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BCR::ABL1-INDUCED LEUKEMOGENESIS: IMPACTS OF MIR-495 AND CIRCADIAN CLOCK

*Impact de miR-495 et de l'horloge circadienne dans la leucémogénèse
induite par BCR::ABL1*

Thèse de doctorat de l'Université Paris-Saclay

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Thérapeutiques (Inserm, Université Paris-Saclay)**, sous la direction de
Ali TURHAN, PU-PH Université Paris-Saclay et directeur Inserm, le
co-encadrement de **Jérôme ARTUS**, MCU Université Paris-Saclay, et
Adlen FOUDI, chargé de recherche, ATIP-Avenir – Inserm

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Titre : Impact de miR-495 et de l'horloge circadienne dans la leucémogénèse induite par BCR::ABL1

Mots clés : Leucémie myéloïde chronique, BCR::ABL1, Leucémogénèse, MicroRNA-495, MDR1, Horloge circadienne

Résumé : La leucémie myéloïde chronique (LMC) est un néoplasme myéloprolifératif caractérisé par la présence d'une protéine de fusion chimère anormale BCR::ABL1. Le traitement de cette maladie a été révolutionné par l'introduction à la clinique, des inhibiteurs de la tyrosine kinase (ITK) ciblant BCR::ABL1. Néanmoins, le problème de la résistance aux ITK est la préoccupation clinique actuelle, qui est apparue comme un défi du paradigme thérapeutique. Ainsi, les objectifs de mon projet étaient d'étudier le rôle potentiel de miR-495-3p dans des modèles de leucémogénèse induite par BCR::ABL1. Le deuxième volet consistait en l'analyse des gènes de l'horloge biologique dans des modèles de cellules transformées par BCR::ABL1 et dans des cellules primaires de patients. (1) Ainsi au cours de la première partie de ce travail j'ai pu montrer que l'expression de miR-495-3p était diminuée dans les modèles cellulaires exprimant BCR::ABL1. Dans les expériences de surexpression, j'ai pu montrer que miR-495-3p modifiait la croissance des cellules leucémiques et la résistance à l'Imatinib.

Cet effet a également été retrouvé avec le traitement au Ponatinib du variant mutant T315I résistante à l'Imatinib. Nos résultats ont montré que miR-495-3p régulait la sensibilité aux ITK via la régulation de MDR1. De plus, les cibles prédites de miR-495-3p sont fortement exprimées chez les patients en crise blastique impliquées dans la phosphorylation des protéines et associées à un mauvais pronostic. (2) Dans la deuxième partie de mon travail, j'ai analysé l'impact de BCR::ABL1 sur la dérégulation de l'expression des gènes de l'horloge dans la LMC. Nous avons mis en évidence une corrélation inverse entre l'expressions du gène de l'horloge *BMAL1* avec celle de *BCR::ABL1*. Cette dérégulation modulée par BCR::ABL1 fait intervenir la voie de signalisation MAPK/ERK. Au total, nos données démontrent que la dérégulation de miR-495-3p et l'expression du gène de l'horloge *BMAL1* pourrait intervenir dans la physiopathologie de la LMC. Ces résultats pourraient permettre d'envisager de nouvelles stratégies thérapeutiques dans le futur.

Title : BCR::ABL1-induced leukemogenesis: impacts of miR-495 and circadian clock

Keywords : Chronic myeloid leukemia, BCR::ABL1, Leukemogenesis, MicroRNA-495, MDR1, Circadian clock

Abstract : Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm characterized by the presence of an abnormal chimeric fusion protein BCR::ABL1. The treatment of CML has been revolutionized by the advent of BCR::ABL1-targeted tyrosine kinase inhibitor (TKI) therapies. Nonetheless, the problem of TKI resistance is the current clinical concern, which has emerged as a challenge of the evolving therapy paradigm. Thus, the objectives of this project were to explore the potential role of miR-495-3p and the core clock genes in the pathophysiology of BCR::ABL1 induced leukemogenesis. My goals were (1) To investigate the altered expression and potential role of miR-495-3p in CML. In the first part of my work, I have shown that miR-495-3p expression was down-regulated in BCR::ABL1-expressing cellular models. Overexpression of miR-495-3p was shown to impair leukemic cell growth and Imatinib resistance.

This impact was also found with Ponatinib treatment in the cells expressing T315I-mutations generating resistance to all TKI's except Ponatinib. Our result showed that miR-495-3p regulated TKI chemo-sensitivity in CML via regulating MDR1-mediated drug efflux activity. Furthermore, predicted miR-495-3p targets were found to be upregulated in patients in blast crisis associated with a worse prognosis. (2) To analyze the impact of BCR::ABL1 on the deregulation of core clock genes in CML. To this end, we showed an inverse correlation between the expression of the core clock gene *BMAL1* and that of *BCR::ABL1*. Our results suggest that BCR::ABL1-modulated deregulation may function via the MAPK/ERK signaling pathway. Altogether, our data demonstrate that the deregulation of miR-495-3p and core clock genes might be involved in BCR::ABL1-induced leukemogenesis and these results might lead to novel therapeutic strategies.

SYNTHÈSE

La leucémie myéloïde chronique (LMC) est un néoplasme myéloprolifératif malin clonal caractérisé par la prolifération incontrôlée de cellules myéloïdes. La LMC a pour origine l'oncoprotéine chimérique BCR::ABL1, qui résulte d'une translocation entre les chromosomes 9 et 22 t(9;22)(q34;q11.2) dans les cellules souches hématopoïétiques et est présente chez plus de 95 % des patients atteints de LMC. Principalement, BCR::ABL1 possède une activité tyrosine kinase constitutivement active qui modifie drastiquement les propriétés cellulaires telles que la prolifération, la différenciation, la survie et la stabilité génétique. Le traitement de la LMC a grandement bénéficié du fait que les cellules cancéreuses sont dépendantes de l'expression de l'oncogène BCR::ABL1, de sorte que l'inhibition pharmacologique de BCR::ABL1 empêche la croissance des cellules leucémiques. L'Imatinib (IM) est le premier inhibiteur de tyrosine kinase (ITK) à avoir été autorisé en clinique après que son efficacité ait été validée dans différents modèles expérimentaux précliniques.

Au début des années 2000, l'introduction de l'IM en clinique a modifié l'histoire naturelle de la maladie. Ainsi, l'IM et le développement d'ITK de 2ème et 3ème génération ont permis d'améliorer efficacement la survie des patients atteints de LMC, qui se traduit par une espérance de vie normale des patients répondant à ces traitements. Néanmoins, malgré les progrès significatifs réalisés dans le traitement de la LMC au cours des dernières décennies avec l'arrivée des ITK, un patient sur quatre (20 à 30 %) devra changer de traitement soit parce qu'il ne tolère pas les effets secondaires du médicament soit parce qu'il développe une résistance au traitement. Les mécanismes moléculaires à l'origine de cette résistance sont d'origine variée et peuvent notamment faire intervenir l'amplification de *BCR::ABL1* ou l'apparition de mutations dans la séquence codante de l'oncogène *BCR::ABL1*. Parmi ces mutations, la mutation T315 est particulièrement préoccupante puisqu'elle confère une résistance à tous les ITK approuvés en première ligne mais reste toutefois sensible au Ponatinib. Un autre mécanisme-clé de la résistance aux ITK dépend fortement de la biodisponibilité du médicament, de son accessibilité à sa cible BCR::ABL1 et de la régulation par des transporteurs membranaires entre afflux et efflux des médicaments dans la cellule. Ainsi, l'altération de la protéine MDR1 (P-gp, ou ABCB1), une pompe d'efflux de tous les ITK approuvés

pour la LMC, diminue l'efficacité du traitement et participe à un mécanisme de résistance indépendant de BCR::ABL1.

Des données expérimentales récentes suggèrent une implication du *miR-495-3p* dans la chimiorésistance dans plusieurs cancers. Il a été démontré qu'il cible directement la partie 3'UTR du gène *ABCB1/MDR1* et que son expression est réduite dans des lignées cellulaires et chez des patients résistants aux médicaments. Dans la LMC, la lignée cellulaire K562 résistante aux médicaments exprime des niveaux plus faibles de *miR-495-3p* et la surexpression de *miR-495-3p* restaure leur sensibilité à l'agent chimiothérapeutique Adriamycine. Cependant, on ignore si l'expression du *miR-495-3p* est affectée chez les patients atteints de la LMC et si *miR-495-3p* affecte l'efflux des ITK médié par MDR1, ce qui pourrait entraîner une résistance au traitement.

Au cours de mon travail de thèse, je me suis intéressé au rôle potentiel du miR-495-3p dans la chimiosensibilité aux ITK de la LMC et j'ai cherché à déterminer le circuit moléculaire sous-jacent impliqué. Dans un premier temps, nous avons d'abord remarqué que l'expression du *miR-495-3p* était diminuée dans plusieurs lignées cellulaires exprimant BCR::ABL1 *in vitro*. Des expériences de perte de fonction ont révélé que l'inhibition du miR-495-3p stimulait la prolifération cellulaire associée à une diminution de la proportion des cellules en phase G0/G1 et à une résistance accrue à l'IM. Inversement, la surexpression du *miR-495-3p* entrave la croissance des cellules leucémiques et la résistance à l'IM dans les cellules leucémiques exprimant BCR::ABL1. Cet effet a également été démontré dans la résistance au Ponatinib du variant T315I de BCR::ABL1 résistant à l'IM. Au niveau fonctionnel, nous avons également démontré que miR-495-3p cible l'ARNm de *MDR1*, ce qui module finalement l'activité d'efflux des médicaments.

Sur la base de l'analyse des données des patients, nous avons découvert que les cibles prédites du miR-495-3p sont codées par des gènes dont l'expression est augmentée chez des patients avancés dans la maladie en phase de crise blastique. Parmi ces cibles, on retrouve un certain nombre de protéines impliquées dans la phosphorylation des protéines qui pourraient avoir une pertinence clinique importante dans la pathogenèse leucémique et le mécanisme de résistance aux médicaments. Nous avons également constaté que la surexpression des gènes cibles de miR-495-3p est associée à un pronostic plus défavorable dans la cohorte de patients atteints de leucémie myéloïde aiguë (LMA).

En conclusion, nos résultats démontrent que la régulation négative de l'expression du *miR-495-3p* est impliquée dans le phénotype malin des cellules BCR::ABL1. Sa régulation à la hausse pourrait surmonter la résistance aux ITK de la LMC en ciblant l'activité d'efflux de médicaments de MDR1 et le mécanisme de résistance aux médicaments. Nous proposons que *miR-495-3p* pourrait être utilisé comme biomarqueur et pourrait constituer une cible thérapeutique potentielle dans le cas de patients chimiorésistants atteints de la LMC.

Par ailleurs, L'hématopoïèse est un processus continu qui assure la production de l'ensemble des cellules sanguines fonctionnelles tout au long de la vie d'un individu. Il s'agit d'un processus étroitement régulé sous l'influence de nombreux signaux intrinsèques et extrinsèques. Il a également été démontré que les rythmes circadiens peuvent jouer un rôle sur l'hématopoïèse et que la perturbation des rythmes circadiens augmente le risque de contracter diverses maladies chroniques, dont des hémopathies malignes. Dans le contexte de la leucémie, une expression altérée des gènes impliqués dans l'horloge moléculaire à l'origine de ces rythmes a été signalée dans plusieurs types de leucémie, notamment la leucémie myéloïde chronique (LMC). D'autres recherches ont suggéré que l'horloge circadienne joue un rôle dans la leucémogénèse. Dans des cellules leucémiques de type LMA, la perturbation expérimentale de l'horloge circadienne inhibe le maintien des cellules souches leucémiques et induit la différenciation cellulaire. Chez les patients atteints de LMC, l'expression quotidienne de *Bmal1* est perturbée, ce qui soulève la question de savoir si l'horloge circadienne est fonctionnelle. Il est intéressant de noter que plusieurs tyrosine-protéines kinases, dont ABL1 et BCR, ont été proposées pour moduler l'horloge circadienne. Si la principale région d'interaction d'ABL1 et de BCR est conservée dans la protéine de fusion BCR::ABL1, les gènes centraux de l'horloge peuvent interagir physiquement avec BCR::ABL1, l'oncogène associé à la LMC. Cependant, l'effet de BCR::ABL1 sur la dérégulation des gènes de l'horloge centrale dans la LMC et l'impact de la perturbation du rythme circadien dans la pathogénèse de la LMC demeurent inconnus.

Dans cette deuxième partie de mon travail de thèse, j'ai testé si l'expression de l'oncoprotéine BCR::ABL1 perturbait l'horloge circadienne dans la LMC. Dans les modèles de lignées cellulaires de LMC et les échantillons de patients, nous avons trouvé une corrélation inverse entre l'expression de l'oncogène *BCR::ABL1* et celle des gènes de l'horloge centrale. Grâce à un système d'expression

inductible TET-OFF de *BCR::ABL1* ou en modulant l'activité de l'oncoprotéine par traitement à l'IM, nous avons également démontré que cet effet sur l'expression des acteurs de l'horloge est réversible. Par ailleurs, des expériences complémentaires semblent indiquer que l'effet de *BCR::ABL1* sur la dérégulation des gènes de l'horloge centrale fait intervenir la voie de transduction du signal des MAPK/ERK.

Ainsi, ces travaux suggèrent que la dérégulation du rythme circadien pourrait être un autre mécanisme important dépendant de *BCR::ABL1* qui régit la pathogenèse et l'homéostasie de la LMC plutôt que chaque composant du circuit de l'horloge moléculaire fonctionnant individuellement comme facteurs de transcription, comme cela a été étudié précédemment. L'ensemble de ces résultats révèle une importance insoupçonnée des gènes de l'horloge circadienne sur la leucémogénèse dont les mécanismes moléculaires sous-jacents et les conséquences phénotypiques restent à élucider.

**BCR::ABL1-INDUCED LEUKEMOGENESIS:
IMPACTS OF MIR-495 AND CIRCADIAN CLOCK**

Presented by Yutthana Rittavee

Public Discussion (21 November 2022)

Inserm U1310

Supervised by Dr J Artus, Dr A Foudi and Prof A Turhan

Université Paris Saclay

Ecole Doctorale N° 582

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ABBREVIATIONS:

3'UTR	3' untranslated regions of the mRNA
ABC	ATP-binding cassette
ABCB1	ATP Binding Cassette Subfamily B Member 1
ABL1	Abelson murine leukemia viral oncogene
ADM	Adriamycin
ALL	Acute lymphocytic leukemia
Allo-HSCT	Allogeneic hematopoietic stem cell transplantation
AML	Acute myelogenous leukemia
AP	Accelerated phase
AR	Androgen receptor
ATP7A	ATPase copper transporting
B-ALL	B-lymphoblastic leukemia
BC	Breast cancer
BCR	Breakpoint cluster region
BP	Blast crisis phase
BTKi	Bruton's tyrosine kinase inhibitor
CCG	Clock-controlled gene
CD	Crohn's disease
CLL	Chronic lymphocytic leukemia
CML	Chronic Myeloid Leukemia
CMR	Complete molecular remission
CN-AML	Cytogenetically normal AML
CP	Chronic phase
CR	Complete remission
CSC	Cancer stem cell
CXCL12	c-x-c motif chemokine ligand 12
DLBCL	Diffuse large B-cell lymphoma

DMR	Deep molecular response
EC	Esophageal cancer
ELN	European Leukemia Net
FDA	Food and Drug Administration
FISH	Fluorescence in situ hybridization
GBC	Gallbladder cancer
GC	Gastric cancer
HCC	Hepatocellular carcinoma
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cell
IFN- α	Interferon-alpha
IRIS	The International Randomized Study of Interferon and STI571 study
LAC	Lung adenocarcinoma
lncRNA	Long-noncoding RNA
LSC	Leukemic stem cell
MAPK	Mitogen-activated protein kinase
MAT1A	Methionine adenosyltransferase 1A
MDR	Multidrug resistance
MLL	Mixed-lineage leukemia
MMR	Major molecular response
MPN	Myeloproliferative neoplasm
MPP	Multipotent progenitor
miR	microRNA
mRNA	Messenger RNA
NSCLC	Non-small cell lung cancer
NT	Nucleotide
OC	Ovarian cancer
OncomiR	Oncogenic microRNA

PB	Peripheral blood
PC	Prostate cancer
P-gp	P-glycoprotein
Ph1	Philadelphia chromosome
PI3K	Phosphoinositide 3-kinase
RNAi	RNA interference
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SCLC	Small cell lung cancer
SCN	Suprachiasmatic nuclei
SLC	Solute carrier transporter
SP	Side Population
STAT	Signal transducer and activator of transcription
STI	Signal transduction inhibitor
TFR	Treatment-free remission
TKI	Tyrosine kinase inhibitor
TNF- α	Tumor necrosis factor alpha
TTFL	Transcriptional-translational feedback loop
WT	Wild-type

CHAPTER 1: Chronic Myeloid Leukemia (CML)

Chronic Myeloid Leukemia (CML), also known as chronic myelogenous leukemia, chronic myelocytic leukemia and chronic granulocytic leukemia, is a clonal hematological malignancy currently classified in the group of myeloproliferative neoplasms (MPNs). CML is a slow-progressing blood and bone marrow disorder characterized by the excessive production of myeloid cell lineages in the bone marrow. CML piques the interest of clinical hematologists, clinical laboratory experts, molecular biologists, and oncologists. In brief, to the clinical hematologist, CML is a prevalent hematologic disease that necessitates a thorough assessment of both clinical and diagnostic information in order to make accurate therapy decisions and ensure quality patient care which has been revolutionized by the use of targeted tyrosine kinase inhibitor (TKI) therapies. To the scientific professional, CML is acknowledged to be first cancer directly related to a genetic mutation, resulting in a reliable diagnostic method. Meanwhile, the significance of a single mutagenic event on signal transduction pathways resulting in malignant transformation intrigues molecular scientists. As the leukemogenic transformation in CML is sufficiently established by consistent occurrence of the t(9;22) (q34;q11) translocation, the multistep processes of oncogenesis required in most other types of cancer appeared not to be needed in CML but recent evidence suggests that there are a plethora of signaling pathways involved which are activated especially in leukemic stem cells independent of BCR::ABL1 and providing, therefore, oncologists with a novel carcinogenic paradigm.

