CHAPTER 5

DISCUSSION

5.1 In-silico analysis

In silico analysis is a computational analysis that involves different database utilisations, such as DIANA TOOLS, pSRNATarget, TargetRank and RNA22. These databases are used to determine miRNA target genes, miRNA folding energy, heteroduplex structure, and miTG score.

From the analysis, it was observed that ten miRNAs of human and plant sources were able to interact with the *ABL1* gene of CML. Manifestation of the Philadelphia (Ph) chromosome with the translocated genes of *BCR-ABL1* is the main feature of CML. Hence, *ABL1* was chosen as our primary target gene due to its critical role in the CML pathway and pathogenesis. From Table 4.1, five plant miRNAs identified were the cpa-miR8154, mtr-2654, sof-miR59e, osa-miR1858 and osa-miR1858b. These plant miRNAs were observed to have a strong relation with *ABL1* with a psRNATarget expectation score of 4 to 4.5 out of the maximum score of 5 (Dai & Zhao, 2011). The folding energy of -24.4 kcal/mol for osa-miR1858a/b derived from plants (*Oryza sativa*), indicates that these miRNAs possess a more stable secondary structure (Saiyed et al., 2023).

Meanwhile, it was revealed that hsa-miR-891a-3p (Table 4.2) has the highest miTG score of 0.99 from DIANA TOOLS, indicating a higher chance of correct prediction (Maragkakis et al., 2009). However, another miRNA, hsa-miR-3131, demonstrated outstanding folding energy of -24.6 kcal/mol and a miTG score of 0.97.

As a result, miR-891a-3p, miR-3131, and osa-mir1858a/b were chosen due to their excellent association with the *ABL1* gene of CML. Subsequently, these three miRNAs were submitted for further analysis to assess gene enrichment and associated pathways.

Gene enrichment analysis of miR-891a-3p (cluster 8) showed biological processes related to post-transcriptional gene silencing by RNA with cellular compartments associated with RISC-loading complex and endoribonuclease complexes (Table 4.7). Post-transcriptional gene silencing is the term used to describe the silencing of genes at translational levels. Genes that are involved in the mentioned processes are NRDE, AGO3, AGO1 and RAN. miRNA is a short nucleotide that is attached to the complementary mRNA target sequence at the 3'UTR. MiRNA can suppress translation or degrade the complexes, thus preventing the mRNA from being translated.

Because of these features, miR-891a-3p was assumed to be able to target the *ABL1* gene by interacting with AGO proteins such as AGO3 and AGO1. The AGO protein functions by preventing the degradation of RNA, favouring one strand of a miRNA over the other, spatially organising the miRNA to bind complementary sequences, and assisting in the identification and detection of RNA targets within cells. Specifically, AGO3 can promote cleavage of target RNA in situations where the match between miRNA is entirely complementary (Kilikevicius et al., 2022). Still, most miRNAs can inhibit protein translation with just a partial complementary match because of the seed region, which is located at positions 2-7 or 8 on the miRNA. These main factors control miRNA-mRNA target recognition (O'Brien et al., 2018).

Furthermore, RAN belongs to the RAS oncogene family. This gene controls DNA synthesis, cell cycle progression, and the translocation of RNA and proteins through the nuclear pore complex. Its expression is typically elevated in cancer. Therefore, miR-891-3p's ability to target this gene may be advantageous in limiting the growth of CML cells. Additionally, for each miRNA, the first-degree interaction

partners (first neighbours) of ABL1 were identified from the protein–protein interaction network to filter genes that are closely associated with ABL1 and may be involved in key biological processes. MiR-891a-3p *ABL1* first neighbour nodes demonstrated critical pathways such as MAPK signalling, PI3K-Akt, Ras signalling, apoptosis, and microRNA in cancer. As a result, mir-891a-3p shows a high level of potential for involvement in critical processes aimed at CML's *ABL1*.

Furthermore, for plant miRNA, osa-miR1858a/b gene enrichment analysis did reveal intriguing and significant gene ontologies and pathways. Cluster 3 of osa-miR1858 a/b was observed to be involved in multiple key pathways, including FoxO, PI3K-Akt, ErbB, MAPK, Wnt, Jak-Stat, p53, Ras signalling, and, most significantly, CML and cell cycle pathways. *ABL1's* first neighbours of osa-miR1858a/b also demonstrated important cancer-related pathways, including focal adhesion, Ras signalling, microRNA in cancer, and many others. These results for osa-mir1858a/b's cluster 3 and *ABL1* first neighbours indicated that this plant miRNA might have a significant impact on CML cell viability and proliferation. This is because signalling pathways such as PI3K-Akt and MAPK promote tumour growth and are accountable for cell differentiation and proliferation. Therapeutically targeting the effectors of these pathways is a highly promising approach (Kim & Choi, 2010; Noorolyai et al., 2019).

On the other hand, miR-3131 did not appear to be associated with many cancer-related biological processes or pathways. The clustering revealed two clusters, but only cluster 2 produced gene enrichment data, primarily related to cell adhesion molecules and localised at the cell surface and plasma membrane. The genes associated with mir-3131 cluster 2 include NLGN3, CNTNAP2, and IL1RAPL1. These genes play a role in neurodevelopmental and neuropsychiatric disorders by affecting synapse formation, signalling, and neural network stability, which are essential for normal brain function (Liu et al., 2022; Ramos-Brossier et al., 2014; St. George-Hyslop et al., 2022). Although

there is no direct link between these genes and CML, there may be a potential association between the functions of NLGN3 and CNTNAP2 in cell signalling and adhesion, as well as the advancement and spread of tumours.

