CHAPTER 2

LITERATURE REVIEW

2.1 Chronic Myeloid Leukaemia (CML) Pathogenesis

Chronic myeloid leukaemia (CML) is a clonal myeloproliferative disorder. It originates from an abnormal pluripotent bone marrow stem cell. The genetic abnormality of the BCR-ABL1 fusion oncogene located on the Philadelphia (Ph) chromosomes. The gene fusion happened due to the reciprocal translocation between chromosomes 9 and 22, t(9;22) (q34; q11), and CML has been regarded to be the first malignancy related to genetic abnormalities (Amir & Javed, 2021). This gene results in the highly stable and constitutively active tyrosine kinase activity that plays a significant role in the pathogenesis of CML, leading to abnormal growth, altered apoptosis and longer survival of CML clones (An et al., 2010; Clarke & Holyoake, 2017; Zhu et al., 2022). CML is categorised into three phases: the chronic phase (CP), acute phase (AP) and blast phase (BP). The expansion of the granulocytic cell manifests CML. The chronic phase usually takes 3-4 years, and it can further develop into the blast phase if added with other genetic problems and left untreated. CML accounts for approximately 15% of adults and occurs in about 1–2 cases per 100,000 individuals annually. The average age is 57 years and it is more prevalent in men than in women (ratio 1.4:1) (Amir & Javed, 2021; Martins et al., 2021).

Imatinib is currently used as the frontline treatment in CML. Imatinib can induce apoptosis of BCR-ABL1 cells by targeting the tyrosine kinase, thus acting as a tyrosine kinase inhibitor (TKI). Several studies have shown that imatinib can restore

the life expectancy of CML patients to that of the general population (Bavaro et al., 2019; Poudel et al., 2022). However, a new problem emerged as there are cases of imatinib resistance and the persistence of BCR-ABL-positive cells. Imatinib resistance is caused by the point mutations at the BCR-ABL gene, thus impairing the binding of the drug to the target protein. Hence, second and third generation TKIs were developed to target imatinib-resistant cells, especially those with the T315I mutation. Nevertheless, these drugs have severe side effects and do not fully eradicate the CML cell.

Several mechanisms lead to TKI resistance. For instance, the BCR-ABL dependent pathway is when the BCR-ABL kinases were overexpressed due to amplification thus leading to a resistant scenario. Besides that, a mutation in BCR-ABL resists the TKI binding due to ABL conformation change. Meanwhile, the BCR-ABL independent pathway significantly contributeto TKI resistance. The initiation of other signalling pathways, for example, the LYN and HCK pathways, made the cell continuously proliferate and resulted in TKI resistance (Poh et al., 2015; Zimmerman et al., 2010). Moreover, the overexpression of efflux transporters, like ABCB1 and the downregulation of influx transporters, like OCT, resulted in a decrease of TKI levels in cells and reduced efficacy of TKI, and finally lead to TKI resistance (Amir & Javed, 2021; Grassi et al., 2019; Yap et al., 2017). Resistance against imatinib is an emerging problem in the treatment of CML. Mutations, involvement of BCR/ABL-independent signalling pathways, and poor accumulation of imatinib in cells remain the ultimate challenges in managing CML. Hence, finding alternative treatment strategies for CML to achieve remission-free treatment is urgent and in demand.

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2.2 The Molecular Mechanism of Chronic Myeloid Leukaemia

2.2.1 The ABL gene and protein

The human ABL gene, evolutionarily related to the Abelson murine leukaemia viral oncogene, is responsible for encoding a nonreceptor tyrosine kinase essential in various cellular processes (Deininger et al., 2000; Laneuville, 1995). This widely expressed protein exists in two splice variants and is equipped with domains facilitating interactions with many proteins, which regulate cell cycle dynamics. Notably, within quiescent cells, ABL's activity is modulated through its association with the retinoblastoma (Rb) protein, effectively keeping its tyrosine kinase function in check. The liberation and subsequent activation of ABL during the G1 to S phase transition underscore its contributory role in cell cycle control and DNA synthesis preparation. Although the c-ABL protein plays a key role in regulating cell division, its complete absence in mouse studies surprisingly does not trigger the excessive cell multiplication often associated with cancer, suggesting the existence of compensatory regulatory mechanisms within the cell (Holyoake, 2001). Instead, the deficiency manifests in developmental impairments and compromised immunity, underscoring c-ABL's critical function in normal physiological development (Laneuville, 1995). ABL1 is ubiquitously expressed as a 145-kilodalton protein with two isoforms obtained from the first exon's alternative splicing. The protein contains three SRC homology domains (SH1-SH3) near the NH2 terminus. The tyrosine kinase functional domain is the SH1 domain, while the SH2 and SH3 domains are the sites for interaction with other proteins. At the 3'end of the protein, nuclear organisation signals and the DNA binding besides the actin-binding domain are found. Overall, the ABL protein is crucial in integrating

signals from various extracellular and intracellular environments that influence the cell cycle and apoptosis (Van Etten, 1999).

2.2.2 The BCR gene and protein

The BCR gene, found on chromosome 22, produces a 160-kDa protein commonly present in the body but with uncertain functions (Deininger et al., 2000; Goldman & Melo, 2003). This protein, localised in the cytoplasm in resting cells, shifts to a perichromosomal area during cell division, suggesting a potential role in managing the cell cycle (Holyoake, 2001). In terms of structure, the BCR protein has a serine-threonine kinase domain, a coiled-coil domain for dimerisation, a central region with domains affecting Rho GTPases, and a C-terminal with GTPase activity (Deininger et al., 2000). It also involves several signalling pathways, including actin polymerisation and neutrophils oxidative burst (Voncken et al., 1995). Moreover, the C-terminus has GTPase activity for Rac that regulates actin polymerisation plus the NADPH oxidase activity in phagocytic cells. BCR can be phosphorylated on several tyrosine residues, primarily the tyrosine 177 that binds to Grb-2 and activates the Ras pathway (Ma et al., 1997). Despite these insights, the precise role of BCR in Ph-positive leukaemia is still not fully understood, which may be attributed to the redundancy in cellular pathways that can mask the effects of individual genes, the limitations of translating findings from animal models to human conditions, and the variability in genetic makeup across different individuals that results in a spectrum of disease presentations and therapeutic outcomes.

2.2.3 BCR-ABL fusion oncogene

The BCR-ABL1 protein isoform produces depend on the BCR breakpoint's location. If the breakpoint takes place at the 5.8 kb area spanning BCR exons 13 or 14 with ABL exon 2, it becomes the major breakpoint cluster region (M-bcr) and produces the e13a2/e14a2 (b2a2 or b3a2) type fusion transcript which encodes the 210-kd chimeric protein ($P210^{BCR-ABL}$) mRNA. This condition happens in almost 90% of CML patients and approximately one-third of patients with Ph-positive acute lymphoblastic leukaemia (ALL) (Deininger et al., 2000; Poudel et al., 2022). Meanwhile, fusion transcript type is the $P190^{BCR-ABL}$ which produces a 190-kd protein. This breakpoint occurs further upstream in the 54.4-kb region of the alternative BCR exon e2' and e2, which is called the minor breakpoint (m-bcr) and produces the e1a2 mRNA which is rare in CML but relatively common in acute lymphoblastic leukaemia (Flis & Chojnacki, 2019). Another rare breakpoint cluster region is the μ-bcr identified downstream of exon 19 of BCR and produced a 230-kd fusion protein, namely the $P230^{BCR-ABL}$. μ-bcr is linked to the rare Ph-positive chronic neutrophilic leukaemia producing the e19a2 fusion transcript (Figure 2.1).

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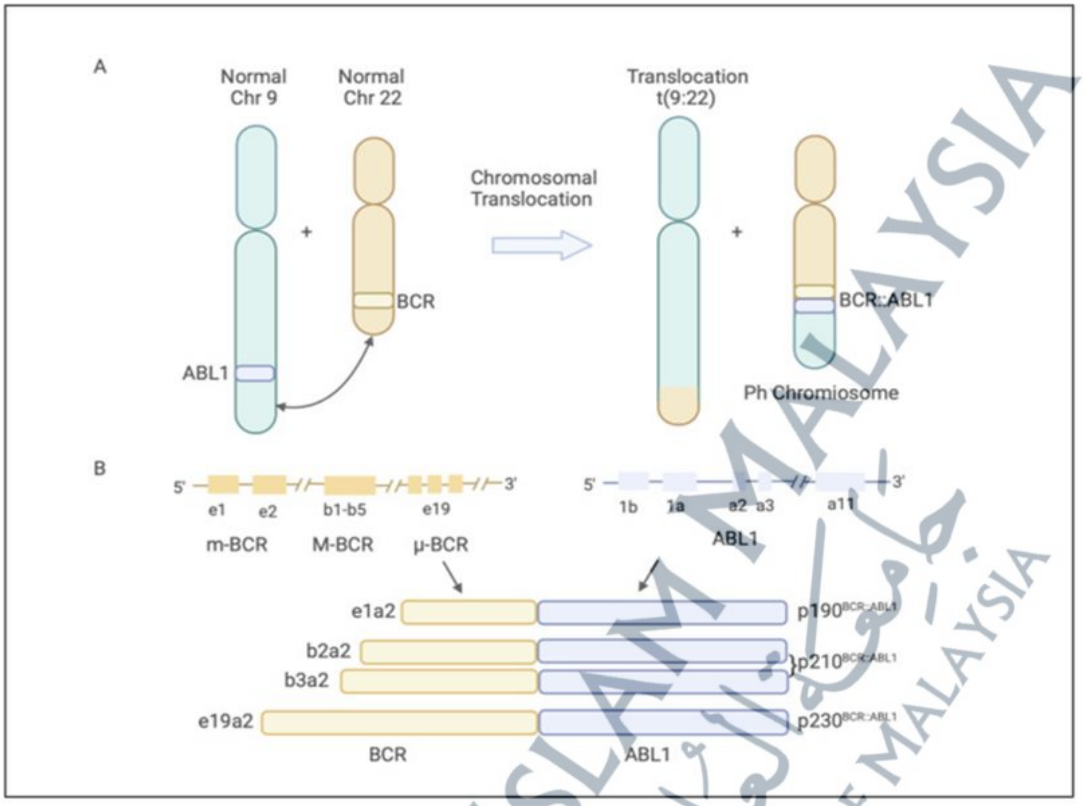
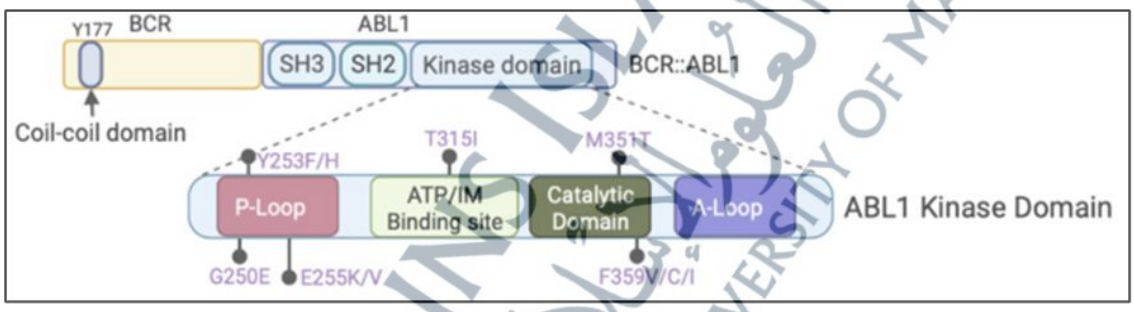


Source: (Poudel et al., 2022)

Figure 2.1: (A) The translocation of chromosomes 9 and 22, t (9;22) (q34; q11) forming the Philadelphia (Ph) chromosome. (B) The location of the break within the BCR gene determines the formation of distinct BCR-ABL1 fusion gene transcripts.

The BCR-ABL1 fusion gene gives rise to the BCR-ABL1 protein, which encompasses distinct domains derived from BCR and ABL1. The BCR region of this chimeric protein plays a crucial role in regulating its enzymatic activity and serves as the binding site for various interacting partners. Notably, the coil–coil domain located in the N-terminal segment of BCR is responsible for mediating the oligomerisation and constitutive activation of BCR-ABL1 activity. Conversely, the ABL1 component of the BCR-ABL1 protein comprises an SRC-homology-2 (SH2) domain, an SH3 domain, and a kinase domain (Soverini et al., 2018). Under normal circumstances, ABL1 possesses a myristoylated N-terminal region, which governs the auto-inactivation of its kinase activity. However, during the fusion process with BCR, the myristoylated N-

terminal region is relinquished (Hantschel et al., 2003; Poudel et al., 2022). The BCR-ABL1 kinase domain encompasses key motifs essential for its catalytic function, including the phosphate binding loop (P-loop), the ATP/imatinib binding site, the catalytic domain, and the activation loop (A-loop). Activation of BCR-ABL1 occurs upon ATP binding to the active site within the ABL1 kinase domain, facilitating the transfer of a phosphate group to its downstream substrates. However, the introduction of tyrosine kinase inhibitors (TKIs) competes with ATP for binding to the active site, leading to the inhibition of BCR-ABL1 activation and subsequently impeding the progression of leukaemia.



Source: (Soverini et al., 2018)

Figure 2.2: Structural arrangement of the BCR and ABL1 components within the BCR-ABL1 protein.

Figure 2.2 illustrates the structural arrangement of the BCR and ABL1 components within the BCR-ABL1 protein. It highlights the N-terminal coil–coil domain of BCR, which includes a crucial tyrosine residue at position 177 (Y177). Additionally, it showcases the presence of an SRC-homology-2 (SH2) domain, an SH3 domain, and a kinase domain within the ABL1 component. Notably, the ABL1 kinase domain exhibits important features such as the P-loop, the binding site for

ATP/imatinib, the catalytic domain, the A-loop, and mutations that have clinical significance, impacting the functionality of the kinase domain (Soverini et al., 2018).

A t(9;22) chromosomal translocation or detecting the BCR-ABL fusion gene in leukocytes is sufficient evidence to establish a diagnosis of CML in patients with chronic myeloproliferative disease. However, intriguingly, studies utilising a highly sensitive two-step reverse transcription and polymerase chain reaction assay have unexpectedly identified BCR-ABL mRNA in the leukocytes of healthy individuals. The initial study reported BCR-ABL fusion transcripts in 30% of normal adults (Biernaux et al., 1995), which was subsequently confirmed by the second study detecting hybrid BCR-ABL mRNA with e13a3 or e14a3 junctions in 27% of the healthy individuals tested. Furthermore, using primers specific to the e1a2 junction, BCR-ABL mRNA was found in 69% of the individuals (Bose et al., 1998).

