Research Synopsis on

Ablation of insulin like growth factor I receptor (IGF-IR) attenuate neuroblastoma tumorigenesis

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Doctor of Philosophy (Ph.D.) in Zoology

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INTRODUCTION

Neuroblastoma(NB) is a pediatric cancer of young children arising from the embryonic sympathoadrenal lineage of the neural crest(Louis & Shohet, 2015).The tumor derives from the developing sympathetic nervous system and most primary tumors occur within the abdomen, with at least 50% arising from the adrenal glands.(Mueller & Matthay, 2009).The disease is clinically heterogeneous, with the likelihood of cure varying greatly according to age at diagnosis, extent of disease, and tumor biology. This extreme clinical heterogeneity reflects the complexity of genetic and genomic events associated with development and progression of disease (Capasso & Diskin, 2010) .

Neuroblastoma is often described as enigmatic and unpredictable because it is associated with contrasting patterns of clinical behavior: life threatening progression, maturation to ganglioneuroblastoma or ganglioneuroma, and spontaneous regression (Schwab et al., 2003). It occurrence is more common in boys as compared to girls (ratio 1.2:1)

NB is a polymorphic cancer with a variety of mutagenic mutations. One family of factors potentially involved in neuroblastoma metastatic progression is the insulin-like growth factor (IGF) family(Golen et al., 2006).The insulin-like growth factor-I receptor is a transmembrane tyrosine kinase receptor which regulates growth, development and metabolism by binding of the IGF-I ligands.(Belfiore et al., 2009; Khandwala et al., 2000).The IGF family consists of two ligands, IGF-I and IGF-II, whose known actions occur through type I IGF receptor (IGF-IR) activation and signaling (LeRoith et al., 2021). IGFs have a significant effect on cell development. IGFs, especially IGF-1, are responsible for most of the activities of pituitary growth hormone. IGF-IR can stimulate a wide variety of responses in cells, including cell proliferation, cell differentiation, changes in cell size, cell adhesion and motility, and resistance to apoptotic stimuli (Barnes et al., 2007).

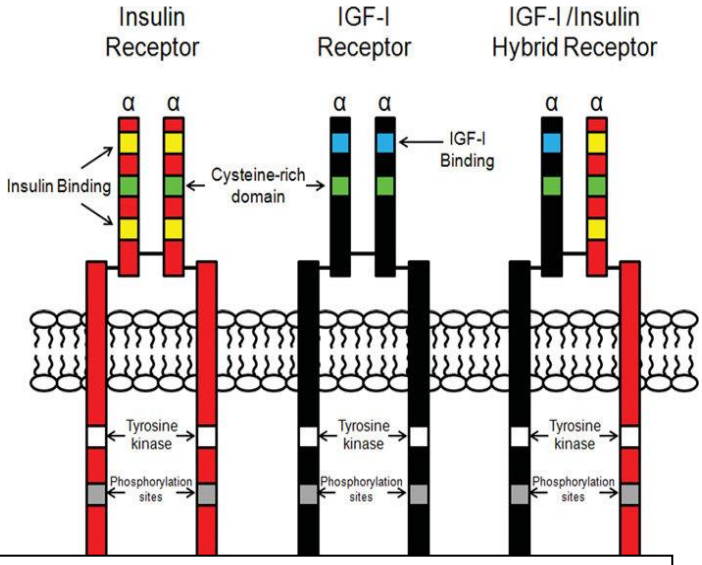


Figure-1: Structure of Insulin like growth factor -I receptor and Hybrid receptor.

IGF-I receptor is highly homologous to insulin receptor. IGF-I receptor is a disulfide bond-conjugated tetramer which consists of two α subunits that are extracellular proteins binding to specific ligands and two β subunits that are transmembrane proteins possessing tyrosine kinase activity shown in **Figure 1 (**Steele-Perkins, et al. 1988; Werner, et al. 1989).This system consists of soluble ligands (including IGF I and IGF II), cell surface transmembrane receptors (including IGFI receptor (IGF-IR) and IGF II receptor (IGF-IIR)) and soluble binding proteins (IGFBP1 (IGF binding protein-1) through IGFBP-6). The biological activities of IGFs are mediated by cell surface receptors and modulated by complex interactions with binding proteins (Roith & Le Roith, 2003; Denley et al., 2005; Sachdev & Yee, 2007). IGFBPs and IGFs comprise a major superfamily of protein hormones that regulate mitogenesis, differentiation, survival, and other IGF-stimulated events in both normal and cancerous cells (Baxter, 1994; Renehan et al., 2004).

Both preclinical research and clinical investigations have implicated the IGF type I receptor (IGF-IR) and its ligands IGF-I and IGF-II in the development and progression of a number of human cancers (Pollak et al., 2004; Larsson et al., 2005; Mitsiades & Mitsiades, 2005). IGF-IR can stimulate a wide variety of responses in cells, including cell proliferation, cell differentiation, changes in cell size, cell adhesion and motility, and resistance to apoptotic stimuli.).The conversion of epithelial cells into mesenchymal cells, an “epithelial–mesenchymal transition” (EMT), is central to many aspects of embryonic morphogenesis and adult tissue repair, as well as a number of disease states(“Molecular Organization of Cells,” 2020). Increased IGF-I, IGF-II, and IGF-IR expression is present in neuroblastoma (Leinninger et al., 2003; Cynthia et al., 2006), and IGF-I or IGF-II coupled to IGF-IR promotes neuroblastoma cell survival and growth whereas IGF-IR inhibition induces tumor regression in mice (Liu et al., 1997; Meyer et al.,

2001). High expression of IGFs and IGF-IR has also been associated with tumor metastatic potential (Reinmuth et al., 2002; Zeng et al., 2003; Larsson et al., 2005).