Experimental validation is always necessary to confirm the findings of *in-silico* analysis. The presence of many algorithms across multiple platforms leads to a high occurrence of false-positive results, which in turn makes in-silico analysis data susceptible to biases. Therefore, we performed additional validation to demonstrate that the chosen miRNA had a specific impact on CML cell lines, specifically the K562-s and K562-r.

5.2 MiRNA Transfection Efficiency and Binding Site Validation

Transfection efficiency can be validated by determining the intracellular miRNA level following the transfection of exogenous miRNA mimics or inhibitors. One of the widely used techniques for determining the amount of miRNA in cells following transfection is qPCR (Thomson et al., 2013).

TaqMan probe measurements of the transfected miRNA (Figures 4.10 and 4.11) showed an increase of all miRNAs of >1000-fold, which was significantly higher than the most abundant endogenous miRNAs. Such a marked increase is likely due to the efficiency of the lipid-based transfection method used in this study (Lipofectamine), which enables intracellular miRNA levels to rise to thousand-fold. This high delivery efficiency is consistent with previous reports showing that lipofectamine-mediated transfection is straightforward and highly reproducible (Wang et al., 2023). Moreover, previous studies observed that microRNAs introduced into cells via Lipofectamine or RNAi-max localised predominantly within the cells or near lysosomes (Jin et al., 2015; Wang & Wu, 2009). The observed elevation in miRNA levels following qPCR analysis

is thought to be the result of miRNAs remaining in vesicles and subsequently being amplified via qPCR after lysis (Thomson et al., 2013).

Figures 4.10 and 4.11 also show that miRNA expression levels varied between cell types. In K562-s, miR-3131 expression exhibited the highest level, whereas in K562-r cells, osa-miR1858a/b expression was the highest. This is because transfection efficiency is strongly influenced by cellular conditions, transfectant concentration, the type of transfection reagent, and the execution of the transfection procedure (Wang et al., 2023). Nevertheless, it was observed that each miRNA level was elevated more in k562-r compared to k562-s.

This result demonstrates that the miRNA transfection in k562-r was better than that of k562-s. There are several potential causes for this, including cellular traits, cell adhesion abilities, cell membrane conditions, and the existence of receptors on the surface of the cell (Kim & Eberwine, 2010). Moreover, adhering to a surface may activate cellular pathways that increase the uptake of foreign molecules, including miRNA, due to the proximity between cells. According to Wang et al. (2021), cell lines that exhibit more adhesion to the culture plate tend to improve transfection efficiency, and interestingly, Kaehler et al. (2022) have shown that cells resistant to tyrosine kinase inhibitors (TKIs) possess this characteristic. Additionally, Jo et al. (2020) and Windisch et al. (2019) found that integrins are one of the transmembrane proteins that help cells adhere to their targets by forming groups of focal adhesion proteins connected to the cytoskeletal framework. However, even though the miRNA level inside both cells is significantly higher, it does not indicate miRNA's functionality. Simply said, even though qPCR is a valid technique to measure total miRNA amount, this did not measure the actual amount of functional miRNA.

Hence, validation of miRNA-mRNA binding is required to further validate and confirm miRNA-mRNA target interaction. The current study utilised the biotin pull-

down assay to validate miRNA-mRNA target binding. Two out of three miRNAs could proceed through this method except for the osa-miR1858a/b due to the inability to prepare the biotin tag of plant miRNA by the supplier.

From the results of Figure 4.14 and Figure 4.15, miRNA target binding was successfully detected only in K562-r cells. In these cells, the expression of the 3'UTR of *ABL1* was significantly higher in the treated group compared to the control groups for both miRNAs. This suggests that the miRNA–mRNA interaction may be more prominent or accessible in the resistant cell line compared to the sensitive counterpart. These findings parallel with previous studies where miR-192 and miR-34a target genes were successfully identified in porcine endometrial epithelial cells and HEK293T cells, respectively, using a similar methodological approaches (Awan et al., 2018; Li et al., 2023). Furthermore, Wani et al. (2008) found that the biotin pull-down assay can be used in other cell lines, provided that transient transfection is sufficiently efficient. Additionally, the authors have reported successfully utilising this method in HEK293T, HeLa, and MCF7 cell lines (Wani & Cloonan, 2014).

On the contrary, in k562-s, the interaction between miR-3131 and target mRNA failed to be validated through this method. MiR-891a-3p showed a trend of a higher level of expression of the 3'UTR *ABL1* compared to the control lysate, but it was insignificant. The possible reason for the inability to validate miRNA to target mRNA in the K562-s cell may be attributed to the absence of a seed sequence that is complementary between the miRNA and the 3'UTR recognition site (Kuhn et al., 2008) since it did not work well in some cells (Tan & Lieberman, 2016). Moreover, certain studies proposed that the biotin component at the 3' end of the miRNA hinders its interaction with AGO2. Therefore, the co-immunoprecipitation of biotin-labelled miRNA with AGO2 was not seen (Guo & Steitz, 2014). It is unknown how different cell contexts affect the set of miRNA-regulated genes or the amount to which each gene

is regulated. Transcripts that are controlled in one cell type may not be identified by pulldown in another cell type because the miRNA is less abundant, the target is not expressed, or other expressed transcripts compete for miRNA binding (Tan & Lieberman, 2016; Tay et al., 2014).

Upon further examination, it was observed that the miRNA level in k562-r cells was elevated compared to k562-s cells, as depicted in Figures 4.10 and 4.11. These findings suggest that the proficiency of miRNA transfection in k562-r was superior to that in k562-s. It might help explain why the biotin pull-down assay did not show evidence of miRNA-mRNA target binding in k562-s. Furthermore, the inability to validate miRNA-mRNA interaction through this technique in the k562-s cell line does not necessarily mean that miRNA did not attach to the target mRNA. Hence, it is encouraged to utilise another validation method to make sure miR-3131 binds to 3'UTR ABL1 in future.

