expressed miRNA in cancers tissues. Luciferase assay for PEX5, GHR and AXIN2 showed a significant decrease in luciferase production in the presence of both miR-195 and PEX5-3'UTR, GHR-3'YTR and AXIN2-3'UTR. The results emphasize that both PEX5, GHR or AXIN2 are real targets for miR-195. This confirms and verifies the prediction method that were used on this study and our previous studies. These genes are suggested to have a major role in regulation of Wnt signaling pathway, epithelial-mesenchymal transition, AKT Signaling Pathway, peroxisomal protein import and cell longevity.

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Table (1): Primer sequence of different 3'UTRs.

Name	Sequence
AXIN2-F	5' CCTGGGGTCTGGCTTTGGTGAA 3'
AXIN2-R	5' GCTTGAGCCCTCAATATAGGGCGA 3'
PEX5-F	5' CACCTGGAGGGATCCCCGCTT 3'
PEX5-R	5' TGGAATCCTGGGACCATGGTAGTTT 3'
GHR- F	5' CTCGAGTGACAGGATGGGGTAT 3'
GHR- R	5' CAGCTGAAACTGCCAGACACAA3'

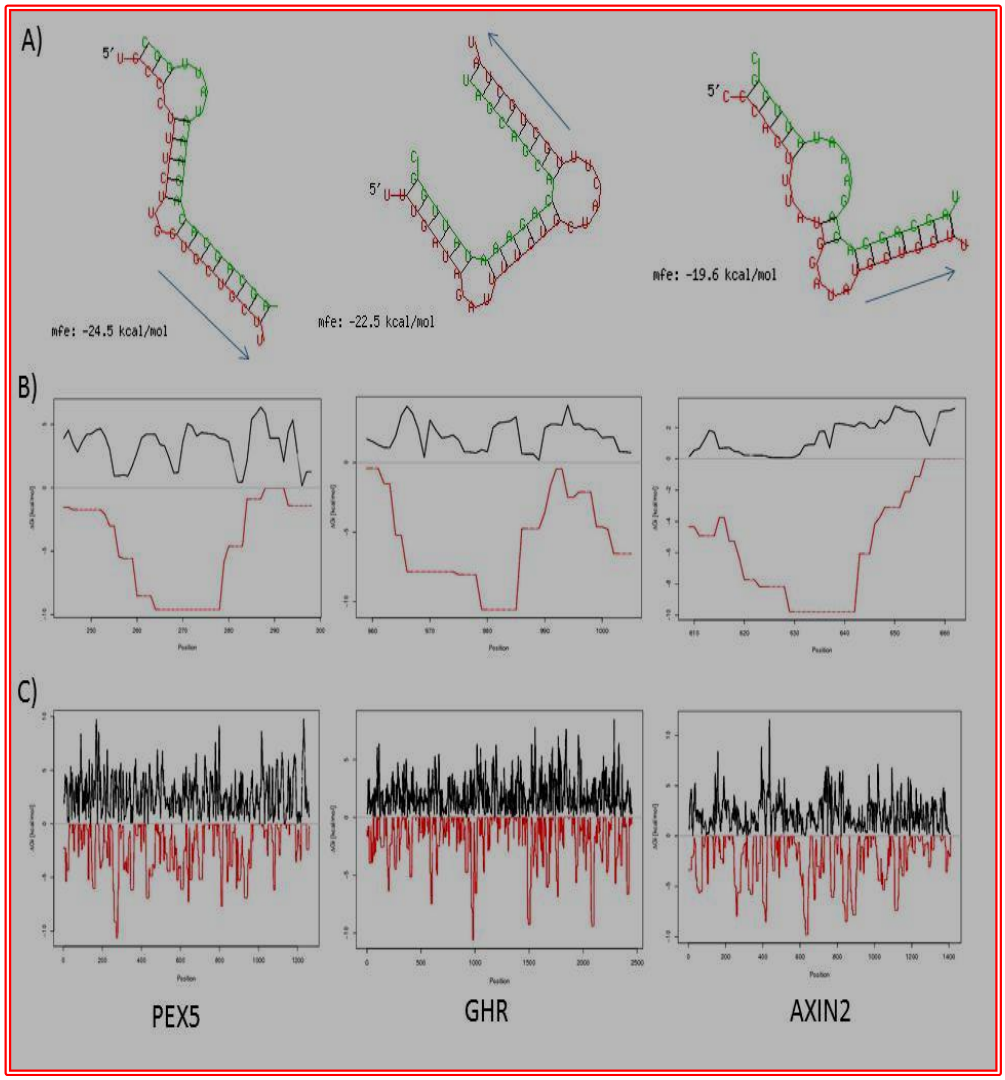

Fig. (1): The hybridization of miRNA-195 and their target genes. (A) shows the hybridization using RNAhybrid between miRNA (green) and PEX5, GHR and AXIN2 (red) with mfe -24.5, -22.5 and -19.6 kcal/mol., respectively. (B & C) shows the interaction free energy (RED) Gi and the energy needed to open existing structures in the longer sequence (BLACK) for the target region using RNAup (B) the optimal position of seed region while (C) shows all the possible positions at the 3'UTR region.

