5 SUMMARY

Cancer is characterized by uncontrolled cell growth. Due to these property, cells subsequently acquire invasive properties and the disease manifests itself from benign stage to incurable metastatic stage (Ayob and Ramasamy, 2018). There are few cancers those have embryonic origin, neuroblastoma (NB) being one of them. NB is a cancer that arises from the neural crest cells. Neuroblast cells of adrenal medulla differentiate into chromaffin cells, adrenergic neurons, schwann cell etc. Neuroblast cells remain undifferentiated and turns into NB cancer. It is observed that to resemble the post-mitotic character of neurons, NB cells are differentiated by different terminal differentiation agents. It has been reported that differentiated NB cells are more neuronal-like based on their changes in morphology, reduced cell proliferating rate, and expression of neuronal markers.

It is known that aberrant expression of growth factors with unstoppable phosphorylation of kinases, results in NB cells attaining metastasic neoplasmic character which is incurable. IGF1 (insulin-like growth factor 1) is one of the growth factor. It is proved that IGF-1R through the activation of PI3K/AKT and MAPK signals causes invasive properties of tumour (Graham et al., 2008; J. Ma et al., 2010b). In vitro finding of Chunfang et al (2019) suggested that amplification of PI3K, AKT and ERK promotes the proliferation and invasion of cancer cells whereas inactivation of these kinases altered EMT and leads to cancer regression (C. Wang et al., 2019). Oncogenic network mediated IGF-IR/PI3K/AKT signalling causes constitutive activation of YAP1 signalling, which in turn plays a fundamental role in cell proliferation and survival of tumour (Berthold et al., 2022).

Overexpression of myc has been shown in resistant NB cancer. Some studies also demonstrates that the myc oncogene also plays a crucial role in the stemness traits, especially self-renewal and multilineage differentiation which turns into aggressive NB. Attenuation of IGF-IR is associated with downregulation of myc (Wijesinghe et al., 2021). In addition, the overexpression of myc in tumors is linked to a poorer prognosis for NB patients. IGF IR inhibition promotes apoptosis through the downregulation of myc and other antiapoptotic pathways including bcl-2 and the potentiation of proapoptotic signals such as p53 (Sa and Das, 2008; Prakobwong et al., 2011; Hosseini, Zand and Cheraghpour, 2019).

p53 expression and epithelial to mesenchymal transition is a correlative phenomenon. On EMT transition, p53 expression is downregulated. MYC and MDM2 (murine double minutes 2) amplification is common phenomenon that leads inhibition of p53 (Chen et al., 2009). p53 is a main safeguard as a tumour suppressor that protects cells against genome instability and malignant transformation. Inhibition of myc/mdm2 expression elicits overexpression of p53 that leads to tumour regression (Zafar, Wang, Liu, Xian, et al., 2021).

Reduction of IGF expression is concomitant with increased levels of differentiation markers. Conversely, markers of epithelial-to-mesenchymal transition (Vimentin, Snail-2, Zeb1, Zeb2 and N-Cadherin) and stemness (OCT-4, SOX-2, ABCG2 and Nanog) is decreased (Vella et al., 2019). Regulation of IGF-1-PI3K/Akt-Bax/Bcl-2 pathway-mediated gene expression controls mitochondrial dependent apoptosis (Zhang et al., 2020).

Not only mRNA expression levels but miRNA is also associated for cancer pathogenesis. Plethora of biological activities are regulated by miRNA via modulation of expression of target genes. Each cancer has a clear miRNA signature that is so-called “miRNome”, which is specific for every tumour type and is associated with clinical-pathological features of the tumors (Ding et al., 2020; Catellani et al., 2021). Up-regulation of miR-5094 is associated with decreased transcription of

down-stream Igf-1 and Bcl-2 (Ding et al., 2020). miRNA-338-3p in breast cancer cells activates the receptor of IGF-1 and promotes breast cancer angiogenesis through IGF-1/IGF-1R/ERK pathway (Zhang et al., 2020). In cancer the migration, invasion, proliferation and inhibition of apoptosis in vitro and in vivo is enhanced by exo-miR-15b-3p, by cleaving Caspase-9 and Caspase-3 expression (Wei et al., 2020).

Findings of Li et al., 2018, identified a negative feedback regulation between let-7e and IGF-1/IGF-1R, suggesting that let-7e could be used in IGF1R-targeted therapeutics in anticancer therapy (Li et al., 2018). Analysis of Gérard et al., 2019 shows that the switch from a quiescent to a proliferative state depends on the relative levels of Let-7 and several cell cycle activators including CDK (Gérard, Lemaigre and Gonze, 2019). Upregulation of IGF-1R reversed the inhibitory effect of Let-7g-5p on epithelial-mesenchymal transition (Zhao et al., 2019).