Similarly, this approach cannot validate the plant miRNA (osa-miR1858a/b). The Luciferase test is an additional technique that may be used to validate miRNA-mRNA targets. Nevertheless, only a small number of studies were able to use luciferase assays as miRNA-mRNA validation methods from plant-based miRNA (Chin et al., 2016; M. Li et al., 2019). The majority of previous research on the cross-kingdom approach of plant miRNA transfection into animal cells used bioinformatics, qPCR, high-throughput sequencing, protein expression, and cell proliferation assays to determine miRNA effects on the target cells (Gismondi et al., 2021; Mohamad & Elias, 2021; Saiyed et al., 2023; Zhang et al., 2023) with only a few studies able to utilise luciferase assay as a miRNA-mRNA validation method.

However, different validation methods could not be performed during this study due to time and financial constraints. However, miRNA's target genes validation, cell viability, cell cycle and protein expression were employed to further determine the

effects of miRNA transfection in CML cells, which were discussed in the subsequent subtopic.

5.3 Effects of miRNA Transfection on the *BCR-ABL1* and *ABL1* Gene

The characteristic genetic abnormality of CML is the t (9;22) (q34; q11) translocation. This translocation generates the *BCR-ABL1* fusion gene, which is a core molecular event in the development and progression of CML (Rittavee et al., 2023). This fusion gene induces genomic instability, stimulating signalling pathways of cell proliferation and cell differentiation and inhibiting apoptosis (Yacob et al., 2022). The development of the tyrosine kinase inhibitor (TKI), imatinib, allows patients with CML to experience near-normal life expectancy. However, point mutations or activation of BCR-*ABL1* independent pathways had caused resistance issues in imatinib treatment. Point mutations within the BCR-ABL1 kinase domain, such as T315I, E255K, and Y253H, confer resistance to imatinib by altering critical amino acids in the ATP-binding site. These structural changes either sterically block imatinib from binding or stabilize the active conformation of the kinase, reducing the drug's affinity. As a result, ATP can still bind and activate BCR-ABL1, leading to persistent downstream signalling and uncontrolled proliferation of leukemic cells despite imatinib treatment.

Hence, microRNA is proposed as an alternative molecular targeted therapy inhibiting the *BCR-ABL1* expression. It is widely accepted that miRNA can bind to target genes at 3'UTR and inhibit mRNA expression. Current study in-silico findings showed that all miRNAs can bind to the *ABL1* gene via complementary binding (Table 4.1 and 4.2). Since *BCR-ABL1* has the same 3'UTR as *ABL1,* we transfected all miRNA mimics into CML cell lines K562-s and K562-r to determine the expression of *BCR-ABL1* and *ABL1*. *BCR-ABL1* is the primary target gene representing the abnormal gene in CML, while *ABL1* is the proto-oncogene that is present in all cells functioning in regulating the tyrosine kinase activity.

According to Figure 4.18, hsa-miR-3131 decreased the expression of *BCR-ABL1* and *ABL1* genes in k562-s compared to imatinib and the untreated group. This study showed that miR-3131 could bind and reduce the expression of both *ABL1* and *BCR-ABL1* mRNA of k562-s. However, a similar impact was not seen in groups treated with miR-891-3p and osa-mir1858a/b. In addition to miR-3131 targeting both *BCR-ABL1* and *ABL1*, imatinib can also influence both genes' expression. While its primary function is to inhibit the BCR-ABL1 fusion protein in CML cells, imatinib also targets the kinase domain of the normal *ABL1* gene, potentially affecting its expression (Barnes et al., 2005; Dasgupta et al., 2016; Milojkovic & Apperley, 2009). This dual action is important to consider when assessing gene expression in treated versus untreated cells, as the drug's influence on normal ABL1 might differ depending on the cellular context and resistance mechanisms (Dasgupta et al., 2016). Meanwhile, in k562-r, mir-3131 and mir-891a-3p can only decrease the expression of *ABL1* and not the fusion *BCR-ABL1* gene compared to imatinib and untreated groups (Figure 4.19). The results showed that transfection of miRNAs could not decrease the oncogene *BCR-ABL1* mRNA expression in k562-r.

This observation of differences in both genes' expression between k562-s and k562-r might be attributed to several factors. K562-r cells might have developed a resistance mechanism independent of *BCR-ABL1*. Hrdinova et al. (2021) showed that imatinib-resistant k562 cell lines (K562-IR) can establish resistance mechanisms independent of mutations in the BCR-ABL1 kinase domain. Specifically, in k562-IR cells, sequencing revealed no mutations in the kinase domain. Mutations in this domain are a common cause of resistance in numerous cases. Instead, these cells exhibit increased amplification and overexpression of the *BCR-ABL1* gene, suggesting that the resistance mechanism may involve other pathways (Hekmatshoar et al., 2018).

Additionally, the activation of alternative signalling pathways, DNA methylation/hypermethylation, transcriptional control, genetic mutations, and complex regulatory networks were also responsible for resistance properties in k562-r cells (Alves et al., 2021).

