Document heading doi: 10.21276/apjhs.2019.6.1.24 **Research Article**

miRNA: An Alternative Therapy for Chronic Myeloid Leukemia

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Abstract

Chronic myeloid leukemia is a myeloproliferative neoplasm that results due to the expression of BCR/ABL P210 oncoprotein in hematopoietic stem cells. This oncoprotein causes constitutive expression of tyrosine kinase and disrupts normal signaling pathways of cell cycle progression and differentiation. The use of targeted therapy, tyrosine kinase inhibitors (TKIs), although proved to be useful, poor drug response, drug resistance, adverse side effects, high cost has posed a severe threat to CML patients. This has shifted the attention of the scientific community to search for a more viable alternative that can tackle not only TKI related problems but also modulate the leukemogenic signaling pathways by silencing Bcr/Abl expression at the transcript level. MicroRNAs provide a beautiful avenue of therapeutic research in haematological malignancies including CML. They are promising prognostic as well diagnostic molecules, and with rapid advancement in miRNA based research and underlying signaling network in CML, the use of miRNA as prognostic, diagnostic biomarker or as a therapeutic agent alone, or in combination with TKIs is sure to see the light of the day. This paper is an endeavour to synthesize our present knowledge about CML, the signaling pathways involved in the disease and importance of microRNA in CML emphasizing on why microRNA is a better alternative than the currently available TKIs.

Keywords: Chronic myeloid leukemia, tyrosine kinase inhibitors, MicroRNA, BCR-ABL protein, Signaling Pathways, Stem cells, Therapy

Introduction

Leukemia: Du and Chen defines leukemia as a clonal malignant hematopoietic stem cell (HSC) disease [1]. It belongs to the category of cancer that usually begins in the blood-forming cells of the bone marrow, and once the leukemic cells overcrowd the normal cells, the disease spreads to blood. It is characterized by the abnormal proliferation of blood precursor cells of myeloid or lymphoid origin [2]. The enormous number of immature blood cells disrupts the production of functional blood cells. Leukemia is categorized according to cell type (myeloid/myelogenous orlymphocytic/lymphoblastic) and cellular maturity (acute or chronic). There are four major types of leukemia, viz: Acute Myelogenous Leukemia (AML), Acute Lymphocytic Leukemia (ALL), Chronic Myelogenous Leukemia (CML) and Chronic Lymphocytic Leukemia (CLL).

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The incidence of leukemia: Globally it is the sixth most life-threatening cancer whose incidence rate has not decreased over the years. According to the World Health Organization (WHO) databases, the global incidence of leukemia varies geographically with a male-to-female ratio of 1.4:1 [3]. It occurs in adults above 55 years and children below 15 years. Leukemia is among the most prevalent childhood cancer. In India, the female to male ratio is 1:0.9 to 1:5.6 with maximum cases in North India and a minimum in the North East. These statistics may not be absolute because of issues like under-reporting. Thus the quality and inclusiveness of such reports in registries are important [4, 5].

Risk Factors in Leukemia: Leukemia is a genetic disease which means that mutation(s) caused in the target gene(s) may or may not be heritable. Although the exact cause of leukemia is not known several studies, have shown that there may be a genetic predisposition to developing leukemia and factors such as [6, 7]

a. regular cigarette smoking,
b. chemicals like benzene mostly present in gasoline, rubber, and shoe-making plants,

c. chemotherapeutic drugs like alkylating agents, platinum agents, topoisomerase II inhibitors,
d. genetic disorders such as Down's Syndrome, Klinefelter syndrome, Fanconi anemia,
e. blood disorders like myelodysplasia, polycythemia vera, primary thrombocythemia
f. gender (males are slightly more prone to develop leukemia)
g. race (European people have a greater tendency to get CLL)
h. family history (applies to cases of CLL)
i. viral infection (infection with human T-cell lymphoma/leukemia virus-1 increases the risk of ALL)
j. age (risk of developing AML and CLL increases with age) contributes to the occurrence of the disease.

Besides genetic factors, epigenetic factors like DNA methylation, histone modifications also play an integral role in the progression of leukemia [8].

Philadelphia (Ph) chromosome: Boveri stated that cancer cells contain 'growth stimulatory chromosomes' that play a pivotal role in malignancy. Janet Rowley has also emphasized the fact [9]. One such chromosome is the Philadelphia chromosome, first identified by Rowley.Double-stranded breaks (DSBs) followed by balanced reciprocal translocation on the long arm (q) of chromosome 9 proximal to Abelson murine leukemia viral oncogene homolog (Abl) gene and on long arm of chromosome 22 near Breakpoint Cluster Region (Bcr) gene results into a shortened chimeric chromosome 22 called Philadelphia chromosome (named after its place of discovery) [9, 10, 11, 12] (Figure 1) and reciprocal Abl/Bcr on chromosome 9 [12,13]. The fusion is between 3' region of Abl and 5' region of Bcr [12, 14].Abl/Bcr gene is transcriptionally active, yet no functional role of it has been identified inCML, and no ABL/BCR protein has been found [12], whereas the discovery of Bcr/Abl gene by Nowell and Hungerford in 1960 has revolutionized the concept of hematopoietic malignancies.The Bcr/Abl oncogene (now officially called Bcr/Abl1)was discovered in 1985**.**