Post-transcriptional level of mRNA is regulated by miRNA through inhibition of translation. Expression of miRNA is altered during cancer pathogenesis. Tumour suppressors are downregulated whereas oncogenic activators are up regulated. If any drug, via inhibition of target specific kinase, is capable of balancing the miRNA levels like the healthy cells, it has much promising anticancer activity as well as it can show inhibitory effect to other kinase phosphorylation.

Picropodophyllotoxin is plant based IGF-IR specific inhibitor whose overexpression is noticeable in neuroblastoma cancer. PPP is a small molecule inhibitor of the IGF-IR with potential antineoplastic activity. As PPP is a plant-based compound, one can postulate that its toxicity is less in comparison to other chemotherapy drug like cisplatin, fluorouracil. For cancer like NB where patients are children, it is important to develop a drug which is originated from plant with promising effect of cancer regression. Therefore, we aimed to examine how inhibition of IGF-IR

modifies tumorigenic signatures pattern of neuroblastoma cells with a general goal to understand the role of PPP as potential therapeutic target for NB tumour.

In vitro cell culture-based techniques were used to analyse the effect of PPP as IGF-IR inhibitor on NB cell line, SH-SY5Y. The IGF-IR is a transmembrane protein whose cytoplasmic tyrosine kinase domain activates the PI3K/AKT and MAPK signalling pathways (Clemmons, 2007). Research on PPP and selective inhibition of IGF-IR is proved in many cancers where candidature of IGF-IR amplification is first. But in NB, ALK is the first transmembrane candidature for tumorigenesis. Hence, research proposed to evaluate PPP affinity to bind with ALK as PPP is ATP binding competitor. Docking result of ALK shows more binding affinity (-8.8 kcal/mol) to PPP in comparison to IGF-IR (-7.5 kcal/mol). These result was further supported by gene and protein level expression.

Expression of IGF-IR was assessed at transcript and protein level as well as localization was done after PPP treatment. Transcript level of expression was observed 0.093±0.003 fold decreased. Protein expression was also found 0.348±0.003 fold decreased. Localization of IGF-IR protein was measured using ICC and fluorescent intensity was analysed using ImageJ software wherein expression of IGF-IR by PPP shows lower expression at cellular level in SH-SY5Y cell line.

Owing to the high degree of ATP binding site homology between the tyrosine kinase domains of the IGF-1R and IR (Insulin receptor), the design of small molecule inhibitors with strict selectivity for IGF-1R is must because IR is vital protein to regulate glucose metabolism and cell survival. If selective inhibitor of IGF-IR binds to IR, imbalance of glucose metabolism enhances aberrant cell death. We have studied IR expression of protein on PPP treatment. Result suggests there is basal level of low expression of IR which was non-significant.

ALK is known surface transmembrane receptor which is amplified in neuroblastoma cancer. Resistance and cancer aggression is much common in ALK dependent NB cells. We have selected

ALK for the study as molecular docking result suggested higher binding affinity of PPP at its ATP domain. On exposure of PPP to SH-SY5Y cells, mRNA expression was assessed downregulated by 0.624±0.034 fold. Protein level expression was analysed using western blot and 0.70±0.032 fold decreased expression was noted. This result proved that PPP has binding affinity with ALK and can work as dual inhibitor for ALK and IGF-IR receptors.

The IGF-1R/IR/PI3K/AKT pathway is aberrantly activated in many cancer and can promote cell proliferation and chemotherapy resistance (Davaadelger et al., 2016). We have examined transcript level expression of PI3K, AKT and CDK kinases after IGF-IR inhibitor treatment. Expression of PI3K, AKT and CDK was fold downregulated by 0.07433±0.0043, 0.04±0.0045 and 0.036±0.0047 respectively. PI3K/AKT pathway is an intracellular signal transduction pathway that promotes proliferation, cell survival, growth and angiogenesis in response to IGF-IR signalling (Yin et al., 2010; Hua et al., 2021; Xue et al., 2021). Activation of IGF-IR tyrosine kinases results in autophosphorylation of tyrosine residue of PI3K/AKT. PI3K/AKT phosphorylation inactivates pro-apoptotic factors such as BAD, BAX and procaspase-9. Inactivation of proapoptotic factor in cancer cells results in cancer cells acquiring resistance against death (Au-Yeung et al., 2017; Thorpe et al., 2017; Shorning et al., 2020). There is abundant cross-talk between IGF-IR and PI3K/AKT pathways in cancer progression, modulation of this crosstalk on exposure of PPP alters the immortal state of cancer cells and leads to programmed cell death.

It has been shown that PI3K/Akt signalling pathway significantly elevates the half-life of MYC through negative feedback. Mitogenic stimulations can promote production and stability of Myc and activation of Ras which increases MYC protein stability by ERK-mediated pathways (Sears et al., 2000; van der Noord et al., 2019; Ahmadi et al., 2021). PI3K downfold expression is in support of MYC down fold expression that suggests antiproliferative activity of PPP via suppression of MYC expression on down stream of IGF-IR signalling. PI3K is upstream regulator

of NFκB. p65 subunit of the NFκB translocate and promotes the activity gene those regulates cell proliferation. In present study, transcript level of NFκB expression was observed down regulated (Fig. 3.8).