Current findings may have been influenced by genetic polymorphisms and differences in the cellular environment between k562-s and k562-r. A previous study revealed that a higher number of mutations were identified in the group of patients exhibiting resistance, with a rate of 63%, compared to the control group (Kustova et al., 2022). In terms of cell line sensitivity, k562-r might develop mechanisms that make them less susceptible to miRNA-mediated control of *BCR-ABL1* which can directly counteract the regulatory impact of transfected miRNAs on *BCR-ABL1* expression. Prior research has found that polymorphisms in microRNA-binding sites, as well as homologous microRNA-binding sites and genomic variations, might impact the regulation of gene expression by microRNAs. This, in turn, can modify the risk of developing leukaemia by interfering with miRNA-mediated regulation (Dzikiewicz-Krawczyk et al., 2014; Giurgiu et al., 2023; Xi et al., 2022). The current miRNA used in this study was unable to directly target or inhibit the *BCR-ABL1* gene in k562-r cells, but they may affect processes such as prevention of apoptosis, induction of autophagy, altered expression of drug transporter protein, and inactivation of DNA repair machinery, which could lead to drug resistance in k562-r cells independently of *BCR-ABL1* (Hekmatshoar et al., 2018). Therefore, it is strongly advised to conduct additional research to confirm the primary cause of variations in *ABL1* and *BCR-AL1* gene expression between sensitive and resistant CML cells.

In addition, the group that received imatinib exhibited elevated levels of both *ABL1* and *BCR-ABL1* gene expression in k562-r. This may be due to the activation of compensatory mechanisms in the cell to maintain stable gene levels, along with the

presence of a significant amount of *BCR-ABL1* resulting from the Philadelphia chromosomes (Loscocco et al., 2019). Short-term treatment with imatinib can trigger feedback loops that transiently increase the expression of *ABL1* and *BCR-ABL1*, thus sustaining the oncogenic signalling necessary for cell survival and proliferation (Lu & Huang, 2021). This result may work against what imatinib is supposed to do, which is to lower the activity of these genes. However, in long-term treatment, typically after 6 months, imatinib is expected to decrease *BCR-ABL1* expression, which is a standard prognostic marker in CML patients receiving TKI therapy (Zaker et al., 2023). This reduction in *BCR-ABL1* transcription is crucial for the effective management of CML and is associated with improved patient outcomes, as it signifies reduced oncogenic activity and better therapeutic response.

5.4 Effects of miRNA Transfection on ABL1 Protein Expression

MiRNAs primarily facilitate post-transcriptional control by forming complete or imperfect complementary binds at target sites. Huang et al. (2019), found that when there is a full complementary pairing between miRNA and target genes, mRNA is degraded. However, when there is inadequate complementary pairing, it may contribute to translational inhibition.

The current study showed that there were no noticeable changes in the concentration of ABL1 protein when the expression of the *ABL1* and *BCR-ABL1* genes were reduced by introducing miR-3131 into K562-s cells. In k562-s, it is assumed that miR-3131 might bind to target genes completely and cause degradation of mRNA without affecting ABL1 protein translation. This finding was supported by the in-silico results, where the folding energy of miR-3131 bound to 3'UTR of ABL1 (-24.6 kcal/mol) showed the strongest folding energy compared to other miRNAs.

However, in K562-r cells, resistance mechanisms may cause the inability to alter gene and protein expression directly. The resistance mechanism may involve alterations in significant pathways and gene expression profiles that confer resistance to miRNA-mediated downregulation of the *BCR-ABL1* gene and protein. Similar results were shown in K562-r cells treated with quercetin, which decreased *BCR-ABL1* mRNA expression without significant changes in BCR-ABL and p-BCR-ABL protein expression (W. Li et al., 2019).

5.5 Effects of miRNA Transfection on CML Cellular Functions

MiRNAs are non-coding RNA consisting of 20–22 nucleotides that regulate gene expressions by either stopping translation or breaking down mRNA by attaching to the 3'UTR region. They play crucial roles in various cellular processes, including cell proliferation, differentiation, and death (Avvari et al., 2022; Hwang & Mendell, 2006; Silva Rodrigues et al., 2018). The miRNA-mRNA association would hypothetically suppress *BCR-ABL1* production, thereby reducing and inhibiting CML cell growth (Mohamad & Elias, 2021). In the context of cancer, miRNAs have been shown to act as oncogenes or tumour suppressors, influencing tumorigenesis by regulating cell proliferation, differentiation, and death (Avvari et al., 2022). Previous studies reported that miRNAs have significant roles in the progression of certain types of cancer including cervical cancer (Chen et al., 2020) and acute myeloid leukaemia (Ufkin et al., 2014). Moreover, miRNA is observed to be involved in an axis which indirectly regulates CML cell proliferation. For instance, miR-212/ABCG2 axis is reported to contribute to the development of IM-resistant leukemic cells (Kaehler et al., 2017). ABCG2 is a drug efflux transporter that plays a critical role in pharmacoresistance by actively pumping chemotherapeutic drugs, including TKIs like imatinib, out of cancer

cells, thereby reducing drug efficacy and allowing cancer cells to survive treatment (Jing et al., 2021).

Figure 4.22 displayed a notable decline in cell viability and proliferation of imatinib-resistant cells (k562-r) by over 50% when transfected with osa-miR1858a/b, and around 40% decrease when transfected with miR-3131 and miR-891a-3p. Furthermore, miRNA transfection resulted in a maximum 20% reduction in cell viability and proliferation in imatinib-sensitive cells (K562-s) compared to untreated cells. Altogether, these results imply that miRNA transfection has a more significant effect on the resistant cell line (K562-r), suggesting that miRNA may influence pathways associated with the development of resistance in cancer cells. This result highlights the influence of miRNAs on important pathways linked to drug resistance, including apoptosis, cell cycle regulation, and drug efflux mechanisms (Navabi et al., 2022). The varying effects of miRNA transfection on cell viability and proliferation in sensitive and resistant cell lines emphasize the complex relationship between miRNAs and the molecular mechanisms involved in drug resistance development.