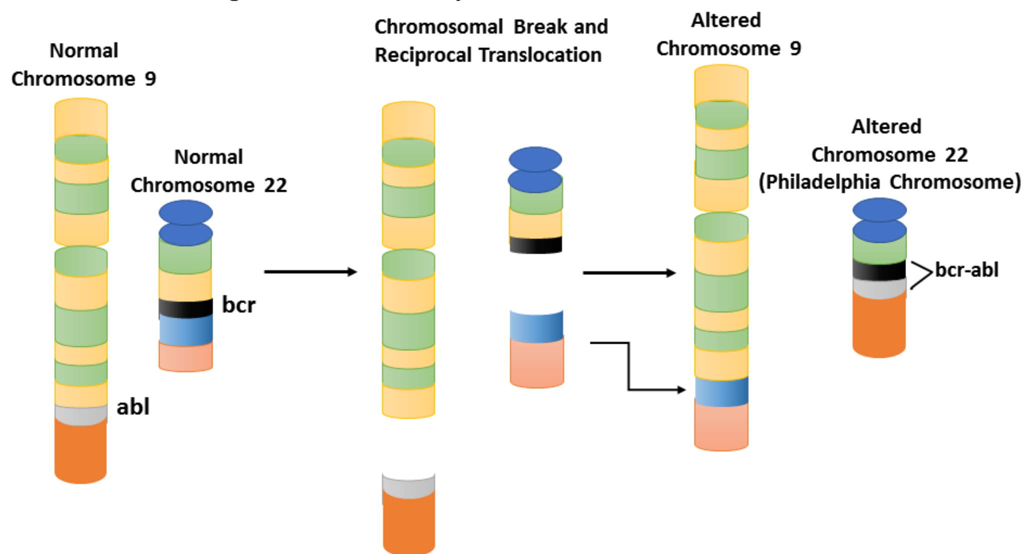

Figure 1: Formation of Philadelphia chromosome

The frequency of such DSBs is enhanced by ionizing radiation [11]. A typical cell being intolerant to DSB activates the cellular repair machinery which then joins the broken ends. Non-homologous end joining (NHEJ) though not exclusive but predominant in eukaryotic cells is the possible mechanism of repair[15, 16]. After translocation, NHEJ results in $22q^-$ and $9q^+$.It is of particular note here that homologous sequences have also been identified in chromosomes 9 and 22 and in interphase (S and G2 phases) they may be present in proximity which might facilitate their recombination [11, 13, 14, 17]. Thus, it can be said that the translocation is not a random event.

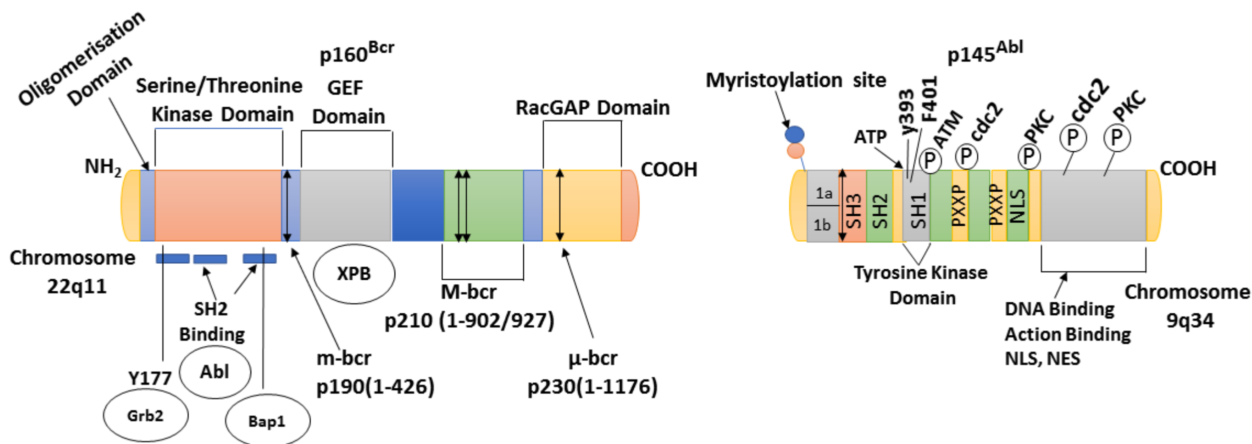

Figure 2: Detailed features of Bcr and Abl genes.

Interestingly, Bcr/Abl transcripts can also be detected in healthy individuals if the PCR technique used is sensitive enough but these individuals do not develop Leukemia. This can be perhaps because of a smart immune surveillance mechanism in these individuals [14].

Molecular structure of Bcr and Abl genes: The Bcr region contains coiled-coil oligomerization domain (amino acid residues from 1 to 69) that forms homotetramers, functionally essential domains like the serine/threonine kinase domain that undergoes self-phosphorylation,guanine exchange factor (GEF) domain, guanosine triphosphatase activating protein (GAP) domain which regulates $p21^{Rac}$ for cytoskeletal reorganization, SH2 domains (two of which interacts with Abl gene). There is a tyrosine residue at 177th position (towards N-terminal of BCR portion of BCR/ABL protein) that communicates with Grb-2 (that initiates the Ras signaling pathway) and its phosphorylation is essential for leukemogenesis[14].

The Abl gene encodes Src-homology (SH) domains, a tyrosine kinase domain, DNA-binding domain, actin-binding domain, nuclear localization signals and proline-rich regions (SH3 motifs), and nuclear export signal (at the C-terminus). The four SH3 motifs interact with the SH3 domains of Crk, Nck, GRB2, etc. which mediates the downstream pathways [18]. Within the tyrosine kinase domain (SH1 domain) there is a tyrosine residue at 393rd position that is the primary autophosphorylation site [11, 17] (Figure 2).

Usually, Bcr encodes a serine/threonine kinase which interacts with actin, GTP and lipids. Abl encodes for a protein tyrosine kinase that transmits signals from ligand stimulated growth factor receptors to influence cytoskeletal changes mainly. The chimeric Bcr/Abl oncogene encodes for an active and constitutive tyrosine kinase that phosphorylates target proteins to facilitate the expansion of hematopoietic stem cells and progenitor cells. The constitutive expression occurs because the myristoylated cap region (which helps in auto-inhibition of tyrosine kinase) at the N-terminal of Abl1b gene is replaced by the Bcr gene[11, 19, 20](Figure 2).Abl1b and Abl1a are the results of alternative splicing of Abl1 (or Abl) gene at the first exon. Abl1a is not myristoylated and is also expressed at lower levels. It is still not clear what takes over the function of myristoyl cap in Abl1a [18]. Most of the auto-phosphorylation occurs at the tyrosine residues of BCR segment of BCR/ABL protein, but the tyrosine kinase activity is attributed to the kinase domain of the ABL portion [14].