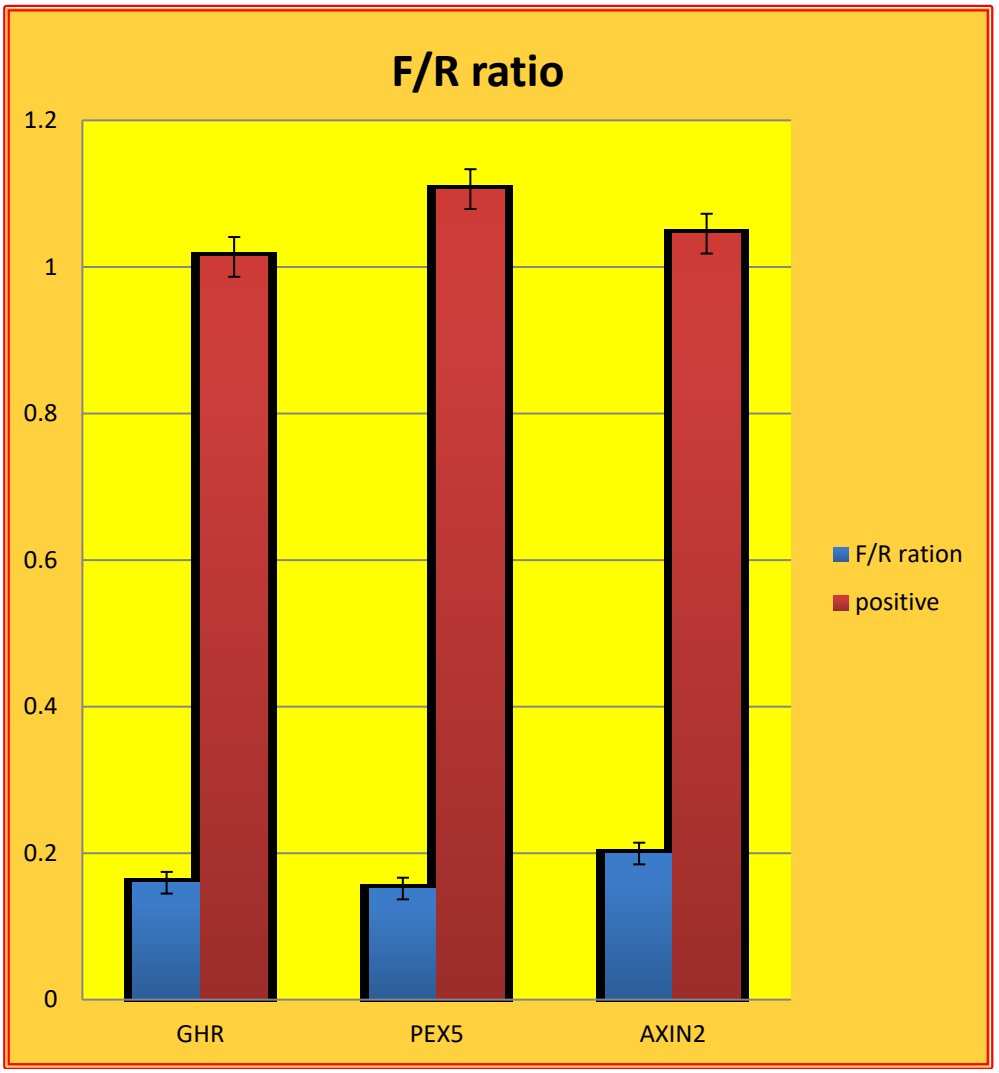

Fig. (2):. The difference in luciferase assay measurements for both transfected cells. Low expression of luciferase in cells transfected with pEGP-miRNA-195 and pmirGLO-3'UTR-GHR, pmirGLO-3'UTR-PEX5 and pmirGLO-3'UTR-AXIN2 while high expression of luciferase in cells transfected with both Null-pRGP-miRNA-195 and pmirGLO-3'UTR-GHR, pmirGLO-3'UTR-PEX5 and pmirGLO-3'UTR-AXIN2.