1.1 The brief history of CML

CML has a long and illustrious history, as well as extraordinary discoveries. The earliest scientific description of leukemia is attributed to John Hughes Bennett in 1845 (Bennett 1845). However, individuals with undefined but comparable symptoms can be documented in French literature as early as the starting of the nineteenth century. It is probable that some of the earlier patients had CML. The first description happened to be published in 1844 in Paris by Alfred Donné, the founder of French clinical microscopy. In his manuscript *Cours de Microscopie*

Complémentaire des Études Médicales (Course of Microscopy Complementary to Medical Studies), he briefly explained his observations with his photomicrography methods of the blood collected from two clinical cases of patients and discovered a maturation arrest of white blood cells (Donné 1844). Within the first set of microphotographs of human blood cells and platelets, this could be described as the first possible microscopic appearance of leukemia despite the lack of details (**Figure 1**). His findings constituted for the first time that leukemia has been related to aberrant blood pathology although he called it an unknown disease.

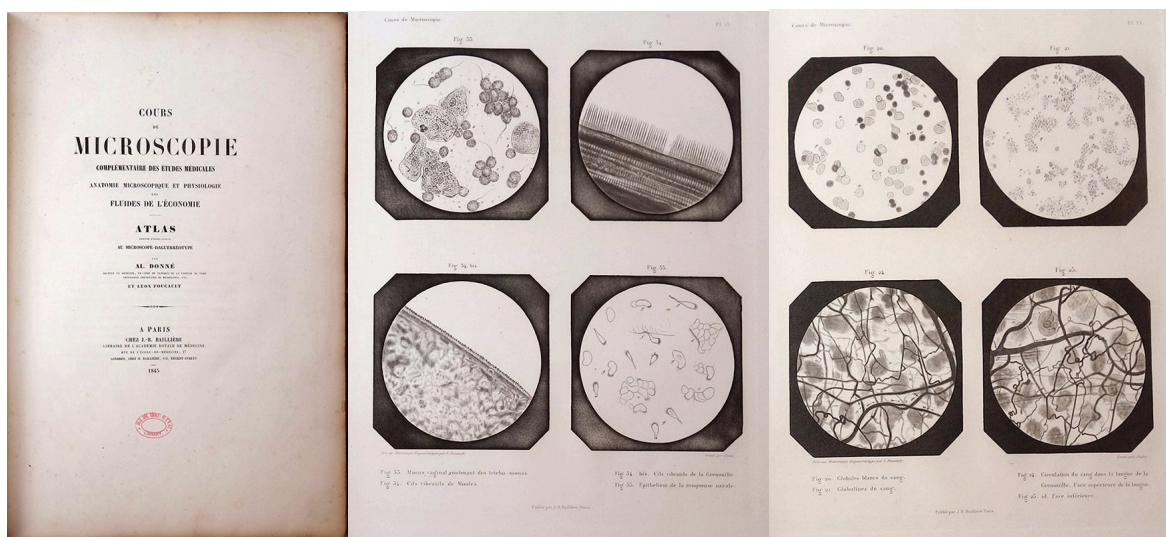


Figure 1: Cours de microscopie. Text and atlas (Donné 1844)

Later on, John Hughes Bennett, who was one of Donné's foreign students and a pathologist at the Royal Infirmary Edinburgh, published the first case reports of patients in 1845 (Bennett 1845). About six weeks after the first leukemia case was reported, Rudolf Virchow, a graduate of the Berlin Army Medical School, published the second one (Virchow 1845). The two separate pathologists reported similar observations such as splenomegaly, an enlarged liver, and leukocytosis symptoms. Bennett's statement was more comprehensive and scientific in a narrative, giving him the distinction of giving the first published recognition of leukemia. The symptoms he stated, alongside the comprehensive post-mortem investigation, would now be diagnosed as CML. The disease, on the other hand, was poorly understood. Virchow was the

first to coin the name "leukemia" (Leukämie), which means "white blood" to suggest that the symptoms were caused by abnormal hematopoiesis (Virchow 1847). In a similar sense, Bennett created the term "leucocythaemia" or "increased white cell blood" (Bennett 1845). In 1856, it was Virchow who was credited with concluding that the sickness was not due to an infectious action, but instead by the tissue that generated white blood cells. He also distinguished between two different types of chronic leukemia, the splenic and the lymphatic forms, which we nowadays refer to as leukemia and lymphoma, respectively (Virchow 1849). However, the precise definition of leukemia remained elusive. The first living patient with leukemia was diagnosed in 1846 by Dr. Henry Fuller, a physician at St George's Hospital in London, who observed a large proportion of abnormal granular colorless globules by microscopic examination of the blood (Fuller 1846). In 1850, he also documented the first case of pediatric leukemia, in a 9-year-old girl (Fuller 1850). On a tissue scale, Ernst Neumann, a professor of Pathological Anatomy at Königsberg, proposed a possible tissue of origin of the disease to be bone marrow and called it "myeloid leukemia" (Neumann 1878). How the cells managed to pass through the bone and reach the circulating blood system, however, remained a question.

Nikolaus Friedreich initially categorized malignancies in 1857. It was established that there are acute and chronic types of leukemia. He provided in-depth information on a case that he first introduced the term "acute leukemia" to describe (Friedreich 1857). Paul Ehrlich's invention of a triacid stain in 1877 made it easier to divide leukemia into the lymphoid (lymphocytes or non-granular cells) and myeloid (granulocytes) groups (Ehrlich 1877). His methods marked the beginning of the morphological revolution in hematology. Ehrlich also recognized the primitive cell as the ancestral cell in which subsequent cells within the various cell lineages originated. He defined it as a cell in a semi-transformed condition. This was possibly the earliest mention of the stem cell ideology and perhaps the first description of an ancestor cell in the hematopoietic series. Leukemia was categorized into four types only in 1913: chronic lymphocytic leukemia (CLL), CML, acute lymphocytic leukemia (ALL), and acute myelogenous leukemia (AML).

1.2 Philadelphia chromosome

A definite diagnosis became 82 years later in the city of Philadelphia, with the identification of recurrent cytogenetic abnormalities characterized by the presence of an abnormal chromosome, named Philadelphia chromosome (Ph1) (Nowell and Hungerford 1960). Chromosomal analysis research started in the 1940s, but it wasn't until the 1950s that the technique was implemented to address leukemias and neoplastic disorders. This chromosomal translocation is known to be strongly associated with CML and is found in CML in 90 to 95 percent of the cases (Kurzrock et al. 1988). In some CML patients, The Ph1 chromosome can be "masked" by complex rearrangements but molecular biology allows the diagnosis to be made. The Philadelphia chromosome had been called a "minute" chromosome. Later, the term was abbreviated to Ph1 to denote the first chromosome in what scientists anticipated would be a series of connections between persistent chromosomal abnormalities and cancers. The Philadelphia chromosome, however, remained the only chromosomal defect that was reliably linked to a particular neoplastic malignancy for more than 10 years.

At first, it was believed that the Philadelphia chromosome was only a deletion of chromosome 21 (Nowell and Hungerford 1960) and even thought to be chromosome Y due to the limitation of low chromosomal resolution from the cytogenetic technique at that time (Tough et al. 1961). When banding techniques were developed, it was shown that the Philadelphia chromosome occurred in fact in chromosome 22. Furthermore, it was only in 1973 that Janet Rowley of the University of Chicago demonstrated that the Philadelphia chromosome was not solely a deletion of the long arm of chromosome 22, instead it was a reciprocal translocation between chromosomes 9 and 22 (Rowley 1973). Chromosome 22 is fragmented right below the centromere, while chromosome 9 is fractured at its long arm's distal end. The little fragment of chromosome 9 gets translocated to chromosome 22, resulting in a much shorter chromosome 22. In contrast, chromosome 9 has been extended by removing a tiny section from its distal end and adding a larger segment.

A few years later, Nora Heisterkamp and John Groffen showed that the human homolog

of the Abelson murine leukemia viral oncogene (*v-Abl*), *ABL1*, was located in the region of chromosome 9, which translocates to chromosome 22 (Bartram et al., 1984; Heisterkamp et al., 1983). The translocation of the *ABL1* proto-oncogene was detected only in patients positive for the Ph1 chromosome (Bartram et al. 1983). Further, a small region of up to 5.8 kb on chromosome 22 was identified, within which all of the translocation breakpoints were occurring and which was named the “breakpoint cluster region” (BCR) (Groffen et al. 1984). BCR later became the denomination of the gene on chromosome 22 fusing with *ABL1*. The majority of CML cases are due to a BCR::*ABL1* breakpoint. This fusion gene encodes a chimeric BCR::*ABL1* protein; continuous activation of the BCR::*ABL1* would result in uncontrolled myeloid cell proliferation. This is the first neoplastic pathology to be defined by a direct link between a disease and a biological marker (**Figure 2**). Although it is uncommon, the Philadelphia Chromosome can also be found in AML and B-lymphoblastic leukemia (B-ALL) patients and is associated with a poor prognosis (Harrison 2001). The most logical explanation for these results is that a large number of these individuals may reflect the blast crisis phase of an undiagnosed CML. As one of the most well-established oncogenes, the encoded BCR::*ABL1* has been confirmed to exhibit an exacerbated tyrosine kinase activity and be the central driver in the leukemogenesis process of most CML cases (Zhao et al. 2002). Overall, it is a reciprocal translocation between chromosomes 9 and 22 [t(9;22)] that occurred in hematopoietic stem cells (HSCs) which are at the apex of the hematopoietic hierarchy (Rowley 1973; Heisterkamp et al. 1983; Bartram et al. 1983). The Philadelphia chromosomal translocation has now been identified as t(9;22) (q34;q11).

	1844	The first description by Alfred Donné
Two separate detailed reports of patients by Rudolf Virchow and John Bennett	1845	
	1845	The term "leukemia" which means "white blood" by Rudolf Virchow
The first live patient with CML diagnosis by Dr. Henry Fuller	1846	
	1878	Bone marrow as a possible tissue of origin of the disease by Ernst Neumann
The discovery of Philadelphia chromosome	1960	
	1973	The discovery of a reciprocal translocation between chromosomes 9 and 22 [t(9;22)]
Bone marrow as a possible tissue of origin of the disease by Ernst Neumann	1978	
	1983	The identification of ABL1 gene translocation from chromosome 9 to 22 by Nora Heisterkamp and Jim Groffen
The identification of the translocation breakpoints of BCR gene	1984	
	2002	BCR-ABL has been confirmed to be the central driver of most CML cases

Figure 2: Historical milestones in CML discovery

1.3 Epidemiology of CML

CML consists of about 15 percent of all leukemias affecting individuals essentially adults but occurring also albeit much rarely in children and infants (Jabbour and Kantarjian 2022). The annual incidence of CML has been reported to range from 0.7–1.0 per 100,000 people in several European nations, 1.0–1.4 in Thailand, to 1.75 in the United States (Jootar 2012; Höglund et al. 2015). According to the European Treatment and Outcome Study for CML (EUTOS) and the Studying First-Line Treatment of CML in a Real-world Setting (SIMPLICITY) registries, the

median age at diagnosis of CML is approximately 56 to 57 years in Western countries (Hehlmann et al., 2019; Hoffmann et al., 2015) and less than 50 years in developing countries (Jootar 2012; Malhotra et al. 2019). After 2000, the annual fatality rate decreased to around 1 to 2 percent (**Figure 3**, Explorer 2021; Jabbour and Kantarjian 2022). The American Cancer Society estimates that the recent 5-year relative survival rate of adult patients ages 20 and older with CML is 70 percent (Siegel et al. 2022).

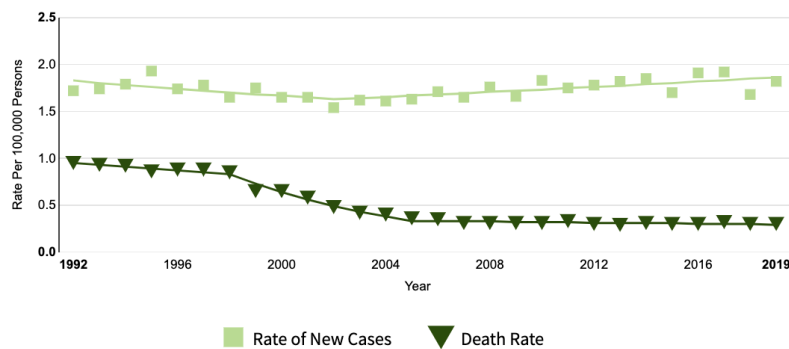


Figure 3: new cases and death rates per 100,000 Americans (Explorer 2021)

This survival rate is currently 3-times higher than in the mid-1970s (22 percent) because of the vast development of targeted therapies (**Figure 4**, Explorer 2021), which is discussed further in the devote chapter (chapter 2). Recent data from a highly documented Swedish cancer registry shows that the survival of CML patients responding to therapies is similar to that of healthy persons of the same age and sex (Bower et al. 2016)

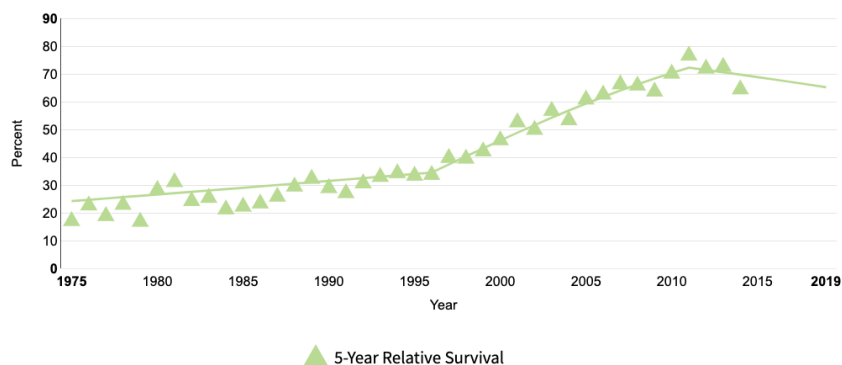


Figure 4: 5-Year Relative Survival Percent from 1975–2014 (Explorer 2021)

The etiology of CML remains largely unknown. The only known risk factor is ionizing radiation as exemplified by the increased incidence of CML in atomic bomb survivors (Heysselet al. 1960). There was also no significant family heritable aggregation of CML in research based on the Swedish Cancer Registry and Multigenerational Registry (Björkholm et al. 2013). In a few studies, it has also been suggested that smoking or exposure to benzene may cause CML, although further research is needed (Vlaanderen et al. 2012; Musselman et al. 2013). Yet, the prognosis of CML may be influenced by non-disease-related variables and the investigation of CML's etiology may be aided by an examination of its epidemiological aspects. For instance, by aging, several studies have found that elderly CML patients (>60 to 70 years) had a worse survival rate than younger ones (Brunner et al. 2013; Fisher et al. 2013). Males are more likely than females to develop CML, with male-to-female ratios ranging from 1.2 to 1.7 in various studies. These findings showed that the prevalence of CML was sex-related, and those female hormones may operate as a preventive factor in the development of CML. In younger age groups, however, the gender disparity in occurrence is less noticeable (Berger et al. 2005; Höglund et al. 2015). Additionally, the majority of the countries with the greatest prevalence of CML were coastal or island states. Particularly, Ethiopia has a much greater prevalence than other countries (**Figure 5**). Additional hints about the etiology of CML may emerge from a thorough analysis of the environmental conditions in these locations (Lin et al. 2020).

Fredriksen et al. found a 1.6-fold increased risk of developing a secondary malignancy in 1,026 patients with CML diagnosed between 1977 and 2008 (Frederiksen et al. 2011). According to research published in 2016, secondary malignancies and cardiovascular problems account for 50% of CML patients' deaths rather than the disease itself (Pfirrmann et al. 2016).