These significant findings suggest that the generation of specific oncogene alone is inadequate for malignant transformation. Instead, it may indicate that nonhomologous recombination events involving specific chromosomes are common within hematopoietic cells, indicating a basal level of genomic instability. Significantly, many BCR-ABL transcripts detected in the healthy population exhibit abnormal junctions and are translated into truncated non-functional proteins. However, the "correct" leukaemia-type BCR-ABL genes are also present in these individuals but do not develop into CML. It is suggested that in such cases, the BCR-ABL fusion genes that arise in progenitor cells may be incapable of clonal expansion. Alternatively, BCR-ABL-positive cells are believed to be recognised and eliminated by the immune system before clonal expansion can occur. Hence, it is suggested that BCR-ABL alone cannot induce CML without additional genetic alterations. Notably, the reverse transcription and polymerase chain reaction techniques employed in these studies demonstrate exceptional sensitivity, and

the prevalence of BCR-ABL-bearing leukocytes in the general healthy population is extremely low, estimated to be no more than 1 to 10 cells per 10^8 white blood cells (Melo & Deininger, 2004). The presence of the BCR-ABL oncogene is indispensable for the initiation, persistence, and advancement of chronic myeloid leukaemia (CML), particularly during the transition from the chronic phase to the blast phase. This oncogene induces genomic instability and stimulates cellular proliferation by activating specific signalling pathways (Huang et al., 2019) The chimeric BCR-ABL1 protein, harbouring a constitutively active tyrosine kinase, exerts its oncogenic effects by perturbing and reprogramming downstream signalling cascades. Consequently, it disrupts vital cellular processes such as proliferation, differentiation, and survival, thereby playing a pivotal role in the pathogenesis of CML (Srutova et al., 2018).

The BCR-ABL1 fusion gene results from a reciprocal translocation between chromosomes 9 and 22, known as the Philadelphia chromosome, which fuses the 5′ end of the BCR gene to the 3′ end of the ABL1 gene. The most common fusion transcript in chronic myeloid leukaemia (CML) is the p210 BCR-ABL1, which includes BCR exons e13 or e14 fused to ABL1 exon a2 (Melo, 1996). Importantly, although the fusion transcript contains coding exons from both genes, the 3′ untranslated region (3′UTR) of the ABL1 gene is retained in the BCR-ABL1 mRNA, downstream of the ABL1 stop codon (Poudel et al., 2022). This region is not involved in protein coding but plays essential roles in post-transcriptional regulation, including mRNA stability, localization, and microRNA (miRNA) binding (Mayr, 2017).

Therefore, despite the chimeric nature of *BCR-ABL1*, its 3′UTR remains derived from *ABL1*, making it a potential target for regulatory mechanisms involving miRNAs that specifically bind to the ABL1 3′UTR (Bueno et al., 2016). This is mechanistically

important in studies such as the present research, which investigates the role of synthetic miRNAs in regulating BCR-ABL1 transcript levels and their effects on downstream gene and protein expression in CML cells. Targeting the 3′UTR of ABL1 offers a potential strategy to suppress both the BCR-ABL1 fusion transcript and the wild-type ABL1 mRNA, since the ABL1 3′UTR is retained in the fusion transcript.

2.2.4 Alterations induced by BCR-ABL in chronic myeloid leukaemia.

In BCR-ABL1 expressed cells, the adhesion of CML progenitor cells to the bone marrow stroma cells and extracellular matrix is decreased. In normal haematopoiesis, the adhesion of progenitor cells to the stroma is vital for regulating haematopoiesis, providing a means of anchoring progenitors and exposing them to specific signals that determine their fate. However, in CML, the disrupted adhesion properties of progenitor cells result in the liberation from regulatory signals and impaired targeting of the bone marrow, leading to immature cells in the peripheral blood (Windisch et al., 2019). The interaction between stroma and progenitor cells involves beta-integrins, and it is noteworthy that CML cells express an adhesion-inhibitory variant of beta-1 integrin, distinct from normal progenitors. This aberrant integrin expression and function in CML cells affect the transduction of signals that regulate proliferation. BCR-ABL1, a prominent protein associated with CML, further contributes to the disturbance of signal transduction processes within the cell. Additionally, the highly phosphorylated protein Crkl, influenced by BCR-ABL1, plays a role in cellular motility and adhesion by interacting with focal adhesion proteins such as paxillin, focal adhesion kinase (Fak), p130Cas, and Hef1 (Salgia et al., 1996). Understanding the consequences of the adhesion defect in CML remains a complex task. While evidence supports the influence of BCR-ABL1 on integrin function, the precise biological implications still need to be

fully elucidated. Interestingly, specific cellular systems suggest an enhancement, rather than reduction, of integrin function by BCR-ABL1 (Bazzoni et al., 1996). Further investigations are needed to unravel the intricate molecular mechanisms underlying CML cell's disrupted adhesion and integrin signalling.

In addition to altered adhesion properties, studies have reported that the expression of BCR-ABL1 in cells inhibits apoptosis even after removing the growth factor (Darji & Bharadia, 2016; Sirard et al., 1994). This phenomenon primarily depends on the tyrosine kinase activity of BCR-ABL and its association with the activation of Ras activation, an essential protein involved in cell signalling (Cortez et al., 1995; Nath et al., 2017). The inhibition of apoptosis in response to DNA damage has also been observed in cell lines carrying the BCR-ABL1 oncogene (Burke, 2010; Glowacki et al., 2013). The underlying biological mechanisms for these phenomena are not yet fully understood, but it is known that BCR-ABL1 can interfere with the release of cytochrome C from mitochondria, potentially blocking caspase activation. This disruption in caspase activation affects the Bcl-2 family of proteins, leading to the upregulation of Bcl-2, and is believed to be mediated through the Ras and PI3K pathways (Soverini et al., 2018). The expression of Bcl-xL, another protein involved in apoptosis, which STAT5 activates, is also influenced by the BCR-ABL1. Nevertheless, BCR-ABL1 phosphorylates the pro-apoptotic protein Bad, thus preventing its function. Raf-1, a protein downstream of Ras, may carry this phosphorylation. This cell survival signal mediated by the BCR-ABL1 is at least half controlled by the Bad gene by modulating Raf-1 localisation to the mitochondria. Overall, the inhibition of apoptosis and promotion of cell survival signals mediated by multiple signals initiated by BCR-ABL1 suggest a dual role, wherein it exhibits both anti-apoptotic effects and provides a proliferative stimulus, thereby contributing to cell survival and potential disease

progression. The expression of BCR-ABL1 also influences the activation of mitogenic signalling pathways, which will be further elaborated in the following subtopic.

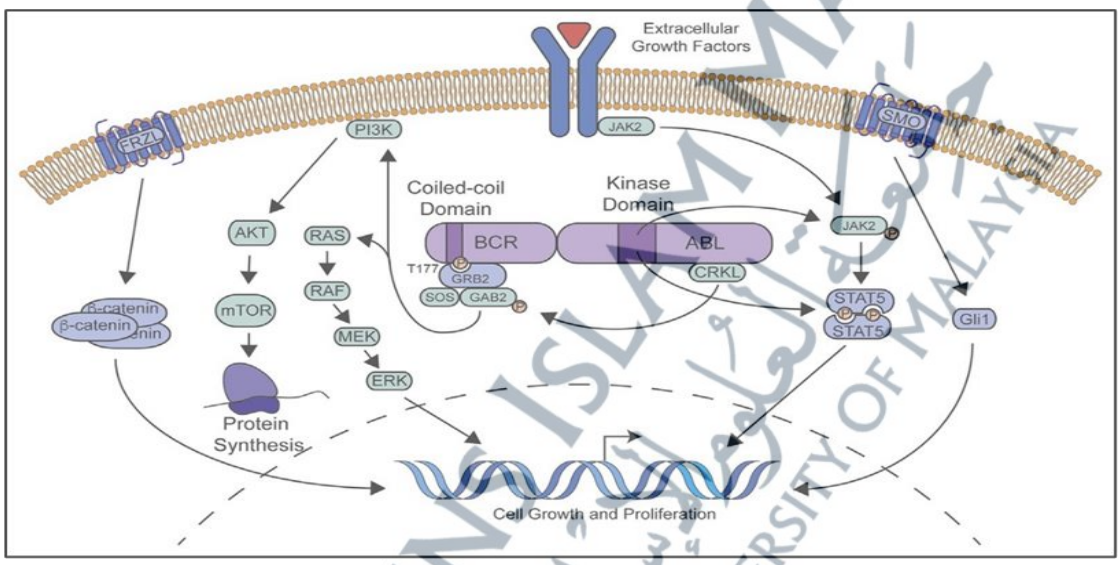
2.2.5 Signalling pathway of oncogenic BCR-ABL1

The BCR-ABL1 fusion protein of CML can activate diverse mitogenic signalling pathways and lead to abnormal cell adhesion, increasing proliferation and apoptosis inhibition. One implicated pathway is the Ras and the mitogen-activated protein kinase (MAPK) pathways. A study by Cilloni and Saglio (2012) mentioned that the ABL protein physiologically shuttles between the nucleus and cytoplasm; meanwhile, the oncoprotein that binds with BCR will remain in the cytoplasm and interact abnormally with other cytoplasmic molecules. These interactions cause the disruptions of several critical cellular processes, such as the perturbation of Ras-mitogen-activated protein kinase (MAPK), which leads to increased proliferation (Cilloni & Saglio, 2012). The Bcr-Abl exerts its influence by directly binding to proteins involved in activating Ras. It begins with the autophosphorylation of tyrosine 177, facilitating the recruitment of the adapter molecule Grb-2. Grb-2, along with Sos protein, promotes the conversion of Ras from an inactive GDP-bound state to an active GTP-bound form (Melo & Deininger, 2004). In addition to Grb-2, BCR-ABL1 can be activated by two other adapter molecules, namely Shc and CrkL. The interaction between Shc and the BCR-ABL1 fusion protein occurs at its SH2 domain, whereas CrkL is believed to bind to the SH3 domain, although this interaction is not well-established and may be specific to fibroblast cells (Deininger et al., 2000). Moreover, the direct association of CrkL with BCR-ABL1 does not seem critical for the transformation process in myeloid cells (Heaney et al., 1997). The activated Ras

recruits the serine-threonine kinase Raf-1, which undergoes tyrosine phosphorylation and transports it to the plasma membrane, initiating a signalling cascade through the mitogen-activated protein kinase (MAPK) pathway. This cascade involves the activation of various mitogen-activated protein kinases, including ERK1/2 (extracellular signal-regulated kinases) and JNK (stress-activated protein kinases). Ultimately, these pathways regulate gene transcription (Al Hamad, 2021). Furthermore, recent research has demonstrated that Grb-2 facilitates Ras/Erk activation and recruits the scaffolding adapter Gab2 (Million & Van Etten, 2000). Gab2, when phosphorylated by Bcr-Abl, triggers the activation of phosphatidylinositol 3-kinase (PI3K)/Akt and Ras/Erk pathways. In-vitro studies observed that myeloid progenitor cells lacking Gab2 are resistant to the transformation by Bcr-Abl, meaning that these cells do not undergo the abnormal changes associated with Bcr-Abl-induced transformation. On the other hand, when Gab2-deficient lymphoid progenitor cells are examined, their transformation by Bcr-Abl is impaired, suggesting that Gab2 plays a more critical role in the transformation of myeloid cells compared to lymphoid cells (Alves et al., 2021).

This finding is consistent with the previous research mentioning that when a specific mutation is introduced in Bcr-Abl (replacing tyrosine 177 with phenylalanine), the ability of Bcr-Abl to induce myeloid leukaemia in a mouse model of CML is prevented, hence supporting the notion that intact tyrosine 177 and the involvement of Gab2 are essential factors in developing myeloid leukaemia (Ding et al., 2015). Nevertheless, BCR-ABL1 has activated multiple types of mitogen-activated protein kinases, including ERK1/2 and JNK (Al Hamad, 2021). Nevertheless, another pathway that BCR-ABL activates is the Jak-Stat pathway. The phosphorylation of signal transducer and activator of transcription (STAT) family transcription factors has been observed in BCR-ABL-positive cell lines and primary CML cells. Notably, in Bcr-Abl-

expressing myeloid cells, phosphorylation of STAT5 appears to be mediated by the Src family kinase Hck, which binds to specific domains of Bcr-Abl (Poh et al., 2015). Activation of STAT5 by p210Bcr-Abl contributes to malignant transformation and anti-apoptotic effects by upregulating the transcription of Bcl-xL. However, the essentiality of STAT5 for leukaemia induction by Bcr-Abl in mice remains uncertain, casting doubt on its physiological relevance (Sillaber et al., 2000).



Source: (Braun et al., 2020)

Figure 2.3: Downstream activation of the molecular pathways associated with BCR-ABL1.

Figure 2.3 demonstrates the downstream activation of the molecular pathways associated with BCR-ABL1. Following the dimerisation of BCR-ABL1, autophosphorylation occurs at tyrosine 177 of BCR. This event creates a docking site for the GRB2/GAB2/SOS complex, which subsequently triggers the activation of multiple signalling pathways, such as PI3K/AKT and MAPK. Simultaneously, autophosphorylation of specific residues within the BCR-ABL1 kinase domain activates the JAK/STAT pathway, potentially activating JAK2 and direct phosphorylation of STAT5. In instances of BCR-ABL1 TKI resistance, extracellular growth factors can exploit the JAK/STAT pathway to sustain cellular growth.

Furthermore, leukaemia stem cells may rely specifically on WNT/β-catenin and SHH/SMO signalling to ensure survival in the presence of BCR-ABL1 kinase inhibition (Braun et al., 2020).

Another two pathways that are implicated by BCR-ABL1 expression are the phosphatidylinositol 3 kinase (PI3K) and MYC pathway. The PI3K pathway is essential for the proliferation of BCR-ABL-positive cells. Bcr-Abl forms a complex with PI3K, $p120^{Cbl}$, and adaptor molecules (such as Crk and CrkL) to activate this pathway (Skorski et al., 1995). Activated PI3K then stimulates Akt, a serine-threonine kinase which exerts antiapoptotic effects by phosphorylating Bad, preventing its binding to anti-apoptotic proteins. Increased phosphorylation of Bad is observed in BCR-ABL1-positive cells, suggesting reduced apoptosis. However, there are indications of additional survival pathways beyond Bad (Neshat et al., 2000). Furthermore, Myc overexpression is found in various types of human malignancies. Myc acts as a transcription factor, mediating gene expression changes in response to mitogenic signals (Deininger et al., 2000). It specifically targets genes involved in cell cycle regulation and apoptosis. In the context of BCR-ABL1, Myc enhances fibroblast transformation by BCR-ABL1, with its full activation requiring the SH2 domain and the C-terminus of the BCR-ABL1 fusion protein (Evan et al., 1992). Jak2 has been identified as playing a role in Myc induction by BCR-ABL1, potentially by promoting Myc mRNA expression and stabilising the Myc protein (Melo & Deininger, 2004).

In summary, BCR-ABL1, through its tyrosine kinase activity, engages multiple signalling pathways with mitogenic potential, including Ras-Raf-MEK-Erk, PI3K/Akt, Jak-STAT and MYC pathways. Understanding the intricate interplay between BCR-ABL1 and these pathways provides valuable insights into the pathogenesis of CML and highlights potential therapeutic targets for intervention.