Table 1: Molecular features of the genes Bcr, Abl and the chimeric Bcr/Abl gene

Feature	Bcr gene	Abl gene	Bcr/Abl gene
Chromosomal location	22	11	22q11
Gene size (kb)	130	230	Variable
Exons	23	11	Variable
Molecular weight of major proteins (kDa)	130 and 160	145	190, 210 and 230 *
Cells in which expressed	ubiquitously expressed#	ubiquitously expressed	hematopoietic stem cells
Cellular location of the protein	Cytoplasm	nucleus and cytoplasm @	Cytoplasm

#Highest in the brain and hematopoietic stem cells
@In neurons and hematopoietic stem cells, mostly cytoplasmic [11]

*There are also a few other proteins that have not been given size because they have not been demonstrated on a gel.

BCR/ABL variants: There are distinct forms of Bcr/Abl gene depending on the point of break and fusion, each associated with different types of leukemia. Three of such clinically significant variants identified till date are P190 also referred by some researchers as P185 [14] (found in 20-30% of ALL), P210 (associated with 95% of CML) [21] and P230 (present in chronic neutrophilic leukemia) [9, 17]. Such variants have a distinct C-terminal due to variations in the break-point region,butcommon N-terminal derived from Abl gene. Sometimes the break is 5’ to exons 1a and 1b and sometimes within intron 1 of Abl gene. But exon 2 (a2) of the Abl gene which encodes the SH1 kinase domain is always present in the chimeric Bcr/Abl gene [14].The breakpoints in major (M) breakpoint cluster region between either exons 13 and 14 (b2), or exons 14 and 15 (b3) of Bcr gene and its fusion to breakpoints between exon 2 and 3 (a2) (mostly) of the Abl gene produces the b2a2 or b3a2 mRNA that is translated into P210 BCR/ABL protein. Similarly, P190 BCR/ABL results from the e1a2 mRNA formed by fusion between breakpoint in minor (m) Bcr gene and a2 and P230 BCR/ABL is formed from the translation product of e19a2 mRNA which involves micro (μ) BCR gene [10, 11, 12]. Besides these, there are also some rare variants with a breakpoint between exons 6 and 7 (which forms the P195 protein) and others like the P200 and P225 translated from e8a2 and e18a2 mRNA, respectively. Such variants are the result of alternative splicing within the Bcr gene and between Bcr and Abl genes besides the difference in breakpoint regions of the Bcr gene [14].Although all encode tyrosine kinases, there are differences in their intrinsic activities and also the downstream signaling pathway. For instance, P190 has greater action than P210 as well as P230 and Stat6 can be activated by P190 and not P210. Unlike P210 and P190, P230 BCR/ABL cultures are dependent on cytokine for growth [17]. It is interesting how minor changes in breakpoint location can manifest itself into different types of leukemia. This is why the Bcr/Abl fusion gene has garnered greater attention from the scientific community. Since the presence of Bcr/Abl is highly linked to CML, hence focus is given on Bcr/Abl in association with CML. The idea is to develop the foundation of a precise control technique to “switch off” or down-regulate the constitutive tyrosine kinase activity by regulating Bcr/Abl at the gene, mRNA or protein level. But in this paper, we are emphasizing its down-regulation at the mRNA level.

Chronic Myeloid Leukemia (CML): CML is the result of Philadelphia (Ph) chromosome represented as t(9;22)(q34;q11) [10] and the molecular weight of the fusion protein so formed is 210 kD. This is a type of somatic mutation (translocation) and hence not heritable. Thus CML is a type of acquired genetic disease. However, not all CML has the Ph chromosome; although 2016 WHO report states that these cases possess the same criteria as Ph-positive CML. And hence they are called atypical CML [22, 23]. It is rare with unknown epidemiological indices[23]**.** The 2016 World Health Organization’s revised ‘Blue Book’ of Myeloproliferative Neoplasms (MPNs) features Chronic Myeloid Leukemia [24]. CML was also included in the 2008 WHO Blue Book. MPNs are characterized by the malignant transformation of pluripotent hematopoietic stem cells that maintain their capacity of terminal differentiation. This is why the myeloid committed precursors form a big pool, and all stages of myeloid maturation are identified. In CML the white blood cells (WBCs) outnumber the other blood cells. Initially, these WBCs function relatively normally but as the condition progresses immature WBCs or myeloblasts (also called blasts) accumulates in the blood and bone marrow. However, Chasseriau et al. also mentions that in CML there is also increase in megakaryocyte and erythroid lineage in peripheral blood and myeloid hyperplasia in bone marrow [12, 25]. The reason is evident since the translocation occurs in HSCs; as a result Ph chromosome is also found in neutrophils, megakaryocytes, erythroid precursors, B-cells, sometimes T-cells and monocytes. Stromal cells of the bone marrow are not affected [26]. However, they affect malignant hematological cells by positively regulating anti-apoptotic proteins [27].

Although in 90% to 95% of the cases of CML, the presence of Bcr/Abl oncogene is verified [9, 10, 17], however many recent studies show an age-related correlation to the presence of Bcr/Abl oncogene transcript in peripheral blood. This is perhaps because with age DNA damage accumulates and the presence of secondary mutations further motivates leukemia in CML patients [9, 28].

Clinical manifestation of CML: A complete blood count (CBC) shows elevated platelet count (frequently above 10^6 per μl) with a few functional platelets, and cells of various granulopoietic stages, basophil, eosinophil and megakaryocyte, WBC count is also increased. The peripheral blood smear appears to be like a bone marrow aspirate.