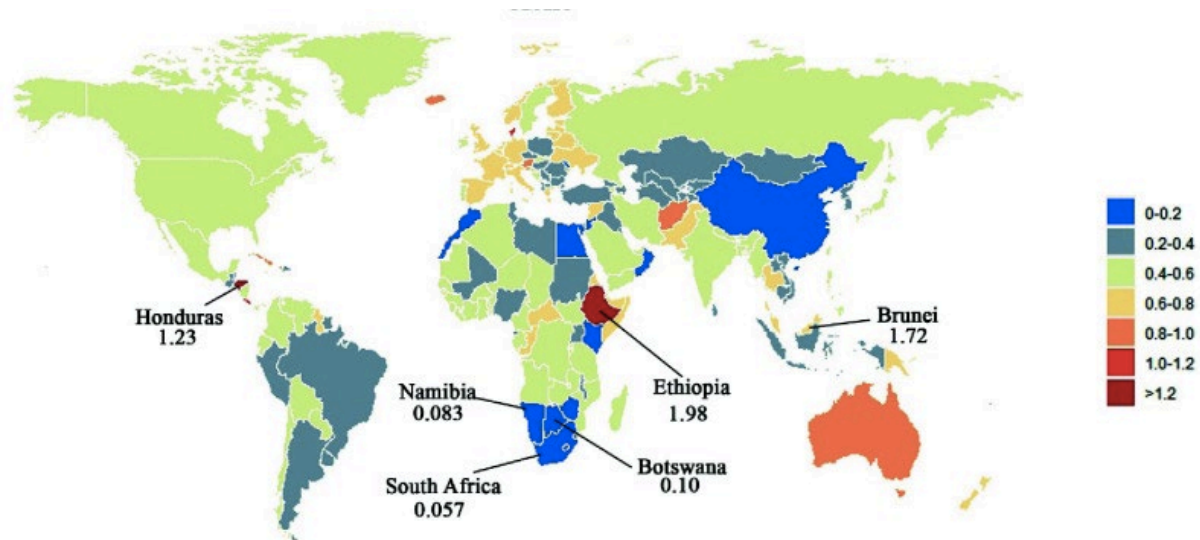


Figure 5: Age-standardized incidence rate in 2017. Heat gradient represents from red (highest) to blue (lowest) (Lin et al. 2020)

1.4 Characteristics of CML

In 1951, Dameshek originally identified CML as a myeloproliferative disorder based on evidence of various abnormalities in erythroblasts, granulocytes and megakaryocytes, while no fibroblasts in bone marrow (Dameshek 1951). Among all types of MPNs, CML is the most common type of myeloproliferative disorder and one of the most successful cancer medicine stories of the past century. We now know that the Philadelphia chromosome is the driver oncogene in CML and its occurrence on primitive HSCs. Three pieces of evidence all support this important connection. First, the 9;22 translocation and BCR::ABL1 transcript are present in all CML patients. Second, the breakpoint is different between patients but always the same in different progenitors of the same patients such as megakaryocytes, erythroid progenitors as well as myeloid progenitors (Turhan et al. 1990). Thirdly, clonality analyses using G6PD isoenzymes in female patients with CML have shown that leukemic cells always have the same A or B phenotype (never both). In contrast, somatic tissues such as fibroblasts express both isoenzymes (Fialkow et al. 1967).

From the clinical point of view, the natural history of CML includes three phases starting with an indolent chronic phase (CP) followed by a progression towards an accelerated phase

(AP) and a blast phase (BP) which is acute leukemia. Before the advent of TKI therapies, this natural history was only slightly modified by classical therapies with a median survival of 3-5 years. This natural history has been modified during the last 30 years, by the introduction of novel therapies such as Interferon-alpha (IFN- α), bone marrow transplantations (BMT) and TKIs (See review: Mughal et al. 2016).

The fact that the disease originates in HSCs as a clonal malignancy has been demonstrated by the pioneering work of Fialkow et al (Fialkow et al. 1967). Due to the persistence of leukemic stem cells (LSCs) even in patients with deep molecular responses (Chomel et al. 2011), BP probably originates also in LSCs but its origin in a committed progenitor which has returned to a primitive state via activation of β -catenin (Jamieson et al. 2004; Vetrie et al. 2020). Proliferation is controlled in chronic-phase CML, allowing the leukemic cells to mature normally and respond properly to normal regulators, such as granulocyte-colony-stimulating and macrophage-colony-stimulating factors (G-CSF and G/M-CSF) and to infection (Clarkson et al. 2003). However, in patients resistant to TKI therapies, CML-CP develops into uncontrolled proliferation, loss of differentiation, and lack of responsiveness to normal regulatory mechanisms generally after 5–6 years. This stage of CML, the frequency of which has now been considerably reduced (2 to 3 percent of patients in different series) is known as the acute phase or BP, contributing to a life-threatening incident, and typically features AML or, less often, ALL. In the intermediate of CML progression, an additional phase called AP appears to exist in certain people who are transitioning from chronic to blast periods. By definition, the difference between these late two phases is defined by the blast cell count in the peripheral blood as 5 to 20 percent in AP and >20 percent in BP (**Figure 6**, Losson et al. 2020). The stem cells in which BCR::ABL1 first occurs and causes chronic phase CML are termed the CML LSCs.

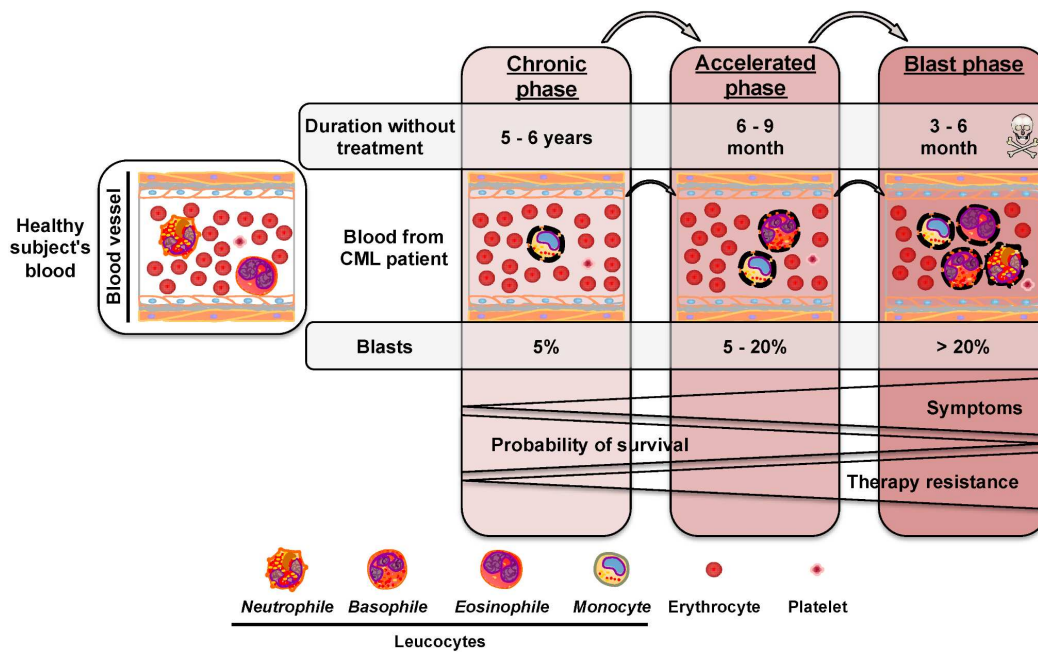


Figure 6: Progression of CML (Losson et al. 2020)

1.5 Hematopoiesis in CML

1.5.1 Background on normal hematopoiesis

The hematopoietic system is structured hierarchically, with HSCs at the top of the differentiation pyramid. According to the classical tree-like model of hematopoietic differentiation, HSCs give birth to offspring that advance through numerous differentiation phases, from lineage-biased yet multipotent progenitors towards lineage-specific committed progenitors and precursors, eventually forming all populations of mature blood cells. This model implies that each progenitor stage has a relatively uniform developmental and differentiation status, and that bifurcating lineage decisions occur either randomly or in response to regulatory cytokines. On another hand, the continuous differentiation model depicts the progressive acquisition of lineage-specific characteristics as well as the presence of transcriptional lineage bias at earlier stages than lineage commitment due to the heterogeneity within each population (**Figure 7**). This system is intended to renew continually, both to maintain steady-state homeostatic replenishment of blood cells and to rapidly boost production to compensate for acute blood loss in the circumstances of physical trauma, sickness, and metabolic or toxic stress.

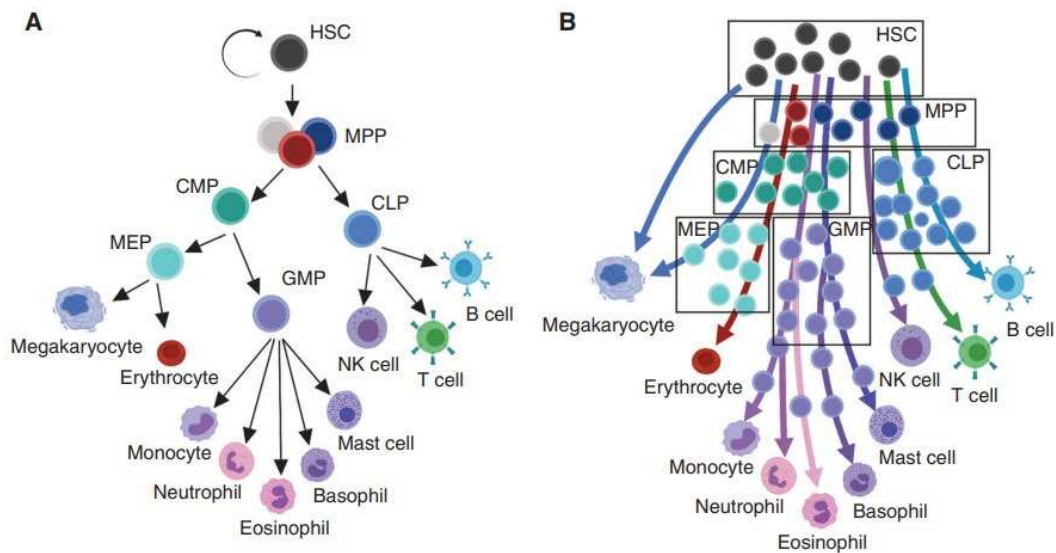


Figure 7: Models of HSC lineage commitment. **(A)** The classical tree-like model. **(B)** The continuous differentiation model (Olson et al. 2020)

HSCs cycle infrequently under homeostatic settings and are mostly in a dormant state known as quiescence (Wilson et al. 2008; Foudi et al. 2009). HSC quiescence acts as a safeguarding mechanism by minimizing replicative stress, which can lead to functional impairment in HSCs, particularly as they age (Flach et al. 2014). In contrast, when self-renewal is emphasized, as a consequence of somatic mutation or environmental stimuli, the HSC compartment becomes hyperplastic as a consequence of enhanced blood production maintenance. The hematopoietic system is then depleted, resulting in cytopenias and, eventually, bone marrow failure. This regulatory axis is similarly disturbed in the setting of malignancies such as MPNs or AML, but with heightened self-renewal and regenerative signaling pathways acting in concert to drive leukemic development (Olson et al. 2020).

1.5.2 Definition of HSC and hematopoietic progenitors

HSCs are distinguished by their multipotency, the capability to grow into all lineages of adult blood cells, and the capability to self-renew throughout the rest of an individual's life. The original

conceptual understanding of the bone marrow as a reservoir of self-renewing and multipotent HSCs comes from the first transplant to treat a patient with aplastic anemia back in 1939 (Osgood et al. 1939). In the 1960s, spleen colony-forming unit (CFU-S) experiments gave additional support to the idea of stem cells with variable myeloid and erythroid cell compositions and self-renewal potential (Till and McCulloch 1961; Becker et al. 1963). Later on, with the advancement of technologies, HSCs were successfully isolated from mouse bone marrow, and the capacity of a single blood-forming HSC to replenish all hematopoietic lineages was shown (Muller-Sieburg et al. 1986; Spangrude et al. 1988; Osawa et al. 1996).

The phenotypic identification of HSCs has advanced significantly, because of a current consensus on defining long-term engrafting (**Table 1**). This significant amount of research, published over the last century, has established our understanding of HSC self-renewal and differentiation capabilities via transplantation experiments and has laid the groundwork for future mechanistic research of HSC biology.

Next, the multipotent progenitors (MPPs), the primary downstream descendent of HSCs, generate all hematopoietic lineages but have lost the potential for substantial self-renewal in transplantation experiments, despite the ability to keep long-term blood production in native state (Busch et al. 2015). These lineage-biased MPPs give birth to more lineage-specific progenitors like common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs). CMPs then mature as megakaryocyte-erythroid progenitors (MEPs) and granulocyte-macrophage progenitors (GMPs), from which all adult myeloid cells are produced. Nevertheless, it has been widely recognized wherein each stem and progenitor population is heterogeneous, and that a large percentage of HSCs generate a lineage-biased instead of balanced output (Dykstra et al. 2007; Morita et al. 2010; Yamamoto et al. 2013).

Cell types	Phenotypic characterization	References
murine HSCs	Lin ⁻ /Sca-1 ⁺ /c-Kit ⁺ /Flk2 ⁻ /CD48 ⁻ /CD150 ⁺ /CD34 ⁻ BM cells	(Cabezas-Wallscheid et al. 2014)
human HSCs	Lin ⁻ /CD34 ⁺ /CD38 ⁻ /CD90 ⁺ /CD45RA ⁻ cord blood and BM cells	(Gentles et al. 2010)
MPP2 (megakaryocyte lineage bias)	Lin ⁻ /Sca-1 ⁺ /c-Kit ⁺ /Flk2 ⁻ /CD48 ⁺ /CD150 ⁺ /CD34 ⁺	(Cabezas-Wallscheid et al. 2014; Pietras et al. 2015)
MPP3 (granulocyte-macrophage lineage bias)	Lin ⁻ /Sca-1 ⁺ /c-Kit ⁺ /Flk2 ⁻ /CD48 ⁺ /CD150 ⁻ /CD34 ⁺	
MPP4 (lymphoid lineage bias)	Lin ⁻ /Sca-1 ⁺ /c-Kit ⁺ /Flk2 ⁺ /CD48 ⁺ /CD150 ⁻ /CD34 ⁺	

Table 1: The phenotypic characterization of HSCs and MPPs

1.5.3 Hematopoietic regulation

1.5.3.1 HSC niche

HSCs are strictly regulated by both intracellularly and extrinsic cues from bone marrow microenvironment known as the HSC niche to ensure their long term functional integrity. For instance, the hypoxic microenvironment regulates HSC quiescence, with stromal and endothelial niche cells supplying quiescence-enforcing and self-renewal stimulating cytokines (Schepers et al. 2015; Wei and Frenette 2018). The c-x-c motif chemokine ligand 12 (CXCL12) and the c-Kit receptor ligand stem cell factor (SCF) have emerged as one of the most critical elements for HSC retention in the niche (Ding et al. 2012).

Niche cells can also promote HSC activation, particularly by secreting pro-inflammatory cytokines, while endothelial cells have been demonstrated to identify infections and activate HSCs to promote emergency hematopoiesis (Boettcher et al. 2014). Moreover, hematopoietic function and the niche are so closely intertwined that a mutation in the niche compartment is capable of inducing leukemogenesis (Kode et al. 2014).

1.5.3.2 DNA Damage and HSC Functional Decline

HSCs are dormant in the G0 cell-cycle stage, which acts as the first protective barrier against genetic instability, metabolic stress, and functional decline. Multiple factors can cause DNA damage and genomic instability; reactive oxygen species (ROS) generated during mitochondrial respiration, replication errors, telomere retention caused by cell division, irradiation, ultraviolet, or exposure to genotoxic agents are examples of intrinsic and extrinsic causes (Rossi et al. 2007; Ito and Suda 2014; Biechonski et al. 2017).

In addition to tight regulation of HSC proliferation, modulation of ROS, a key oxidative stress molecule that causes nucleotide oxidation and DNA breaks, is crucial to preventing DNA damage buildup and HSC functional decline (Yahata et al. 2011). ROS generation in HSCs is controlled by a coordinated antioxidant response and the inhibition of aerobic metabolism (Ito and Suda 2014). Importantly, not only is ROS a mutagen by itself, but its buildup in consequence to replicative stress and unresolved DNA damage plays a key role in the differentiation and eradication of damaged HSCs (Yahata et al. 2011).

1.5.3.3 Metabolism and HSC Activation

Recent research has revealed processes by which HSC metabolic level influences proliferation, differentiation, and self-renewal potential. For example, sustaining a glycolytic metabolic state is essential for HSC quiescence, and animals missing HIF1 α or PDK2/4, which restrict pyruvate transit through the TCA cycle, exhibit higher mitochondrial respiration and ROS production, as well as a loss of HSC quiescence and self-renewal potential (Takubo et al. 2010, 2013). HSCs have higher quantities of mitochondria than other downstream progenitors, although they are often tiny and inactive (Norrdahl et al. 2011). A transition from a quiescent glycolytic state to a pro-proliferative/pro-differentiation state is crucial for HSC cell fate choices and for balancing HSC self-renewal with hematopoietic system regeneration.

1.5.4 Leukemic hematopoiesis in CML

1.5.4.1 CML stem cells

CML has long been noted to be a stem cell disease, but the exact characteristics of these leukemic stem cells have yet to be determined. *In vitro* experiments using long-term culture-initiating cell (LTC-IC) assays initially revealed the presence of malignant multipotent stem cells in CML patients (Chen et al. 1994). The majority of CML progenitors had a greater proliferative capacity than normal progenitors, implying that the majority of CML progenitors were actively cycling (Eaves et al. 1998).