Many cancers are being treated by IGF-IR inhibitors and many inhibitory molecules of the same are under preclinical and clinical trials. One of the known inhibitors of IGF-IR is Picropodophyllotoxin (PPP) with molecular weight 414.41 and Chemical name is (5R,5aS,8aR,9R)-5,8,8a, 9-Tetrahydro-9-hydroxy-5-(3,4,5-trimethoxyphenyl) furo [3',4':6,7] naphtho [2,3-d]-1,3-dioxol-6(5aH)-one. PPP has the ability to inhibit IGF-1R autophosphorylation, as a consequence resulting in inhibiting cell survival and up-regulating apoptosis. Furthermore, PPP was also seen to impede growth in multiple myeloma cell lines, bone marrow stromal cells (Scagliotti & Novello, 2012),uveal melanoma cells (Vasilcanu et al., 2006), 5T33MM mouse model (Menu et al., 2006) and colon cancer cells (Feng et al., 2012).

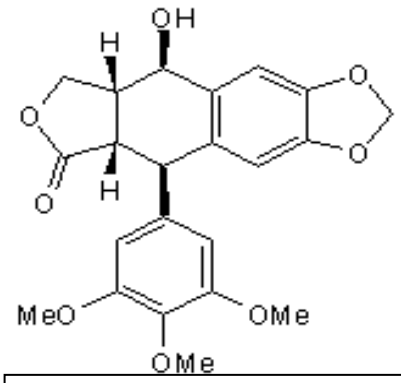


Figure 2: Structure Picropodophyllotoxin

REVIEW OF LITERATURE

IGF-I/ IGF-IR are expressed in a wide variety of tumor types, including neuroblastoma (Cianfarani and Rossi, 1997). IGF-IR activation enhances neuroblastoma cell proliferation and survival, and increased IGF-IR expression increases the protection of neuroblastoma cells from apoptosis induced by chemotherapeutic agents (Golen et al., 2000). IGF-IR overexpression promotes tumor growth, progression, invasion, and metastasis (Foulstone et al., 2005), whereas disruption of IGF-IR expression reverses the transformed phenotype (Adams et al., 2005). Increased IGF-I, IGF-II, and IGF-IR expression is common phenomenon of neuroblastoma (Leinninger et al., 2003), and IGF-I or IGF-II coupled to IGF-IR promotes neuroblastoma cell survival and growth (Kiess et al., 1997) whereas IGF-IR inhibition induces tumor regression in mice (Liu et L., 1998; Cynthia et al., 2006).

In neuroblastoma cells, tumor growth has been linked to both the IGF system and MycN. In addition, c-myc levels are diminished in the presence of IGF-I, which would account for the long-term differentiating effects of IGFs in neuroblastomas (Singleton et al., 1996; Chambery et al., 1999) and conversely, N-myc is associated with tumorigenesis and cell proliferation of NB (Hopewell et al., 1995). IGF-1R activates multiple downstream signaling cascades, including PI3K/AKT and MAPK/ERK signaling pathways, which regulate cell proliferation, differentiation, and survival of NB tumor (Cao et al., 2007; Shi et al., 2013). Results of Kim et al. (2007) suggested that high levels of nuclear NF-κB were found in the majority of primary human and rodent tumor tissue. IGF-IR can activate the NF-κB signaling pathway. IGF-IR can modulate glycogen synthase kinase 3 (GSK-3) activities via Akt; GSK-3 regulates Snail via NF-κB. NF-κB binds to promoter region of snail and increases its activity that signifying a potential IGF-IR– GS3K–NF-κB–Snail signaling pathway via modulation of E-cadherin expression in EMT.

Shi et al. (2013) reported that miR-181 acts as a tumor suppressor by directly targeting IGF-1R, resulting in the inhibition of IGF-1R signaling pathways. In addition, miR-181b overexpression suppressed cell proliferation, migration, and invasion, and attenuated tumor growth in vivo. Shi and colleagues also demonstrated that IGF-IR protein levels were downregulated by miR-145 oligonucleotides. Cell proliferation during invasive state of cancer is regulated by MycN. Crosstalk of IGF-I and MycN is common in many pediatric cancers. MycN expression is regulated by let 7 and also coordinates the metastasis pathways that escort the event to uncontrolled neoplastic disease (de Nigris et al., 2013). *IGF-IR* mRNA is a potential target for miR-7. Ectopic transfection of miR-7 led to a significant reduction in IGF-IR at both the mRNA and protein levels. The miR-7 mediated downregulation of IGF-IR expression attenuated the IGF-I induced activation of Akt (Jiang et al., 2010).

HYPOTHESIS

Present work focuses on the role of IGF-IR in neuroblastoma progression and metastasis. IGF-IR is one of the oncogenes and its expression stabilized by crosstalk of other oncogenes and tumor suppressor gene when tumorigenic cascade occur. In addition, IGF-IR shows epithelial mesenchymal transition which is associated to downregulation of multiple epithelial cell markers and concomitant upregulation of many mesenchymal markers. On suppression of IGF-IR

expression, aggregation of p65 and p50 of NF-κB in nucleus may segregate and translocated in cytoplasm. Down regulation of NF-κB may suppress the expression of snail that inhibits cell detachment by stabilizing the expression of E cadherin and hamper the invasive property of NB cells.

According to our current hypothesis, IGF-IR inhibitor activate PTEN autophosphorylation site and inhibits the upstream and downstream carcinogenic cascade, resulting in neuroblastoma regression. Since IGF-IR plays a role in cancer cell survival, the broad question of proposal is how this IGF-IR inhibition suppresses the malignant potential of NB tumors.

Objectives

The aim of this study is to understand the mechanism of how IGF-IR inhibition leads to neuroblastoma tumour regression, confirming that IGF-IR is one of the most promising therapeutic targets for NB cancer.

a. Comparison of perturbed downstream pathway on IGF-IR suppression

b. Role of miRNAs in neuroblastoma regression on inhibition of IGF-IR

c. Estimation of epithelial mesenchymal transition and anoikis

MATERIAL AND METHODS

Cell culture

The human neuroblastoma SH-SY5Y cell line was obtained from NCCS, Pune and cultured MEM: F12 supplemented with 10%FBS and 1X antibiotic at 37°C in 5% CO_2 incubator.