CML is diagnosed based on the percent abundance of myeloblast cells. These cells are large and are identified because of their lacy chromatin, distinct nucleoli, pale cytoplasm, and high nucleus to cytoplasm ratio and may exhibit Auer rods (clumps

of granular material that arrange as elongated needles in the cytoplasm of blast cells).Accordingly, three distinct phases have been identified so far in CML patients, viz chronic phase (CP), accelerated phase (AP) and blast phase (BP). CP may go undetected, and therefore in such patients, CML is biphasic with only AP and BP at the clinical level [26]. CP is clinically manageable but it being asymptomatic is somewhat scary because once BP ensues, the inevitable result is death [29]. Whenever present, CP is clinically characterized by an elevated count of myeloid cells in bone marrow, spleen, and peripheral blood. The common symptoms include fatigue, natural satiety, malaise, weight loss, pain, bleeding (due to low and non-functional platelet count), and gastrointestinal ulcers (due to elevated histamine released from basophilia). Splenomegaly (in 40-50% cases) and hepatomegaly (in 10% cases) is also observed. WBC count exceeds 100×10^9/L of blood [17, 30].Table 2 shows the WHO criteria for AP and BP**.** It is crucial to identify the correct stage of CML because of its correlation to the kind of treatment that shall best suit the stage and treatment response [21].

Table 2: WHO criteria of AP and BP in CML [26]

Phases	WHO criteria
Accelerated	
Blasts	10-19%
Basophils	≥20%
Platelets	<100 X 10^9/L (unrelated to therapy) or 1000 x 10^9/L (unresponsive to therapy)
Spleen and WBC	Both spleen size and WBC count increases unresponsive to therapy
Blast	
Blasts	≥20%
Extramedullary blasts	Present
Other	Large clusters of blasts in bone marrow biopsy

Treatment Implications: Even before the advent of Tyrosine Kinase Inhibitors (TKIs), non-specific drugs like hydroxyurea (a ribonucleotide reductase used to control abnormal blood count), busulfan (alkylating agent), and interferon-alfa (IFN-α) were used. These did not eliminate the abnormal clones or reduce genomic instability to prevent the onset of blast phase except for IFN-α which worked to provide anti-leukemic immunity. But it had to be stopped due to poor tolerance in patients and toxicity was also reported when given in combination with some other drugs. Allogeneic Stem Cell Transplantation (ASCT) with a cure rate of 70% is the only curative treatment for CML which also faced the limitation of Human Leukocyte Antigen (HLA) matched identical sibling. So Auto-transplantation (use of graft with Philadelphia negative progenitors that can perform normal hematopoiesis) was proposed for such patients, but due to the absence of graft versus leukemia effect and presence of Philadelphia positive cells, it too proved to be a failure [10, 58]. The next stage of CML treatment saw the rise of TKIs. CML is the first disease to be successfully treated with a selective drug designed to inhibit the causative tyrosine kinase. Imatinib mesylate (Gleevec), called initially STI571 and CGP57148B, is the first FDA approved BCR/ABL TKI. Since then many more TKIs have been approved, and a few are currently under clinical trials. Table 3 outlines a comprehensive outlook of the most commonly prescribed TKIs mainly focusing on their drawbacks such as high cost and associated ailments, let alone the resistance that develops due to their continuous use. The molecular mechanism of such resistance the most common being T315I (threonine to isoleucine mutation at the 315th residue of BCR/ABL) is due to variations in the target domain of ABL kinase of BCR/ABL, increase in mRNA and protein level of the oncoprotein [17].

Why microRNA? MicroRNAs being highly potent open the avenues for its use to even act on targets that cannot bereached by the conventional drugs [38].For instance, drugs generally work on proteins with enzymatic activity or those having an accessible conformation, but miRs can be designed to target any gene of interest. Moreover, without causing any harm to the patient (non-invasiveness), the efficiency of the patient to drug response can be evaluated which is a boon to save them from the adverse effects of such drugs and also to select a personalized drug therapy [38, 40]. Based on the miRNA expression profiles early treatment is possible and the stage of cancer, their sub-types and severity can be monitored.

Table 3:Pharmacological properties and a few adverse effects of TKIs [31]

Year of FDA/EMA approval	2001/2001	2006/2006	2007/2007	2012/2013	2012/2013
Approximate cost for one month supply	$11053	$12428	$12433	$13078	$13536
Half-life (hours)	18	3- 5	17	22.5	24
Mode of elimination	~81% in feces, mostly as metabolites	~85% in feces, mostly as metabolites	~93% in feces, mostly as parent drug	~91% in feces	~ 87% in feces
Neutropenia	++++++	++++++	++++++	++++	+++++
Thrombocytopenia	+++++	+++++	++++++	+++++	++++++
Anemia	+++	++++	++++	++++	+++
Abdominal pain	++	++	++	++	++++
Clinical pancreatitis	+++				
Heart failure	++	+++	+++		
Pericardial effusion	++	++			
Fluid retention/edema	++	+++	+	+	++
Fatigue	++	++	++	++	++

Frequency of toxicity + <1 % ++ 1-5 % +++ 5-10 % ++++ 10-20 % +++++ 20-30 % ++++++ >30 %

Hence, search for an alternative has landed researchers on miRNA.

Micro-RNA: Micro-RNAs (miRs) are approximately 19-25 nucleotides long, endogenous, evolutionary conserved RNAs responsible for post-transcriptional regulation of messenger RNAs (mRNAs) [8,21, 32, 33]. In majority of the cases, miRs target the 3' untranslated region (UTR) of the mRNA (seed match sequence) and cause its deadenylation and decapping (if there is perfect complementarity between the two) and induce translational repression (if complementarity is incomplete, mRNA is not degraded) [8, 32, 34, 35, 36]. Interactions of miRs with 5' UTR and coding sequences (induce silencing of mRNA) and promoter (activate transcription of mRNA) has also been observed [35]. They play a crucial role in the developmental processes of the organism and have been found to influence all cancer types investigated to date. The miRs that are transported out of the cell via vesicles or with binding proteins into the extracellular fluid act as messengers of cell-to-cell communication [35]. They are stable and thus can be routinely quantitated and potentially may also serve as disease biomarkers[33]. The human genome contains ~2,500 distinct mature miRs[37, 38] and more than one-third of genes are putative miR targets[25]. In mammals, miRs which are expressed in HSCs are the chief controllers of hematopoietic cell lineage differentiation [32, 34].