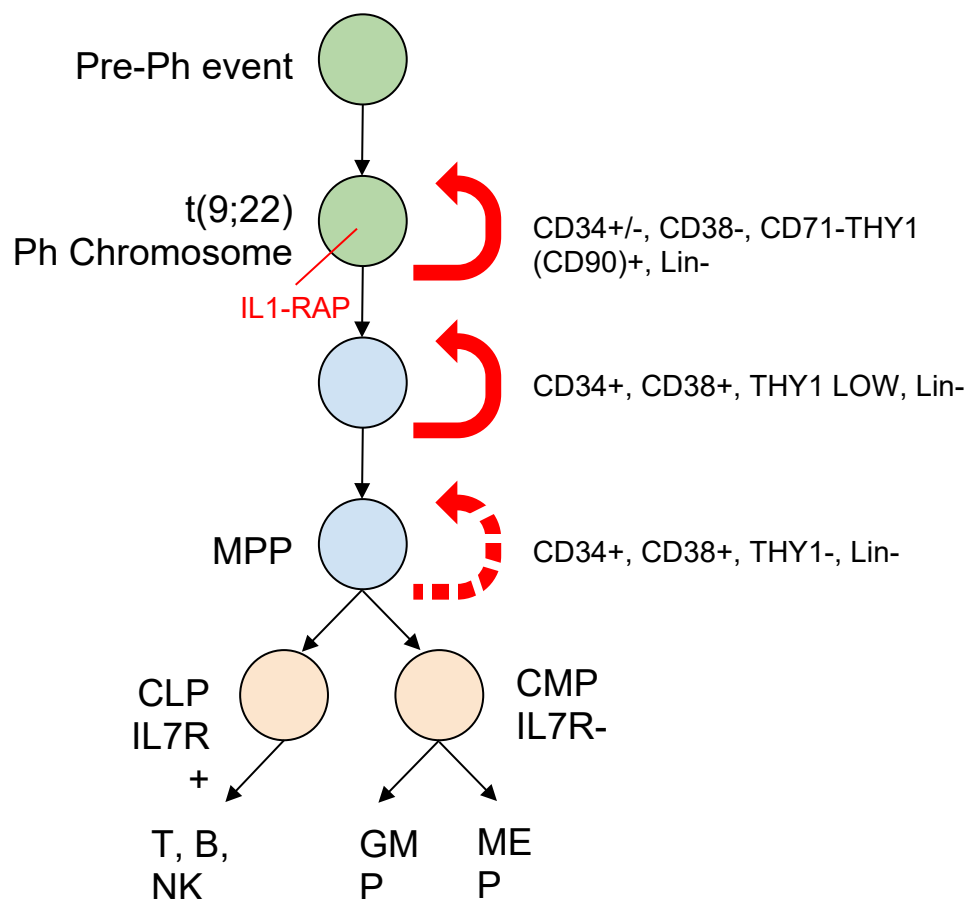


Figure 8: Cell surface characteristics of CML LSCs

As can be seen in **Figure 8**, LSCs from CML and normal HSCs both reside in the CD34+/CD38-/Lin- cell population, have similar self-renewal capability, and can both

differentiate into multiple cell lineages. However, *in vitro* and *in vivo*, there are several distinctions that have allowed them to be distinguished (Mayani et al. 2009). Indeed, different studies have found a number of surface markers preferentially expressed in one of the two populations and potentially able to allow the recognition of CML LSCs, though in some cases, these markers are merely expressed differently or asynchronously by both populations such as; Dipeptidyl peptidase IV (DPP4; CD26) (Herrmann et al. 2014), CD33 (Herrmann et al. 2012), CD36 (Landberg et al. 2018), and Interleukin 1 Receptor Accessory Protein (IL-1RAP) (Järås et al. 2010).

One major characteristic of the CML LSCs is their quiescent nature (Holyoake et al. 1999) and their resistance to TKI (Graham et al. 2002). The latter has been shown by isolating quiescent cells using CFSE assays followed by the analysis of their growth in the presence of high concentrations of Imatinib (Graham et al. 2002).

Studies have demonstrated that LSCs can self-renew in CML and other cancers, resulting in drug resistance and worse progression of the disease (Olsson et al. 2014). This genesis of the disease is widely recognized to be resistant to traditional chemotherapy and may also be somewhat resistant to BCR::ABL1-targeted therapies. Isolation and characterization of distinctions between LSCs and their normal counterparts, as well as a demonstration of total control of leukemia by targeting these cancer stem cells, are all part of the challenge. Recent progress in these fields has uncovered a number of unique target candidates that might pave the way for future therapeutic treatments focused on selectively eliminating LSCs while leaving healthy hematopoietic progenitors. As the features of LSCs are better understood, it will be feasible to properly evaluate existing therapy techniques and devise more effective CML medicines.

1.5.4.2 Deconstructing the molecular pathways leading to leukemia

Transgenic mice models, analogous to normal human epithelial and fibroblast cells, have been utilized to analyze *in vivo* the molecular pathways that lead to leukemic transformation. Cancer

develops in stages, with a growing number of somatic mutations giving birth to a progressively altered clonal population of cells (Hanahan and Weinberg 2011). This multistep model of carcinogenesis was initially proposed to need a clonal event that causes enhanced proliferation, which, when combined with mutations that prevent cellular differentiation, causes transformation. Proto-oncogenes that either inhibit or enhance programmed cell death, or apoptosis, have been discovered to play key roles in oncogenesis as well as hematopoiesis regulation.

Whilst genetic rearrangements and leukemic-associated fusion genes play an important role in determining the type of leukemia by interfering with hematopoietic differentiation programs, they require additional cooperating mutations to cause completely cancerous malignancies. Although the morphologies of leukemias vary, general processes underpinning leukemic transformation include enhanced cell survival, higher proliferation capability, improved self-renewal capacity, genomic instability, and differentiation prevention. Examples of unregulated processes and/or signaling pathways identified in various forms of leukemias are provided (**Figure 9**).

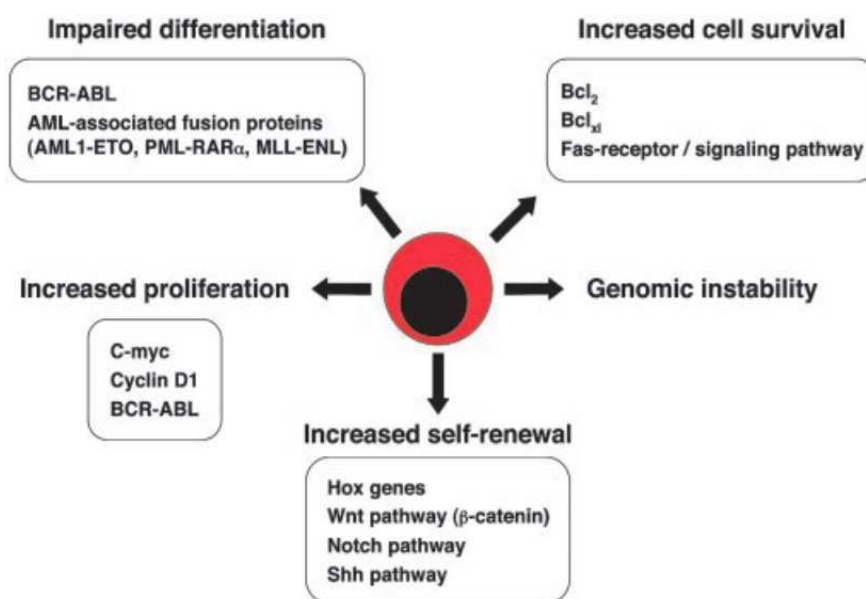


Figure 9: Deregulated pathways leading to leukemia (Passegué et al. 2003)

1.6 Molecular biology of BCR::ABL1

BCR::ABL1 is the hallmark of CML found in all cases of CML. Its presence, along with karyotyping to verify the presence of the Ph1 chromosome, confirms the diagnosis (Quintás-Cardama and Cortes 2006). The in-depth understanding of the CML diagnosis is discussed in detail in Chapter 2.

As described above, *the BCR::ABL1* fusion gene is generated by the juxtaposition of the 5' end of *BCR* gene on chromosome 22 with the 3' end of *ABL1* gene on chromosome 9. Ordinarily, the *ABL* gene encodes p145, a 145-kDa nuclear protein with non-receptor tyrosine kinase activity. It has been demonstrated that the p145 protein migrates between the nucleus and the cytoplasm (Van Etten et al. 1989). This movement has been associated with cellular growth regulation through its association with numerous growth factor receptors such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and colony-stimulating factor (CSF).

The *BCR* gene complex, on the contrary, is made up of at least four distinct genes known as *BCR1*, *BCR2*, *BCR3*, and *BCR4*. The 9;22 translocation mostly involves the *BCR1* gene. *BCR1* is made up of 20 exons with two splice sites and is around 100 kilobases long. A 160 kDa protein (p160), which is ordinarily produced by the gene, is less well understood but it is constitutively expressed in a variety of cell types but is most potent in hematopoietic cells.

Fusion normally occurs when a 140-kb region of the *ABL1* gene exons 1b and 2 with exons 13 and 14 of the *BCR* gene, resulting in messenger RNA (mRNA) transcripts with an e14 and/or an e13 junction, known as the major breakpoint region (M-BCR) in the most CML patients (Li et al. 1999). The second main fusion product is P190 BCR::ABL1, which is the smallest of the fusion proteins and is produced by the *BCR* gene's minor breakpoint region (m-BCR), which produces the transcript e1a2. P230 BCR::ABL1 comes third as a micro breakpoint cluster region (μ -BCR) originating between *BCR* exons 19 and 20 (**Figure 10**). Like so, these three breakpoint cluster portions of the *BCR* gene resulted in three fusion proteins P210, P190, and P230, respectively (Abdulmawjood et al. 2021).

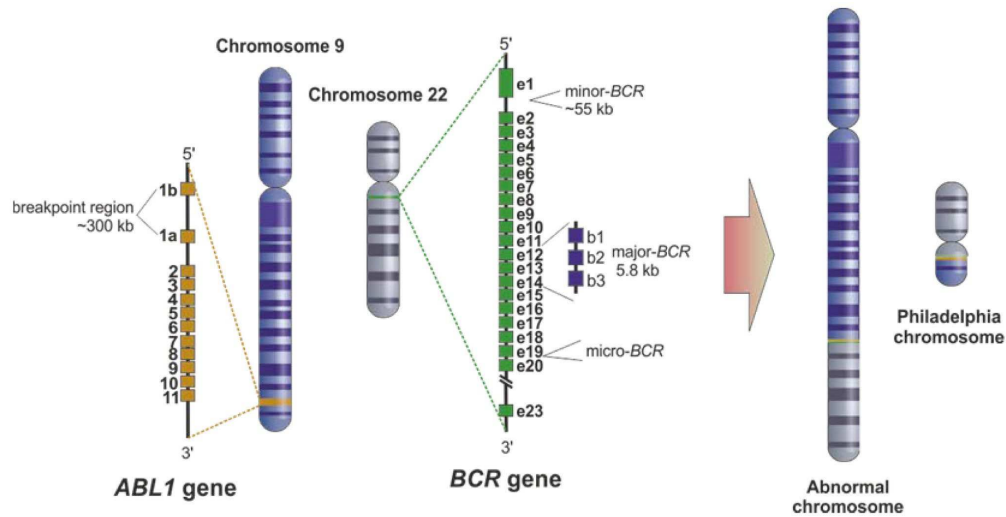


Figure 10: Ph1 and *BCR::ABL1* fusion gene isoforms (Abdulmawjood et al. 2021)

Structurally, the common ABL1 domains, including the SRC homology domains SH2 and SH3, the tyrosine kinase (Y-kinase) domain SH1, and DNA- and actin-binding domains (DBD and ABD), are found in all three BCR::ABL1 fusion proteins. On the other hand, the fusion proteins could comprise some or all of the following BCR domains depending on the BCR breakpoint locations: a dimerization coiled-coil domain (CCI), a Ser/Thr (S/T) kinase, a Rho guanine nucleotide exchange factor (Rho-GEF), a Dbl homology (DH) tandem, a Pleckstrin homology (PH) tandem, a calcium-dependent lipid-binding domain (Cal-B), and truncated RAC-GAP domain (Zhou et al. 2018). For instance, DH and PH tandem domains are present in P210 BCR::ABL1 and involved in the P210 BCR::ABL1 signaling networks, whereas they are absent in the P190 isoform (**Figure 11**, Li et al. 1999; Reckel et al. 2017).

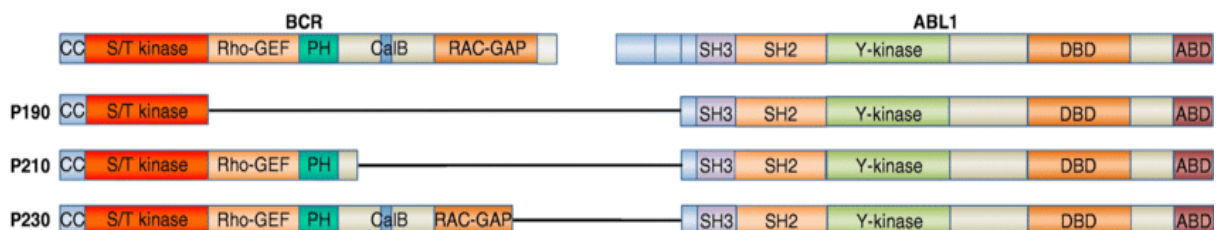


Figure 11: Structures and essential protein domains of various BCR::ABL1 fusion proteins

adapted from (Zhou et al. 2018)

Despite the fact that all three BCR::ABL1 fusion proteins share the same ABL1 domains and constitutively active protein kinase activity compared to wild-type ABL1, they each connect with distinct leukemia and have a very diverse signaling network. The 210 kDa chimeric protein-P210 isoform is the most common and a molecular hallmark of most CML patients, appearing in 98 percent of CML cases but only 20% of Ph1+ B-lymphoblastic leukemia/lymphoma cases (B-ALL). On the contrary, the P190 BCR::ABL1 gene is found in over 80% of Ph1+ B-ALL cases but rarely in CML cases around 1%. Nonetheless, when this isoform is present, it has been linked to an accelerated severity of the disease (Flis and Chojnacki 2019). Furthermore, P210 is thought to have an HSC origin, whereas P190 is thought to have a B cell progenitor origin lacking myeloid involvement (Castor et al. 2005). This could be due to P190's increased intrinsic tyrosine kinase activity, which leads to higher tyrosine phosphorylation of substrates required for lymphoid cell proliferation and transformation (Danial et al. 1995; Ilaria and Van Etten 1996; Reckel et al. 2017). Patients with P190 CML are older, have monocytosis, and have a higher frequency of BP when they first appear. In addition, P190 also has a stronger lymphoid leukemogenic activity than P210 (Lugo and Witte 1989; Kelliher et al. 1991; Voncken et al. 1995).

Because of the low frequency in other unusual transcripts beyond P210 and P190, the clinical significance of those usual is barely known. For example, the rare P230 has been described in the rare form of CML patients who commonly exhibit significant neutrophilic maturation or thrombocytosis without the presence of immature precursors found in peripheral blood (neutrophilic-CML; CML-N) (Pane et al. 1996).

1.7 Leukemogenic effects of BCR::ABL1

The proto-oncoprotein ABL protein is a non-receptor tyrosine kinase that is widely expressed. It typically shuttles between the nucleus and the cytoplasm, but in normal cells, it is mostly found in the nucleus. However, when fused to BCR protein, it mainly localizes to the cytoplasm, in which it can interact with a number of proteins involved in the leukemogenic pathway. The first

exon of BCR encodes an oligomerization domain that increases ABL1's tyrosine kinase activity, promoting dimerization or tetramerization and subsequent autophosphorylation. As a result, BCR::ABL1 can recruit and activate several downstream signal transducers by increasing the number of phosphotyrosine residues as well as the number of binding sites for SH2 domains on other proteins (Pendergast et al. 1991, 1993).

Interactions of both the BCR::ABL1 fusion protein and other cytoplasmic molecules disrupt a wide range of pathways, including some of the most clinically important and well-studied. Three major signaling pathways are tightly regulated by BCR::ABL1 constitutive activations: (1) the Ras–mitogen-activated protein kinase (MAPK) signaling which leads to BCR::ABL1-dependent cellular transformation (Sawyers et al. 1995); (2) the phosphoinositide 3-kinase (PI3K)/AKT mediated signaling (Varticovski et al. 1991; Jain et al. 1996) which promotes cell survival (Skorski et al. 1995) as well as anti-apoptotic activity (Franke et al. 1997); and (3) the signal transducer and activator of transcription (JAK/STAT) pathway (Shuai et al. 1996) which leads to the phosphorylation of STATs and in turn regulates the expression of various pro-survival and pro-proliferative genes (Melo and Deininger 2004). These three cell signaling pathways are at the forefront of BCR::ABL1 mediated pathogenesis and will be discussed further below.

1.7.1 Ras–mitogen-activated protein kinase (MAPK) signaling

The Ras is a small GTP-binding protein and is an important element in the signal transduction of the Ras-MAPK signaling pathway by regulating the activity of Raf which in turn regulates MEK1/2 and then ERK1/2. This pathway is involved in cell proliferation and survival and is activated in the tumorigenesis of numerous cancers (Steelman et al. 2011). In CML, BCR::ABL1 constitutively activates Ras protein via Grb2/Gab2 phosphorylation which allows the functional activation of MEK/ERK pathways leading to the transcription of target genes involved in hematopoietic cell growth and drug resistance while a decrease in the expression of the apoptosis genes (Puil et al. 1994). Disruption of the Ras pathway by a dominant negative mutant

of Ras reduces the phenotype of CML in mice models (Baum and Ren 2008). In combination with an anti-leukemia chemotherapeutic drug (Nilotinib) in mice (Packer et al. 2011), pharmacological inhibition of MEK activity by PD0329501 increased drug-resistant CML cell death, and cancer development regression by locking both MEK1 and MEK2 into a closed, catalytically inactive conformation (Sebolt-Leopold and English 2006).