2.3 Treatment of Chronic Myeloid Leukaemia (CML)

2.3.1 Tyrosine kinase inhibitors (TKI)

The tyrosine kinase plays a key role in diverse biological processes, including growth, differentiation, metabolism and apoptosis (Moradi et al., 2019). Bcr-Abl oncoprotein works by stimulating cell proliferation and inhibits apoptosis in CML. As for the treatment, Imatinib is used as the front-line therapy in CML. Since its FDA approval in 2001, imatinib, an inhibitor of BCR/ABL tyrosine kinase (TKI), has become the established treatment for patients with CML-Ph+. In the past, patients diagnosed with this condition had a survival rate of 7.5 years, but with the advent of imatinib, the average survival time has significantly increased to 17.5 years (Lavrov et al., 2019). Imatinib is a Bcr-Abl targeted agent that acts as a tyrosine kinase inhibitor (TKI). It is a small molecule inhibitor and competitively binds at the ATP-binding site of the Bcr-Abl. This binding prevents the conformational switch to active forms thus inhibiting tyrosine kinase's activity (Bhutra et al., 2014).

The evaluation of treatment efficacy in chronic myeloid leukaemia with Philadelphia chromosome-positive (CML-Ph+) patients relies on assessing hematologic, cytogenetic, and molecular responses. The restoration of typical clinical manifestations characterizes Complete Hematologic Response (CHR), while Complete Cytogenetic Response (CCyR) corresponds to the absence of the Philadelphia chromosome (Ph). Notably, the achievement of a molecular response represents a crucial treatment endpoint, wherein the optimal molecular response is defined as BCR-ABL1 transcript levels ≤ 10% within 3 months, ≤ 1% within 6 months (similar to CCyR), and ≤ 0.1% within 12 months, denoted as Major Molecular Response (MMR) (Yacob et al., 2022).

Treatment progress beyond the optimal response in chronic myeloid leukaemia with Philadelphia chromosome-positive (CML-Ph+) patients is categorised as either warning or failure, indicating the need for careful consideration regarding treatment continuation or modification based on the patient's circumstances. In cases where patients do not respond adequately or experience intolerance to imatinib, administering second and third-generation tyrosine kinase inhibitors (2G or 3G TKIs) becomes necessary. Nilotinib, dasatinib, and bosutinib, classified as 2G TKIs, are now considered front-line treatment options. These agents facilitate a faster achievement of Major Molecular Response (MMR) and elicit significantly deeper molecular responses compared to imatinib. However, it is essential to note that using 2G and 3G TKIs is associated with more severe side effects and clinical limitations without demonstrating improvements in overall survival outcomes (Braun et al., 2020). The BCR-ABL1 transcripts are typically determined through reverse transcription-quantitative polymerase chain reaction (RT-qPCR). This method determines the ratio of BCR-ABL1 transcripts to ABL1 transcripts or a recognised control transcript, as established by the International Randomized Study of Interferon and STI571 (IRIS) trial. The resulting ratio is expressed on the International Scale (IS). The baseline on the scale is set at 100%, as standardised by IRIS. Below this baseline, specific values such as 1%, 0.1%, 0.01% (MR4), 0.0032% (MR4.5), and 0.001% (MR5) correspond to 2-log, 3-log, 4-log, 4.5-log, and 5-log reductions from the IRIS baseline, respectively. These reductions indicate deep molecular responses (DMR), signifying significant decreases in BCR-ABL1 transcript levels.

Following the ratio mentioned, another study has reviewed and recommended a laboratory procedure for scoring the deep molecular response following treatment for CML. A conversion factor is employed to convert laboratory ratios to establish an

International Scale (IS) ratio. This conversion factor is determined by conducting sample exchanges with a well-established reference laboratory or by calibration against the WHO International Genetic Reference Panel. These procedures ensure the alignment of the laboratory measurements with the standardised IS ratio, allowing for accurate and comparable assessment of BCR-ABL1 transcript levels across different laboratories and studies (Cross et al., 2015).

Overall, tyrosine kinase inhibitors from the 1st generation until the 3rd generation have revolutionised treatment for CML. Nevertheless, despite the successful use of these TKIs, there were still cases of TKI resistance due to several factors. Consequently, identifying alternative molecularly targeted therapies has emerged as a potential solution to overcome the resistance challenge.

2.3.2 Imatinib resistance of CML

In most cases, point mutations in the BCR-ABL kinase domain (KD) have been identified as the primary cause of clinical resistance to imatinib (Soverini et al., 2011). About 20-30% of CML patients experience imatinib resistance. Hence, second-generation TKIs like Nilotinib and Dasatinib were introduced, as different Bcr-Abl KD mutations lead to varying resistance levels. It was reported that the imatinib resistance patients with defined Bcr-Abl mutations could be exposed to high risk of acquiring additional mutations, thus increasing resistance toward the second or third generation of TKI therapies (Soverini et al., 2009). These point mutations arise from amino acid substitutions within the BCR-ABL domain. For example, the T315I mutation occurs when threonine is replaced by isoleucine, resulting in drug resistance and reduced life expectancy. While some BCR-ABL KD mutations have not been linked to drug

resistance (Khorashad et al., 2006; Sherbenou et al., 2007), the T315I mutation shows high resistance to imatinib, Dasatinib, and Nilotinib. Thus, for this kind of mutation, Ponatinib was developed. Notably, Nilotinib appears more effective than Dasatinib in patients with V299L, T315A, or F317L/V/I/C mutations (Soverini et al., 2011). Other mutations like the E225V mean glutamic acid substitution with valine, while an E225K represents glutamic acid substitution with lysine. Besides, a Y253H represents tyrosine replacement with histidine, while the H396P represents proline histidine replacement (Sillaber et al., 2000).

Furthermore, it was also mentioned that the imatinib resistance mechanism relates to different target genes such as AKT2, STAM2 and STAT5A (Kaymaz et al., 2015). STAT5A is believed to play significant roles in imatinib resistance during Bcr-Abl-induced tyrosine phosphorylation by accelerating the transformation of haematopoietic cell lines by p210Bcr/Abl (Sillaber et al., 2000). Figure 2.4 shows a map of amino acid substitution that can occur at the Bcr-Abl Kinase Domain. It includes key regions such as the P-loop, which represents the phosphate binding loop, and the SH2 contact and SH3 contact regions that interact with proteins containing SH2 and SH3 domains. Additionally, the A-loop indicates the activation loop. Notably, amino acid positions marked with a star are directly involved in imatinib binding through hydrogen bonds or van der Waals interactions (Soverini et al., 2011).

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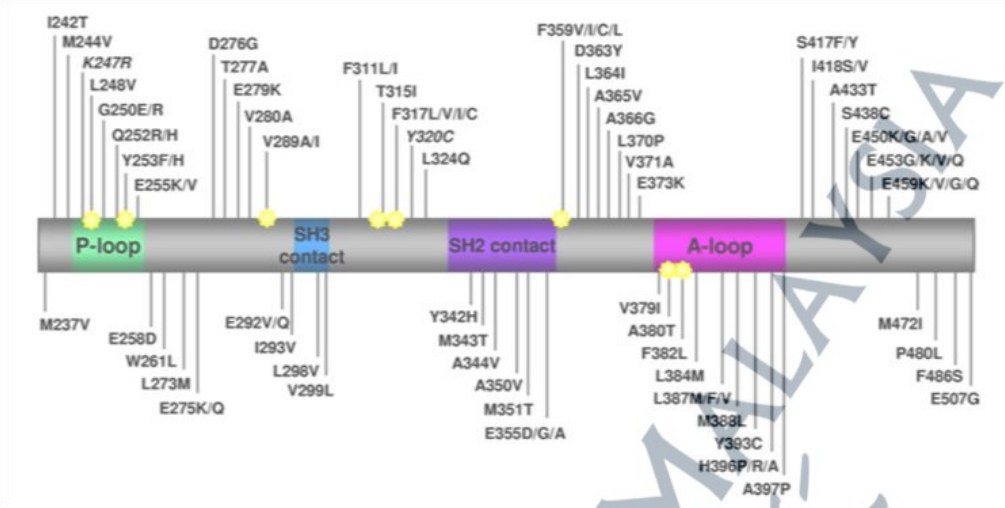


Source: Soverini et al., (2011).

Figure 2.4: The diagram illustrates the amino acid substitutions within the Bcr-Abl Kinase Domain.

Moreover, the crystallographic studies have elucidated the interaction of imatinib with the ABL1 kinase domain. These studies showed that imatinib binds to an inactive conformation of the enzyme, leading to the displacement of the Asp-Phe-Gly (DFG) motif and the adoption of a closed conformation for the activation loop, thereby preventing ATP binding (Nagar et al., 2002). The resistance to imatinib has been observed in chronic myelogenous leukaemia (CML) patients, with mutations occurring in or near the imatinib binding site. Hence, the second and third-generation ABL1 tyrosine kinase inhibitors (TKIs) have been developed to address this resistance. Based on the imatinib scaffold, Nilotinib exhibits higher affinity for the ABL1 kinase domain and demonstrates efficacy against some imatinib-resistant mutations. Dasatinib, a dual SRC/ABL1 inhibitor, binds to the ATP site in an active conformation, exhibiting superior potency compared to imatinib and nilotinib. Nilotinib and dasatinib have partially overlapping resistance profiles, with mutations at specific positions remaining a common challenge, especially the T315I mutation (Braun et al., 2020). Meanwhile, Ponatinib, a highly potent ABL1 inhibitor, was explicitly designed to target the T315I

mutation, which confers resistance to multiple TKIs. However, ponatinib's broad kinase target profile beyond ABL1 has increased the risk of vascular occlusive events. So, Asciminib, an allosteric ABL1 kinase inhibitor, was developed to mitigate off-target toxicities. Asciminib uniquely binds to the myristoylation pocket of BCR-ABL1 and exhibits potency against several BCR-ABL1 point mutations, including T315I. Clinical trials have shown positive responses in patients with chronic-phase CML with 92% complete haematological response (CHR) and 54% complete cytogenetic response (CCR) (Eide et al., 2019; Wylie et al., 2017). However, asiminib resistance might be problematic if the myristoylation pocket mutation occurs (Braun et al., 2020). Nevertheless, a recent study found that 55% of CML patient having asciminib as their treatment had several side effects such as fatigue, thrombocytopenia, anaemia and arthralgias. However, none of the patients experienced cardiovascular events or arterial disease (Pérez-Lamas et al., 2022).

Developing ABL1 TKIs, such as Nilotinib, dasatinib, ponatinib, and asciminib, has addressed resistance issues associated with imatinib in CML treatment. These inhibitors target different conformations and binding sites of the ABL1 kinase domain, providing varying potency and addressing specific resistance mutations. However, further research and clinical investigations are necessary to fully understand the side effects and long-term efficacy of these TKIs, particularly in the context of compound mutations and low-level resistance variants. Hence, lower toxicity or perhaps a combination of TKI drugs with other molecules can help reduce the toxicity of current drugs used in CML treatment.

2.3.3 Independent mechanism of TKI resistance

Despite the tyrosine kinase inhibitors, imatinib-resistant disease with no detectable BCR-ABL KD mutations still occurs. This resistance is believed to be driven by BCR-ABL1 independent pathways, including drug influx-efflux, alternative signalling pathways, DNA hypermethylation and microRNA (miRNA) dysfunction, which contribute to the resistance of CML cells to current treatments (Yap et al., 2017). Additionally, BCR-ABL1 independent mechanisms such as increased genomic instability, modification of drug transporters, alteration of the bone marrow microenvironment, support of leukaemia stem cells, and activation of survival-promoting signalling pathways further contribute to therapy resistance in chronic myeloid leukaemia (Loscocco et al., 2019; Rudich et al., 2022). Notably, recent research has revealed that specific metabolites associated with Glutathione (GSH) metabolism, including L-alanine, 5-oxoproline/pyroglutamic acid, L-glutamic acid, glycine, and phosphoric acid, play a role in imatinib resistance by preventing apoptosis of cancer cells through their reaction with reactive oxygen species (ROS) in mitochondria (Bansal & Simon, 2018; Singh et al., 2022).

However, in addition to activating the independent mechanism of TKI resistance, leukemic stem cells (LSCs) pose a significant therapeutic challenge as they can survive independently of BCR-ABL1 and contribute to relapse following treatment discontinuation. (Corbin et al., 2011). LSCs in chronic myeloid leukaemia (CML) exhibit high resistance to tyrosine kinase inhibitors (TKIs) due to their heterogeneous adaptations, including modifications in the transcriptome, genome and epigenome. Due to the activation of diverse BCR-ABL independent pathways and dysregulation caused by the BCR-ABL1 oncogene in LSCs, it is expected that the metabolic programming of LSCs will be altered, thereby implicating their survival adaptations and resistance to

treatments, which is a recognised hallmark of cancer stem cell biology (Hanahan & Weinberg, 2011; Holyoake & Vetrie, 2017). Unlike normal cells that primarily utilise oxidative phosphorylation in the mitochondria to produce energy from glucose, cancer cells can undergo a metabolic switch, known as the Warburg effect, to a less efficient process called aerobic glycolysis. This metabolic shift involves high levels of glucose consumption in the cytosol, even in the presence of abundant oxygen (Alves et al., 2021).

Nevertheless, another study found that factors such as the bone marrow environment also play a critical role in the survival of LSCs. A hypoxic condition in a bone marrow helps to stabilise the LSC by regulating the HIF-1α which acts as the hypoxia-inducible factor crucial for LSC maintenance, survival, and proliferation (K. Wu et al., 2021; H. Zhang et al., 2012).

Another factor that contributed to the maintenance of the CML-LSC is the activation of autophagy. Autophagy is a conserved self-eating mechanism or self-degradative process which is important in balancing cellular homeostasis when the cell is under stress. Autophagy can limit tumour transformation during the early stage, but it also favours the survival, proliferation, metastasis and resistance to chemotherapy in advanced-stage tumours (Leonardi et al., 2021; Sadri Nahand et al., 2022). Autophagy helps the LSC to adapt during metabolic stress, such as during the TKI treatment, by generating ATP and essential building blocks when oxygen and nutrients are inadequate, thus making it resistant to apoptosis (Rakesh et al., 2022). Moreover, in CML-LSCs, the basal autophagy process is elevated significantly compared to normal hematopoietic stem cells (HSCs) and continues to increase after TKI treatment. This indicates that inhibition of the autophagy process could help enhance TKI-induced cell death in LSCs (Alves et al., 2021).

In conclusion, CML resistance includes the BCR-ABL dependent and the BCR-ABL independent pathways. The BCR-ABL dependent mechanism is related to the mutations at the tyrosine kinase domain, while the independent mechanism involves the activation of alternative signalling pathways that help the LSCs to survive. Hence, it is important to understand the pathways, metabolic alterations, and leukemic stem cell availability to facilitate the advancement of improved therapeutic modalities and strategic interventions that are capable of effectively overcoming resistance and preventing the recurrence of the disease.

2.3.4 Alternative Molecular Targeted Treatment of CML

Failure to fully achieve remission-free treatment using TKI alone has led to the discovery of alternative treatments for CML. These treatments involve the use of alternative drugs known as epigenetics drugs. When combined with TKI, these epigenetics drugs were found to have more positive effects in inhibiting or decreasing CML cell proliferation thus helping in managing the resistance problem (Amir & Javed, 2021). However, the drug combination needs to be optimised accordingly before being administered to patients. Epigenetics drugs are still unable to cater for all of the problems related to TKI resistance yet still may have certain side effects. Hence, scientists are starting to look at other molecules that might help in managing CML. Because of that, microRNA had come into the picture. In cancer, microRNA aberrant expression was related to disease manifestation and pathogenesis. Similar to CML, several microRNAs were found to be significantly expressed or downregulated. These discoveries have made microRNA one of the diagnosis and prognosis tools and the biomarker for monitoring drug sensitivity in patients. Because of the versatility of

miRNAs as biomarkers in CML, it was also suggested that they be used as the treatment. By using miRNA mimics or antimirs, miRNA-related research as treatment is a trend nowadays. Hence, it is urgent to study these miRNAs' mechanisms and actions that might help in inhibit or decrease CML cell proliferation.