Furthermore, the osa-miR1858a/b identified from the microarray data in this study was found to decrease MAP4K1 involved in TKI response and resistance pathway, supporting the possibility of a regulatory role in pathways associated with resistance traits. Previous studies have shown that miRNA transfection not only effectively inhibits cell proliferation but also suggest their potential for further investigation as therapeutic candidates. This aligns with research indicating that miRNAs may act as a sensitizing agent, reducing cell viability in cancer cells.

For instance, miR-227 was observed to increase imatinib sensitivity by targeting the STAT5A in imatinib-resistant CML cells (Kaymaz et al., 2015). Similarly, miR-7 was reported to suppress CML cell proliferation and sensitize cells to imatinib by targeting *BCR-ABL1* and its downstream PI3K/AKT pathways (Gajda et al., 2021;

Jiang et al., 2017). While miR-29b inhibits CML cell growth and development of cell colony by promoting apoptosis through the breakdown of procaspase 3 and PARP (Y. Li et al., 2013).

Previously, we discussed the role of miRNA in cell viability. However, miRNA also plays a significant role in regulating the cell cycle. Specifically, miRNA transfection can affect cell cycle progression by targeting key genes involved in cell cycle regulation. For instance, miR-545 has been shown to target and downregulate the expression of cyclin D1 and CDK4 genes in lung cancer cells (Du et al., 2014). This downregulation of cyclin D1 leads to a decrease in cell proliferation and an increase in apoptosis, ultimately affecting cell viability. Therefore, miRNA transfection can have a dual effect on cell viability and cell cycle progression, making it a potential therapeutic target for cancer treatment.

According to Figure 4.23, in k562-s, transfection of osa-miR1858a/b resulted in cell cycle arrest at the S and G2/M phases compared to control groups. Osa-miR1858a/b induces an increase of 3.9% in cell cycle arrest at the S phase and 2.8% at G2/M compared to untreated cell line. Moreover, Figure 4.24 shows that in the k562-r cell, miR-891a-3p and osa-mir1858a/b caused a 3.9% and 1.8% increase in cell cycle arrest at the G2/M phase compared to the untreated, respectively. Additionally, it also resulted in a 5.5% and 3.4% higher arrest at G2/M compared to imatinib, respectively. However, in both cells, imatinib showed significantly higher cell cycle arrest at G0/G1, which shows an early control of cell proliferation.

These results demonstrated that, in contrast to imatinib, which acts on the cell cycle at an early stage, transfection with these miRNAs causes a cell cycle arrest at a later point. These outcomes were consistent with previous research that discovered miRNAs might cause cell cycle arrest in various cancer cell lines at different phases. While miRNA targeting the G2/M phase may encourage the activation of signalling

pathways leading to apoptosis (Zaker et al., 2023) and miRNA targeting the G0/G1 phase can regulate cell proliferation before DNA replication (Silva Rodrigues et al., 2018). Nevertheless, specific miRNAs, including miR-4779 in colon cancer patients, can also encourage apoptosis by inducing a cell cycle arrest at the G0/G1 phase (Koo & Kwon, 2018). The miRNA's capability to target specific phases in the cell cycle suggests its functional significance in controlling cell proliferation. Prior studies have demonstrated that miR-1972 and miR-582-3p can halt the cell cycle, specifically at the G2/M phase in CML lineage-negative cells and AML cells, respectively. This effect is achieved by targeting CDC25B, CDK1, and Cyclin B1. Both cell lines exhibited an upregulation of P21 expression, an inhibitor of CDK-cyclin, which is a crucial function in controlling the cell cycle by either stimulating or inhibiting the transition from the G2 to the M phase (Agatheeswaran et al., 2016; H. Li, X. Tian, et al., 2019). Inhibition of the G2/M checkpoint is important because it halts cells with DNA damage from progressing into mitosis and enables DNA repair (Ligasova et al., 2023). Additionally, a G2/M phase inhibition may promote apoptosis by activating genes such as p53, c-Myc, and p27 (Abraham et al., 2016; Hu & Huang, 2023; Minciacchi et al., 2021).

Note that both k562-s and k562-r cells exhibited cell cycle arrest at G2/M when transfected with osa-mir1858a/b, a plant miRNA. This outcome demonstrates originality in our research by utilizing plant-derived miRNA to target CML cells. Osa-mir1858a/b's capacity to trigger G2/M phase arrest in CML cells positions it as a promising approach for CML management. Previous study by Marzano et al. (2020) discovered that a combination of plant miRNAs (gma-miR-160, gma-miR-4995, gmamiR-4368, gma-miR-5677, gma-miR-4351, zma-miR-172, and mtr-miR-5754) effectively causes a halt in the G1 phase of colorectal cancer cell line without encouraging apoptosis. Rather, they found that mtr-miR-5754 and gma-miR4995 effectively reduce cell proliferation by targeting oncogenes MALAT1 and NEAT1.

Typically, miRNA that causes a cell cycle arrest at the G0/G1 phase targets genes that are involved in the G1 to S phase transition, like cyclin D, CDK, or activating the Cyclin-dependent Kinase inhibitors (CKIs). By focusing on these genes, miRNA blocks the phase transition.

Osa-miR1858a/b's ability to cause a cell cycle arrest in G2/M suggest a tumour suppressor-like function. Targeting either the G0/G1 or G2/M phases of the cell cycle is advantageous for inhibiting cancer cell growth. While the precise molecular targets remain to be confirmed, the current observations emphasise the potential of osa-miR1858a/b to decrease cell viability and impede cell cycle progression in CML cells, possibly through indirect modulation of the BCR-ABL1 signalling pathway or other oncogenic regulators. These findings highlight osa-miR1858a/b as promising candidates for further investigation as part of potential treatment strategies in CML.