Transcription factor NFκB subunit translocates to nucleus and regulates cell division. CDKs are key regulatory enzymes involved in cell proliferation through regulating cell-cycle checkpoints. NFκB regulates CDK activity (Guttridge et al., 1999; Tchakarska and Sola, 2020). Autophosphorylation of CDK dysregulates cell cycle that leads to tumorigenesis. We have observed down regulation of CDK gene along with inhibition of IGF-IR expression. Tubulin polymerization is one of the important event for mitosis of cell. Tubulin dynamic is regulated with growth factor receptor. IGF-IR is proliferative signaling. IGF/IGF-IR binding passes the signal for cell division on downstream pathways. We have assessed low level of transcript expression of tubulin on exposure of PPP.

Impeded function of p65 subunit of NFκB, suppresses the oncogenic function of NFκB and amplifies the expression of p53 and induces apoptosis in cancer cells (Kong et al., 2020). On inhibition of IGF-IR activity by PPP, transcript level expression of p53 was observed 5.625±0.156 fold increased that suggests loss of power of uncontrolled cell proliferation.

To assess the functional significance of PI3K/AKT pathway in metastasis of cancer induced by insulin or IGF-1 treatment, we also aimed on EMT-related biomarkers to observe anoikis. It is known that IGF-1 is sufficient to down-regulate E-cadherin and up-regulate SNAIL, TWIST and NANOG in NB cancer for its aggressiveness (Zheng et al., 2019). However, when the cells were treated with PPP, the upregulation of E-cadherin and the down regulation of SNAIL, TWIST and NANOG was observed. Results suggested that IGF-IR signaling modulation on exposure of PPP reduced EMT via PI3K/AKT signaling pathways in SH-SY5Y cells.

Downstream of IGF-IR signaling is associated with PI3K/AKT signaling and regulated via non coding miRNA. miRNAs perform important role in post-transcriptional gene regulation. miRNAs

primarily affect gene expression levels via targeting mRNA. The canonical role of miRNA is to influence mRNA via recognition sites in the 3′ untranslated region (UTR), which regulates their stability. Therefore, the relative levels of miRNA, and its effect on mRNA, have a major role in carcinogenesis (Carthew and Sontheimer, 2009; Hill and Tran, 2021). In current study, on exposure of 16nM dose of PPP for 24 hours to SH-SY5Y cell line, miR-223, let-7 was upregulated whereas expression of miR-9 was downregulated.

On exposure of PPP for 24 hours to SH-SY5Y, the acquired characteristic of EMT is lost by cancer cells and they gain MET character. This reduces the rate of proliferation of tumor cells. This phenomenon was observed using scratch assay. Gap was formed when cells were confluence and treated for 24 hours with PPP at 16nm concentration. Inhibition of cell proliferation of NB cell due to PPP treatment resulted in an uncovered gap. This suggests, PPP exposure altered IGF-IR signaling that reduced cell proliferation and induce NB regression.

Upregulation of E cadherin and downregulation of mesenchymal gene inhibits invasive ability of cancer cells and decreases cell proliferation that leads to anoikis. Akt activation is a direct consequence of E-cadherin loss and cancer metastasis (Derksen et al., 2006; Teo et al., 2018). Our result supports this phenomenon of anoikis. During cell death, mitochondrial membrane potential is altered which modulates the expression of BCL2 and BAX. Cancer is characterized by increased activity of BCL2 and low level of BAX. On exposure of PPP to NB cells, expression of BCL-2 was found low whereas the expression of BAX was observed 9.244±0.1359 fold increased. This result is also supported by the data of FACS and AO/EtBr where apoptotic event was prominent on inhibition of IGF-IR signaling. FACS data suggested 49.17% cell death due to PPP exposure. Early and late apoptotic cells were observed prominent in compare to control when treated by PPP (Fig. 3.15).

PPP exposure inhibits phosphorylation of IGF-IR as well as ALK and alters the phosphorylation of PI3K/AKT kinases. On inhibition of kinases activity, downstream proliferation and survival

transcription regulator transcription factor MYC and NFκB expression was down regulated. Due low expression of CDK and TUBULIN, microtubule dynamics was altered. Considering all these molecular cross talk, NB cells lost their mesenchymal characteristics as well as lost the property of uncontrolled cell division. In response, these cells underwent anoikis. Loss of mitochondrial membrane potential via suppression of BCL2 activity that leads to apoptosis is confirmed using FACS and AO/EtBr staining.

In conclusion, our result suggests PPP a known potent inhibitor of IGF-IR has also binding affinity with ALK. Along with altered activity of these two RTK, change in activity of PI3K/AKT was also modulated that alters the oncogenic cross talk and imply apoptosis in NB cells.