Cytotoxicity Assay

To determine relative cell growth and viability, the MTT assay [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] was performed. SH SY 5Y cells (8000per well) were plated in 96-well plates and incubated overnight for adhesion. Cells were treated by picropodophyllotoxin (PPP), IGF-IR inhibitor, at five different concentrations in triplicate for 24 hours. Cells were incubated with mixture of MTT: complete medium (1:10, v/v) at 37°C for 4 hours. The MTT-containing medium was discarded, and the formazan crystal was dissolved in 200µl of DMSO. OD was measured at 540 nm. The IC50 was calculated as the half maximal (50 percent inhibitory) concentration.

Scratch assay

Cells were cultured in 6 well plate to grow in a monolayer. A sterile pipette tip was held vertically to scratch a cross in each well. The detached cells were removed. Cells were treated by PPP and scratch closure was monitored. The distance migrated by the cells was measured as the difference in distance between the edges of the wound between the time points after 24 hours.

RNA isolation

NB cells were treated by IGF-IR inhibitor for 24 hours at 0.05uM and 0.016uM of concentration. Cells were scraped and centrifuged at 5000 RPM for 3 minutes. Pellet of cells were agitated in 200ul TRIzol® and volume was make up to 700ul with TRIzol. Cells were votex and 300µl of chloroform was added. Sample was centrifuged at 12000 RPM for 15 minutes at 4^{o}C, aqueous phase was collected and isopropanol were added. Sample was incubated for 1 hour at -20^{o} C then centrifuged at 10000 RPM for 10 minutes at 4^{o}C. Chilled ethanol was added to pellet and centrifuged at 10000 RPM for 10 minutes at 4^{o} C. The pellet was air dried for 10-15 minutes and dissolved in 20µl DEPC water.

Quantification of RNA

The BioSpecNanoDrop Spectrophotometer was used to calculate the total RNA concentration. The 260/280 ratio was used to measure purity of RNA and integrity of RNA was observed by 1.5% agarose gel electrophoresis.

cDNA Synthesis

The BIORAD iScript cDNA synthesis kit was used to reverse transcribe 1µg of total RNA into cDNA. 1µg RNA, 4µl iScript reaction mix, 1µl iScript reverse transcriptase, and 14µl nuclease free water made up the reaction mixture. The reaction mix was prepared at 25°C for 5 minutes before being reverse transcribed at 46°C for 20 minutes. It was then held at 95°C for 1 minute to heat inactivate the enzyme. Samples were stored at -20^{o}C for further experiments.

Primer designing

Primers were designed using Primer- 3plus software. Self-complementary of forward and reverse primer was analyzed and obtained from Xcelris. Primers were designed to meet the specific requirements for quantitative RT-PCR. Primers were also designed to produce an amplicon of approx. 100-200 bases in length.

Real time PCR

Real time PCR was performed on cDNA using Power up SYBR Green mix by ThermoFisher SCIENTIFIC and 0.1 µM each gene specific forward and reverse primers were added. The GAPDH gene was used as a reference gene to normalize the relative expression between the samples. Numbers of cycles were adjusted to obtain amplified DNA. The Ct value was derived by subtracting the Ct value GAPDH from that of the target gene. For each target gene Ct values were calibrated against control Ct values.

List of Primers

Gene name	Accession number	Sequence
IGF-IR-F	NM_000875.5	TGT TGA TCG TGG GAG GGT TG
IGF-IR-R		GCA AGG TCT CTG TGG ACG AA
PI3KCB-F	NM_006219.3	GAC TTT GCG ACA AGA CTG CC
PI3KCB-R		AGG TAT GCA TGG CCT CCT TC
AKT-F	NM_005163.2	GGC AAG GTG ATC CTG GTG AA
AKT-R		CGA CCG CAC ATC ATC TCG TA
BAX-F	NM_138764.5	GCC CTT TTG CTT CAG GGT TT
BAX-R		GGA AAA AGA CCT CTC GGG GG
Bcl-F	NM_001707.4	GCC CGA GAA CTA ATG GC TT
Bcl-R		CTC AGG GAC TCA CTC TGC TG
E. Cadherin-F	Z13009.1	CGG ACG ATG ATG TGA ACA CC
E. Cadherin-R		CCA CAT TCG TCA CTG CTA CG
Snail1-F	NM_005985.4	ACC CCA CAT CCT TCT CAC TG
Snail1-R		AGT TCT GGG AGA CAC ATC GG
Nanog-F	NM_001297698.2	GGT GAA GAC CTG GTT CCA GA
Nanog-R		AGGAGGGGAGAGGAAGGATT
Twist-F	NM_001271893.4	GCT ACA GCA AGA AGT CGA GC
Twist-R		GTC ACT GCT GTC CCT TCT CT
GAPDH-F	NM_001289745.3	GAC AGT CAG CCG CAT CTT CT
GAPDH-R		GCG CCC AAT ACG ACC AAA TC

PCR Reaction Mixture & Condition

PCR Reaction Mixture	
Syber green	5ul
Forward Primer	0.5ul
Reverse Primer	0.5ul
cDNA template	1ul
Nuclease free water	3ul

Stage 1(Hold Stage)	Stage 2(PCR Stage)	Stage 3(Melt curve)	Cycles
50.0 °C-02:00 mins	95.0°C-00:15 mins	95.0°C-00:15 mins	40
95.0 °C-10:00 mins	53.0°C-01:00 mins		

MicroRNA scoring

microRNA scoring was performed by mirDB.Specific microRNA was selected based on IGF-IR signaling pathway. microRNA where selected whose scoring was more the 80%. Another software, mirCancer was used to know the which microRNA are expressed in specific neuroblastoma either they are upregulated or downregulated on specific pathway they have targeted.