Micro-RNA Biogenesis: miRs are transcribed by RNA polymerase II either intragenic (from introns and few exons of a protein-coding gene) or intergenic (from their promoters independent of another gene) regions. Processing is performed by RNA polymerase II/III co- or post-transcriptionally. The 5' end of the mature miR has about eight bases at the 5' end called the seed sequence or region [37] and those miRs which have similarity in seed sequence are transcribed as a long transcript and belong to a family [35].

In canonical pathway, after transcription of the DNA sequence into primary miR, DiGeorge Syndrome Critical Region 8 (DGCR8) – an RNA binding protein and Drosha (a ribonuclease III) processes it into a pre-miR (hairpin RNA). The latter is then exported by exportin (XPo) 5/Ran GTP into the cytoplasm where Dicer (Type 2 RNase III endonuclease whose activity is enhanced by Transactivation response element RNA-binding protein, TRBP) removes the 3' loop, and mature miRNA duplex is formed. Of the duplex, the strand that is loaded into Argonaute (AGO) protein using ATP (based on lower 5' uracil) is called the guide strand, and the other is the passenger strand (generally degraded or can become functional) [25]. The complex of guide strand and AGO so formed is called the minimal miR-induced silencing complex (mi-RISC). The guide strand now interacts with target mRNA based on complementarity, and the result can be incomplete or perfect complementarity. The consequences of such interactions are explained above. The above is the dominant pathway. There

exists the non-canonical pathway as well. In this pathway, Mirtrons are produced from introns of mRNA while splicing and without Drosha cleavage exported to the cytoplasm by XPo1 (Drosha/DGCR8-independent pathway). If Drosha is involved, then the processed pre-miR is transported via XPo5 to the cytoplasm and AGO cleaves one strand. Finally, the mi-RISC is formed [21, 35, 39].It is of particular note here that the entire process of miR biogenesis and miR-mRNA interaction is tightly controlled both spatially and temporally in different organisms which means microRNA pattern reflects the physiology of a cell which can serve as a biomarker in cancer including CML.

MicroRNA and BCR/ABL protein: Use of microRNA as a prognostic and diagnostic biomarker as well as a therapeutic agent is gaining greater importance as the number of researches being carried out every year is increasing rapidly. They play a pivotal role in cancer both as a tumor suppressor as well as a tumor promoter. They can further modulate the tumor milieu and immune system because of their ability of intercellular communication [40]. The gradual inclination towards microRNA is not only because the current drug therapies available to treat CML are less effective with myriads of side effects but also because microRNA has the intrinsic potential of RNA interference (RNAi). Moreover, they induce either resistance or sensitization to drugs. MicroRNA being the icon of RNAi technology can be effectively applied to degrade or block the mRNA of tumor suppressor proteins and inhibit the cascade of downstream signaling pathways of BCR/ABL that ultimately result in leukemogenesis. The fact that miR therapies are currently under clinical or pre-clinical trials undeniably shows their great potential to treat cancer.The following account focuses on the significance of a few miRs as a master regulator of Bcr/Abl mRNA. Although the importance of miR as a biomarker is not discussed at depth in this account separately but examining its expression profile in connection with its therapeutic potential surelyserves the other purpose as well.

MicroRNAs directly targeting Bcr/Abl mRNA: The presence of specific sequences in Bcr/Abl mRNA provides an efficient means to control it by miR[10]. Differential expression of many miR in CML as compared to normal cells show that these miRs regulate the progression of leukemia**.** Table 4 shows the identified miR that directly targets the mRNA of the oncogene or Abl.

Table 4: Features of miRthat targetsBcr/Abl and/or Abl mRNA.

MicroRNA	Location	Target	Role	Expression level	Reference
miR-29b	7q32.3	Abl	Anti-proliferative and pro-apoptotic effect on cells	↓	[41]
miR-320a	8p21.3	Bcr/Abl	Inhibits invasion, Anti-proliferative and pro-apoptotic effect on cells	↓	[41]
miR-30a miR-30e	6q13 1p34.2	Abl Bcr/Abl	Regulator of cell cycle progression	↓	[41]
miR-424 (oncogene)	Xq26.3	Bcr/Abl	Inhibits apoptosis and promotes cell proliferation	↑	[41]
miR-7	9	Abl	Induces Apoptosis	↓	[42]
miR-23a	19	Bcr/Abl	Targets BCR/Abl/PI3K/AKT/MMP9 signaling pathway	↓	[42]
miR-143	5	Bcr/Abl	Induces apoptosis in K562 cell line	↓	[42]

BCR/ABL has been the target of the TKIs as well because this oncoprotein activates many of the downstream pathways that inhibit apoptosis and cell differentiation, induces cell proliferation and migration [29].The theoretical promise of miR as a therapeutic agent to target Bcr/Abl mRNA does have specific technical issues. For instance, the half-life of P210 BCR/ABL protein is more than 24 hours which means that prolonged ex-vivo culture is needed to induce death in most leukemic cells. Besides, studies show that Bcr/Abl mRNA and protein is not expressed in all CML stem cells and so residual leukemic cells would soon take over the former[10]. Circulating microRNA levels are extracted from venous plasma, or serum or exosomalmiRs in peripheral blood and often several miRs are reported to express differently in plasma, serum or exosomes. Thus, choice of a proper selection method for certain

miRs should not be denied [43].It is crucial that downstream targets be also targeted by microRNA, and a combinatorial approach is made into treating CML.