5.6 Effects of miRNA Transfection on The Gene Expression Profiling

For DEGs identification, not all treatment groups were sent for microarray due to limited resources. Hence, one human miRNA (hsa-miR-3131) and one plant miRNA (osa-mir1858a/b) were selected based on the promising results from the cellular and molecular results. MiR-891a-3p was not included in the present microarray analysis and therefore was not subjected to expression or mechanistic analysis in this study.

MiRNAs treatment in both cells was hypothesized to affect genes that are related to CML pathogenesis other than BCR-ABL1. MiRNA regulates gene expression by binding to the 3'UTR, thereby either suppressing translation or enhancing mRNA degradation. Utilizing miRNA treatment in CML cells will certainly impact genes associated with crucial pathways and biological functions.

The microarray results indicated that no differentially expressed genes (DEGs) were identified in the groups treated with miRNAs compared to the untreated group.

This might be due to certain factors such as a low sample size (Schurch et al., 2016), sample preparation issues (Rodriguez-Garcia et al., 2023), or technical and biological variabilities (Ho et al., 2008; Liu et al., 2023). Nevertheless, we thoroughly validate the chosen genes by considering their fold change, ensuring they exceed a threshold value of 2, regardless of whether they are upregulated or downregulated. Selected genes based on previously mentioned criteria in sub-heading 4.6.2 were exported in the DAVID database for gene ontology (GO) and pathways selection. In k562-s, four genes were selected to be further validated which are the MYC, TNF, CSF1, and IL6 and in k562-r, IL1B, TERT, MAP4K1, and PAK1 were chosen.

From Figure 4.27, in k562-s transfected with miR-3131, TNF was significantly upregulated compared to the control untreated group ($p<0.05$). Additionally, in the same cell when treated with osa-mir1858a/b, CSF1 was found to be upregulated while IL6 was downregulated compared to the untreated group. However, in k562-r, the IL1B was upregulated while MAP4K1 was downregulated in miR-3131 and osa-mir1858a/b treated groups, respectively (Figure 4.28).

After being treated with miR-3131, the k562-s cells showed an upregulation of the TNF gene expression. TNF, or tumour necrosis factor, is a key player in inflammatory reactions and is involved in several clinical diseases. It functions as a crucial controller of immunological responses, playing both inhibitory and stimulatory roles in various cellular processes (Shen et al., 2019). Within the context of cancer, TNF has been linked to the promotion of tumour growth by its ability to augment cell proliferation and advancement. This cytokine serves as a crucial agent in the development of long-lasting inflammation that is linked to the formation and progression of cancer (Chu, 2013).

Our findings align with previous studies suggesting complex interactions between *BCR-ABL1* and TNF regulation. *BCR-ABL1* suppression, as observed in our

study following miR-3131 transfection, was associated with a significant upregulation of TNF expression. This observation is consistent with Carra et al. (2016), who demonstrated that *BCR-ABL1* can modulate TNF-α receptor expression, potentially affecting TNF signalling pathways. Such findings highlight the intricate molecular mechanisms underlying CML and underscore the potential therapeutic implications of targeting these pathways. Moreover, the concept of compensatory mechanisms may explain the observed increase in TNF expression following *BCR-ABL1* suppression. Cells may activate alternative signalling pathways or adaptive responses to compensate for the loss of *BCR-ABL1* signalling, thereby influencing TNF expression levels. Previous studies have demonstrated that the activation of compensatory signalling pathways occurs after inhibiting *BCR-ABL1* with imatinib treatment (Burchert et al., 2005). This activation leads to continued cell proliferation and an early development of resistance to imatinib. Understanding these compensatory mechanisms could provide valuable insights into the dynamics of TNF regulation in CML and facilitate the development of more effective therapeutic strategies. Additionally, it is necessary to conduct more research to understand the precise processes that connect the inhibition of *BCR-ABL1* to the overexpression of TNF. Elucidating these pathways could offer valuable understanding into innovative therapeutic approaches aiming at regulating TNF signalling to alleviate cancer-related inflammation and tumour advancement.

Upon transfection with plant-based miRNA, osa-mir1858a/b, two genes associated with the PI3K-Akt and MAPK signalling pathways were observed to be influenced in similar cells (k562-s). When compared to untreated k562-s cells, the expression of colony-stimulating factor (CSF1) is increased, while the expression of Interleukin-6 (IL6) is decreased after miRNA transfection. The increase in CSF1 expression following transfection with osa-mir1858a/b is an interesting discovery. CSF1 is a cytokine that is essential for the growth and activity of macrophages and other

myeloid cell (Irvine et al., 2009; Stanley & Chitu, 2014). Within the context of chronic myeloid leukaemia CML, there is a connection between CSF1 and the advancement of the disease. This is because CSF1 promotes the survival and pro-tumoral functions of tumour-associated macrophages (TAMs) (Guo et al., 2020). The upregulation of CSF1 following osa-mir1858a/b transfection suggests that mir1858a/b is influencing the tumour microenvironment by promoting the recruitment and activation of tumour-associated macrophages (TAMs), potentially leading to the observed decrease in cell viability to only 80% and cell cycle arrest at G2/M compared to untreated cells. Nevertheless, the downregulation of IL6 expression after osa-mir1858a/b transfection is also important. IL6 is a cytokine involved in inflammation and immune response (Jiang et al., 2018). In CML, IL6 has been linked to the disease's progression. *BCR-ABL1* activity regulates IL6 expression, resulting in a paracrine feedback loop that sustains CML progression (Reynaud et al., 2011). The reduction in IL6 expression by osa-mir1858a/b may indicate that this miRNA is inhibiting the pro-tumoral activities of IL6 in CML cells, which could contribute to the anti-tumour effects observed. Furthermore, osa-mir1858a/b is a miRNA derived from plants. Previous studies observed that ginger-derived plant vesicles containing miRNAs can enter mammalian cells, be absorbed by intestinal cells, and exhibit anti-inflammatory characteristics, indicating cross-kingdom control (Yin et al., 2022). The discovery that osa-mir1858a/b can target IL6 and decrease its expression is a breakthrough in the possible use of plant miRNA for addressing CML.