Sr.Number	microRNA	Target	Scoring
1.	hsa-miR-15-5p	Akt serine/threonine kinase 3	98
		Cyclin dependent kinase 17	89
		Phosphoinoside 3 kinase regulatory subunit1	88
2.	hsa-miR-223-3p	Insulin like growth factor I receptor	88
		Cyclin dependent kinase 17	89
3.	hsa -let 7a	Phosphoinositide 3 kinase regulataory subunit 4	98
		Insulin like growth factor I receptor	80

4.	hsa-miR-9-3p	Cyclin dependent inhibitor 1 C	84
		Phospatidylinositol 4,5 Bisphosphate 3 kinase	88

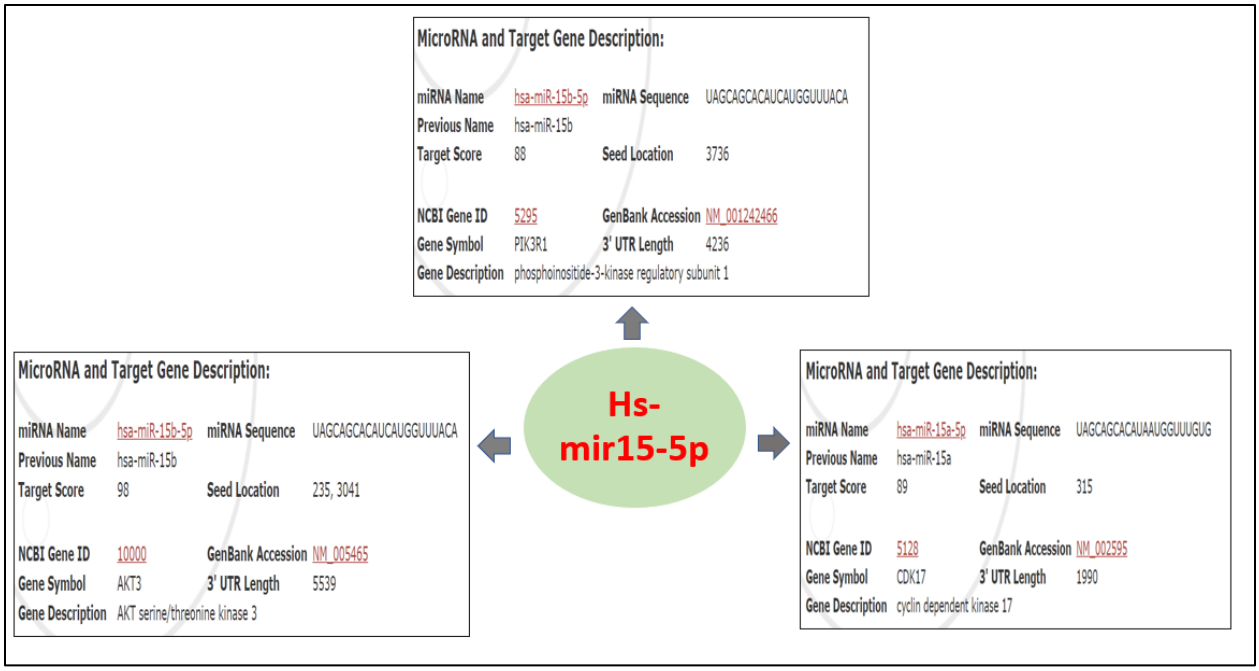
MicroRNA Primers

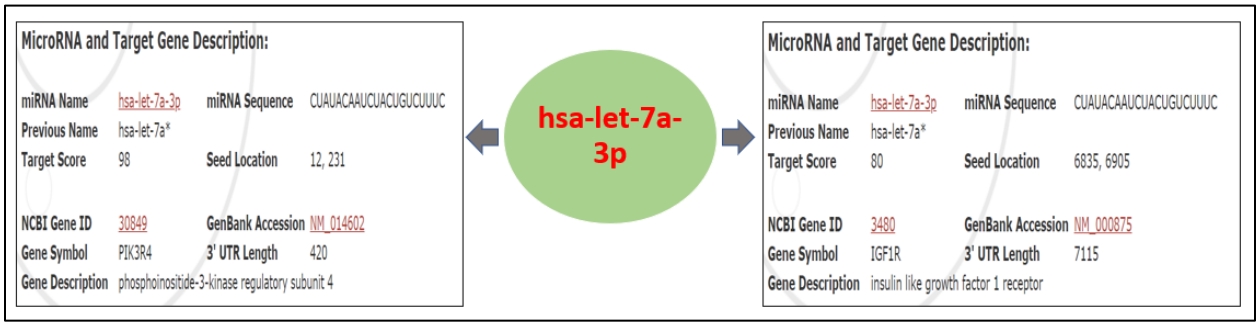
On the bases of scoring and docking results miRNA was selected. Primers were designed using sRNAPrimerDB.

Name	**Reverse Transcriptase Primer**	**GC**
hsa-miR-9-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCATAC	54
hsa-miR-15a-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCACAAA	54
hsa-miR-223-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGGGGT	58
hsa-let-7a-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACTAT	52

Primer for RT PCR

Name	**RTPCR Primer**	**GC**
Universal primers	GTCGTATCCAGTGCAGGGT	58
hsa-miR-9-5p	AGCGAGGCTCTTTGGTTATCTAG	47.83
hsa-miR-15a-5p	AACGCACTAGCAGCACATAAT	42.86
hsa-miR-223-3p	AACGGCTGTCAGTTTGTCAA	45
hsa-let-7a-5p	AAGCGACCTGAGGTAGTAGGT	52.38
U6	F- GCTTCGGCAGCACATATACTAAAAT	40
	R- CGCTTCACGAATTTGCGTGTCAT	48