2.4 The microRNA

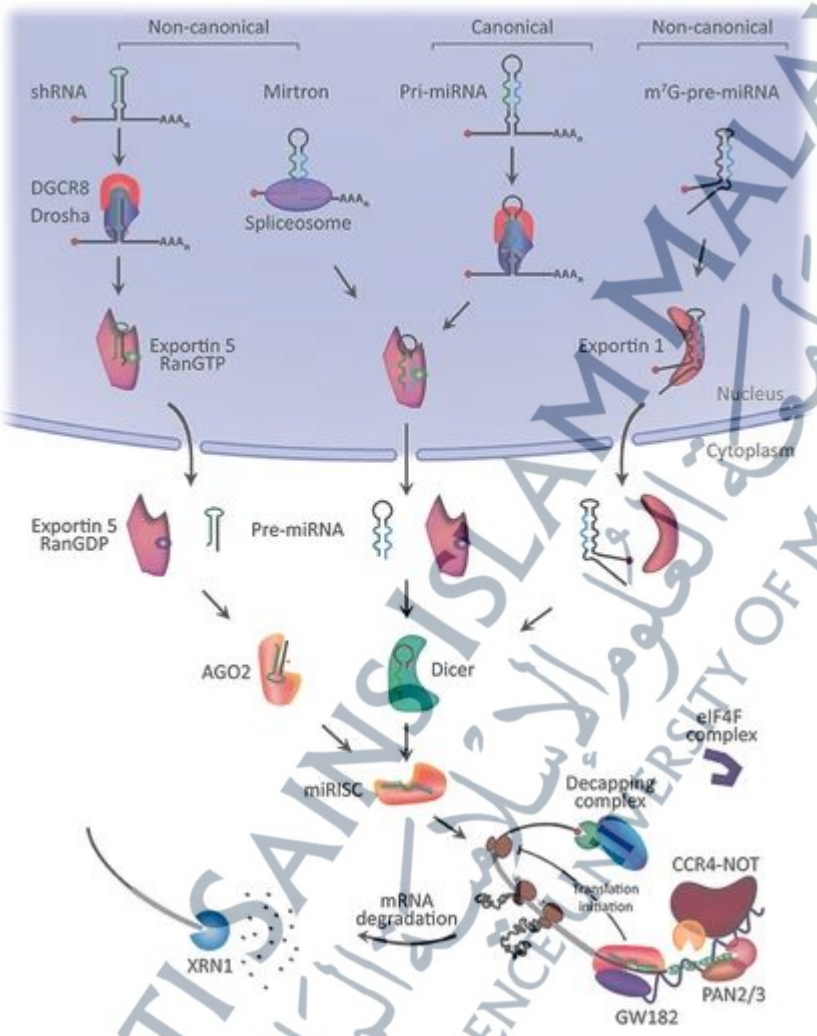
MicroRNA is linked to many human diseases and is currently sought as a clinical diagnostic and therapeutic target. The miRNAs are small endogenous non-coding RNA that function in regulating gene expression (O'Brien et al., 2018). MiRNA affects gene expression (post-transcriptionally) and acts as a gene silencer by inhibiting translation and accelerating target RNA degradation by binding to the 3'UTR of the target gen. The miRNA is the alternative molecularly targeted treatment for cancer nowadays, and a current study is trying to propose it as the alternative targeted treatment for CML. MiRNA is a non-coding RNA, about 22 nucleotides in length, and can be found in animals, plants and viruses (Samad & Kamaroddin, 2023). It functions by base pairing with the mRNA molecules. The mRNA molecules that are attached with miRNA will be silenced by (1) cleavage of the mRNA strand into two pieces, (2) destabilisation of the mRNA through shortening of its poly(A) tail, and (3) by making the mRNA translation into protein less efficient (Macfarlane & Murphy, 2010). MicroRNAs (miRNAs) play a vital role in the normal development of animals and contribute to a range of biological processes. The abnormal expression of miRNAs has been correlated with numerous human diseases, especially cancer. In malignancies, the oncogenic miRNA (oncomiRS) is overexpressed, while the tumour-expressed miRNA is downregulated (Radhi et al., 2022). Several investigations into the relationship

between miRNAs and cancer suggest their potential utility as oncogenes or tumour suppressors for enhancing the identification, diagnosis, and treatment of various cancers (Macfarlane & Murphy, 2010; Yacob et al., 2022).

2.4.1 MiRNA biogenesis

Since miRNA discoveries in 1993, miRNA has been recognised as an important molecule for gene regulation (Lee et al., 1993). MicroRNAs (miRNAs) can modulate the expression of a wide array of genes, ranging from tens to hundreds, which are integral to various cellular functions and metabolic activities (Kilikevicius et al., 2022). MiRNA can be synthesised in two ways; canonical or noncanonical pathway. Briefly, the canonical miRNA biogenesis pathway involved miRNA transcription, processing by Drosha and Dicer and lastly, the RNA Induced Silencing Complex (RiSC) loading and target repression (Hammond, 2015; O'Brien et al., 2018). It begins with the pri-miRNA transcript spliced, capped and polyadenylated by the enzyme Drosha producing the pre-miRNA. This process is associated with RNA-binding protein DGCR8 (Han et al., 2004). Later, the process continues by exporting the pre-miRNA into the cytoplasm using Exportin5 (Yi et al., 2003). Pre-miRNA undergoes Dicer cleavage and becomes duplex RNA. This step is aided by RNA binding protein the TRBP. Duplex RNA then binds to Argonaute 2 (Ago2) protein and is loaded into the effector complex of RNA-induced silencing complex (RISC). The Ago2 protein binds to the “seed” region of miRNA at nucleotide 2-8 thus leading to mRNA target repression or dissociation (Lewis et al., 2003). The miRNA has a resemblance with the small interfering RNA (siRNAs) of the (RNAi) pathway but differs in terms of their origin, biogenesis, specificity of action and roles in cellular physiology (Chakraborty et al., 2017). MiRNA words are

derived from the regions of RNA transcripts that fold back to form short hairpins. The application of miRNA-based therapeutic agents (mimics and antagonists) is of current interest thus making it a novel therapeutic tool for cancer treatment.



Source: (O'Brien et al., 2018)

Figure 2.5: Canonical and non-canonical pathways of microRNA (miRNA) biogenesis and function. MiRNAs are processed from primary transcripts via canonical (Drosha/DGCR8) or non-canonical (shRNA, mirtron, or m^{7}G-capped) pathways. Mature miRNAs, incorporated into the miRISC complex, regulate gene expression by promoting mRNA degradation or inhibiting translation

Meanwhile, miRNA can also be produced through non-canonical pathways. The pathways can be classified into two categories: those independent of the Drosha/DGCR8 complex and those that bypass DICER (Saiyed et al., 2022). One such

pathway involves the processing of small hairpin RNA (shRNA) by the DGCR8/DROSHA microprocessor complex, after which it is transported to the cytoplasm via Exportin 5/RanGTP. The pathway then continues with AGO2-dependent but DICER-independent cleavage (O'Brien et al., 2018). Distinctly, the Mirtron and M^7G-pre-miRNA pathways circumvent the DGCR8/DROSHA complex yet rely on DICER. While mirtrons utilize Exportin 5/RanGTP for cytoplasmic transport, M^7G-pre-miRNAs depend on Exportin 1 (Stavast & Erkeland, 2019). Irrespective of the specific route taken, all pathways culminate in the formation of the functional miRNA-induced silencing complex (miRISC). In most cases, miRISCs target and bind to mRNA, leading to translational inhibition, though in certain instances, they can also instigate mRNA degradation through the action of the exoribonuclease XRN1.

2.4.2 MiRNA mechanism of action

MiRNAs act by targeting multiple genes within a particular pathway, resulting in a broader, yet specific response (Gajda et al., 2021). It contains both binding sites for regulatory proteins as well as microRNAs. The binding of miRNA to the 3'UTR can decrease gene expression of various mRNAs by inhibiting the translation and degradation of the transcript. Besides that, 3'UTR also has silencer regions that bind to repressor protein, thus inhibiting mRNA expression. Many 3'UTRs contain AU-rich elements (AREs) that can affect the stability or decay rate of the transcript and later affect translation initiation. Generally, the 3'-UTR plays a crucial role in gene expression. It is crucial in gene expression because it can influence the localization, stability, export and translation efficiency of an mRNA. Furthermore, various sequences

that are involved in gene expressions, such as the (1) poly (A) tail, (2) microRNA response elements (MREs), and (3) A-U rich elements (AREs) are present in 3'UTR.

2.4.3 MiRNA-based therapeutics

MicroRNAs play a vital role in the maintenance of tissue homeostasis exerting dual biological functions: one as tumour-suppressive and the other as an oncogene. A tumour-suppressive miRNA targeted oncogene and is typically downregulated in cancer. Meanwhile, an oncogenic miRNA regulates tumour-suppressive genes and is often overexpressed in cancer (To et al., 2019). Consequently, based on their functions and expressions in cancer, different approaches to miRNA-based therapeutics were developed.

Strategies for miRNA-based therapeutics: Mirna-based therapeutics aim to rectify aberrant miRNA expressions within cells. This includes the reconstitution of endogenous miRNA and the reduction of oncomiRs. This strategic approach employs two primary methods: synthetic miRNA molecules, referred to as miRNA mimics, and oligonucleotide-based miRNA inhibitors known as antimiRs. Specifically, miRNAs are employed either as a restorative agent by utilizing mimics to replace lost specific miRNAs, or as antagonists to inhibit and counteract the overexpressed miRNA responsible for pathological processes (Hanna et al., 2019; Rupaimoole & Slack, 2017). The antimiRs, also recognized as antisense oligonucleotides (ASOs) effectively downregulate oncogenic miRNA. For instance, ASOs possess the capability to hinder the binding of miRNAs to their target mRNA. The restoration of tumour-suppressor miRNAs presents a novel approach through direct transfection of the synthetic miRNA mimic into the cells. These miRNA mimics are chemically synthesised as double-stranded RNA molecules, closely mimicking mature miRNA duplexes. One noteworthy

advantage of utilising miRNA mimics is their potential to target multiple pathways simultaneously. On the other hand, the inhibition of oncogenic miRNAs using ASOs involves preventing miRNAs from binding to their target mRNA. To increase the therapeutic potential, ASOs undergo sugar backbone modification and chemical modification to enhance resistance against nuclease-mediated degradation, while simultaneously increasing their binding affinity to their target (Lennox & Behlke, 2010; To et al., 2019; Watts, 2013). A noteworthy application of miRNA-based therapeutics in the context of CML treatment is evident in a study by Li et al., (2013). Their findings indicate that mir-29b effectively suppresses the proliferation of CML cells by inducing apoptosis via regulation of BCR/ABL1 protein. Additionally, another study conducted by Li et al., (2019) identified a total of 11 key candidate targets and 33 corresponding microRNAs. Notably, these pathways predominantly revolved around PI3K/AKT, Ras, JAK/ STAT, FoxO and Notch signalling pathways. Furthermore, the roles and functions of miRNAs can exhibit intricacies and ambiguity contingent upon the specific contexts. For example, a study conducted by (Zhang et al., 2018) unveiled miR-126 significant involvement in sustaining the persistence and stemness of leukemic stem cells (LSCs). Interestingly, the same miRNA also emerges as a noteworthy prognostic biomarker for gauging the responsiveness of imatinib treatment, as demonstrated by (Ali Beg et al., 2021). These seemingly contradictory findings offer a comprehensive perspective on miR-126's dual role in CML as a molecular regulator of LSC behaviour and a potential clinical tool for treatment assessment. Generally, the findings from previous studies have categorized miRNAs into two fundamental groups. MiRNAs that exhibit differences from the standard control group are classified as biomarkers or prognostic markers. Meanwhile, miRNAs that are found to inhibit cell growth progression and promote apoptosis are recognized for their therapeutic potential. Table 2.1 presents a

comprehensive list of miRNAs associated with various pathological conditions and their potential roles as biomarkers, prognostic markers, and therapeutic agents.

While miRNA-based therapeutics showed great potential for targeting diseases like CML, its application in other diseases is currently in clinical trials. Since the first approval of siRNA treatment by the FDA in 2018 (Adams et al., 2018), miRNA has emerged as another RNA-based therapeutics. Based on Table 2.6, three miRNA mimics are in clinical trials. Two of them, miR-16 and miR-29 are in Phase I and Phase II while another miRNA, miR-34a was terminated due to a severe immune response and caused the death of four patients (Hong et al., 2020). For miRNA inhibitors (antimiR), several miRNAs are in Phase I and Phase II clinical trials. Among them is the inhibitor for miR-21 (RG-012). RG-012 is known as Lademirsen and acts by silencing metabolic pathways of ATP generation, ROS production and inflammatory signalling (Samad & Kamaroddin, 2023).

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Table 2.1: The list of miRNA's roles and functions.

Mirna	Findings	Target gene	Function	References
Let7	• Dysregulated in blast crisis of CML • Low expression of Let7 leads to self-renewal capacity, relapse and therapy resistance	ADAR1	• Biomarker • Potential therapeutic target	(Zipeto et al., 2016)
Mir-101	• Tumour suppressor • downregulating Jak2 expression and sensitizing K562 cells to imatinib.	JAK2	• Potential therapeutic target	(Farhadi et al., 2016)
miR-126-3p (miR-126)	• Regulates LSC dormancy & engraftment potential • Downregulated in LCSs but overexpressed in the surrounding leukemic niche • Mir-126 expression is higher after imatinib treatment	PI3KR2 SPRED1	• Potential therapeutic target • Prognostic biomarker	(Zhang et al., 2018) (Ali Beg et al., 2021)
miR-141-5p	• Downregulated in CML patients and K562 cells but elevated after treatment with TKI. • Tumour suppressor	RAB32	• Potential therapeutic target • Diagnosis biomarker	(Bao et al., 2019)
miR-142-5p	• Downregulated in non-TKI responders CML patients' blood & bone marrow	ABL2 cKIT MCL1 SRI	• Prognosis biomarker	(Klumper et al., 2020)
miR-153-3p	• Downregulated in imatinib-resistant patient • Reduced imatinib induces apoptosis	Bcl-2	• Potential therapeutic target	(Li et al., 2020)
Mir-181a	• Downregulated in CML patients and K562 cell	PPF1A	• Potential therapeutic target	(Gu et al., 2019)

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Table 2.2: The list of miRNA's roles and functions (continued).