Meanwhile, in k562-r, MAP4K1 was observed to be significantly downregulated in cells after osa-mir1858a/b transfection. MAP4K1 is a serine/threonine kinase that plays a crucial role in immune regulation and the progression of cancer. It sends signals downstream to modify the MAPK pathway, which has an impact on the growth and survival of cells (Hu et al., 1996). Prior studies

have shown that a higher level of MAP4K1 is associated with poor prognosis in acute myeloid leukaemia (AML). Conversely, inhibiting MAP4K1 activity impedes the growth of AML cells by causing them to pause in the G0/G1 phase. This effect is achieved by regulating the expression of p21 and p27 (Ling et al., 2021). MAP4K1 exerts control over DNA repair and damage pathways, influencing the cell cycle and ultimately influencing AML progression. It also functions as a reliable marker of poor AML prognosis and contributes to the development of resistance to tyrosine kinase inhibitors (TKIs) (Knight et al., 2021). Our study demonstrates the potential therapeutic applications of osa-mir1858a/b in CML. The finding of this study shows that Osa-mir1858a/b significantly reduces the k562-r cell viability to 50% and induces a cell cycle arrest at the G2/M phase compared to the imatinib and untreated groups. This reduction in cell viability was accompanied by a decrease in the expression of MAP4K1, a protein involved in various cancer progression (He et al., 2021; Knight et al., 2021; Sun et al., 2023; Zhao et al., 2021). Despite not directly affecting *BCR-ABL1* expression, Osa-mir1858a/b's inhibition of MAP4K1 suggests that it may play a role in regulating cell cycle and cell viability in CML cells, especially the resistant type. The potential therapeutic application of osa-mir1858a/b in CML is promising. As a tumour suppressor miRNA, osa-mir1858a/b targets the MAP4K1 pathway and inhibits CML progression. Notably, osa-mir1858a/b of plant-based origin, demonstrated its ability to influence gene expression in CML cells, highlighting its potential as a promising candidate for CML treatment. While we did not directly prove cross-kingdom transfer, our findings suggest that this plant-derived miRNA can modulate the expression of genes from a different biological kingdom.

On the other hand, transfection of hsa-miR-3131 did cause an upregulation of IL1β in k562-r. IL1B is a cytokine which involved in inflammatory response, cell proliferation, differentiation and apoptosis (Zheng et al., 2018). In cancer, IL1B exerts

multiple effects on various aspects of the immune system, including immune cells, angiogenesis, cancer cell proliferation, migration, and metastasis. The mature bioactive form of IL1B is synthesized in response to inflammatory signals, largely by myeloid cells, including monocytes, macrophages, and neutrophils. It has been observed that IL1B is increased in multiple myeloma patients (Litmanovich et al., 2018; Rébé & Ghiringhelli, 2020). This indicates that IL1B might play a significant role in CML progression perhaps by maintaining the tumour microenvironment and activating various signalling pathways, such as NF-κB and MAPK, which are critical for cell growth and survival (de Mooij et al., 2017; Litmanovich et al., 2018). The observed capacity of hsa-miR-3131 to reduce cell viability indicates its potential as a therapeutic intervention for CML. The absence of any alteration in the expression of *BCR-ABL1* or the progression of the cell cycle by miR-3131 in k562-r indicates that it impacts cell viability via alternative signalling pathways. The notable increase in IL1B expression indicates that IL1B is involved in facilitating these effects, potentially by stimulating inflammation that negatively impacts the survival of leukaemia cells. As stated by Kouroumalis et al. (2023), inflammation in bowel diseases impacts intestinal cell survival by elevated death of intestinal epithelial cells (IECs) possibly via apoptosis, necroptosis and pyroptosis pathways. Hence, further research should focus on elucidating the exact pathways through which miR-3131 exerts its effects and the role of IL1B in this process. Investigating apoptotic, markers and conducting functional studies on IL1B will provide deeper insight into the therapeutic potential of miR-3131 in CML.

5.6.1 Enrichment and pathways analysis of upregulated and downregulated genes.

Microarray is a high-throughput technology that is used to simultaneously identify and analyse gene expression. The platform facilitates the testing of a substantial number of differentially expressed genes (DEGs) in a comparison between two groups. It aids in the identification of mutations, biomarkers, and genes that are linked to chemoresistance (Wan et al., 2020). The outcomes of microarray experiments are influenced by factors such as the size of the sample, the way the sample is prepared, and the number of replications performed. It is desirable to have higher sample numbers, with at least three replicates for studies based on cell lines, when trying to discover differentially expressed genes (DEGs) between groups (Jaksik et al., 2015). However, due to some limitations and challenges, we can only provide two replicates per group in the current study.