MicroRNAs regulating downstream targets of BCR/ABL:

The most directly linked domains to Abl kinase constitutive activation are the coiled-coil (CC) domain (at the N-terminal) and a tyrosine residue at 177th position (Y177) of the BCR portion of the chimeric gene [13, 20,44]. As already mentioned the CC domain helps in dimerization (two monomers dimerize) or tetramerization (dimers stacking) of the oncoprotein which increases the kinase activity making the cell resistant to imatinib[13, 17, 20]. It also causes autophosphorylation of Y177 allowing occurrence of myelogenous disease similar to leukemia by BCR/ABL downstream interactions that is responsible for the expansion, proliferation, and survival of leukemic cells. The Y177 is the site where downstream signaling molecules like GRB2, GRB10, 14-3-3 interact [18]. The investigation of such signaling pathways thus becomes the pre-requisite before the miRs that regulate them are identified. It is noteworthy that a significant number of such interactions between BCR/ABL and other proteins remain to be revealed. Bcr/Abl encodes for a cytoplasmic tyrosine kinase that is oncogenic only when expressed in HSCs with self-renewal potential. The HSC transforms into leukemic stem cell (LSC) that generate an enormous number of leukemic progenitor cells during the chronic phase which loses their capacity to self-renew and finally differentiate into mature cells. The expansion, proliferation, genetic as well as epigenetic aberrations, arrested differentiation and drug resistance are what guide the transition from chronic to blast phase [57].Cardama and Cortes [18] provided a model to explain the interplay of several components in the transformation of HSC to LSC and transition through phases of CML. In the model, events described are Bcr/Abl expression (e.g. by expression of ICSBP or loss of JUNB), activation of anti-apoptotic and anti-senescence genes (e.g. TERT), evasion of immune system, arrested differentiation (e.g. by silencing of C/EBPα), loss of tumor suppressors and activation of self-renewal pathway (e.g. β-catenin) [18]. BCR/ABL kinase perhaps reduces the fidelity of DNA repair mechanisms and allows point mutations and deletions to take place in LSCs. A few of the pathways involved in leukemogenesis and transforming activity are discussed here.

Pathway involved in disruption of cellular differentiation: Y177 of Bcr/Abl is the primary docking site for the SH2 domain of GRB2 (Growth factor receptor-bound protein 2). The interaction recruits SOS (son of sevenless) which is a guanine nucleotide exchanger of RAS. RAS activation is shown to be mediated by Shc proteins [14, 36]. Shc protein interacts with the SH2 domain of GRB2 and Shc/GRB2/SOS is as well involved in RAS activation [36]. RAS and GAB2 (GRB2-associated binding protein 2) are activated via phosphorylation of the tyrosine kinase. GRB2/GAB2 complex results in constitutive activation of PI3K/AKT (phosphatidylinositol 3-kinase/Protein kinase B) (not shown in the figure) and ERK (extracellular signal-regulated kinase) in primary myeloid cells [10, 18, 20]. Besides, CRKL (Crk-like protein), an adaptor protein of SH2/SH3, also activates RAS as well as JUN and is oncogenic that is important in fibroblast transformation by BCR/ABL. Farnesyl-transferase inhibitors have been shown to block mutant as well wild-type RAS signaling pathway. GAB-/- mice fail to undergo differentiation [18]. The RAS/RAP1/MEK/ERK is the central pathway involved in signal transmission from cell-surface receptors to transcription factors in the nucleus. GRB2/GAB2 activates B-RAF kinase that is an effector molecule of RAP1 (RAS-related protein). This, in turn, activates MEK/ERK pathway that stabilizes hnRNP-E2 (heterogeneous nuclear ribo nucleoprotein E2). hnRNP-E2 is not detected in CP but only in BP which suppresses the mRNA levels of C/EBPα (CCAAT/enhancer binding protein) as a result of which myeloid precursors fail to differentiate into granulocyte-monocyte progenitor cells[20, 45]. This occurs during the CP to BP transition. Consistent with these facts Yin et al. (2017) found that C/EBPα is expressed in CP but lost in BP of CML [18, 46]. In P210 BCR/ABL expressing cells, the effects of C/EBPα is partly dependent on the repression of GATA-2 (GATA-binding factor 2, a transcription factor that is critical for self-renewal, maintenance and differentiation of HSCs[47]. The above effects related toC/EBPα deregulation is shown to be reversed by imatinib. Scherr et al.(2006) used RNAi to reduce GAB2 protein expression in CML CD34+ cells and found that that inhibited Bcr/Abl dependent proliferation [48].

Pathways for cell survival and proliferation: Interaction of Bcr/Abl with cytokine receptors activates JAK1-3 (Janus Kinase) which further activates STAT1, 3, 5 and 6 (Signal transducers and activators of transcription). JAK2/STAT5 pathway directly leads to cell survival by transcriptionally activating BCL-XL (B-cell lymphoma--extra-large, anti-apoptotic protein). Besides, BCR/ABL directly abrogates the transcription of ICSBP (Interferon

Consensus Sequence Binding Protein) and releases its inhibitory effect on anti-apoptotic genes helping in cell survival [18]. Activation of JAK2 directly phosphorylates Y177 and hence stabilizes and enhances BCR/ABL signaling pathways which is indispensable for leukemogenesis.Moreover, JAK2 activates c-MYC oncogene and protects the oncoprotein from degradation. In contrast, C/EBPα causes transcriptional repression of c-MYC. But since, C/EBPα is lost during BP, so c-MYC is overexpressed, and this activates Survivin via JAK2/PI3K pathway which leads to cell proliferation and survival [20]. JAK2 knockdown or inhibition inhibits STAT5 and RAS/PI3K.

PI3K/AKT pathway also activates MDM2, the chief negative regulator of TP53 (Tumor suppressor protein 53). TP53 is critical in determining the cell's fate and sensitivity to treatment. TP53 inactivation/disruption which frequently occurs in CML and most of the cancers causes unavailability of P21 (it acts as a stop signal for cell division) that results in the escape of cell cycle checkpoints and uninterrupted proliferation. This pathway also induces activation of SKP2 (S-phase kinase-associated protein 2), a ubiquitin E3 ligase which is turn activates AKT (positive feedback loop) and also targets cell cycle control elements like P21 resulting in progression of CML. SKP2 is an established proto-oncogene. SKP2 inhibitors are novel therapeutic cancer strategy currently under investigation [59].