MicroRNA and Target Gene Description:
miRNA Name hsa-miR-15b-5p miRNA Sequence UAGCAGCACAUCAUGGUUUACA
Previous Name hsa-miR-15b
Target Score 88 Seed Location 3736
NCBI Gene ID 5295 GenBank Accession NM_001242466
Gene Symbol PIK3R1 3' UTR Length 4236
Gene Description phosphoinositide-3-kinase regulatory subunit 1
Hs-mir15-5p
MicroRNA and Target Gene Description:
miRNA Name hsa-miR-15b-5p miRNA Sequence UAGCAGCACAUCAUGGUUUACA
Previous Name hsa-miR-15b
Target Score 98 Seed Location 235, 3041
NCBI Gene ID 10000 GenBank Accession NM_005465
Gene Symbol AKT3 3' UTR Length 5539
Gene Description AKT serine/threonine kinase 3
MicroRNA and Target Gene Description:
miRNA Name hsa-miR-15a-5p miRNA Sequence UAGCAGCACAUAAUGGUUUGUG
Previous Name hsa-miR-15a
Target Score 89 Seed Location 315
NCBI Gene ID 5128 GenBank Accession NM_002595
Gene Symbol CDK17 3' UTR Length 1990
Gene Description cyclin dependent kinase 17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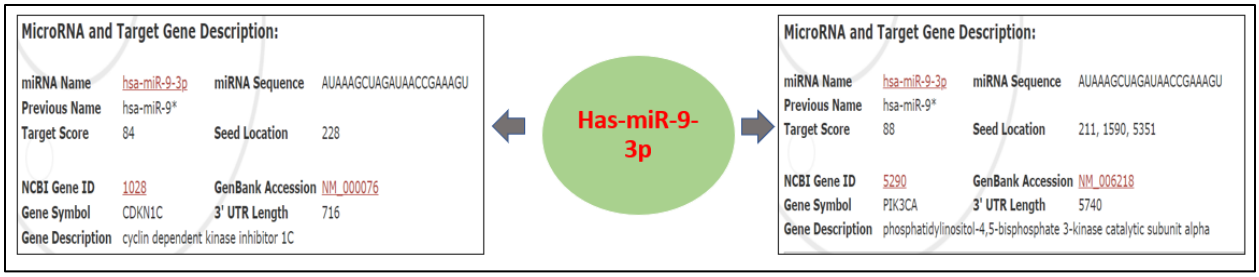
Previous Name hsa-let-7a*
Target Score 98 Seed Location 12, 231
NCBI Gene ID 30849 GenBank Accession NM_014602
Gene Symbol PIK3R4 3' UTR Length 420
Gene Description phosphoinositide-3-kinase regulatory subunit 4
hsa-let-7a-3p
MicroRNA and Target Gene Description:
miRNA Name hsa-let-7a-3p miRNA Sequence CUAUACAAUCUACUGUCUUUC
Previous Name hsa-let-7a*
Target Score 80 Seed Location 6835, 6905
NCBI Gene ID 3480 GenBank Accession NM_000875
Gene Symbol IGF1R 3' UTR Length 7115
Gene Description insulin like growth factor 1 receptor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Previous Name hsa-miR-9*
Target Score 84 Seed Location 228
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Gene Symbol CDKN1C 3' UTR Length 716
Gene Description cyclin dependent kinase inhibitor 1C
Has-miR-9-3p
MicroRNA and Target Gene Description:
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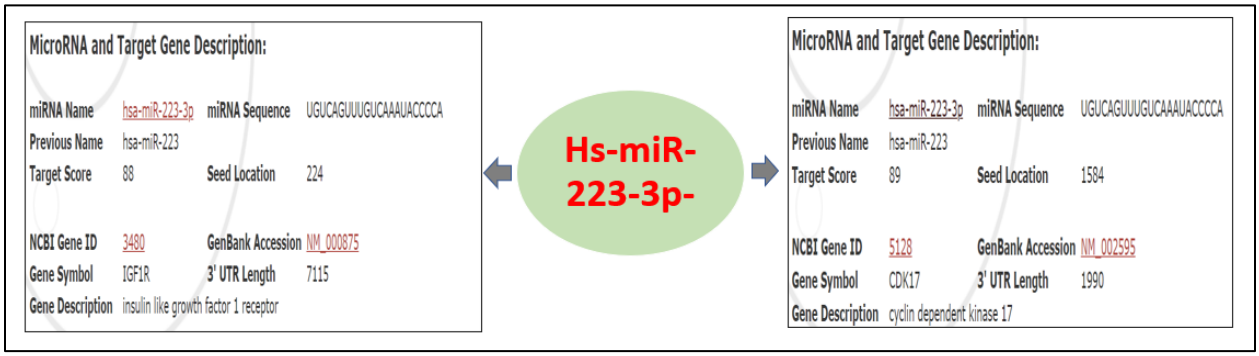
Previous Name hsa-miR-9*
Target Score 88 Seed Location 211, 1590, 5351
NCBI Gene ID 5290 GenBank Accession NM_006218
Gene Symbol PIK3CA 3' UTR Length 5740
Gene Description phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha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Previous Name hsa-miR-223
Target Score 88 Seed Location 224
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Gene Symbol IGF1R 3' UTR Length 7115
Gene Description insulin like growth factor 1 receptor
Hs-miR-223-3p-
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miRNA Name hsa-miR-223-3p miRNA Sequence UGUCAGUUUGUCAAAUACCCCA
Previous Name hsa-miR-223
Target Score 89 Seed Location 1584
NCBI Gene ID 5128 GenBank Accession NM_002595
Gene Symbol CDK17 3' UTR Length 1990
Gene Description cyclin dependent kinase 17