Mirna	• Findings	Target gene	• Function	References
Mir-181c	• mir-181c expression were different between different p210 BCR-ABL transcript levels patients	BCR-ABL1	• prognosis biomarker	(Radhi et al., 2022)
miR-185	• tumour suppressor • downregulated in CD34+ cells from non-TKI responders CML patients	PAK6	• prognosis biomarker • Potential therapeutic target	(Lin et al., 2020)
Mir-196b	• Tumour suppressor • Heavily methylated in CML patients	BCR-ABL1 HOXA9	• Potential therapeutic target	(Liu, Zheng, et al., 2013)
miR-199a/b- 5p	• Downregulated in imatinib resistance K562 cell • Promotes protective autophagy	WNT2	• Potential therapeutics target	(Chen et al., 2018)
Mir-203	• Downregulated in CML causes LSCs persistence • prevent apoptosis and allow stem cell renewal	Survivin Bmi-1	• Potential therapeutic target • Biomarker	(Zhang et al., 2016)
Mir-203-5a	• miR-203a-5p reduced imatinib resistance by regulating Glutathione (GSH) metabolism.	GSH metabolism	• biomarker	(Singh et al., 2022)
miR-21	• Oncogene • It protects CML LSCs following treatment with imatinib • AntagomiR-21 increases the efficiency of imatinib and promote apoptosis	PI3K/AKT pathway	• Potential therapeutic target	(Wang et al., 2015)
Mir-212	• Dysregulated during the conversion of treatment naïve cells to imatinib resistance cells.	ABCG2	• Prognostic marker	(Kaehler et al., 2017)

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Table 2.3: The list of miRNA's roles and functions (continued).

Mirna	• Findings	Target gene	• Function	References
miR-214	• Downregulated in imatinib-resistant patients and K562R cell • Increased drug efflux transporter in CML	ABCB1	• Potential therapeutic target	(Jin et al., 2018)
miR-221	• Tumour suppressor • Downregulated in imatinib-resistant patients • Increased survival & decrease apoptosis	STAT5	• Potential therapeutic target	(Jiang et al., 2019)
Mir-23a	• Low expression in CML • High expression-induced apoptosis	BCR-ABL1	• Potential therapeutic target	(Xishan et al., 2014)
miR-29a-3p	• Overexpressed in CML LSCs, stop apoptosis • antagomir-29a-3p desensitize CML to imatinib	TET2	• potential therapeutic target	(Salati et al., 2017)
Mir-29b	• downregulated in CML patient • overexpressed miR-29b promotes apoptosis and slow proliferation rate of cell	BCR-ABL1	• Potential therapeutics target • biomarker	(Y. Li et al., 2013)
Mir-30a	• Target Beclin-1, ATG5 and promote intrinsic apoptosis. • Downregulated in CML and contribute to imatinib resistance	BCR-ABL1	• Biomarker • Potential therapeutics target	(Liu, Song, et al., 2013; Yu et al., 2012)
Mir-320a	• Downregulated in CML and causes low survival rate in patients	BCR-ABL1	• potential therapeutic target	(Xishan et al., 2015)

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Table 2.4: The list of miRNA's roles and functions (continued).

Mirna	• Findings	Target gene	• Function	References
miR-328	• Downregulated in the CML blast phase leads to CML survival. • Restoration of miR-328 induces apoptosis in CML cell	hnRNPE2	• Potential therapeutic target	(Eiring et al., 2010)
miR-342-5p	• Downregulated in CML patients • Promotes cell cycle, therapy resistance	CCND1	• Potential therapeutic target	(Y. Y. Wu et al., 2021)
miR-378	• Overexpressed in the bone marrow of CML patients • Promotes self-renewal & pluripotency of LSCs in CML	FUS1	• Biomarker • Potential therapeutic target	(Ma et al., 2019)
miR-379-5p	• Downregulated in bone marrow microenvironment • Reduce the efficacy of imatinib treatment	AKR1C3	• Potential therapeutic target	(Pan et al., 2021)
Mir-424	• Downregulated in CML and cause imatinib resistance • Overexpression able to sensitive K562 cell to imatinib	BCR-ABL1	• Biomarker • Potential therapeutics target	(Hershkovitz-Rokah et al., 2015)
Mir-409-5p	• Downregulated in paediatric CML and CML cell	NUP43	• Potential therapeutic target	(Liu et al., 2019)
miR-494-3p	• Downregulated in CML LSCs • Increased miR-494-3p expression enhances the effectiveness of TKI in inducing apoptosis in CML LCS	c-Myc	• Potential therapeutic target	(Salati et al., 2017)

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Table 2.5: The list of miRNA's roles and functions (continued).

Mirna	• Findings	Target gene	• Function	References
Mir-495-3p	• mir-495-3p was lower in BCR-ABL1 expressing cellular models in-vitro • Overexpression of mir-495-3p hindered CML cell growth and TKI resistance in T315I-mutant cells	MDR1	• Biomarker • Potential therapeutics target	(Rittavee et al., 2023)
miR-577	• Downregulated in mononuclear cells of CML patients • Desensitize CML to imatinib treatment	NUP160	• Potential therapeutics target	(Zhang et al., 2019)
miR-660-5p	• overexpressed in CML LCSs • protects CML LCSs by binding to its target • regulates cellular response to hypoxic environment	EPAS1	• biomarker	(Salati et al., 2017)
Mir-96	• tumour suppressor • Downregulated in CML blast phase compared to chronic phase • Promotes CML-BP cell proliferation and cell differentiation	BCR-ABL1	• Prognosis biomarker • Potential therapeutic target	(Huang et al., 2019)

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Additionally, the latest use of miRNA-based therapeutics includes artificial miRNA, also known as amiRNA. AmiRNA is a combination of siRNA sequences and scaffolds of the pri-miRNA transcript. Currently, two amiRNAs are undergoing clinical trials: AMT-130 and BCH-BB694. AMT-130 is targeting the huntingtin (HTT) gene of the Huntington disease (Keskin et al., 2019). Meanwhile, BCH-BB694 is used in sickle cell disease (SCD), targeting the BCL11A gene that allows erythroid lineage-specific knockdown (Esrick et al., 2021).

Table 2.6: miRNA-based therapeutics in clinical trials.

Mirna-approach	Notable miRNAs	Clinical trial	References
miRNA mimic	miR-16, miR-29b, miR-34a	• MesomiR 1 (miR-16 mimic): Ongoing trial, tumour growth inhibition. • Remlarsen (miR-29 mimic, MRG-201): Potential for fibrosis inhibition, promising preclinical data. • MRX34 (miR-34a): terminated.	(van Zandwijk et al., 2015), (Gallant-Behm et al., 2019) (Hong et al., 2020)
miRNA inhibition	miR-21, miR-92a, miR-103/107, miR-122, miR-132, miR-155	• RG-012 (lademirsen, miR-21 inhibitor): Histological improvements in Alport mice. • MRG-110 (miR-92a inhibitor): Enhanced angiogenesis • RG-125 (AZD4076, miR-103/107 inhibitor): Impact on non-alcoholic steatohepatitis in diabetes patients • miR-122 inhibitors (miravirsen, RG-101): Promising Hepatitis C V viral load reduction • CDR132L (anti-miR-132): Positive safety and cardiac function outcomes • MRG-106 (anti-miR-155): cutaneous T-cell lymphoma cellular proliferation reduction.	(Kashtan & Gross, 2021) (Gallant-Behm et al., 2018) (Huang, 2017) (Deng et al., 2020) (Batkai et al., 2021) (Querfeld et al., 2016)
amiRNAs	miHTT (AMT-130), BCL11A (BCH-BB694)	• AMT-130: Potential for Huntington's disease symptom reduction, preclinical outcomes. • BCH-BB694: Targeting BCL11A for sickle cell disease treatment, early clinical stage.	(Keskin et al., 2019). (Esrick et al., 2021)

Nevertheless, miRNA-based therapeutics also can be achieved via the use of a combination of conventional drugs and miRNA, a combination of miRNA and siRNA and the use of miRNA sponges (Diener et al., 2022). The success of miRNA-based therapies depends on efficiently delivering miRNA molecules to the target cells or tissues. MiRNAs are fragile molecules prone to degradation when exposed to various biological conditions. Hence, a safe and efficient miRNA delivery system is still a significant challenge in miRNA-based therapeutics. Currently developed delivery systems for miRNA use viral and non-viral vectors. Both approaches pose their advantages and limitations in gene transfer. In viral vectors, utilising genetically modified viruses can assist in miRNA delivery efficiently and enhance the gene expression levels for a more extended period. The viruses were genetically modified to reduce toxicity and allocate space for integrating of desired genes. Different types of viral vectors, including retroviral (RV), lentiviral (LV), adenoviral (Ad) and adeno-associated viruses (AAVs) and bacteriophage-based virus particle (VLP) vectors, have been developed for specific transgenes and targeted cell types (Samad & Kamaroddin, 2023). The viral delivery system has advantages in efficiently transferring desired oligonucleotides into different tissue types and driving elevated gene expression levels. However, it also has limitations such as high immunogenicity, toxicity and size limitation. Specific properties of the transgene, vector dose and serotype, administration route, host species, and presence of pre-existing neutralising antibodies may influence the development of an immunological response against viral vectors (Dasgupta & Chatterjee, 2021). Due to the adverse effects of the viral delivery vectors, the non-viral delivery system has emerged as an alternative to viral-based delivery methods. These systems are less toxic, more biocompatible, and can successfully deliver miRNA into cells without being degraded by nucleases (Diener et al., 2022). Some examples of non-

viral delivery systems include lipid-based nanocarriers, polymer-based approaches, inorganic compounds, and extracellular vesicle carriers. Lipid-based nanocarriers are the most widely used non-viral delivery methods. They can form complexes with nucleic acid and have a high affinity for cell membranes. Compared to viral-based delivery systems, these non-viral approaches offer potential advantages in terms of reduced immunogenicity, toxicity, and size limitations. However, further research is needed to optimise these non-viral delivery systems and evaluate their efficacy in clinical settings.

Taken together, miRNA-based therapeutics are currently in high demand and gaining attention for their potential to regulate mRNA or gene dysregulation in diseases, especially cancer. However, this technique requires further research and exploration, particularly in developing an efficient delivery system to achieve high loading capacity, stability, enhanced half-life in circulation, minimal toxicity, and prevention of rapid cargo degradation. Furthermore, future studies should prioritise characterising disease-specific markers on target tissues and exploring new targeting ligands to enhance the efficacy of miRNA therapeutics.

2.4.4 Plant microRNA: A novel therapeutics tool for cancer therapy

Recently, there have been increased reports that miRNAs could be transmitted from species to species to mediate cross-kingdom regulation, especially between plants and animals (Elias et al., 2020; Ma et al., 2017; Mohanty et al., 2022). Plant miRNA previously is believed to be degraded by digestion and cannot survive in animals; hence the possibility of taking plant miRNAs using dietary ways was ignored. However, recent researchers have found that plant miRNAs are more stable than animal miRNAs

due to chemical modification that can help them to remain stable in acidic environments, like in the stomach (L. Zhang et al., 2012). It was found to be circulated in the bloodstream (Liang et al., 2015), breast milk (Lukasik et al., 2017) and various tissues and organs (Jia et al., 2015). Additionally, other studies have also suggested that plant miRNAs might have health benefits in humans by potentially helping with conditions like cardiovascular disease, cancers, chronic inflammation and lung fibrosis (Cavalieri et al., 2016; Du et al., 2019; Hou et al., 2018; Saiyed et al., 2022). Generally, plant miRNAs are involved in signal transduction, stress responses, plant development and secondary metabolites synthesis (Djami-Tchatchou et al., 2017; Nandakumar et al., 2021). For instance, in tomato and banana plants, the endogenous miRNA manages the advancement of fruits & their ripening (Bi et al., 2015). Plant miRNA synthesis is not so different from animal miRNA biogenesis pathway. It starts with the transcription of primary miRNA (pri-miR) by the RNA polymerase II/III enzyme. In the nucleus, pri-miRNAs are converted into stem-loop pre- miRNAs by DICER-LIKE 1 (DLC1). Pre-miRNAs were then transported into the cytoplasm by the plant exportin. Mature miRNAs are formed from pre-miRNAs' strands and methylated by a small RNA methyltransferase called HUA Enhancer 1 (HEN1) (Yu et al., 2005; Yu et al., 2017). Lastly, the response to miRNA silencing is carried out by AGO-containing RNA-induce silencing complex (RISC) proteins that work on the guide miRNA strand. During the maturation process of the pre-miRNA, it undergoes modification of the single 2'-O-methylation on its 3' end (Li et al., 2005). This criterion gives plant miRNAs more stability and less susceptibility to RNAse degradation and can withstand low pH conditions and human phagocytosis processes (Mohanty et al., 2022; Yu et al., 2005). The biogenesis and acting mechanism of miRNAs display a high degree of similarity between animals and plants, making it possible for cross-kingdom interaction.

Moreover, the evolutionary divergence in the composition and function of miRNA processing machinery also contributed to possible interactions between species. Due to evolutionary conservation, it is believed that plant miRNA has the functional homology to their mammalian counterparts that help regulate human genes during cell line transfection (Pirro et al., 2016).

The miRNAs are believed to function by joining in with a specific gene-targeted to mediated regulatory pathway, thus affecting significant biological functions. Scholars viewed this discovery as an underpinning for novel platforms in diagnosing, prognosis, and treating diseases, including cancer. However, others consider that the mentioned results are doubted and claimed as a "false positive effect" of the experiments (Pinzón et al., 2017) . Nevertheless, evidence reveals miRNAs that may target endogenous genes are rising in numbers. Like miRNAs in animals, plant miRNAs could be the potential source of the inhibitor. It can act in two ways. The first is by acting as an inhibitor (synthetic RNAs) by reversing miRNA's action by upregulating targeted mRNA. Secondly, the plant miRNA mimics (synthetic miRNAs) inhibit translation or degrade the target mRNAs (Rufino-Palomares et al., 2014). In target recognition, plant miRNAs bind to their target by complete binds within the seed regions, thus triggering mRNA degradation instead of translational repression like in animal miRNAs (Gismondi et al., 2021). Plant miRNA can function as a regulatory factor across species. They started in 2009 when researchers from Monsanto Company discovered that various endogenous plant small RNAs were found to have perfect complementarity to human genes and those of other mammals (Ivashuta et al., 2009). It was followed by Zhang and team in 2011, who claimed that plant miR168a could inhibit the expression of LDLRAPI1 in mammalian liver cells via gastrointestinal tract absorption. According to Zhang et al., (2012), functional studies in vitro and in vivo