1.7.2 Phosphoinositide 3-kinase (PI3K)/AKT mediated signaling

Generally, The PI3K pathway communicates extracellular signals to regulate transcription factor activation, enabling cell growth and survival while suppressing cell death in human cancers (Zhao et al. 2006). Meanwhile, AKT is a downstream mediator of PI3K and has a number of targets involved in protein synthesis, cell proliferation, and autophagy. It's also a proto-oncogene accompanied by downstream targets, one of which is mTOR (Sheng et al. 2011). One of the first studies in this field suggested that the transformation of hematopoietic cells by BCR::ABL1 required PI3K/AKT-dependent pathway (Skorski et al. 1997). Following that, a study revealed that PI3K/AKT is critical for CML maintenance and that disrupting this signaling can stop BCR::ABL1 leukemogenesis and kill primary CML cells (Klejman et al. 2002).

Moreover, this pathway is also related to autophagy which occurs in response to cell stress (i.e., a decrease of BCR::ABL1 signaling) in order to put the cell into quiescence instead of apoptosis, and it can be restored when the environment improves. Recent research has found that while BCR::ABL1 inhibits autophagy via mTOR, TKI treatment restores it, potentially blocking leukemia cells to be protected and therapeutic resistance (Sheng et al. 2011). Therefore, when autophagy and BCR::ABL1 are inhibited together, the elimination of primitive CML cells is significantly improved compared to the BCR::ABL1-targeted drug alone. As a result, this strategy looks to be a promising way to counteract unwanted autophagy suppression (Salomoni and Calabretta 2009).

1.7.3 Signal Transducer and Activator of Transcription (JAK/STAT) pathway

STAT proteins are a family of cytoplasmic transcription factors that are activated when Janus kinases (JAKs) or Src family kinases (SFKs) phosphorylate them in response to cytokine signaling, including IL-2, IL-3, IL-4, IL-7, and IL-15; G-CSF and GM-CSF; erythropoietin and thrombopoietin; growth hormone; and prolactin (Hennighausen and Robinson 2008). There are seven mammalian STAT proteins known so far: STAT1 through STAT4 as well as STAT5A, STAT5B, and STAT6 (Darnell Jr et al. 1994). They have a role in a variety of cellular activities, including proliferation, survival, apoptosis, and differentiation (Benekli et al. 2003). In hematopoietic cells, STAT1, STAT3, and STAT5 are detected as transcription factors (de Groot et al. 2000) and play a key role in hematopoietic cell proliferation, differentiation, cell cycle and apoptosis (Chai et al. 1997). In leukemia, signaling from JAK/STAT pathway is frequently increased (Lin et al. 2000). It was also discovered that JAK2, STAT1, STAT3, and STAT5 are constitutively active in CML cell lines (Chai et al. 1997). Constitutive or unregulated activation of STATs would result in cellular transformation, including leukemogenesis (Garcia and Jove 1998; Bowman et al. 2000).

JAK/STAT signaling is another important BCR::ABL1-downstream signaling pathway that governs CML growth, survival, and treatment resistance (Bousoik and Montazeri Aliabadi 2018; Amarante-Mendes et al. 2022). BCR::ABL1 kinase activity directly stimulates JAK2/STAT activation in cellular models of CML, promoting proliferation by triggering the transcription of target gene codings such as cyclin D1 or c-Myc and cell survival by triggering the overexpression of anti-apoptotic proteins like MCL-1, BCL-x1, and BCL-2 (Chai et al. 1997; Warsch et al. 2013).

STAT5 is constitutively active in CML cell lines and in primary CML cells (Chai et al. 1997; de Groot et al. 2000). In *Stat5* knockout mice, BCR::ABL1 expression is not sufficient to induce leukemogenesis indicating a key role of STAT5 in the pathogenesis of CML (Hantschel et al. 2012; Walz et al. 2012). For STAT3, its activation causes TKI resistance in both TKI-resistant leukemic stem and progenitor cells in CML (Eiring et al. 2014). JAK1/STAT3 signaling

that is induced by external factors has also been found to mediate stem cell persistence in CML (Kuepper et al. 2019). Single-cell mass cytometry revealed a significant increase of phosphorylated STAT3 in nilotinib-treated CML patients. This discovery may have prognostic implications (Gullaksen et al. 2017).

Since the JAK/STAT pathway has been shown to play a vital role in myeloid differentiation, various treatment approaches have been developed to target this mechanism. Interestingly, it has been demonstrated that inhibiting JAK2 or STAT5 causes apoptosis in BCR::ABL1-positive cell lines and primary cells derived from either imatinib-sensitive or -resistant CML patients (Hoelbl et al. 2010; Gallipoli et al. 2014; Samanta et al. 2009). In mouse xenografts, TKI plus Ruxolitinib (a JAK2 inhibitor) combination treatment has been shown to decrease the number of leukemic stem cells (Gallipoli et al. 2014). Furthermore, according to Yagi et al., the combination of Tofacitinib (a JAK1 inhibitor) and Imatinib together caused anti-tumor effects in CML cells (Yagi et al. 2018).

1.7.4 Other signaling pathways

The WNT/ β -catenin signaling system is required for normal cell self-renewal, but its disruption has also been linked to cancer development including leukemia (Eiring et al. 2015; Fetisov et al. 2018). Direct binding and phosphorylation of β -catenin by BCR::ABL1 leads to its stability and nuclear translocation/accumulation (Coluccia et al. 2007; Luu et al. 2004), which has been linked to the growth and survival of leukemic stem cells in CML patients (Hu et al. 2009). Moreover, CML stem and progenitor cells in BP had overexpression of β -catenin. Pharmacological and genetic suppression of β -catenin reduced the ability of those cells to self-renew (Zhou et al. 2017a). Importantly, it has been demonstrated that blocking β -catenin via a specific inhibitor, AV65, causes apoptosis in CML cells, even those with the BCR::ABL1 mutation (Nagao et al. 2011).

Protein phosphatase 2A (PP2A) is a tumor suppressor and serine-threonine phosphatase that negatively controls mitogenic and survival signals emitted by the PI3K/AKT

and RAS/MAPK pathways (Janssens and Goris 2001). It regulates β -catenin, as well as apoptosis and the maintenance of G1/S cyclin levels throughout cell cycle progression (Ciccone et al. 2015). PP2A is downregulated by BCR::ABL1 in CML patients, especially during BP (Neviani et al. 2005). Notably, imatinib or PP2A-activating drug treatments result in BCR::ABL1 inactivation and degradation, and hence loss of tumorigenic activity in BCR::ABL1-positive cells and TKI-resistant quiescent CML stem cells, but not normal quiescent HSCs (Neviani et al. 2013).

Furthermore, MYC is also activated by BCR::ABL1, which is linked to drug resistance, abnormal DNA synthesis, and genomic instability (Albajar et al. 2011). The level of MYC in CML at the time of diagnosis may be a good predictor of treatment efficacy and advancement in BP (Albajar et al. 2011). BCR::ABL1 reportedly targets p27, a nuclear cell cycle inhibitor, at numerous levels, which promotes cell-cycle progress. BCR::ABL1 activity not only suppresses or enhances the degradation of p27 (Jonuleit et al. 2000) but also increases p27 protein localization, which has been shown to promote oncogenesis (Agarwal et al. 2014). Conversely, BCR::ABL1 is a negative regulator of ABL1 with a pro-apoptotic activity (Dasgupta et al. 2016). The BCR::ABL1 oncoprotein is widely attributed to genomic instability, making CML cells more vulnerable to subsequent genomic abnormalities, which is a primary driver of cancer development. Altogether, BCR::ABL1 is a multipurpose signaling protein that disrupts a variety of physiological activities (**Figure 12**).

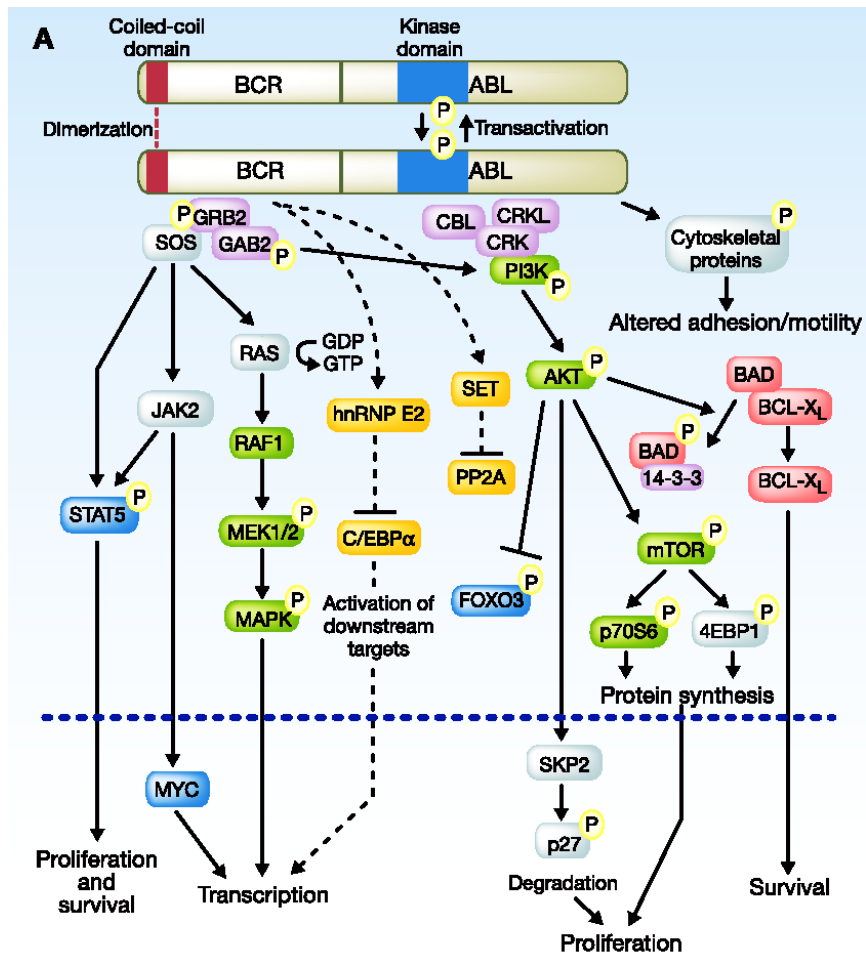


Figure 12: BCR::ABL1 downstream signaling pathways *adapted from* (O'hare et al. 2010)

CHAPTER 2: CML treatment

2.1 Detection and diagnosis

As described previously, CML is a triphasic disease. Most (90 to 95 percent) of CML patients are diagnosed during an early stage known as CP. About 50 percent of individuals are diagnosed incidentally while asymptomatic (Jabbour and Kantarjian 2022). In this case, the disease is found through routine physical examinations or blood testing for another reason. When symptoms present, clinical characteristics are typically non-specific. Fatigue, weight loss, and night sweats are common constitutional symptoms in CML patients. Hemorrhage brought on by platelet dysfunction and signs of anemia could also happen. Left upper quadrant abdominal pain and fullness can result in splenomegaly, which is frequently presented in 46 to 76 percent (Savage et al. 1997). It is also rather possible to have hepatomegaly (10 to 20 percent), which like splenomegaly, indicates extramedullary hematopoiesis. Mature granulocytes are still formed during this stage, however, there is an expanded pool of myeloid progenitor cells in the peripheral blood (PB).

With CML phase transformation, symptoms include fever, arthralgias, pain from splenic infarction, and headaches are increasingly frequent. The majority of individuals progress into AP before developing BP, although 20 to 25 percent of patients develop BP without AP warning signs. AP can be subtle or manifest as worsening anemia, splenomegaly and organ infiltration. BP manifests morphologically as acute leukemia with bleeding, fever, infections and worsening constitutional symptoms. At the cellular level, myeloid and/or lymphoid differentiation stops and immature blasts accumulate in the bone marrow spreading then to tissues and organs (Jabbour and Kantarjian 2022).

The diagnosis of typical CML is straightforward and consists of conventional cytogenetics demonstrating (1) the presence of the Ph1 abnormality, the t(9;22)(q34;q11), in the context of persistent unexplained leukocytosis (or rarely thrombocytosis) or (2) or the Ph-related molecular BCR::ABL1 alterations using fluorescence in situ hybridization (FISH) or molecular analyses like Reverse transcription polymerase chain reaction (RT-PCR) (Kantarjian

et al. 2008). This research methodology topic has been adequately documented, as detailed by a review (See further; Jabbour and Kantarjian 2022). While 85 percent of individuals have a conventional t(9;22)(q34;q11), a small percentage of patients have variant translocations, which can be simple (involving chromosome 22 and another chromosome distinct from chromosome 9) or complicated (involving one or more chromosomes in addition to chromosomes 9 and 22) (Chen et al. 1993).

2.2 Treatment options for CML in the pre-TKI era

In brief, common chemotherapies for cancer like Busulfan, Hydroxyurea, Cyclophosphamide and Vincristine were once used as the first line of treatment for CML in previous decades. Allogeneic hematopoietic stem cell transplantation (allo-HSCT) was then used as the second line of treatment for CML. For a variety of hematological cancers, this is thought to be a potentially curative therapy to restore hematopoiesis (Gyurkocza et al. 2010). However, allo-HSCT is constrained by donor availability, transplant-related mortality and early morbidity, hence it is now regarded as a third-line treatment for the majority of pediatric cases. For these reasons, BCR::ABL1 inhibitors could therefore be considered as a focused therapy for CML, eliminating all the side effects of conventional chemotherapy and graft-versus-host disease, one of the main drawbacks of allo-HSCT.

CML patients now have a variety of therapeutic choices. Most CML patients can expect to live a normal life and to be functionally cured if they keep going with TKI-based treatment, are cooperative with treatment, and are carefully monitored for indications of resistance (see below).

2.2.1 Conventional therapies

From 1865 through the start of the 20th century, arsenical preparations were used as the first method of treatment for leukemia. The earliest published account on the use of arsenicals is attributed to Lissauer, albeit comprehensive documentation of his work is lacking. A 32-year-old

lady who Lissauer treated using Fowler's solution, an alkaline concoction of arsenic trioxide and tincture of lavender, and who was likely suffering from leukemia, healed well enough to leave the hospital but later relapsed in the absence despite continuous therapy (Lissauer 1865; Manley 2022). It's also noteworthy to mention that Arthur Conan Doyle, more known for chronicling Sherlock Holmes' adventures, wrote about the use of arsenic as a leucocythemia (leukemia) treatment in *The Lancet* in 1882 (Doyle 1882). Then, for almost 40 years, arsenic trioxide was used as a leukemia treatment until being gradually replaced, initially by splenic X-ray irradiation in 1902. It remained the normative treatment for more than 50 years.

The first successful therapy for CML utilized splenic x-rays and traditional chemotherapeutic medicines, primarily Busulfan and Hydroxyurea. This medication helped to reduce myeloid cell development and considerably improved the quality of life during CP. However, it did not prevent or slow the progression to AP and BP and had a limited effect on overall survival. The second therapeutic breakthrough was the introduction of IFN- α treatment. Beginning in the early 1980s, IFN- α was the first medication to increase hematological remission with a partial or full cytogenetic response (Talpaz et al. 1986; Yoffe et al. 1987). It also resulted in a better long-term prognosis (Kantarjian et al. 2003). It demonstrated efficacy in a large number of patients and was more successful in low-risk diseases (Talpaz et al. 1991). However, the best therapeutic window for utilizing IFN- α is uncertain and it has multiple undesirable side effects (Talpaz et al. 2015). These include "flu-like" signs and symptoms such as aches and pains in the muscles and bones, fever, headaches, tiredness, nausea, and vomiting. Patients using this medication may have difficulty focusing and thinking clearly. Additionally, interferon can reduce blood cell numbers. Although side effects persist as long as the medication is taken, they may get easier to deal with time and usually disappear when the treatment stops. However, some patients find it difficult to manage these adverse effects on a daily basis and may have to cease their therapy as a result. The discovery of a class of small molecules that target the tyrosine kinases, especially BCR::ABL1, at the end of the 20th century, or about 30 years ago, quickly replaced all of these treatments.

2.2.2 Stem cell transplants

Allo-HSCT has long been regarded as the only therapy capable of curing CML (van Rhee et al. 1997). This assumption was supported by data showing that allo-HSCT can provide long-term independence from cytogenetic or hematologic illness recurrence without the requirement for maintenance treatment. Allo-HSCT transplantation was proven to be effective at eradicating Ph1+ cells, first when the donor HSC came from an identical twin (Fefer et al. 1979) and then from HLA-matched siblings or matched donors (Goldman et al. 1982; McGlave et al. 1982).

Nevertheless, allo-HSCT is largely limited by the availability of donors, transplant-related mortality, early morbidity, and the operation cost so it is considered a third-line treatment. Therefore, the aforementioned IFN- α was made available to patients who were ineligible for allo-HSCT (Talpaz et al. 1983). The proportion of CML patients receiving allo-HSCT in the CP has declined dramatically since the discovery of TKIs.

The necessitate of allo-HSCT practice begins to rise again since around 2 percent of patients develop resistance to several TKIs annually. TKI resistance confers a poor prognosis and will be discussed in detail in subsequent sections. When patients advanced into AP or BP after the treatment of at least two TKIs, allo-HSCT becomes more significant (Jabbour et al. 2011).