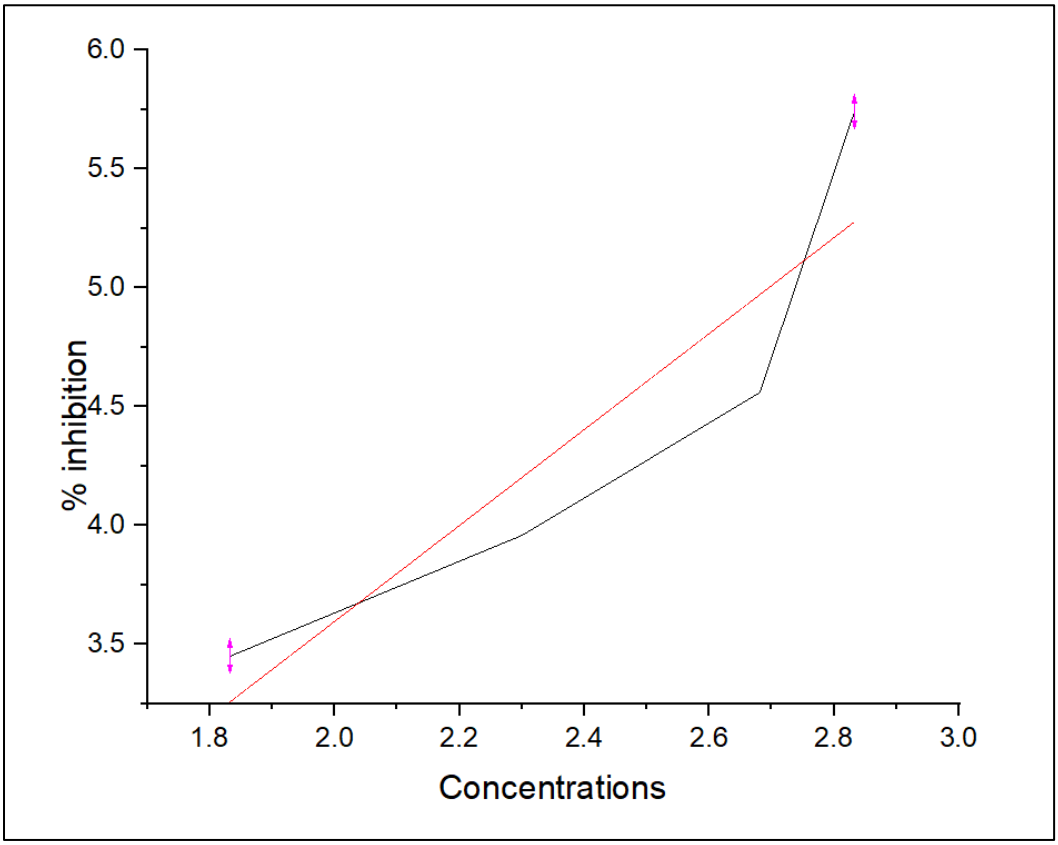
Western Blotting

Cells lysate was prepared in RIPA buffer and Protein concentration was measured by BCA method. Separation of protein was done by SDS-PAGE then electroblotted onto PVDF membranes. After blocking for one hour in BSA, a membrane was incubated with primary anti IGFIR antibody for overnight. Exposed to secondary HRP conjugated antibody for two hours detection of chemiluminescence of targeted protein will be performed thereafter.

RESULTS AND DISCUSSION

Cytotoxicity assay

SH SY 5Y cells were incubated with different concentrations of IGF-IR inhibitor PPP for 24 hours in triplicate wells, and IC50 value was calculated. Calculated IC50 value was 0.501 uM (501nM).



Graph 1: Represents Percent inhibition vs concentration of PPP after 24 hrs of incubation

Scratch assay

Figure 4 indicates that PPP treated cells inhibit the scratch closure. After 24 hours of incubation with PPP, rate of cell proliferation was observed significantly low which was resulted in gap with formed scratch. The Observed scratch closure of SH SY 5Y non treated cells was 1.454 mm whereas the closure was 1.885 mm in treated cells. PPP might target tubulin and inhibits polymerization of microtubule which inhibits cell proliferation (Choi et al., 2015; Li et al., 2019). This might resulted into high gap of scratch closure in drug exposed cells.

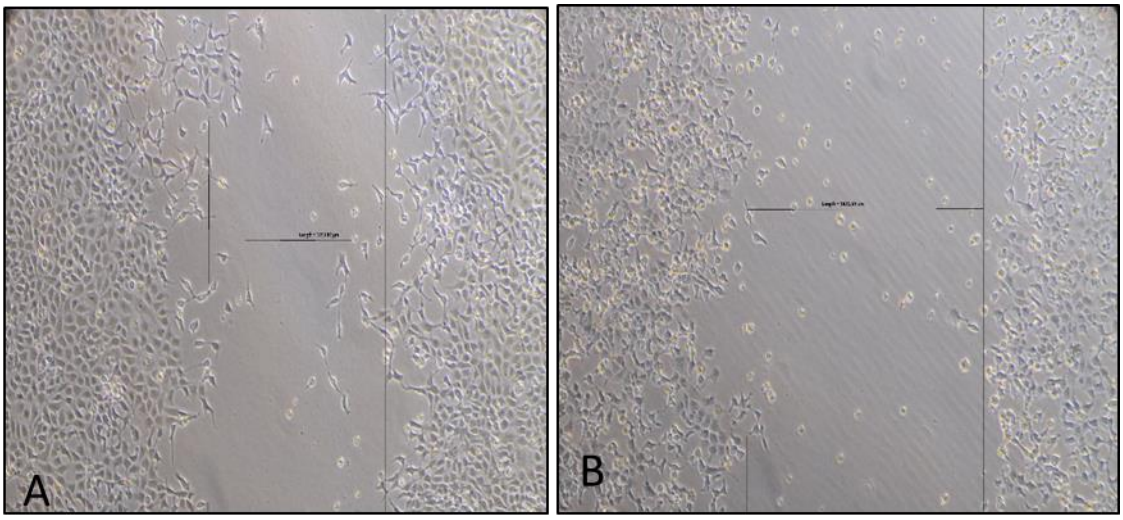


Figure 4 Scratch closure in SH SY 5Y untreated and drug exposed cells after 24 hours

Gene Expression analysis

IGF1R, a well-known tyrosine kinase receptor, regulates various normal developmental pathways and malignant properties, such as proliferation, migration, invasion, and chemoresistance across several types of cancer (LeRoith 2011). SH-SY5Y cells were treated with PPP for 24 hours. After the exposure of PPP, it was observed that IGF-IR transcript expression was down regulated (0.0093 fold change) on expression of PPP to SH SY 5Y (Figure 5). The resulted alteration was observed in translation level where protein expression value was also measured low after PPP treatment of 24 hours.