demonstrated that MIR168a could bind to the human/mouse low-density lipoprotein receptor adapter protein 1 (LDLRAP1) mRNA, inhibit LDLRAP1 expression in the liver, and consequently decrease LDL removal from mouse plasma. Besides, a plant-derived miRNA mimic, miR159, was found in human serum and was inversely correlated with the incidence and progression of breast cancer in humans (Chin et al., 2016). Additionally, mir171vr, which is an isoform of the plant mir171, was found to be targeting GNA12 in HEK293 cells and able to regulate cell growth and metabolism via the mTOR pathway. Moreover, the same miRNA stability was tested and it showed that it can withstand pH 2 to 7 and a high temperature until 100°C. miR171vr showed a highly conserved sequence that can be found in 14 plant species such as *Malus domestica* (apple), *Oryza sativa* (rice), *Zea mays* (corn), *Theobroma cacao* (cocoa), *Cucumis melon* (melon) etc which most of them are foods (Gismondi et al., 2021). These studies have found evidence of plant miRNAs in the blood and tissues of animals and humans, indicating that they can withstand the digestion process and enter the bloodstream. However, there is also conflicting evidence, with other studies failing to find any transfer of plant miRNAs from the diet, as shown in Table 2.3. From Table 2.3, most past research only focused on the bioinformatics approach and in-silico study of plant miRNA-target interaction in human genes, and many plant miRNAs have not yet been validated through experimentation. Meanwhile, it has been suggested that plant miRNAs can be taken via dietary intake in daily life since the discovery of plant miRNA delivery through diets by Zhang and his colleagues in 2012. It is believed that plant miRNAs can be delivered interspecies into the cells through the use of extracellular vesicles or exosomes (Valadi et al., 2007). Numerous previous findings mentioning the use of plant-derived nanoparticles (PDNPs), plant-derived extracellular vesicles (PDEVs), plant-derived exosome-like nanoparticles (PELNs), small extracellular

vesicles (SEVs) or exosome-like nanoparticles are referring to the similar mechanism of delivery of these plant miRNAs. For example, PELNs of Ginger (*Z. officinale*), were found to contain 27 highly expressed miRNAs that are involved in the inflammatory regulation and cancer-related pathways. These miRNAs could be absorbed by the intestinal cells using caveolin-mediated endocytosis and micropinocytosis (Yin et al., 2022). Additionally, another approach to delivering plant miRNA into the cells by using SEVs was discussed by Ozkan et al. (2021). Garlic (*Allium sativum*)-derived SEVs were found to successfully inhibit human kidney carcinoma cell proliferation and induce apoptosis via an intrinsic pathway (Ozkan et al., 2021). These findings gave insight into the potential of plant miRNA delivery into human cells. However, some findings cannot find a significant amount of plant miRNA in human serum or blood indicating the failure of miRNA delivery into the circulatory system via diets (Table 2.3). Despite these arguments, plant miRNA can potentially regulate gene expression in human or animal models. Depending on the animals' models and types of plants, miRNA introduced into the cells is the major factor contributing to miRNA detection and its effects on human gene expression. It is because a lot of evidence shows the ability of these plant miRNAs to target human genes and regulate biological processes such as cell proliferation, viability and cell cycle (Elias et al., 2020; Mohamad & Elias, 2021; Saiyed et al., 2023; Zhang et al., 2023). However, most of the available data was collected from computational methods and *in vitro* studies. Hence, validation of these findings is a major need. Moreover, the delivery of these plant miRNAs employing food ingestion is still very early to be confirmed. Hence, there is an urgent need for further study to validate the hypothesis of cross-kingdom interaction mediated by plant miRNA and miRNA delivery mechanism in different types of cells especially in cancer.

In summary, miRNA-based therapeutics from plant miRNAs remain controversial within the scientific community. Even though, there is a growing body of evidence mentioning the presence of plant-derived miRNA in human cells, tissues, and blood, these discoveries often faced great challenges in terms of reproducibility. The inconsistent findings from different studies contribute to the complexity and nuances associated with plant miRNA's potential as a therapy in humans. Hence, ongoing studies are still in progress and there is a lot of room for new research to be done related to plant-based miRNAs.

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Table 2.7: Summary of experimental results on plant miRNAs.

Mirna	Method	Findings	References
Lb-mir166a	*In vitro, in vivo*	Lb-miR166a can regulate various genes in tumour cells, inhibiting renal cell carcinoma proliferation, promoting apoptosis and inhibiting metastasis.	(Zhang et al., 2023)
miR167, miR1525, miR756 (*C.longa*)	*In silico*	miRNAs are the critical regulator of the tumour microenvironment. SARS-cov2 and Influenza A are connected to miR1525 and miR756 by regulating ZFP36L and ETV5 respectively.	(Saiyed et al., 2023)
Plant miR171	*In silico, in vitro*	Mir171 targets GNA12 in HEK293 cells and modulates the mTOR pathway. Can potentially be used to treat disorders where GNA12 is overexpressed.	(Gismondi et al., 2021)
Mtr-miR5754 Gma-miR4995	*In silico, in vitro*	Plant miRNAs target MALAT1 and NEAT1 in HCT116 cells and reduce their proliferation.	(Marzano et al., 2020)
Mir167e-5p	*In vitro, in silico*	Plant miR167e-5p inhibits enterocyte cell proliferation by targeting β-catenin.	(M. Li et al., 2019)
plant mir156a	*In vitro, in vivo*	miR156 targets JAM-A/F11R of inflamed human endothelial cells, reducing monocyte adhesion and suppressing the progression of atherosclerosis.	(Hou et al., 2018)
Plant mir159	*In vitro, in vivo*	miR159 targets TCF7 of breast cancer cells thus reducing its proliferation.	(Chin et al., 2016)

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Table 2.8: Summary of experimental results on plant miRNAs (continued).

Mirna	Method	Findings	References
HS-Mir2911	*In silico, in vitro, in vivo*	Honeysuckle miR2911 target various Influenza A viruses by inhibiting PB2, and NS1 protein expression.	(Zhou et al., 2015)
Osa-Mir168a	*In vitro, in vivo*	Mir168a can bind to LDLRAP1 mRNA, inhibit their expression in the liver and decrease LDL removal from mouse plasma.	(L. Zhang et al., 2012)
Bra-miR156g-5p & Myseq-330	*In silico, in vitro*	plant miRNA overexpression does not alter the viability, apoptosis, clonogenicity and migration of pancreatic cancer cells,	(Xiao et al., 2020)
miR156a, miR164a and miR167a	*In vivo, in vitro*	No evidence of increased levels of corn miRNAs in whole blood or tissues after supplementation of corn miRNAs in the diet was observed in a mouse model.	(Huang et al., 2018)
Olive oil, beer	*Pilot study*	Plant-based processed products such as beer and extra virgin oil do not contain miRNA and are unable to be detected in human plasma after 2 hours of ingestion.	(Mico et al., 2016)
miR172, miR166, 167, and 168	*In vivo*	RT-qPCR results indicated late amplification of the plant miRNA, but the quality of data was low and inconsistent with specific, reliable detection. Insignificant evidence for the presence of these plant miRNAs in non-human primate blood following dietary intake of a plant miRNA-rich substance.	(Witwer et al., 2013)

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Table 2.9: Summary of experimental results on plant miRNAs (continued).

Mirna	Method	Findings	References
miR156a, miR159a, miR169a	*In vivo*	Plant miRNAs were undetectable in human plasma, mice and recipient honeybee tissues. Diet-derived miRNA is an ineffective mechanism to deliver plant miRNAs.	(Snow et al., 2013)
Osa-miR-168a	*In vitro, in vivo*	This paper replicates the experiments conducted by Zhang et al. (2012). It conducted a well-controlled mouse feeding study using both standard chow and purified synthetic chow diets. The analysis of small RNAs from the plasma and liver of mice fed on balanced rice chow and rice chow did not reveal any measurable uptake of rice grain miRNAs, including osa-miR168a. There was no apparent uptake of osa-miR168a in the liver or plasma of animals fed any of the diets. These findings demonstrate that dietary exposure to osa-miR168a does not impact plasma LDL levels. Additionally, they indicate that the changes in plasma LDL levels reported by Zhang and colleagues resulted from nutritional imbalances between the test and control groups rather than an RNAi-mediated effect of consuming osa-miR168a in rice.	(Dickinson et al., 2013)

*HCT116- colorectal carcinoma, Lb- *Lycium barbarum* -, Mtr- *Medicago truncatula* , Gma- *Glycine max*, HS- honeysuckle, Osa- *Oryza sativa,* Bra- *Brassica rapa*

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2.5 MicroRNA Target Prediction via In-silico Analysis

In the past decade, significant advancements have been made in detecting single-stranded non-coding RNA, explicitly focusing on the small RNA with 20-24 nucleotides, called the microRNA. MicroRNA target prediction is essential to predict the miRNA target before validating it through experimentation. The early assumption is crucial as experimentation of validating miRNA target is laborious, time-consuming and expensive (Khatun et al., 2022). Computational prediction or *in silico* analysis is an alternative approach that offers an additional way of gaining insights and knowledge. Moreover, both computational and experimental data are mutual as they work together to enhance overall understanding (Rincón-Riveros et al., 2021). Additionally, results from this computational prediction help generate hypotheses about potential interactions and can be put to the test in a laboratory setting using either *in vitro* or *in vivo* models. Previous research highlights the use of various specific software, databases, or algorithms relevant to miRNA research, each offering unique features and advantages for miRNA target prediction and *in silico* analysis. These tools offer many functionalities, such as predicting potential miRNA binding sites on target mRNAs, assessing the thermodynamic stability of miRNA-mRNA interactions, and providing information on miRNA-mediated gene regulation.

Basically, in miRNA target identification, one common approach is the homolog-based method. This method primarily relies on identifying miRNAs based on their homolog conservation. The conserved homolog sequences of various species are efficient in minimizing the false positive results. However, this method might have higher false negative results. This is because it may not effectively detect non-conserved and species-specific mRNA (Huang et al., 2017). Another approach to predicting

miRNA targets is the sequence-based method. This method analyses the genetic sequences and does not rely on conservation status. It analysed the genetic sequences using a specific algorithm to predict miRNA targets. Some common algorithms and methods used in sequence-based miRNA target prediction include TargetScan, miRanda, PicTar, PITA, RNAhybrid, miRDB, DIANA-microT and MirTarget2. Among these, DIANA-microT has garnered significant attention, establishing itself as a pioneer in the field of computational miRNA target prediction. This application operates on an algorithm that employs diverse parameters calculated for individual miRNAs. It then combines both conserved and non-conserved miRNA recognition elements (MRE) to yield a definitive final prediction score, which correlates with the fold change in protein output. This score, termed the miTG score, measures the probability of a miRNA associating with and influencing a particular mRNA of a gene. A higher miTG score suggests a greater possibility of a true interaction (Maragkakis et al., 2009; Tastsoglou et al., 2023). A summary of the different algorithms used is listed in Table 2.4. Nevertheless, these approaches could produce a false positive if it relies solely on genetic sequences, and it may also lead to false negatives in certain cases. That's why, certain algorithms have a combination of a few features to increase their sensitivity and specificity.

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Table 2.10: MiRNA target prediction algorithms and tools.

Tool name	Parameters and features considered in prediction	References
DIANA-microT	Specifically trained on miRNA recognition elements (MRE) in 3'UTR and CDS region. Combines machine learning and thermodynamic parameters to predict miRNA targets.	(Paraskevopoulou et al., 2013)
TargetScan	Combines sequence-based and thermodynamic features to predict miRNA binding sites, and considers seed sequence matching, site conservation, and structural accessibility.	(Agarwal et al., 2015)
miRanda	Uses dynamic programming to align miRNA sequences with potential target miRNA sequences, considering sequence complimentary and thermodynamics.	(John et al., 2006)
PicTar	Predict the miRNA binding site by considering the conservation of the target site across multiple species.	(Krek et al., 2005)
miRDB	Uses machine learning and deep learning techniques to predict miRNA targets based on seed sequence complimentary, target site conservation, target site abundance and free energy of binding.	(Chen & Wang, 2020)
PITA	Calculates the minimum free energy of binding between miRNAs and their target sites, incorporating RNA duplex thermodynamics.	(Kertesz et al., 2007)
miRWalk	Integrates predicted and experimentally verified miRNA target interactions based on seed sequence complementarity, site conservation and site accessibility.	(Sticht et al., 2018)
RNA22	Identify miRNA binding sites based on seed sequence complementarity, target site conservation and thermodynamic stability of duplex. Offers high sensitivity.	(Loher & Rigoutsos, 2012)
RNAhybrid	Calculates the minimum free energy of hybridization between a miRNA and a target mRNA, considering sequence complimentary.	(Kruger & Rehmsmeier, 2006)
psRNAtarget	Analyse complementary matching between sRNA and target using a scoring scheme. Evaluate target site accessibility using unpaired energy (UPE).	(Dai et al., 2018)

Furthermore, recent advancements have ushered in machine learning-based approaches that use extensive datasets to enhance the accuracy of miRNA target predictions (Khatun et al., 2022). These tools initiate a prediction process by retrieving miRNA target sequences from databases such as Refseq and NCBI. Subsequently, they isolate miRNA sequences while filtering out homolog sequences. Following this, information extraction occurs in various methods, including homolog-based, sequence-based, structure-based and NGS-based techniques, and comes out with mathematical models and prediction outcomes. This approach relies on diverse input features to represent miRNA target binding, encompassing factors like binding type and conservation across species. Moreover, machine learning needs a substantial dataset that encompasses both positive and negative samples derived from experimental data sources. This approach also requires a large and diverse dataset for training because more data samples improve the predictive power of the models (Huang et al., 2017). For instance, RNAhybrid and psRNATarget employed the support vector machine (SVM)-based features, tailored for miRNA target prediction in mammals and plants, respectively. These tools exemplify the application of machine learning techniques in generating predictions based on the input features and training data (Baloch & Din, 2021).

Several databases that contain interactions of the miRNA and mRNA target genes or proteins have been developed. These databases employed an automatic or manual text mining of previously reported data from the literature. A few of them may include additional information such as sequences and computational prediction algorithms for analysing the interaction between miRNA and target. These target prediction databases rely on the data sources. The typical data sources can be in the form of gene expression data, protein-protein interaction database or genomic

sequences. For the identification of miRNA targets in humans and plants, there were a few recently developed open-source platforms. For example, mirTarbase, compiled the miRNA-target interaction (MTIs), which had been validated by experimentation (Huang et al., 2020). While in plants, the plant microRNA database (PMRD) may serve as a database that consists of a collection of 8433 miRNAs taken from 121 plant species (Zhang et al., 2010). Additionally, miRbase is the largest and the most widely used database that comprises more than 30,000 entries of miRNA hairpin structures from 271 organisms. Besides, miRbase also includes a wide range of additional information on published microRNAs, including the sequences, biogenesis precursors, literature references and links to third-party information about miRNA genes and sequences such as NCBI (Kozomara et al., 2019). However, the databases are prone to bias because of the poor quality of RNA sequences from the next-generation sequencing data that were not validated with experimentation (Ludwig et al., 2017).

Besides miRNA target prediction, bioinformatic tools are pivotal in elucidating the broader functional implications of miRNA dysregulation. One key aspect is the identification of gene ontology (GO) terms and pathways intricately involved in diseases, particularly cancer. By employing *in silico* analysis, researchers can systematically categorize target genes based on their functions and the biological processes they regulate. For instance, a miRNA may be found to significantly impact cell proliferation pathways or modulate crucial signalling cascades involved in cancer progression. This knowledge deepens our understanding of the molecular underpinnings of diseases and offers potential therapeutic insights. By assessing the enrichment of miRNA target genes in specific pathways, scientists can discern the functional consequences of miRNA dysregulation. This analysis provides a comprehensive view of the molecular networks orchestrated by miRNAs and highlights

potential intervention points for therapeutic strategies. For example, miRNAs regulating apoptosis pathways have garnered significant attention due to their potential role in cancer therapy (Elias et al., 2022; Navabi et al., 2022). Pathway analysis has been an important instrument in identifying these miRNAs and elucidating their impact on vital cellular processes.