2.3 Targeted therapies

2.3.1 Imatinib, the first-generation TKI

The importance of BCR::ABL1 kinase activity in malignant transformation offered the rationale for therapeutic targeting. Ciba-Geigy (now Novartis) scientists created a range of chemicals with tyrosine kinase inhibition activity. Using this library, screening procedures found lead compounds with action by inhibiting phosphorylation of proteins involved in cell signal, c-KIT tyrosine kinase and Platelet-Derived Growth Factor Receptor through competitive inhibition at the ATP-binding site of the BCR::ABL1 oncoprotein (**Figure 13**, Druker et al. 1996). Hereby, Imatinib (STI-571; Gleevec) was developed in 1992 as a consequence of optimization to

improve oral bioavailability. Imatinib was also the first clinically utilized signal transduction inhibitor (STI). It works by interacting with the BCR::ABL1 kinase domain in order to inhibit the transfer of a phosphate group to tyrosine on the protein substrate, resulting in the alteration of the activity of several genes responsible for the regulation of the cell cycle, cell adhesion, cytoskeleton structure, and, eventually, apoptotic death of Ph1+ cells (Deininger 2008). Imatinib generics have recently become available after the expiration of Gleevec's patent and were reported on the comparable efficacy and safety compared with Gleevec (Danthala et al. 2017; Abou Dalle et al. 2019).

By blocking the transmission of proliferative signals to the nucleus, cells altered by BCR::ABL1 exhibited a dose-dependent reduction of proliferation and induction of apoptosis. These findings were verified in primary CML and Ph1+ ALL cells, as well as committed progenitors from CML patients (Druker et al. 1996; Schindler et al. 2000). At doses up to 1 μ M, Imatinib had minimal effect on normal hematopoietic colony formation. The effect of Imatinib treatment was further confirmed *in vivo* using BCR::ABL1 xenografts murine models in which leukemia progression was suppressed (le Coutre et al. 1999). These preclinical works were instrumental to set the stage for clinical trials in BCR::ABL1-positive leukemia patients.

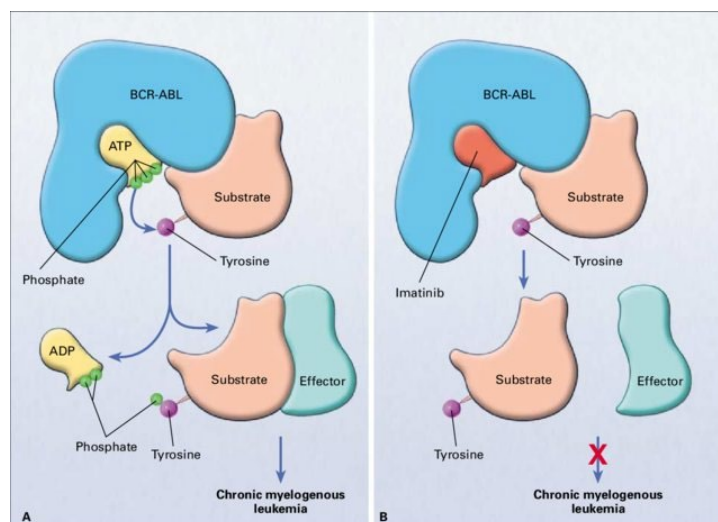


Figure 13: Mechanism of BCR::ABL1 activity and Imatinib-induced inhibition

(Savage and Antman 2002)

Imatinib phase 1 clinical trial began in 1998 in individuals with CML who were resistant or intolerant to IFN- α (Druker et al. 2001). In December 2002, Imatinib mesylate was the first TKI to be approved by the Food and Drug Administration (FDA) as first-line therapy for the treatment of CML patients in the CP by obtaining better results compared to those who had been treated with hydroxyurea and IFN- α . The International Randomized Study of Interferon and STI571 (IRIS) study is regarded as a key clinical trial for TKIs with CML by comparing Imatinib 400mg daily *versus* a combination of IFN- α and cytarabine. This investigation studied 1,106 CML patients in the CP and found Imatinib to be vastly superior in all major endpoints (Hughes et al. 2003; O'Brien et al. 2003).

Currently oral TKI therapies represent the first line therapy in all for CML patients. The frontline treatment could be Imatinib mesylate at 400 mg/day or second generation therapies such as Nilotinib or Dasatinib. Concerning Imatinib, at the 10-year follow-up, only 48 percent of participants in the IRIS study were still on medication. According to the current European Leukemia Net (ELN) standards, roughly 15 to 20 percent of CML patients do not obtain an optimal response to this drug (Stagno et al. 2008; Baccarani et al. 2013; Hochhaus et al. 2017).

The appearance of resistance or intolerance phenomena of some patients to Imatinib principally showed the need to develop the second generation of TKIs. A patient is considered Imatinib resistant if they have increased white blood cell or platelet counts, a poor or failed cytogenetic response, progression to more severe phases, or having more than 30 percent in Ph1+ cells in peripheral blood or bone marrow. The molecular mechanisms leading to Imatinib resistance will be discussed in Chapters 2.8.1 and 2.8.2. Intolerance is a result of Imatinib side effects such as a rise in blood bilirubin, headache, rash, vomiting, cardiotoxicity, neutropenia, obesity, and abdominal pain (Chen et al. 2008; Cavalluzzi et al. 2020). The 2nd generation of TKI includes Dasatinib (Shah et al. 2004), Nilotinib (Weisberg et al. 2005) and Bosutinib (Golas et al. 2003).

2.3.2 Second-generation TKIs

2.3.2.1 Dasatinib

Dasatinib (earlier BMS-354825) was originally mentioned in the literature in 2004 (Lombardo et al. 2004). 2 years afterward, Dasatinib is the first second-generation TKI approved by FDA in 2006. *In vitro* studies showed 325 times more efficient Dasatinib inhibiting activity than Imatinib against cells expressing wild-type BCR::ABL1 and Imatinib-resistant mutants, with the exception of T315I (isoleucine replaces threonine in position 315 of BCR::ABL1) (**Figure 14**, Lombardo et al. 2004; O'Hare et al. 2005; Tokarski et al. 2006). Based on X-ray analysis, it was observed that Dasatinib binds the ABL protein in a less strict conformation compared to Imatinib and also targets src family kinases.

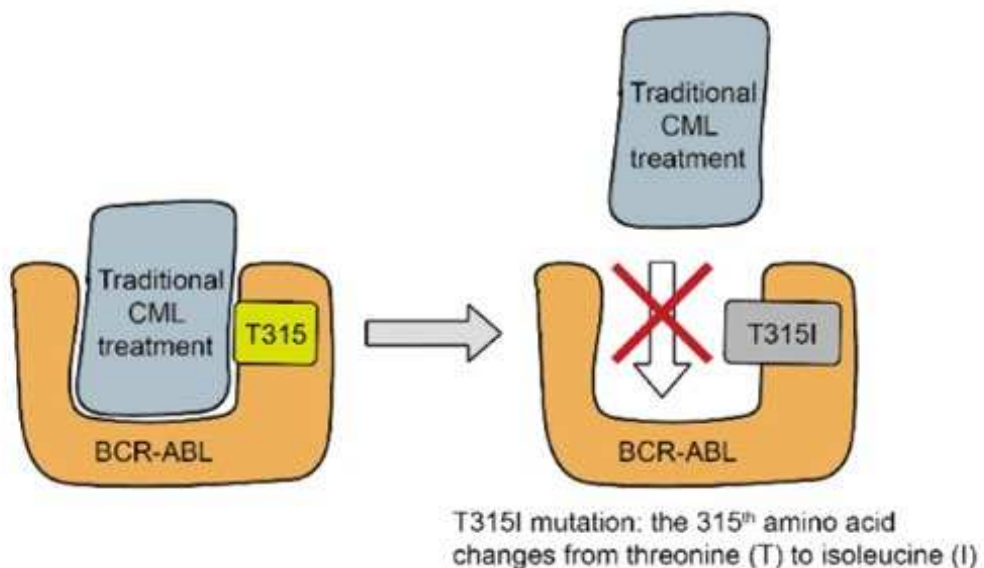


Figure 14: A T315I mutation in the BCR::ABL1 protein where traditional kinase inhibitors such as imatinib, dasatinib, or nilotinib cannot bind to its ATP-binding pocket (Thurston and Pysz 2021)

Dasatinib is active at lower dosages (100 mg daily) with a superior safety profile in early clinical studies (Talpaz et al. 2006; Shah et al. 2016). Patients treated with Dasatinib had fewer transformations to AP or BP than those treated with Imatinib (4.6 *versus* 7.3 percent). Dasatinib

and imatinib, on the other hand, had equal 5-year survival rates (91 and 90 percent) (Cortes et al. 2014).

Dasatinib's most severe side effects are hematological toxicity (including neutropenia and thrombocytopenia), liver problems, diarrhea, headache, peripheral edema, and hypocalcemia. Furthermore, pleural effusion is a clinical problem that may necessitate the use of diuretics as well as thoracentesis or pleurodesis (Hartmann et al. 2009).

2.3.2.2 Nilotinib

Nilotinib is another second-generation TKI and is structurally similar to Imatinib. *In vitro*, its specificity for the ATP binding site of BCR::ABL1 is 30-50 times greater (Weisberg et al. 2005). Nilotinib, like Dasatinib, was first shown to trigger hematologic and cytogenetic responses in patients who had failed Imatinib. It can also overcome some BCR::ABL1 mutations (L248V, G250E, Q252H) apart from T315I (Redaelli et al. 2009; Bose et al. 2013). Nonetheless, unlike Dasatinib, it has no effect on src family kinases at recommended doses. A major complete cytogenetic remission (no cells in the bone marrow have the Philadelphia chromosome) was observed and a reduction in the percentage of patients who proceeded to the AP of CML after an annual treatment with Nilotinib in comparison with Imatinib (Saglio et al. 2010).

While Nilotinib medication was generally well tolerated, there was an elevated risk of accumulating vascular events while on treatment. So, Nilotinib treatment is not appropriate for those who have vascular risk factors or metabolic dysfunctions. Other prominent common side effects are headache and skin rashes (20 to 30 percent mild to severe; eased by dosage decrease), self-limited increases of indirect bilirubin (10 percent), blood sugar spikes (10 to 20 percent), and pancreatitis (rarely at 1 to 2 percent).

2.3.2.3 Bosutinib

Bosutinib was approved by the US FDA and the European Medicines Agency in 2012 and 2013, respectively, for the treatment of adult CML patients who had developed resistance or

intolerance to prior therapy. In 2018, Bosutinib was recently tested as first-line therapy for the CP of CML (Cortes et al. 2018). It was initially authorized for people with CML who were resistant or intolerant to initial treatment. 536 individuals newly diagnosed with CP were randomized to receive 400 mg of Bosutinib every day or Imatinib in a global phase III research. At 12 months, a major molecular response (MMR, at least 1,000 fold or 3 log reduction of BCR::ABL1 level) rate at the primary endpoint was considerably greater with Bosutinib compared to Imatinib (47 *versus* 37 percent) (Cortes et al. 2018). For its side effects, Bosutinib was associated with liver function abnormalities via elevated serum transaminases (alanine and aspartate aminotransferase), as well as diarrhea, while cardiac and vascular toxicity was rare.

2.4 Third-line generation TKI (mutation T315I)

T315I is the most frequently reported mutation found in resistant cases (between 4 and 20 percent) (Gorre et al. 2001). This mutation, known as a "gatekeeper" mutation, gives resistance to all frontline TKIs. It has resulted in the development of Ponatinib, which is currently the only TKI effective against T315I CML.

Ponatinib is the first-line therapy for patients with CML-CP who have the T315I mutation, supplemented by allo-HSCT if a donor is present and desired response rates are not reached. It was synthesized as a ligand of the ATP binding domain of BCR::ABL1 in a closed conformation. Ponatinib has very low side effects occurrence at 15–30 mg/day, with a vaso-occlusive disease, skin rashes, and thromboembolism being seen on rare occasions (Cortes et al., 2013). However, its use drew attention to the appearance of an important side effect, which is the inhibition of parental cell Ba/F3 proliferation (Azam et al., 2006). Notably, Ponatinib still kept its activity in other CML mutations, such as M244V, G250E, and Q252H.

A ponatinib real-life insight from Italy was recently published, in which 666 CML patients (515 CP, 50 AP and 101 BP patients) were medicated with ponatinib 45 mg daily. Due to adverse effects, 20 percent of patients had their doses reduced. Ponatinib was terminated in 22 percent of patients (7 percent had intolerance, and 9 percent had resistance). In CML-CP, the

median duration to therapy termination was 47 months. This study validates ponatinib therapy's great effectiveness and safety in CML (Breccia et al. 2022).

2.5 Asciminib

Patients who develop resistance or intolerance to existing treatment choices have few, often ineffective, therapy alternatives and typically proceed to advanced phases (Chopade and Akard 2018; Soverini et al. 2018). As a reason, there is a high priority need to investigate innovative therapeutics. Asciminib is an investigational drug that is an effective, specific and allosteric inhibitor of BCR::ABL1 (Wylie et al. 2017; Schoepfer et al. 2018; Hughes et al. 2019). It uses a unique mechanism of activity, specifically targeting the myristoyl-binding pocket of ABL1 (Figure 15).

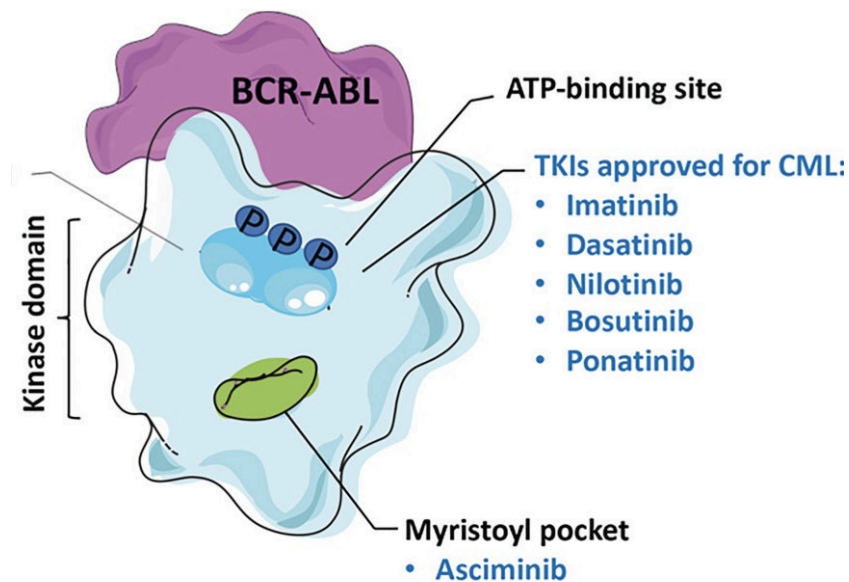


Figure 15: Asciminib binds to the myristoyl pocket of the BCR::ABL1 kinase

(Andretta et al. 2021)

The results of the phase I Asciminib monotherapy trial demonstrate that Asciminib is a potential therapeutic for later-line therapy in CML-CP patients, including those who carry the T315I mutation (Hughes et al. 2019). At the normal dose of 40 mg twice daily, Asciminib has

superior tolerability in contrast to traditional TKIs, with an early signal indicating decreased rates of cardiovascular toxicity compared to second- and third-generation TKIs (Shanmuganathan and Hughes 2022).

2.6 Selecting a frontline therapy

The International Scale (IS) was developed as a tool for monitoring treatment results and interpreting molecular reactions. Progress toward these objectives can be measured using (1) hematologic response [white blood cell and platelet counts; the presence of myelocytes plus metamyelocytes and basophils; the absence of blasts and promyelocytes in peripheral blood and extramedullary involvement (Baccarani et al. 2006)], (2) cytogenetic response [percent Ph1+ metaphases (Kantarjian et al. 2003)] and (3) molecular response [A mathematical log reduction of decreased BCR::ABL1 level by a certain amount from the standard baseline].

Following Imatinib failure, patients are subjected to detailed bone marrow examination including cytogenetics and molecular testing with mutation screening. The next TKI treatment options are chosen depending on the disease phase, the mutation profile, and the comorbidities of the patients.

In the first year, patients are checked every three months, and thereafter every six months. Monitoring cytogenetic response with peripheral blood FISH and PCR is an alternative to bone marrow examinations. If a patient is responding well and the FISH testing at 6 or 12 months is negative, and/or BCR::ABL1 transcripts are less than 1 percent, it may be safe to skip subsequent marrow examinations, since the patient is likely to be in stable condition. On the other hand, patients having up to 10 percent BCR::ABL1 transcripts at 6 months, up to 1 percent BCR::ABL1 transcripts at 12 months or loss of response at any time point are candidates for therapy adjustment (**Figure 16**).

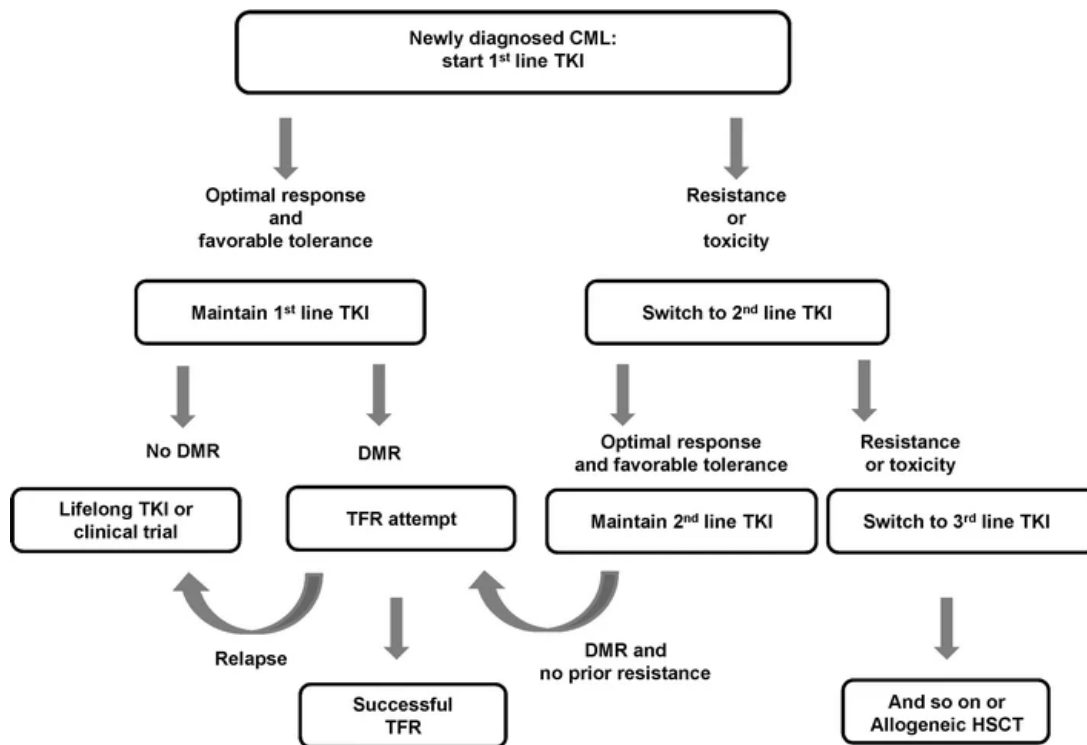


Figure 16: Principle of CML management integrating treatment-free remission (TFR) in clinical practice (Rea and Cayuela 2018).