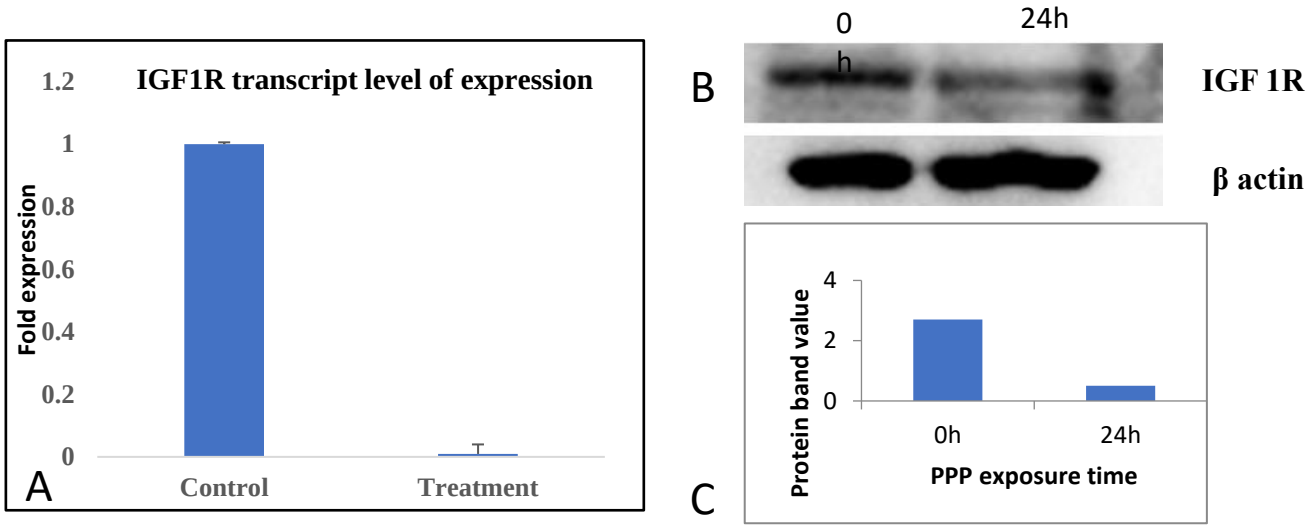


Figure 5: Expresesion pastern of IGF 1R after 24 hours of PPP treatment A) Transcript level of fold change, B) western blot result on protein level of expression change, C) Value of protein band

IGF 1R receptor inhibitor, PPP, is ATP competitive inhibitors might inhibit the activation of insulin receptors due to the high homology of ATP binding sites of IGF-IR and IRK (Favelyukis et al., 2001; Bao et al., 2010). PPP inhibits Tyr1136 phosphorylation in the activation loop of IGF-IR kinase, which contributes to stabilize the conformation of the activation loop ((Eva Surmacz 2000; Menu et al., 2006). It has been shown that PPP also down-regulates IGF-IR leading to the apoptosis (Girnita et al. 2004; Vasilcanu et al., 2004; Bao et al., 2010).

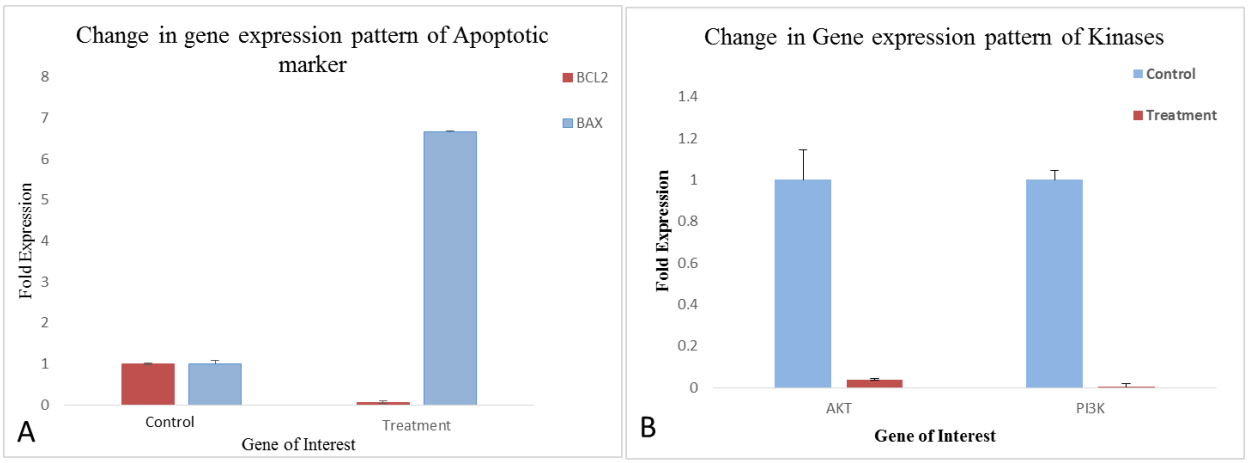


Figure 6: Change in transcript level after 24 hours of PPP treatment to SH SY 5Y A) Altered expression of mitochondrial membrane potential marker; B) Change in transcript expression of Kinases

PI3K 3CA (phosphatidylinositol 3-kinase, catalytic, α-polypeptide), the gene encoding the p110α subunit, are frequently mutated or amplified in the most common cancers, such as breast cancers colon cancer, gastric cancer, cervical cancer, prostate cancer, and lung cancer (Yang et al., 2019). The transcript expression of AKT was observed down regulated by 0.0389 fold change and PI3K was down regulated by 0.0073 fold change. The percent inhibition of AKT expression was 96.11. Kinases like AKT and PDK1 binds to the lipid products of PI3K and thereby localize to the cell membrane to activate cell growth and cell survival pathways (Manning and Cantley 2007). On Tyr1136 of IGFIR dephosphorylation might resulted into dephosphorylation of kinases which may inhibit the cascade of cancer progression. Altered phosphorylation pattern of PI3K and AKT on treatment of PPP, inhibits cellular proliferation and trigger apoptosis through cell cycle arrest. PPP has been shown to associate with inhibition of the phosphatidylinositol 3-kinase/Akt and vascular endothelial growth factor pathways and activation of the extracellular signal–regulated kinase pathways (Vasilcanu et al. 2004; Economou et al. 2008).