Our data suggest that miR-3131 and osa-miR1858a/b did exert influence on several genes, particularly those that are pivotal in fundamental biological processes and pathways. Despite the lack of significant differentially expressed genes (DEGs) from the microarray data, a few genes linked to specific biological processes and pathways were selected for further confirmation using quantitative polymerase chain reaction (qPCR) based on their fold change in the microarray result. Earlier in Table 4.9, the initial *in-silico* analysis of hsa-miR-3131 target genes resulted in the identification of two clusters, which consisted of three genes, namely NLGN3, IL1RAPL1, and CNTNAP2. It is assumed that these genes are located on the cell surface and involved in KEGG pathways related to cell adhesion molecules (CAMs), as well as the synaptic system. However, following the microarray analysis, it was discovered that both upregulated and downregulated genes of miR-3131 play significant roles in several essential activities and pathways in different cell lines (k562-s and k562-

r). For example, when mir-3131 was introduced into the k562-s cells, proteins were found to be involved in repairing DNA double-strand breaks, regulating the G2/M cell cycle checkpoint, controlling the development of blood cells, and facilitating the movement of molecules across cell membranes (Table 4.27 – 4.33).

In addition, while the initial *in-silico* analysis predicted potential osa-miR1858a/b binding sites in certain genes based on their 3'UTR regions, the microarray data reflected changes in global gene expression profiles, which are influenced by various regulatory mechanisms beyond miRNA binding. To investigate whether the differentially expressed genes (DEGs) identified in the microarray are direct targets of osa-miR1858a/b, we performed a follow-up analysis using TargetRank and psRNATarget. However, no 3'UTR binding sites were predicted for IL6 or MAP4K1, two genes downregulated in the microarray analysis, suggesting that their downregulation might be mediated through indirect mechanisms.

This highlights the complex regulation of gene expression. MiRNAs may affect genes indirectly by targeting upstream components in signalling pathways, leading to downstream changes. Future experiments, such as luciferase reporter assays, will be required to confirm the direct interactions between miRNAs and other identified target genes.

As observed following microarray analysis, the disparities between early *in-silico* prediction and actual data highlight the significance of validating *in-silico* data using experimental methods (Riolo et al., 2020). Computational prediction of miRNA target genes can be challenging due to potential incorrect targets and the limitations of prediction techniques (Fridrich et al., 2019). Combining results from many prediction approaches can enhance the precision of computer-based analysis (Oliveira et al., 2017). Issues related to the specificity of sequences and the stability of mRNA structures can compromise the accuracy of miRNA target prediction. The disparities between *in-silico*

and microarray findings may stem from the constraints of *in-silico* prediction and the necessity for empirical confirmation (Roberts & Borchert, 2017). Furthermore, even though no DEGs were detected from the microarray analysis, further validation using quantitative real-time PCR (qPCR) is highly recommended for the validation of selected genes (Gao et al., 2021; Kazmierczak et al., 2018). No DEGs were detected, perhaps due to only subtle changes occurring between the miRNA-treated and untreated groups. These subtle changes might not be able to be detected in the microarray experiment, as the microarray platform is not as sensitive as qPCR (Berthuy et al., 2016). Hence, further validation with qPCR might perhaps identify genes affected by the treatments (Yacob et al., 2022), providing additional support for their biological relevance despite the small sample size in the microarray experiment. Future studies with increased sample sizes and biological replicates would be necessary to fully elucidate the transcriptional changes induced by the treatment and identify all differentially expressed genes with high confidence.

5.7 Novelty of The Research

In this section, the novel contributions of this study are summarised in terms of theoretical advancements and methodological innovations. This study introduces several innovative aspects to understanding miRNA-mediated regulation in CML. We identified novel miRNAs, such as hsa-miR-3131, hsa-miR891a-3p, and the plant-derived osa-mir1858a/b, which target the 3'UTR of the *ABL1* gene. This finding is particularly significant due to plant-derived miRNAs' novel concept of cross-kingdom regulation. The selection of osa-miR1858a/b was motivated by in silico analyses predicting strong and conserved binding sites within the *ABL1* 3′UTR, as well as previous reports suggesting that certain dietary plant miRNAs can remain stable during digestion, enter mammalian circulation, and modulate endogenous gene expression. Plant miRNAs offer unique chemical stability due to their 2′-O-methylation at the 3′

end, which increases their resistance to degradation by exonucleases. This biochemical property, combined with their potential to modulate disease-related targets, makes them intriguing candidates for therapeutic exploration. Including osa-miR1858a/b in the present study not only allowed assessment of a non-human-derived regulatory molecule in CML but also broadened the scope of miRNA research to explore unconventional, cross-kingdom therapeutic approaches.

Additionally, the study reveals differential gene expression regulation by these miRNAs in imatinib-resistant versus sensitive cells, highlighting the complex role of miRNAs in drug resistance. Methodologically, the combination of *in-silico* analysis, biotin-pulldown experiments, and cell functional assays used in this study offers a comprehensive approach to validating miRNA-mRNA interactions.

Aspect of novelty	Description
Theory/ knowledge	
Identification of novel miRNAs	Discovery of hsa-miR-3131, hsa-miR891a-3p, and plant-derived osa-mir1858a/b as regulators of *BCR-ABL1*.
Cross-kingdom regulation	Demonstration of plant miRNAs affecting human gene expression and cell viability.
Differential regulation in drug resistance	Observation of miR-3131 and osa-mir1858a/b having distinct effects in imatinib-resistant (K562-r) versus sensitive (K562-s) cells.

Identifying these miRNAs opens new avenues for targeted therapeutic strategies in CML. The novel concept of cross-kingdom regulation by plant miRNAs suggests potential applications in biomedicine and agriculture, offering a unique perspective on gene regulation. Additionally, understanding the differential effects of miRNAs in drug-resistant versus sensitive cells provides insights into overcoming drug resistance, a significant challenge in cancer treatment. The methodological approaches used in this

study set a precedent for future research aiming to validate miRNA interactions and their functional implications.