IKK-β (inhibitor of nuclear factor kappa-B kinase subunit beta) activated via the PI3K/AKT pathway phosphorylates IκBα which is an inhibitor of NF-κB (Nuclear Factor kappa-light-chain-enhancer of activated B cells). Once IκBα has phosphorylated it dissociates from its target, NF-κB. NF-κB then migrates to the nucleus and protects the cell from apoptosis, induces cell proliferation and cell death evasion [49]. NF-κB is considered to be a partner of BCR/ABL and is constitutively activated in most of the cancers thereby providing an attractive target of cancer therapy including CML.The FoxO3a (Forkhead box O3) is a transcription factor that triggers apoptosis by upregulating apoptotic genes. It regulates the Notch signaling pathway (a highly conserved pathway that regulates quiescence in adult stem cells) and suppresses cell cycle. AKT can directly phosphorylate it and thus mediate its interaction with 14-3-3 nuclear export protein so that it is not able to re-enter the nucleus. Phosphorylated FoxO3a is also the target of ubiquitin ligase E3 ligase and is finally degraded [50]. IKK-β can also phosphorylate it. C/EBPα is a positive regulator of FoxO3a, but it is lost in BP. This in turn causes FoxO3a reduction and ultimately the reduction of TRAIL (Tumor Necrosis Factor Related Apoptosis-Inducing Ligand). Hence, apoptosis is inhibited. Figure 3 portrays the most studied signaling pathways involved in leukemogenesis in CML.

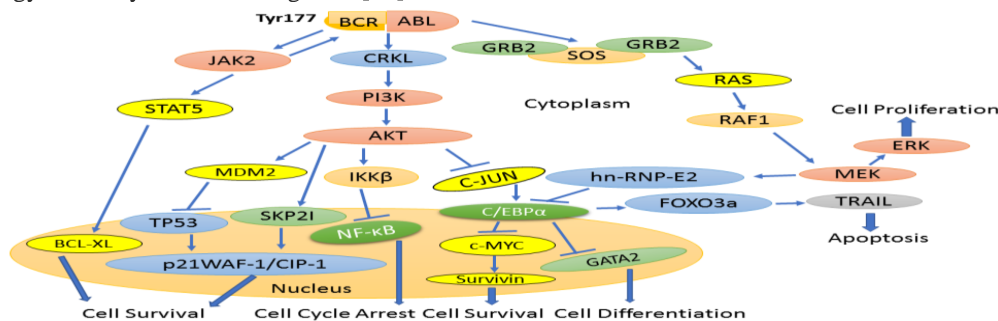

Figure 3: Downstream signaling pathways of BCR/ABL in CML.Peach coloured shapes indicate kinase; yellow coloured shapes indicate established oncoproteins; blue coloured shapes indicate established tumor suppressor proteins, and other colours indicate proteins present in normal cells. Arrow indicates activation whereas line with a bar indicates inhibition. (adapted from Kang et al. [20] and redrawn).

The following tables 5 to 10 show the various microRNAs that naturally target signaling molecules mentioned in the above pathways [21,36, 41, 42]. Target detection methods that are mostly used are qPCR, western blot analysis, luciferase activity assay, microarray expression profiles. MicroRNA based bioinformatics tools like TargetScan, miRbase, and miRNAMap are readily available and user-friendly target prediction tools that generally search for the seed region match. To establish the potentiality of microRNA, multiple functional analysis supported by experiments in cell lines as well as patient samples is required. The following account mentions the most studied microRNAs involved in CML after thorough experimentation.

Table 5: MicroRNA targeting RAS in CML

MicroRNA	Chromosomal Location
miR-124-3p	8
miR-1271-5p	5
miR-145-5p	5
miR-148a-3p	7
miR-152-3p	17
miR-183-5p	7
miR-19a-3p	13
miR-196a-5p	17
miR-200b-3p	1
miR-200c-3p	12
miR-218-5p	4
miR-22-3p	17
miR-29a-3p	7
miR-429	1
miR-506-3p	X
miR-96-5p	7
miR-98-5p	X

Table 6: MicroRNA targeting GAB2 in CML

MicroRNA	Chromosomal Location
miR-125a-5p	19
miR-218-5p	4

Table 7: MicroRNA targeting GRB2 in CML

MicroRNA	Chromosomal Location
miR-124-3p	8
miR-1271-5p	5
miR-128-3p	2
miR-141-3p	12
miR-153-3p	2
miR-182-5p	7
miR-200a-3p	1
miR-216a-3p	2
miR-27a-3p	19
miR-27b-3p	9
miR-3681-3p	2
miR-96-5p	7

Table 8: MicroRNA targeting STAT5 in CML

MicroRNA	Chromosomal Location
miR-141-3p	12
miR-194-5p	1
miR-200a-3p	1
miR-23a-3p	19
miR-23b-3p	9
miR-23c	X
miR-130a-5p	11
mir-150-5p	19
mir-2278	9

Table 9: MicroRNA targeting PI3K in CML

MicroRNA	Chromosomal Location
miR-124-3p	8
miR-130a-3p	11
miR-135a-5p	3
miR-130-3p	11
miR-135a-5p	3
miR-137	1
miR-15a-5p	13
miR-153-3p	2
miR-16-5p	13
miR-181a-5p	9
miR-19a-3p	13
miR-195-5p	17
miR-199-3p	19
miR-200b-3p	1
miR-200c-3p	12
miR-218-5p	4
miR-25-3p	7
miR-27a-3p	19
miR-29a-3p	7
miR-30-5p	6
miR-301-3p	17
miR-32-5p	9
miR-363-3p	X
miR-365a-3p	16
miR-367-3p	4
miR-424-5p	X
miR-429	1
miR-454-3p	17
miR-497-5p	17
miR-506-3p	X
miR-7-5p	9
miR-9-5p	1
miR-92a-3p	13