Epithelial mesenchymal transition (EMT) initiates and facilitates the invasion-metastasis process of human cancer. Mesenchymal gene expressions are key regulator for invasion of cancer. Down regulation of ECM and up regulation of mesenchymal molecules are commonly observed in cancer progression. (Katoch et al., 2021). Result of present study represents that the altered EMT transition pattern on PPP exposure that might be resulted in to anoikis and leads to programmed cell death. Altered mitochondrial membrane potential is one of the markers for apoptosis which is supported by the expression pattern of Bax and Bcl2 transcript after 24 hours of PPP exposure. Bcl2, anti-apoptotic marker expression was observed downregulated (0.0618 fold change, 99.32% inhibition of transcript level) whereas Bax expression was observed up regulated (6.6 fold expression change) (Tsujimoto 1998; Porter and Jänicke 1999; Choi et al. 2014).

Another genes are also hampered by the treatment of PPP for 24 hours such as snail, Nanog,Twist and Bax. As compared to control snail,Twist and bax are upregulated. The fold change increased for twist is 2380.73 ,for snail 8203 and for nanog it was 449.19. Snail, Twist and Slug are potential regulators of cell adhesion and migration. Slug, Snail, and Twist are

transcription factors that regulate the expression of tumor suppressors such as E-cadherin.(Martin et al. 2005)

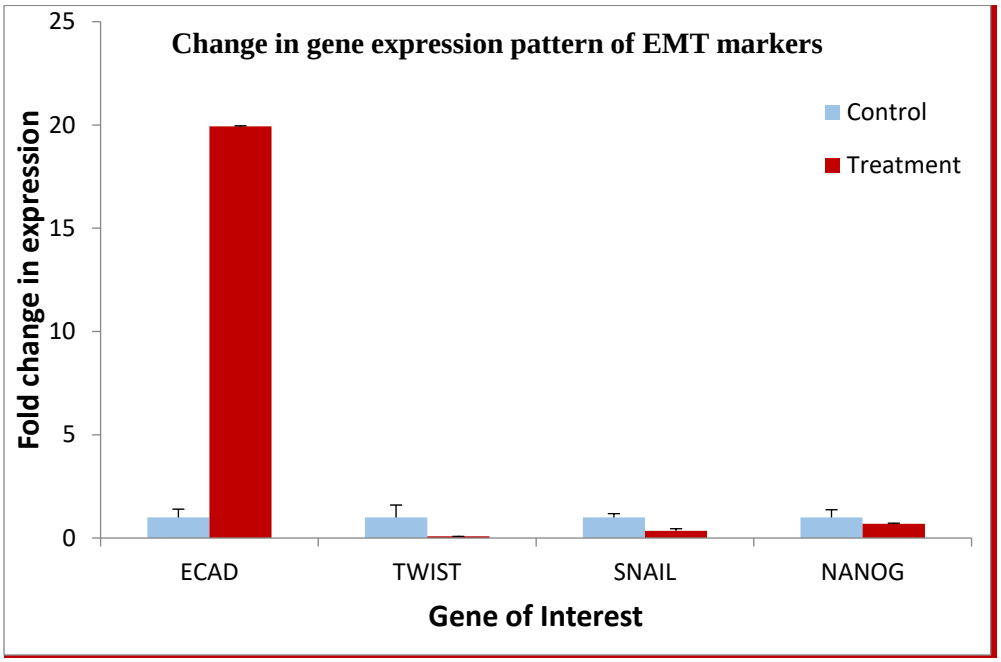


Figure 6: Gene expression pattern of EMT markers after 24 hours treatment of PPP to NB cells

The SH-SY5Y cells were given the PPP treatment, which resulted in lower Bcl 2 levels and greater Bax levels. Bcl2 and Bax mRNA transcript levels were investigated. The proteins Bcl2 and Bax are responsible for maintaining the permeability of the mitochondrial membrane. In cancer, Bcl2 is an antiapoptotic protein whose excessive expression makes cells resistant to cell death. Activated Bax oligomerizes to form pores in the mitochondrial outer membrane, resulting in the release of cytochrome c. Released cytosolic cytochrome c leads to caspase activation and subsequent cell death (Tsujimoto 1998; Porter and Jänicke 1999; Choi et al. 2014).

Conclusion

The current research highlights one of the pathways to suppress IGF-IR signaling to induce tumor regression neuroblastoma. The selective inhibitor of IGF-IR, PPP was able to inhibit the IGF-IR signaling in SH-SY5Y cells and cells morphology was disturbed after the exposure of PPP to the SH-SY5Y cells.

The results were confirmed by the change in gene which are hampered after the treatment. IGF-IR and other kinases such as PI3K and AKT were downregulated. Different cell adhesive markers such as E.Cadherin, snail, nanog and twist were hindered. Overexpression of IGF-IR was observed in different cancer such as breast, lung, colon prostate and bone cancer, therefore suppressing the expression the of IGF-IR can also hamper the other signaling molecules in IGF-IR pathway. Wound healing suggests the migration pattern was changed after the exposure of PPP. Apoptotic activator gene Bax was significantly upregulated after the treatment with the selective inhibitor of IGF-IR, leading the cells towards apoptosis. Anti-apoptotic gene BCL 2 was downregulated which is again a favorable phenomenon in tumor regression. Protein expression was low when treated with PPP for 24 hours suggesting that PPP can suppress the IGF-IR signaling

Neuroblastoma is an aggressive childhood cancer that is often resistant to the most aggressive chemotherapeutic and surgical interventions. Neuroblastoma is a heterogenous disease and thus specific target treatment is required since the present cure rate is very low This study can help to provide specific target therapy to the neuroblastoma patients.

Thus, targeting IGF-IR by treating it with specific inhibitors PPP can lead to apoptosis of tumour cells led to regression of Neuroblastoma and can thus serve as a novel therapeutic.

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Date: 02/09/2021 **Signature of the candidate**

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Guide