By utilizing multiple microarray datasets and performing bioinformatics analysis, researchers can identify differentially expressed microRNAs and their target genes. For instance, a previous study identified twelve hub genes, including HLA-DQA1, MPL, BANK1, and TCL1a that were associated with dasatinib resistance in CML and the functional enrichment analysis found these genes were involved in immune response, signal transduction and cell communication (Luo et al., 2021). The same study further identified the hsa-miR28-5p, IL7 and MALAT1 network as the potential prognostic markers in CML patients. Additionally, bioinformatics analysis can play a crucial role in identifying potential therapeutic targets and pathways for treating leukaemia stem cells (LSCs) in CML. Key target genes can be identified by constructing protein-protein interaction networks and subsequent module analysis and topology analysis. The previous study identified 11 key candidate targets and 33 corresponding miRNAs. The identified miRNAs were observed to be involved in the pathways of PI3K/AKT, Ras, JAK/STAT, FoxO and Notch signalling pathways which are involved in cancer progression and apoptosis (H. Li, L. Liu, et al., 2019).

Moreover, bioinformatics analysis helps identify potential therapeutic targets and pathways by analysing gene expression profiles, protein-protein interactions, and pathway enrichment, providing a foundation for developing targeted therapies in CML (Mohamad & Elias, 2021; Soltani et al., 2017). Nevertheless, in the study of imatinib resistance, integrating three different datasets facilitated a regulatory network analysis

encompassing miRNAs, genes, and transcription factors. A sub-network centred on MYC emerged as a key component within this network. This sub-network included the mir-17 family, mir-29a, and hsa-miR-451a, which collectively contributed to regulating ABCB1 and BCL2 expression, ultimately playing a pivotal role in developing imatinib resistance (Soltani et al., 2017).

Addressing the complexities of miRNA target prediction poses many challenges, mainly because computational predictions are prone to false positives and negatives. Such predictions heavily rely on factors like evolutionary conservation, seed region emphasis, limited databases and algorithms. The emphasis on sequence complementarity without considering the actual biological functionality may lead to over-predictions, thereby adding complexity to the research process. The current reliance on evolutionary conservation of binding sites, an important feature of many prediction algorithms, might lead to the neglect of important species or context-specific interactions (Quillet et al., 2020). Given their ability to target multiple genes and vice versa, the vast regulatory networks in which miRNAs operate further amplify the complexity. While several databases and tools have emerged to address these challenges, the variability between different algorithms often leads to discrepancies in predicted targets for the same miRNA. Such inconsistencies necessitate the employment of consensus methods or a combination of multiple tools, further complicating the prediction process.

In summary, both bioinformatics and in-silico analysis are instruments for advancing miRNA research, particularly in identifying miRNA targets, conducting pathway analysis and exploring gene ontology. While data from *in silico* analysis provided invaluable insights into miRNA-mediated regulation of biological processes, researchers must be aware of this method's inherent biases and limitations. Therefore,

experimental validation remains the most reliable approach to verify predicted miRNA-target interaction and elucidate their functional relevance. Despite the challenges, the collective effort of researchers in this field has advanced our understanding of miRNA-mediated biological regulation and opened up exciting new possibilities for developing novel therapeutic therapies.

2.6 Experimental Strategies for microRNA Target Validation

MicroRNA-target validation is an essential process to confirm the binding of miRNAs with mRNA and their consequent impact on biological systems. This validation is required because specific criteria must be met to confirm this interaction. Experimental validation of miRNA targets poses some of the most fundamental challenges in miRNA biology. Selecting appropriate methods for target validation is pivotal to accurately confirm the miRNA-mRNA interaction. The choice of the most suitable method for validating miRNA-mRNA interactions is nuanced and heavily dependent on research objectives, available resources, and the required level of detail. MiRNAs, typically 22-24 nucleotides in length, recognized their target mRNAs through base pairing, specifically in the 2-8 nucleotide region known as the 'seed region'. This region attaches to the complementary sequence in the 3'untranslated region of the mRNA (Kuhn et al., 2008). The seed pairing can manifest in several ways:

1. 8-mer site: A perfect Watson-Crick match from nucleotide 2 to 8 of the miRNA seed, with an adenine (A) opposite at mRNA position 1.

2. 7-mer site: A Watson-Crick match from nucleotide 2 to 8, without adenine at mRNA position 1.

3. 7-mer A1 site: A Watson-Crick match from nucleotide 2 to 7, with an adenine opposite at mRNA position 1.

4. 6-mer site: Match pairing at position 2-7 or 3-8 of the seed region (Riolo et al., 2020).

However, it's important to note that an optimal seed match may not always accurately reflect the dynamics between miRNA and its target. Experimental validation is crucial to conclusively establish the interaction between miRNA and mRNA target (Chou et al., 2018). The general approach to miRNA target validation is often guided by several criteria, including miRNA-mRNA interactions, co-expression of miRNA and target mRNA, the effect of miRNA on target protein levels, and the impact of miRNA on target biological functions (Riolo et al., 2020; Ritchie et al., 2013). Each of these criteria is assessed through experimentation, using either direct or indirect methods, to validate miRNA-target interactions. Direct methods typically encompass techniques such as luciferase reporter assays and western blots, which provide tangible evidence of interaction by showcasing diminished protein levels or altered reporter activity upon miRNA binding (Chou et al., 2018; Kuhn et al., 2008). Indirect methods, on the other hand, include transcriptome profiling and sequencing with the in-silico prediction tools. While these offer a broader perspective, they sometimes lack the specificity of direct methods. Moreover, this method is expensive and technically demanding. Nonetheless, it is crucial to note that both direct and indirect methods possess limitations. The complex cellular environment can significantly impact miRNA activity, highlighting the critical need to consider contextual factors during the validation process (Choi et al., 2017). Hence, the meticulous integration of direct and indirect validation strategies tailored to the research question, and the complexity of the biological environment is essential for robust and credible miRNA-target validation. The methodology selection is fine-tuned to align with the research question, often employing a tiered approach.

This may commence with computational predictions, followed by initial validation utilising techniques such as Luciferase Reporter Assays, and ultimately culminating in comprehensive characterization through methods like high-throughput sequencing.

2.6.1 Reporter assay, immunoprecipitation, pull-down assay.

Due to the complexities of miRNA-target interactions, researchers utilised a range of methods, each with its unique attributes and limitations. Among these methods, the Luciferase Reporter Assay is commonly used for initial validation. In this assay, the putative miRNA target site is cloned downstream of the open reading frame of a reporter gene. The luciferase enzyme, encoded by this reporter gene, emits a luminescent signal that can be quantitively measured. The strength of this signal is inversely proportional to the miRNA regulatory effects on its target (Riolo et al., 2020). This quantitative nature of Luciferase reporter assay sets it apart from other methods, providing a tangible metric to gauge the efficacy of miRNA binding. The recombinant plasmid is then transiently transfected into a host cell. The luciferase activity (fluorescent) is measured after 24-48 hours. A reduction in luminescence is indicative of successful miRNA binding, thereby confirming the target interaction (Choi et al., 2017). However, this method can lead to inaccurate assessments of the target since the transfection of miRNA is in high concentration and might create a non-physiological interaction between two molecules of complimentary surfaces, potentially yielding results that do not accurately reflect physiological miRNA-mRNA interactions (Ritchie et al., 2013). Moreover, both miRNA and targeted UTR are overexpressed in a heterologous system that can also lead to non-physiological interactions due to different cofactor environments. Simply said, this assay is conducted in an artificial cell culture system, which may not fully represent

the complexity of miRNA regulation *in vivo*. The transfection variability, stemming from differences in cell lines and transfection conditions, can introduce experimental noise.

Another method based on the identification of RNA molecules that interact with specific proteins, capturing the miRNA-containing protein, is known as RNA immunoprecipitation (RIP). Basically, after miRNA transfection, highly selective antibodies are used to precipitate the RNA binding protein, specifically the AGO proteins, a component in the RNA-induced silencing complex (RiSC). Thus, the miRNA and mRNA associated with the RISC attached to the AGO protein are being precipitated using the antibodies (Riolo et al., 2020). This approach purifies the miRISC-associated with mRNA, which is later identified through qPCR, microarray analysis, or deep sequencing (Ritchie et al., 2013). RIP, however, has the disadvantage of identification of artificial mRNA-miRISC or being unable to detect miRNA-mRNA, which has weak interactions (Riolo et al., 2020).

A further approach used to correct the limitation of the RIP technique, is the Cross-Linking Immunoprecipitation (CLIP). This approach offers the highest degree of precision and detailed mapping, but it is a technically demanding method and time-consuming. CLIP utilised the ultraviolet (UV) light to covalently conjugate protein-RNA interaction. This technique, with its exacting requirements, offers a granular view of miRNA-mRNA interactions, enabling the identification of precise binding sites. The CLIP techniques include the PAR-CLIP (photoactivable-ribonucleic enhanced CLIP), HITS-CLIP (high-throughput sequencing by crosslinking and immunoprecipitation (Li & Zhang, 2019).

Other methods, such as microarray, RNA or deep sequencing are high throughput. This indirect strategy is based on quantifying global expression changes

following treatment with miRNA or antagomirs. The gene expression changes were used as a marker to detect target genes. The miRNA-target genes interaction, however, still needs to be validated using bioinformatics. This method emerges as a method of choice for researchers interested in achieving genome-wide coverage. Besides being technically demanding, these methods are expensive.

Moreover, a more recent approach utilised an affinity-based target gene identification, also known as the pull-down assay, to determine miRNA target interactions. This approach involved transfecting the cells with a 3'-biotinylated synthetic miRNA to capture the miRNA targets. The miRNAs and their associated mRNA are later enriched and purified using the streptavidin magnetic beads. The pulled-down miRNA-mRNA targets were then subjected to further analysis either by qPCR, microarray or sequencing (Li & Zhang, 2019; Ritchie et al., 2013). miRNA pull-down assay allows the investigation of miRNA-mRNA interactions in their native environment. This approach unveils the intricate tapestry of these connections, yet it may encounter challenges when detecting low-abundance targets, potentially mandating supplementary techniques for comprehensive validation.

The successful application of this method is exemplified by the successful identification of binding proteins such as iron regulatory proteins (IRP1 and IRP2), Hu antigen R (HuR), AU-rich element RNA-binding protein 1 (AUF1), and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) with RNA and DNA (Tsuji, 2023). The study optimised the biotinylated RNA or DNA pull-down assays and significantly improved the detection of protein interaction to the cis-acting elements in the human ferritin H gene using streptavidin-agarose and magnetic beads. This enhanced detection method is crucial for understanding the regulation of gene expression through protein-nucleic acid interactions and can study other RNA/DNA-binding proteins. Previous study did

used the same method to identify miRNA target genes complementary to genomic DNA. They mentioned that miR-373 binding sequences were in the promoter, exon, intron and intergenic region (Xun et al., 2019). These findings suggest that miRNAs have a broader regulatory impact on gene expression than previously understood, which has implications for understanding the full scope of gene regulation by miRNAs.

However, because of a poorly understood mechanism and not a well-established area, further research is required to confirm the validity of those findings. Nevertheless, Li et al. successfully identified 1688 potential target genes of miR-192 in porcine endometrial epithelial cells using a miRNA pull-down assay. Through rigorous bioinformatics and empirical validation, their research highlights miR-192's influence on genes like CSK and YY1, which affect vital signalling pathways relevant to embryo implantation (Li et al., 2023). Moreover, another study conducted by Dash et al. introduces an innovative strategy for identifying mRNA targets of cellular miRNAs, further enriching the landscape of miRNA research. This study employed a biotin-based pulldown assay to validate the direct interaction between miR-125b and the 3' UTR of PARP-1 mRNA (Dash et al., 2018). The technique involves transfection of biotinylated miRNA mimics into mammalian cells, then isolating miRNA-mRNA complexes using streptavidin-coated magnetic beads. This method is precise, as evidenced by quantifying target mRNAs through qPCR and its minimal false positive rate thus promoting its potential application in confirming miRNA targets across various cells. This meticulous approach complements sequence-based RNA capture as mentioned in the previous paragraph, offering a potent tool for confirming miRNA targets.

Moreover, in a comparative analysis, the efficacy of two distinct methodologies for identifying targets of miR-34a was evaluated, yielding significant findings. The study demonstrated the effectiveness of the biotinylated miRNA pull-down technique,

where biotin-tagged miR-34a was successfully incorporated into the AGO2 protein, integral to the RNA-induced silencing complex. This method effectively identified a substantial array of potential miR-34a mRNA targets, as confirmed by subsequent luciferase reporter assays (Awan et al., 2018). On the other hand, the miR-34a overexpression approach showed limited capabilities in accurately identifying direct miR-34a targets, as revealed through RNA sequencing. Interestingly, the targets identified by both methodologies showed minimal overlap, suggesting that each method may uncover different aspects of miR-34a's interaction with mRNA. The study thus highlights the superiority of biotinylated miRNA pull-down in isolating miR-34a targets and proposes a synergistic use of both techniques for a more exhaustive exploration of miRNA interactions. This research contributes significantly to the understanding of miRNA target identification, offering insights that could be pivotal for future studies in molecular biology.

Furthermore, previous studies have described the use of Biotin pull-down assay. These papers explain this method's step-by-step protocols, thus providing a comprehensive understanding of miRNA target identification. Phatak and Donahue's approach utilised reverse transcription-polymerase chain reaction (RT-PCR) to pinpoint specific miRNA-mRNA interactions precisely, offering a methodology for examining direct miRNA influences (Phatak & Donahue, 2017). Conversely, Tan and Lieberman's strategy, incorporating RNA sequencing (RNA-seq), provides a more comprehensive array of miRNA-interacting RNA species, both coding and non-coding (Tan & Lieberman, 2016). Additionally, the research conducted by Subramanian et al., concentrating on miR-34a, a miRNA with known tumour-suppressive functions, illustrates the practical application of the biotinylated miRNA pulldown assay in specialised contexts such as oncological studies (Subramanian et al., 2015). Though

varied in their analytical focus and experimental design, each of these methodologies collectively reinforces the biotinylated miRNA pulldown assay as an indispensable tool in molecular biology. This assay facilitates an understanding of miRNA functions and their regulatory roles and underscores the methodological diversity and adaptability necessary for advancing miRNA-related gene regulation research.

In summary, the versatility of all mentioned methods lies in their ability to complement one another, providing a holistic understanding of miRNA-mRNA interactions and pushing the boundaries of knowledge in this ever-evolving field. We summarise each method of miRNA target validation in Table 2.

Table 2.11: MiRNA target validation methods.

Method	Advantages	Disadvantages
Luciferase Reporter Assay	Good for initial validation but cannot capture the complexity of the cellular environment.	Test one interaction at a time, influenced by cellular context, time-consuming and laborious.
RNA Immunoprecipitation (RIP)	Allows for the study of interactions within a natural cellular context	Less sensitive, identification of artificial mRNA-miRISC, capturing indirect associations.
Cross-Linking Immunoprecipitation (CLIP)	Provides high specificity and mapping detail but is technically demanding.	The highest specificity is due to the UV cross-linking. Technically demanding and expensive.

Table 2.12: MiRNA target validation methods (continued).