2.7 Treatment-Free Remission

According to the latest reports, the life expectancy of CML patients responding to therapies is comparable to the population in general (Bower et al. 2016; Maas et al. 2022). treatment-free remission (TFR) is a concept that has been devised after the attempts of stopping TKIs in patients in undetectable residual disease, corresponding to a deep molecular response. Only 20 to 30 percent of patients achieve this level of response with the current therapies. TKI stopping strategies pioneered in France (Rousselot et al. 2007; Mahon et al. 2010) followed by several international trials (**Table 2**) have shown that 50 to 60 percent of patients will present a molecular relapse, suggesting the persistence of a CML LSC in the marrow of these patients. Evidence for the persistence of the LSCs in the bone marrow of patients in deep molecular response has been obtained by using LTC-IC or SCID-repopulating cell assays (Chomel et al. 2011; Chu et al. 2011).

Study	Number	Treatment before discontinuation	Response required for discontinuation	Definition if relapse	TFR % (Median follow-up time)
STIM1	100	IFN then Imatinib for ≥ 3 years	CMR	Loss of MMR or ≥ 1 -log increase in BCR::ABL1	39 % (55 months)
STIM2	200	Imatinib for ≥ 3 years	As for STIM	As for STIM	Preliminary results 46% (95% CI 38-56) at 2 years
ALLG CML8	40		UMRD ≥ 2 years	Loss of MMR or confirmed loss of MR ^{4.5}	45 % (42 months)
According to STIM	80		As for STIM; occasional positive samples eligible	Loss of MMR	64 % (23 months)
ISTAV	112	Imatinib	Undetectable PCR (3 PCRs)		51.9 % at 36 months (Median follow-up 21 months)
EUROSKI	809	Imatinib, Nilotinib, Dasatinib	MR ⁴ for ≥ 1 year; TKI for ≥ 3 years	Loss of MMR	Preliminary results 61% (95% CI 54-68) at 6 months
DESTINY	168		MR ⁴ and stable response under half standard dose for 12 months		In progress

Table 2: Imatinib-discontinuation studies; CMR, complete molecular response (MR^{4.5}); MMR, major molecular response; UMRD, undetectable minimal residual disease; 95% CI, 95% confidence interval.

2.8 Resistance to TKIs

TKI resistance is caused by a variety of pathophysiological processes, ranging from alterations in the molecular therapeutic targets themselves to mechanisms that affect drug concentration or impair the signaling network of leukemic cells (**Figure 17**).

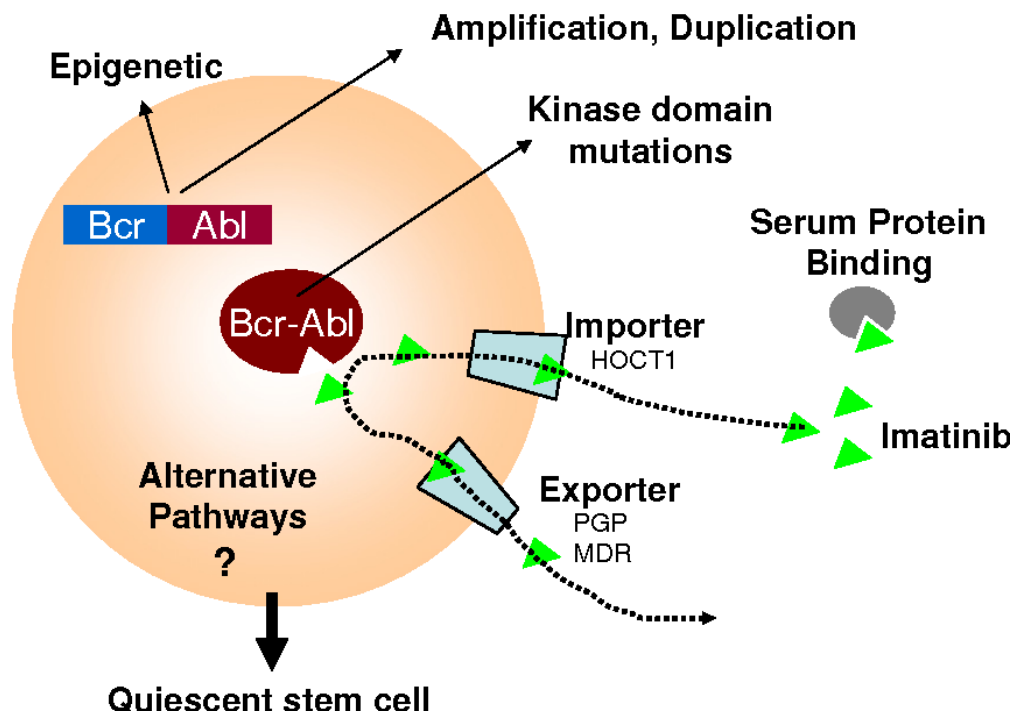


Figure 17: Mechanisms of TKI resistance (Roychowdhury and Talpaz 2011).

2.8.1 BCR::ABL1-dependent TKI resistance

2.8.1.1 Amplification of BCR ABL and Mutation of ABL-kinase domain

The success of TKI therapy is mainly contingent on efficient BCR::ABL1-drug interaction (Apperley 2007). BCR::ABL1-dependent TKI resistance is associated with amplification of *BCR::ABL1* oncogene (le Coutre et al. 2000), high expression levels of BCR::ABL1 mRNA (Vigneri et al. 2017) or emerging mutations in some BCR::ABL1 various domains (Buffa et al. 2014; Braun et al. 2020). These alterations impair TKI affinity to the binding domain as well as change in BCR::ABL1 conformation. T315I was the first BCR::ABL1 mutation identified (Gorre et al. 2001). We can count more than 100 different mutations impacting over 50 amino acids

that can be classified as resistant (T315I), less sensitive (Y235H, F359V, E255K), and sensitive (all other mutations except those mentioned before) (Nardi et al. 2004; Balabanov et al. 2014). Mutations become more common as the disease worsens, occurring in roughly 75 percent of CML patients in BP (Morozova et al. 2015).

The most prevalent TKI resistance mechanism is the emergence of point mutations on the ABL1 kinase domain, which is more common in acquired resistance cases rather than primary resistance cases and is related to a poor prognosis and a greater likelihood of disease progression. Based on their locations, BCR::ABL1 point mutations are categorized mainly into four groups: mutations at the ATP-binding site (P-loop), the drug contact site, the catalytic domain (C-loop), and the activation (A)-loop (Braun et al. 2020) (**Figure 18**). Mutations on P-loop, along with T351I, have been the most often identified in resistant patients, accounting for 36 to 48 percent of cases (Branford et al. 2003; Jabbour et al. 2006).

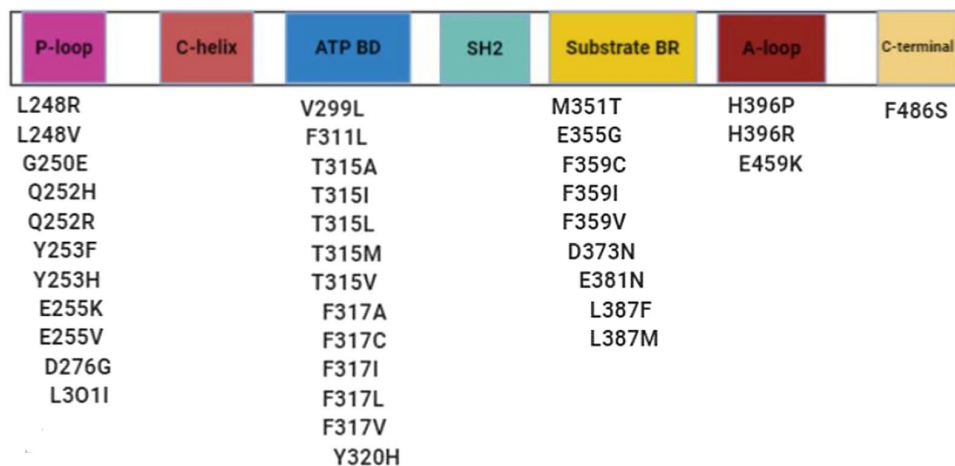


Figure 18: Schematic representation of TKI resistant mutations (Benchikh et al. 2022)

2.8.1.2 Resistance of LSCs

The idea that cancer/leukemia stem cells (CSCs/LSCs) are fundamental for cancer development, therapy resistance, and relapse has driven this field of interest, and the relevance of CSCs has been proven in a range of malignancies (Morrison et al. 1995; Weissman 2000; Al-Hajj et al. 2003). LSCs can self-renew in CML and other malignancies, leading to treatment

resistance and disease progression (Olsson et al. 2014). Since existing leukemia treatments are based on the common biological features of malignant blast cells with proliferative capability, LSCs are typically dormant. As a result, existing therapies do not successfully eradicate both the LSCs and the disease (Holyoake et al. 1999).

Even though the specific molecular mechanism of LSC-mediated resistance to existing treatments has not been properly understood, one significant aspect may be the quiescence of LSC, which permits this population of cells residing in the bone marrow niche to avoid being targeted by current chemotherapies and targeted therapies, resulting in disease relapse (Ito et al. 2008; Kamińska et al. 2008). Several studies reveal that a dormant population of CML stem cells (CD34⁺CD38⁻CD45RA⁻CD71⁻HLA-DR^{low}) with BCR::ABL1 kinase domain mutations, detectable prior to Imatinib therapy commencement, gives rise to leukemia cells that survive because they are fundamentally resistant to Imatinib (Sorel et al. 2004; Jørgensen et al. 2007; Niemann et al. 2007).

2.8.2 BCR::ABL1-independent resistance

TKI therapies have been successful essentially based on the expression of BCR::ABL1 on the target cells. However, it has now been shown that CML stem cells do not only rely on the expression of BCR::ABL1 for their survival. Indeed, it has been shown that CML cells in which tyrosine kinase activity has been inhibited, can survive in the presence of growth factors (Corbin et al. 2011). These findings have generated the concept of “Oncogene Independence” in CML, suggesting that signaling pathways other than BCR::ABL1 could be of importance for eliminating CML stem cells (Chomel and Turhan 2011). In order to uncover signaling pathways other than BCR::ABL1, extensive research has been performed over the last decade with the identification of several important and “druggable” targets.

One other aspect which has emerged for explaining LSC persistence is the influence of the bone marrow niche protecting the most quiescent LSC from the toxicity of TKI (Shah and Bhatia 2018). However, it has also been shown that there is a cross-talk between the “niche”

and the leukemic cells (Chomel et al. 2014) which induces signaling abnormalities (**Figure 19**), transforming the “normal Ph1-negative” niche into an abnormal one (Schmidt et al. 2011). In addition, it has also been recently shown that endothelial cells from the bone marrow niche supply miR-126 to Ph1 LSC to support their growth (Zhang et al. 2018). Data from our group has also demonstrated that an abnormal transcriptome can persist in CML mesenchymal stem cells even in patients at a deep molecular response (Aggoune et al. 2017).

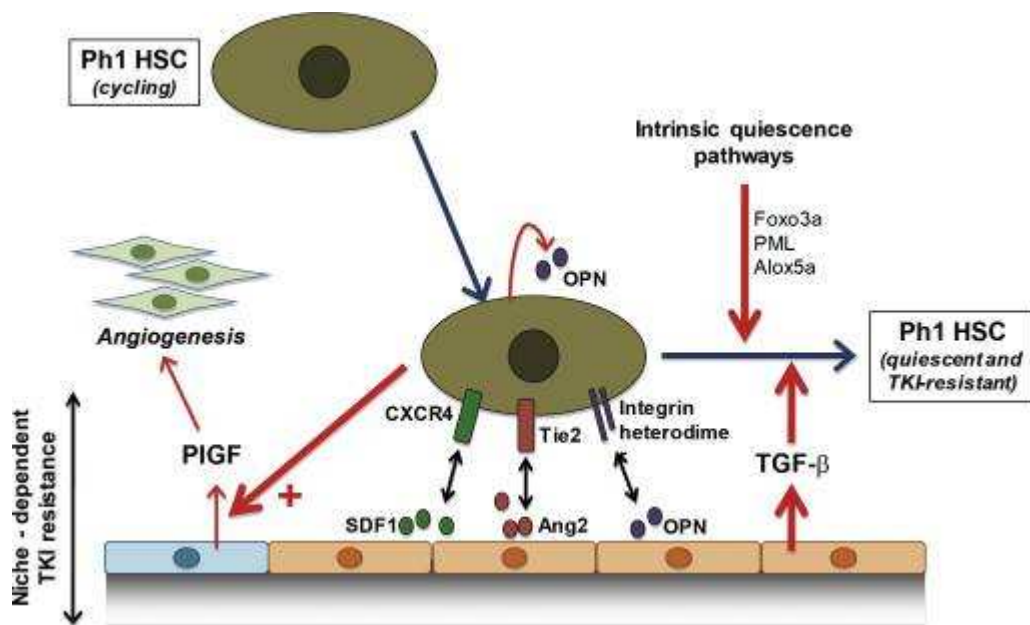


Figure 19: Summarizes niche abnormalities and cross talk in CML
(Chomel and Turhan 2011)

Uncovering BCR::ABL1 independent mechanisms are critical for understanding and identifying the function of additional proteins in treatment response. BCR::ABL1-independent resistance mechanisms comprise aspects such as drug bioavailability, for example, abnormal activity of drug transporters. It is also associated with the activation of alternative signaling pathways, genomic instability and epigenetic alterations (Giustacchini et al. 2017; Pagliaro et al. 2020).

2.8.2.1 Signaling pathways and transcription factors

CML cells may initiate alternative signaling pathways to compensate for the lack of BCR::ABL1 kinase activity in order to counteract BCR::ABL1 inhibition. As a result, despite effective BCR::ABL1 suppression, cells will be capable of growing and surviving. Several mechanisms have been reported, including RAF/MEK/ERK, SRC, JAK/STAT, and PI3K/AKT, resulting in resistance to TKI (**Figure 20**).

Ma et al. reported enhanced activity in the RAF/MEK/ERK signaling pathway responsible for BCR::ABL1-independent Imatinib resistance through CML-LSCs by employing a large-scale RNA interference (RNAi) screen to identify genes implicated in Imatinib responsiveness. Imatinib and Trametinib, a MEK-inhibitor, were recently coupled in a mouse model, successfully eradicating CML-LSCs and demonstrating that this combination should be tested in patients as well (Ma et al. 2014). Zhou et al. demonstrated that combining a WNT/ β -catenin signaling inhibitor with Nilotinib eradicated a CML-resistant cell line as well as primary cells from TKI-resistant patients in BC by suppressing the expression of CD44, MYC, p-CRKL, p-STAT5, and Survivin (Zhou et al. 2017a). SRC proteins, such as LYN and HCK, promote survival by activating AKT and increasing proliferation via activating STAT5. The upregulation of SRC proteins in CML served as the foundation for the discovery and usage of dual SRC/ABL inhibitors such as Dasatinib and Bosutinib (Soverini et al. 2018). STAT5 plays a role in the progression of CML by enhancing cell cycle progression, ROS generation, blocking apoptosis, and increasing P-gp expression (Warsch et al. 2011). STAT5 deficiency has been linked to TKI sensitivity. STAT3, on the other hand, emerges as a key molecule in the resistant phenotype, including TKI resistance (Nair et al. 2012).