Table 10: MicroRNA targeting CRLK in CML

MicroRNA	Chromosomal Location
miR-17-5p	13
miR-20a-5p	13
miR-93-5p	7
miR-106a-5p	X
miR-519a-3p	19
miR-133a-3p	18
miR-302a-3p	4
miR-372-3p	19
miR-373-3p	19
miR-520a-3p	19
miR-183-5p	7
miR-15a-5p	13
miR-16-5p	13
miR-195-5p	17
miR-424-5p	X
miR-497-5p	17

Table 11:Some of the other microRNAs involved in recently discovered signaling pathways are mentioned in the following table [42]

MicroRNA (tumour suppressor)	Target	Role
Mir-155	CEBPβ	Increases apoptosis
Mir-7	Abl, PI3K, AKT	Increases apoptosis and sensitizes K562 cells to imatinib
MIR-186	ROCK1	Inhibits cell proliferation and migration
Mir-570	IRS1, IRS2	Increases apoptosis, suppresses cell proliferation, decreases glucose metabolism
Mir-15a-5p	CXCL10	Suppresses cell proliferation, promotes apoptosis
Mir-150	FOXOB, EIF4B, PRKCA, TET3	Proteoglycan metabolism in cancer, mTOR or Wnt signaling pathway
Mir-139-5p	Brg1	Suppresses differentiation and proliferation
Mir-219	XIAP	Inhibits survival of leukemic cells
Mir-203	Survivin, Bmi-1	In LSCs, the miRNA increases cell proliferation and self-renewal capacity
Mir-130a	BCL-2, MCL-1, XIAP	Promotes apoptosis
Mir-708	IKKβ	Suppresses NFκB pathway
Mir-186	DDX43	Inhibits cell proliferation and increases apoptosis
Mir-202	HK2	Inhibits glycolysis
Mir-181a	RALA	Inhibits growth and apoptosis, sensitizes cells to imatinib
Mir-148b	DNMT1 and DNMT3	Inhibits cell proliferation and invasion
Mir-2278	AKT2, STAM2	Inhibits proliferation of resistant leukemic cells and promotes apoptosis
Mir-486-5p	PTEN, FOXO1	Role in modulation of normal as well as hematopoietic progenitor growth
MicroRNA (Oncogene)		
Mir-17-92	A20 (TNFAIP3)	Regulates NFκB pathway
Mir-301a	TIMP2	Promotes cell proliferation and inhibits apoptosis
Mir-362-5p	GADD45α	Inhibits apoptosis and promotes cell proliferation
Mir-99a	CTDSPL and TRIB2	Inhibits apoptosis and promotes cell proliferation

Target detection methods that are mostly used are qPCR, western blot analysis, luciferase activity assay, microarray expression profiles. MicroRNA based bioinformatics tools like TargetScan, miRbase, and miRNAMap are readily available and user-friendly target prediction tools that generally search for the seed region match. To establish the potentiality of microRNA, multiple functional analysis supported by experiments in cell lines as well as patient samples is required. The following account mentions the most studied microRNAs involved in CML after thorough experimentation.

Mir-150 is found to downregulate in both CP and BP significantly and were not restored in patients resistant to imatinib which implies that it can be used as a biomarker in treatment response [51].

Several studies confirm that Mir-203 expression is significantly down-regulated in CML cell lines but not in other MPNs. Reintroduction of the miR resulted in a marked decrease of the oncoprotein [52]. Thus, mir-203 can be a diagnostic biomarker in CML.

Mir-17/92 cluster includes mir-17, mir-19a, mir-19b and mir-20a. The members of this cluster are shown to overexpress in CML. Specially overexpression of mir-20a only in K562 CML cell line and not in AML cell line shows its potential to be a biomarker of CML [53].Mir-10a targets upstream stimulatory factor 1 (USF1) which is an established transcription factor of cell proliferation upregulated to different extents in CML patients. However, its use as a biomarker of drug response requires further investigation as the results from various CML cell lines do not show consistency [54].Its overexpression in K562 cells is shown to arrest the G1 phase by activation of p21 and p27 [55].Mir-221 expression is found to decrease in patients with imatinib resistance. Its restoration and knockdown of STAT5 in K562 cells reduced BCL2:Bax ratio and increased the sensitivity of the cells to imatinib treatment [56].

Conclusion

In-depth analysis about microRNA expression profile concerning any cancer including leukemia allows it to be used as an antagomir (or anti-miR) or as a miRNA mimic. Anti-miR suppresses the function of endogenous miRNAs thus providing an avenue to inhibit the role of oncogenic miRNAs that are often over-expressed in cancer. This approach is called miRNA inhibition. In contrast, a mimic is used in replacement approach to function as an endogenous miRNA thereby enhancing the effect of tumor suppressor microRNA [60]. Both methods require the introduction of synthetic miRNA [38]. In this regard use of small interfering RNAs (siRNAs) as a therapeutic agent is often discussed. But unlike miRNA, they cannot be used for prognosis and diagnosis and to evaluate treatment response. Besides, both share common hurdle of relative stability. *In vivo* introduction of miRNA can also trigger the innate immunity via Toll-like receptors. However, studies show that these problems are manageable by their smart chemical modification [38, 43].

However, as compared to siRNA the clinical development of miRNA is lagging probably due to uncertainty in their action and specificity. Till date only two of them namely, MRX34 (miRNA-34a mimic) for primary liver cancer and TargomiRs (miRNA-16 mimic) for malignant pleural mesothelioma has reached phase 1 clinical trials. A few of the miRNAs involved in CML are also under pre-clinical phases. It is important to note that miRNAs are advantageous when it comes to the treatment of complex diseases like cancer or neurodegenerative diseases because it involves targeting several genes at once and also miRNA can target, any target gene inaccessible to drugs. Besides, a combinatorial approach to treating CML with TKIs after sensitizing cells to TKIs by use of microRNAs to avoid drug resistance is another promising therapeutic aspect. With the rapid advancement in microRNA research and understanding of signaling network in CML, and simultaneously managing problems of miRNA delivery systems, it can be expected that miRNA therapeutics in the clinic will soon be a reality.

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