Method	Advantages	Disadvantages
High-Throughput Sequencing	Offers genome-wide coverage but is expensive and data-intensive.	Most sensitive, capable of detecting low-abundance targets. Technically demanding and expensive.
Pull-Down Assay	Useful for studying interactions in their native cellular context, cost-effective and less time-consuming	less sensitive in detecting low-abundance targets.
qPCR	to validate findings from other experimental methods like RIP, pull-down assays, or high-throughput sequencing	Cost-effective, highly sensitive and specific.

2.7 Effects of miRNA in Biological Process

Cell functional assays are a valuable tool in understanding the behaviour and characteristics of cells. They allow researchers to assess the specific functions of cells, such as proliferation, migration, differentiation, and response to stimuli. Furthermore, these assays can provide important insights into the efficacy and mechanisms of potential therapeutics or interventions. Cell functional assays serve as critical tools for examining the alterations in cellular behaviour ensuing from miRNA expression modulation. It is significantly important in molecular biology and particularly in miRNA research. By studying how different miRNAs affect cell function, researchers can better understand cellular processes and potentially identify new targets for therapeutic intervention. Through the application of cell functional assays, researchers can dissect the complex influence of miRNAs, enhancing our understanding of their regulatory mechanisms and implications in health and disease. Understanding cell function is critical to cellular research. These cell function assays can be used on multiple instrument platforms, including microscopy, flow cytometry, microplate

reader, and high-content screening (Li & Xia, 2019). Cell viability, proliferation, and apoptosis are generally considered together despite measuring different parameters. A previous study by Ying et al. underscores the regulatory influence of miR-345-5p on cell proliferation and apoptosis by targeting the AKT1/2 signalling pathway (Ying et al., 2019). Given their ability to alter cell survival and growth kinetics, this targeting mechanism places miRNAs as potential therapeutic agents. Integrating miRNA functional studies and cell cycle analysis is critical in understanding the modulation of cellular activities. Cell viability and proliferation assays, such as the MTT or resazurin assays, are fundamental to discerning the effects of miRNA interference on cellular health. When used along with cell cycle analysis techniques, these assays are crucial in tracking the cell cycle progress. Advancements in analytical methodologies, including automated systems and machine learning techniques, are set to enhance the sensitivity and throughput of cell cycle analysis. These assays' sustained refinement and application are vital for revealing the intricate interactions between miRNAs and their corresponding gene targets, providing a better understanding of cellular regulation.

2.7.1 Cell cycle

Cell duplication, known as cell proliferation, originates from the full cycle of cellular activities. During proliferation, cells engage in DNA replication and divide their chromosomes to ensure an equal distribution of the genetic material to each cell. This intricate cycle of growth and division is tightly orchestrated by a network of regulatory signals, including cytokines, mitogens, and growth factors (Orrenius et al., 2011). These molecules interact with receptors on the cell surface, triggering a cascade of signals that culminate in the nucleus to initiate division. The cell cycle is a sophisticated sequence

of phases, culminating in cell division and the generation of new cells. This regulated sequence ensures the precise duplication of DNA and equitable division of cell contents, a fundamental biological process resulting in the multiplication of cells. The cell cycle encompasses a series of regulatory mechanisms that facilitate DNA replication and cell division via mitosis. Key to this regulation is Cyclin-dependent kinases (CDKs), especially the mitotic CDK (CDK1) and interphase CDKs (CDK2, CDK4, and CDK6), which are crucial for advancing the cycle (Hossian et al., 2019; Panda et al., 2020). The Retinoblastoma protein (Rb), upon phosphorylation by the CDK4/CDK6 complex, allows for the cycle to continue, while the activation of CDK1 is vital for the division to occur successfully. The development and clinical trial assessment of numerous CDK inhibitors in recent years underscores their importance in cancer therapy, with some inhibitors gaining approval for breast cancer treatment (Sánchez-Martínez et al., 2019; Shah et al., 2018). CDK1 and CDK4/6, therefore, are particularly significant in managing cancer cell growth.

Internal or external signals can either prompt or deter metabolic events. Internal signals like small cell size, insufficient nutrient supply, uncompleted DNA synthesis, or incomplete kinetochore attachment—can serve as inhibitory signals. Conversely, the presence of ample cyclins, a stable mitosis-promoting factor (MPF), the completion of DNA replication, and the secure attachment of all sister chromatids to kinetochore spindle fibres can trigger the progression of the cycle (Panda et al., 2020). Moreover, the cell cycle is rigorously regulated by a system of checkpoints that pause or postpone the cycle if there are instances of DNA damage, replication errors, or abnormal mitotic events, thus preserving chromosomal integrity. The G1/S checkpoint is a critical control point for the cycle and plays a significant role in tumour development. This checkpoint is governed by the activity of CDK4/6, whose expression levels are typically elevated

in cancer cells. This dependency on CDK4/6 makes these kinases prime targets for inhibitor treatments. Several cell cycle inhibitors have gained FDA approval, including Palbociclib (Turner et al., 2015), Ribociclib (Hortobagyi et al., 2022), and Abemaciclib (Johnston et al., 2020), all of which are utilised in treating metastatic breast cancer. Meanwhile, the G2 checkpoint is regulated by the WEE 1 protein, which inhibits the CDK1 activity, leading to a halt in the cell cycle at the G2/M phase. These proteins function as regulators of the CDKs and act as checkpoints for DNA damage and cell volume (Ghelli Luserna di Rorà et al., 2020). Furthermore, the same kinases verify proper DNA replication and adequate cell size before the cell moves into the next cycle phase. The G2 checkpoint is vital for maintaining the cell cycle's integrity. Cyclin B and CDK1, specific to the G2/M checkpoint, are also integral in cell cycle control. Their decreased activity results in a pause at the G2/M phase, highlighting their importance in cell cycle advancement (Panda et al., 2020). In the context of the G2/M checkpoint, both CDK1 and Cyclin B are pivotal. Activation of CDK1 is crucial for cells with DNA damage to advance past the G2/M checkpoint, underscoring its vital role in cell cycle progression. Figure 2.5 summarises the important regulators involved in a cell cycle.

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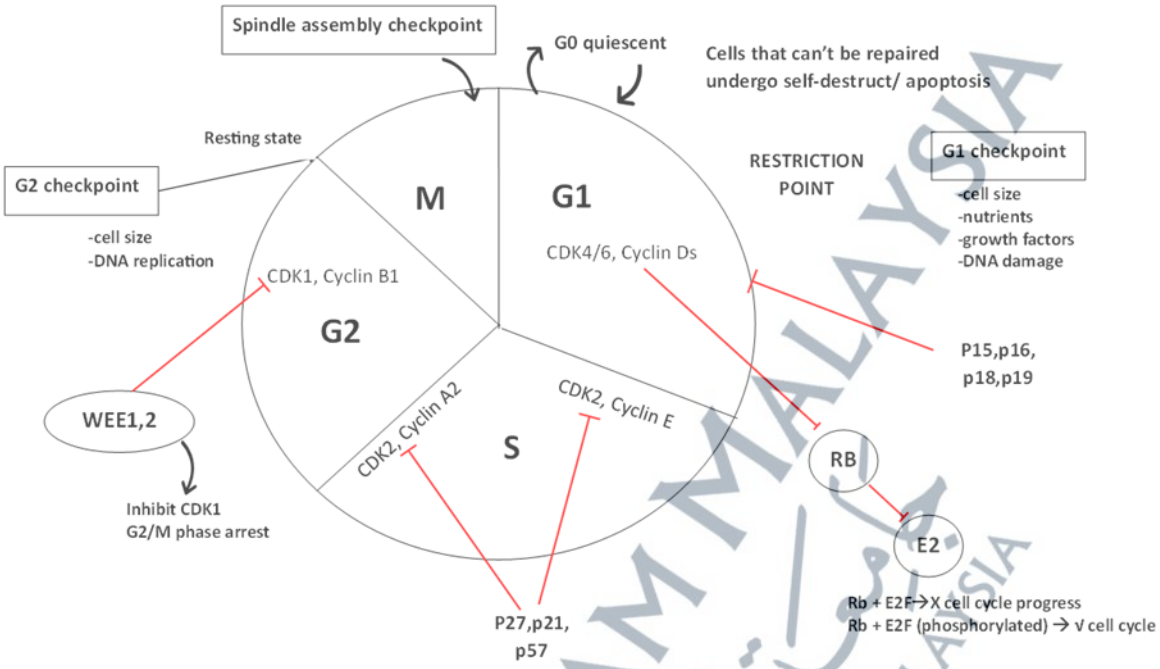

Figure 2.6: The schematic diagram of the cell cycle.

Advancements in molecular biology have underscored the significant role of non-coding RNAs (ncRNAs) in controlling the expression and function of cell cycle regulators by targeting specific molecules involved in cell cycle progression. These ncRNAs, which include microRNAs and long non-coding RNAs, regulate the levels of various cell cycle regulators at the translational, post-translational and transcription levels, impacting multiple signalling pathways and cellular activities. For instance, miR-19a/b and miR-17-92 have been shown to control the cell cycle progression by influencing the expression of WEE1, cyclin G2 and other cell cycle regulators (Brockway & Zeleznik-Le, 2015; Huang et al., 2021). Additionally, miRNAs regulate G1/S transitions by targeting important cell cycle regulators such as CDKs, cyclins and CKIs, thereby affecting cell cycle progression and proliferation. For example, miR-195, miR-365 and miR-159 were found to regulate the G1/S transitions by suppressing the Cyclin D1 and CDK4/6 in gastric cancer (Deng et al., 2013). Nevertheless, a few

miRNAs were found to target and suppress the E2F and control its expression and function. miR-17-15p, miR-20a, miR-125b, miR-210 and miR-195 were found to be negatively regulate the expression of E2Fs (Giannakakis et al., 2008; Huang et al., 2011; Pickering et al., 2009; Xu et al., 2009) in various cancers. These miRNAs are anti-proliferative and act as a tumour suppressor, often altered in cancer.

However, several miRNAs have been shown to promote invasion, metastasis and angiogenesis in cancer cells. For instance, miR-23a/24-2/27a clusters and miR-10b were observed to promote breast cancer cells and liver metastases and angiogenesis by targeting SPRY2 thus activating the p44/p42 MAPK pathway (X. Li et al., 2013). The emerging insights into ncRNAs, especially the miRNAs as both biomarkers for therapy response and targets for new treatments, pave the way for more tailored and precise cancer therapies, merging the study of miRNA mechanisms with cell cycle regulation as a burgeoning field of investigation. In summary, cell cycle analysis is a critical part of cell functional assays in miRNA research, providing insights into how miRNAs regulate cell division and growth, which is essential for understanding their roles in both normal physiological processes and in diseases like cancer.

2.7.2 Cell viability

Apart from the regulatory framework of cell division, cell viability assays serve as a fundamental tool for evaluating the health and survival of cells in response to various conditions, introducing of specific miRNAs in a sample. These assays are based on various function of cells that measures metabolic activity, membrane integrity, ATP levels, or other indicators of cell health, which collectively provide insights into the viability and proliferative capacity of cells (Aslantürk, 2018; Ishiyama et al., 1996).

Techniques such as MTT, XTT, and resazurin reduction assays are commonly used to assess cell metabolic activity, whereas assays like trypan blue exclusion and ATP quantification offer alternative means to evaluate cell health (Präbst et al., 2017). The cell viability methods often involved light microscopy, colourimetric assay, fluorescent assay or specific dyes such as trypan blue in the dye exclusion method. Dye exclusion is the simplest and most widely used method to determine cell viability. Choices of dyes range from trypan blue, Congo red, eosin, and erythrosine B assays (Aslantürk, 2018). Viable and healthy cells should have good cell membrane integrity; thus, the dye cannot penetrate the membrane layers and will have clear cytoplasm. Meanwhile, non-viable cells will use the dye colour and have blue cytoplasm (Strober, 2015). However, this method has several disadvantages, such as experimental procedures unsuitable for processing large samples due to time consumption. Additionally, there is an inability to differentiate between the healthy cells and the cells that are alive but are losing cell function, and this method is not sensitive enough to be used as a measurement for *in vitro* cytotoxicity testing (Kamiloglu et al., 2020).

Meanwhile, colourimetric assays are based on the measuring biochemical markers of metabolic activities. For instance, enzymes such as succinate dehydrogenase are to indicate functional mitochondria in a cell. Commonly used colorimetric assays include the 3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium (MTS), 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8/ CCK-8 kit) and crystal violet (CVS) assays. MTT assays are rapid and simple to perform. However, this assay produces an insoluble product that necessitates a solubilisation step, making it unsuitable for real-time assays. Besides, this assay also leads to cell death due to crystal

formation (Präbst et al., 2017). Integrating of these assays in miRNA research is critical, as it allows for the observation of miRNA impact on cell survival pathways and growth, which is essential in understanding their role in development and disease progression.

A study by Kaehler et al (2017) demonstrated that inhibition of miR-212 in k562 sensitive cells treated with imatinib leads to an increase in ABCG2 expression, thus enhancing cell viability and reduced apoptosis, contributing to early imatinib resistance and tumour progression (Kaehler et al., 2017). Moreover, miR-486-5p and miR-15a-5p were both downregulated in malignant pleural mesothelioma (MPM) and cervical cancer, respectively. Their downregulation leads to increased cancer cell viability and proliferation. For instance, miR-486-5p low expression leads to overexpression of PIM1, which regulates cell cycle progression at multiple checkpoints and thus promotes cell proliferation and viability (Pinelli et al., 2021). Nevertheless, miR-150-5p is downregulated in cervical tissue, negatively regulating the *Yes*-associated preotin1 (YAP1). Upregulation of YAP1 promotes cancer cell viability, migration and invasion and decreased apoptosis in cervical cancer cell (Chen et al., 2020). These studies underscore the potential of miRNAs as therapeutic targets in cancer treatment.

In contrast, several oncogenic miRNAs that play a significant role in promoting cancer cell viability and proliferation have been identified. One of the well-documented examples is miR-21, which is frequently upregulated in various cancer types. miR-21 promotes cell viability by targeting tumour suppressor genes, leading to enhanced cell survival and resistance to apoptosis (Wang et al., 2015). Similarly, miR-155 (Witten et al., 2019), miR-17-92 cluster (Jia et al., 2017), miR-221/222 (Navabi et al., 2022) and miR-10b (Wang et al., 2018) have been implicated in cancer cell viability through the inhibition of critical regulatory pathways, ultimately supporting uncontrolled cell growth. These miRNAs often act by downregulating tumour suppressor genes or key

proteins involved in cell cycle regulation. Understanding the interplay between oncogenic miRNAs and cell viability is crucial for unravelling cancer progression mechanisms. Gaining insight into the role of oncogenic miRNAs in cancer is vital for developing of targeted therapies. These therapies aim to counteract the harmful impact of these miRNAs on cancerous cells, which is a key step in enhancing treatment approaches for individuals diagnosed with cancer.

Hence, selecting of the assay method is critical in determining the type of interaction and impact on cellular viability. Evaluating cell viability is crucial in drug discovery studies, toxicology research, and industrial microbiology analyses. It involves assessing living cells within a population under various stressors or chemical exposures to understand their potential effects on proliferation and inhibition.

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