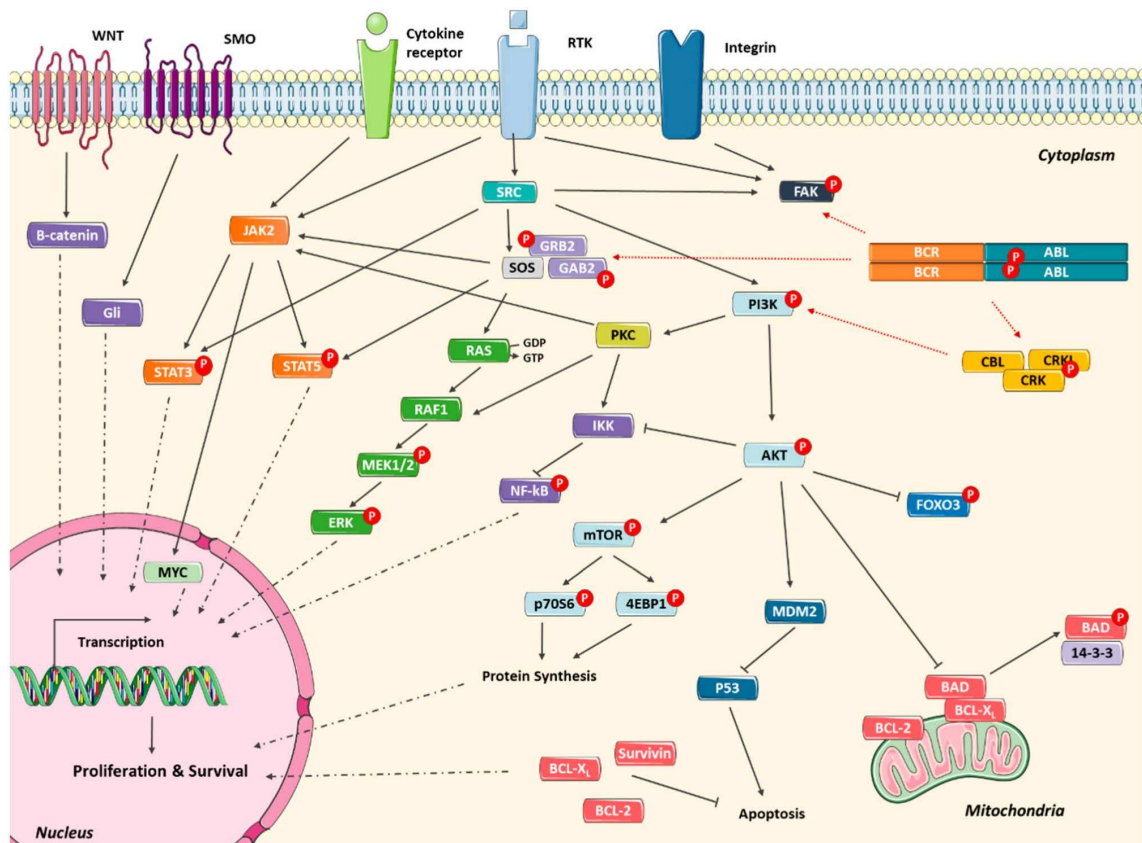


Figure 20: Schematic representation of alternative to BCR::ABL1 signaling network

(Alves et al. 2021)

The molecular clock is thought to regulate immunological, angiogenesis, DNA repair, and cell proliferation processes (Lévi et al. 2010). In fact, several epidemiological studies have connected shift workers' disrupted circadian rhythms to a higher risk of developing cancer (Papantoniou et al. 2018; Salamanca-Fernández et al. 2018). Consequently, it is not unexpected to see that in practically all malignancies, including earlier observations of CML (Yang et al. 2006, 2011) and AML (Yang et al. 2015; Puram et al. 2016), the circadian clock circuitry is altered. They are typically regarded as tumor suppressors even if the underlying mechanism is not completely known. This particular topic will be covered in more detail in the dedicated chapter 4.

A favorable environment for cancer is produced by the disruption of several signaling pathways brought on by BCR::ABL1 or by independent processes, which contributes to the

ineffectiveness of TKIs and, ultimately, to resistance. The argument for novel treatment strategies using additional inhibitors or in conjunction with TKIs is supported by an understanding of this complex.

2.8.2.2 Drug bioavailability

The efficiency of the medicine to reach its molecular target is crucial to the effectiveness of the treatment. Since drug transporters play crucial roles in the control of intracellular drug accumulation, their overexpression has been linked to putative resistance mechanisms. Alteration to these transporters may provide an explanation for the resistance phenotypes brought on by inadequate TKI uptake and/or excessive TKI removal from the cell (DeGorter et al. 2012).

Because the majority of drugs are not ATP-dependent and are transported across cell membranes by solute carrier (SLC) transporters like OCT1, they have a limited capacity to diffuse freely (Nigam 2018). OCT1 is the primary drug transporter involved in the uptake of TKIs and its expression and/or its activity correlates to the levels of therapeutic response. Functional activity of OCT1 in leukemic cells at the time of diagnosis has been noted as a prognostic marker of TKI response (Chhikara et al. 2017). However, there is disagreement over the findings of OCT1 expression and activity because researchers have found no link between OCT1 expression and Imatinib transport or responsiveness (Watkins et al. 2015). Dasatinib might be used instead of other TKIs to overcome lower levels of TKI uptake since it can diffuse through cell membranes (Giannoudis et al. 2008; Hiwase et al. 2008). On another hand, ATP-binding cassette (ABC) transporters mediate the efflux of metabolites, xenobiotics, and chemotherapeutic drugs (Liang et al. 2015). The most well-studied transporter is P-glycoprotein (P-gp, MDR1, ABCB1), whose upregulation has been associated with several chemoresistant malignancies (**Figure 21**, Neul et al. 2016).

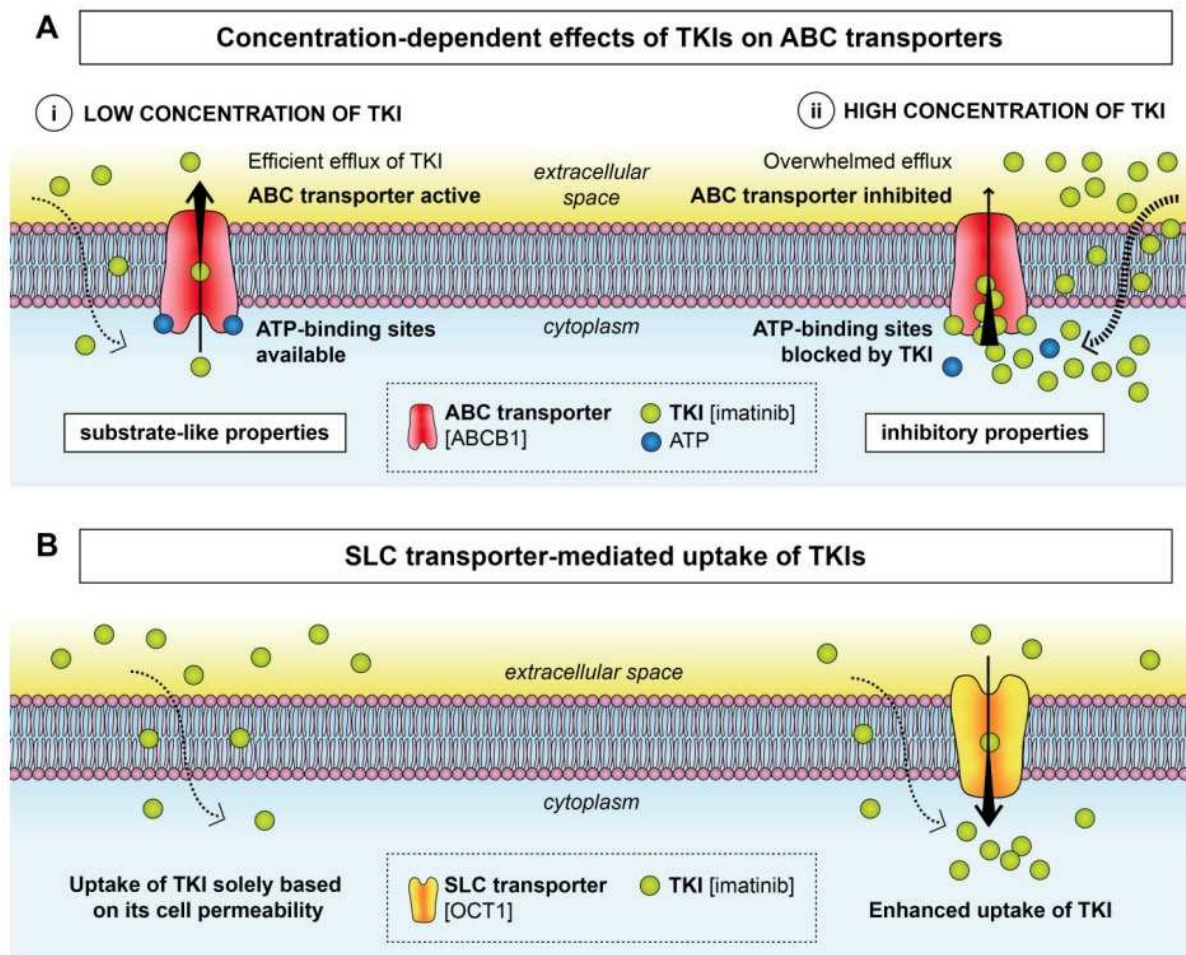


Figure 21: Transport of TKIs by ABC and SLC transporters (Krchniakova et al. 2020)

All TKIs authorized for the treatment of CML are acknowledged P-gp substrates (Eadie et al. 2014). Poor long-term consequences and advanced-phase pathology are linked to high *ABCB1* expression levels (the gene that encodes P-gp) (Eadie et al. 2017; Maia et al. 2018). Intriguingly, Imatinib, Dasatinib, and Nilotinib at higher dosages are able to overcome ABC-related resistance *in vitro*, pointing to a potential role for ABC-transporters in the emergence of resistance (Dohse et al. 2010). Interestingly, CML stem cells have been shown to express low levels of OCT1 and high levels of MDR contributing to their resistance to TKI (Jiang et al. 2007). Therefore, combinations of therapeutically significant TKIs and ABC drug transporter inhibitors have the potential to increase intracellular concentrations of the targeted TKI, overcome MDR, and improve TKI effectiveness (**Figure 22**, Alves et al. 2022).

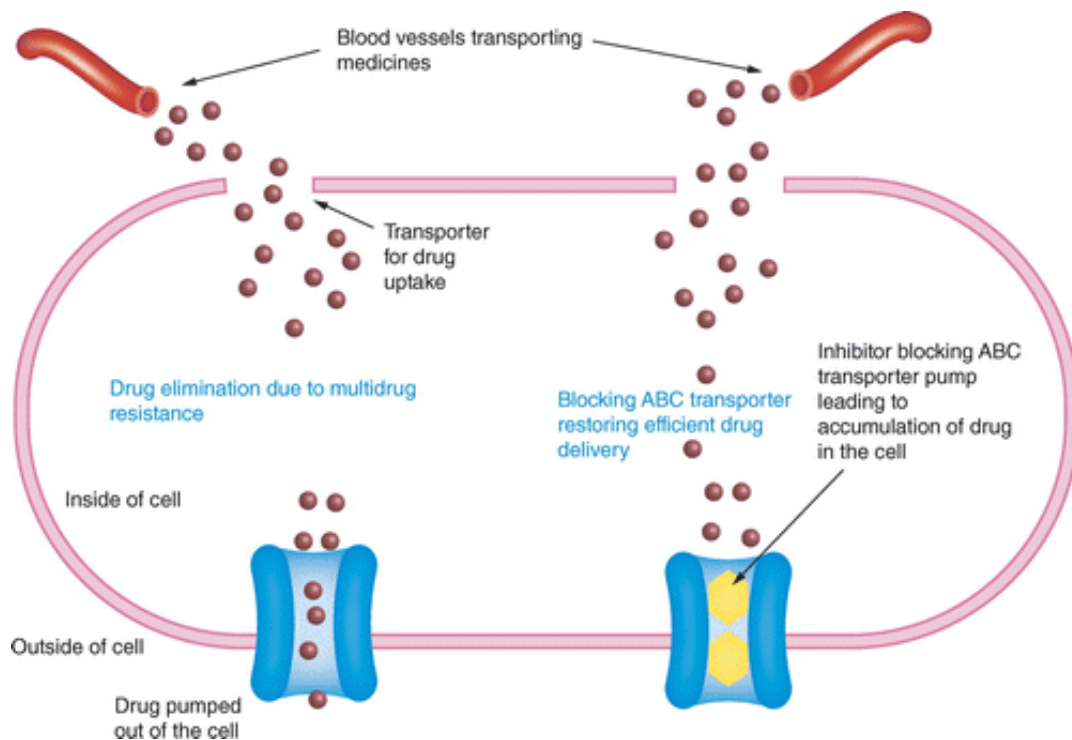


Figure 22: ABC transporters mediate the efflux of chemotherapeutic drugs

(Sharma et al. 2021)

2.8.2.3 Post-transcriptional alterations

The phenotype of CML and its susceptibility to treatment may be also significantly impacted by post-transcriptional alterations in addition to the alteration of BCR::ABL1 itself. The key regulators of these mechanisms are microRNAs (miRNAs or miRs), which have an impact by inhibiting protein synthesis and enhancing mRNA degradation. MicroRNAs could function as either oncogenes or tumor suppressor genes in cancer development and progression (**Figure 23**). In CML, abnormal expression of many microRNAs has been linked to stem cell survival and self-renewal, sensitivity or resistance to TKI treatment, and disease progression (Flamant et al. 2010; Eiring et al. 2014).

When compared to healthy persons, CML patients display strikingly different patterns of microRNA expression. These variations also differ amongst CML patients at different stages of the disease and between those who respond to TKI and those who do not (Machová Poláková et al. 2011; Agatheeswaran et al. 2016). For instance, compared to Imatinib-sensitive cells, the

tumor suppressor miR-451 was significantly suppressed while the oncogenic microRNAs (oncomiRs) miR-21 and miR-26b were increased in Imatinib-resistant CML K562 cells (Alves et al. 2019). BCR::ABL1 directly controls the expression of certain microRNAs via influencing microRNA effectors such as MYC, CCN3, and MYB (Suresh et al. 2011; Firatligil et al. 2013; Srutova et al. 2018). Some microRNAs, such as miR-203, which impacts BCR::ABL1 and has its expression decreased in CML due to hypermethylation, may potentially interact directly with BCR::ABL1 (Chim et al. 2011). The devoted chapter that follows will go into more detail on this specific subject.

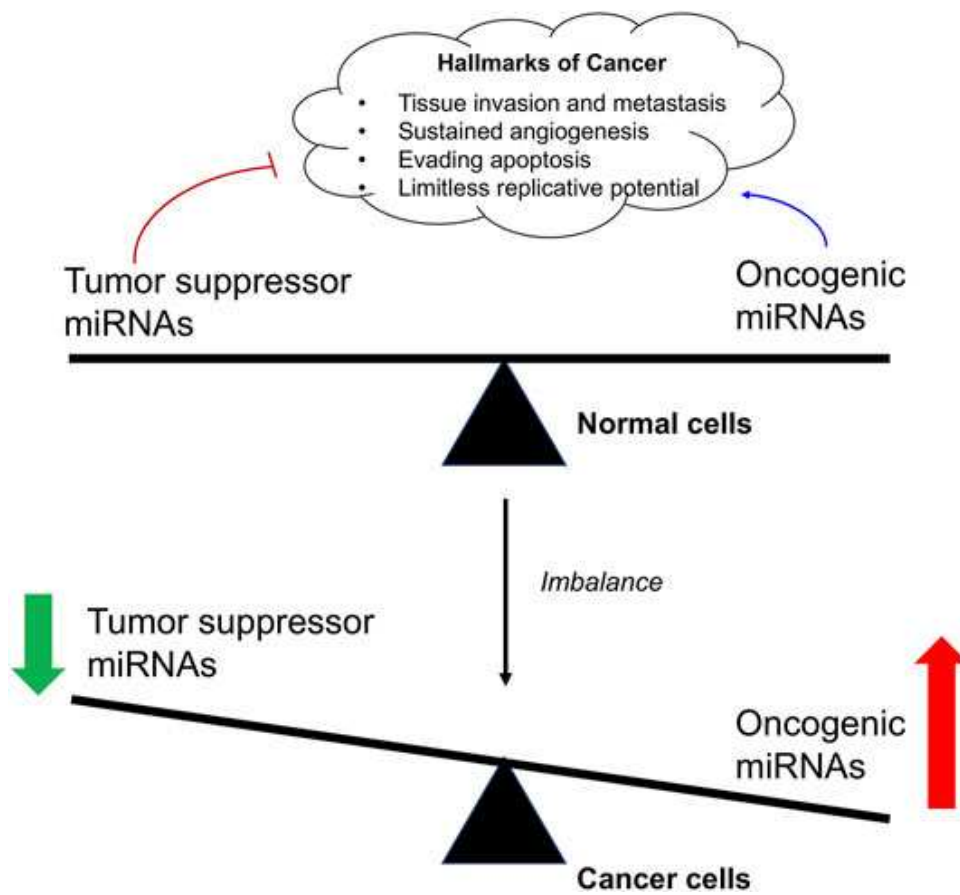


Figure 23: The roles of oncomiRNAs and tumor suppressor miRNAs (Fu et al. 2021)

2.9 Future research of CML treatment

Currently hematopoietic stem cell transplants remain a major potential therapeutic strategy in patients' resistant to TKI therapies, if an HLA-compatible intrafamilial donor or donor from International Marrow Registry are available. The mortality of these therapies has been considerably reduced but the long-term toxic effects (GVHD, immune suppression) remain highly significant (Craddock 2018). This fact emphasizes the importance of developing novel therapeutic solutions to tackle CML resistance. Future research will concentrate on the potential molecular curative treatment for CML in order to obtain a long-term deep molecular response (DMR; defined as the absence of detectable BCR-ABL1 mRNA in blood) and its retention after TKI treatment is stopped. This may be accomplished with only the updated more powerful new generation TKIs or in conjunction with other existing or developing medicines. Challenge existing approaches may increase the elimination of minimal residual disease, perhaps eradicating the need for TKI treatment indefinitely. Uncovering the pathophysiologic processes downstream of BCR::ABL1 may allow the development of novel therapeutics. The following table shows some potential adjuvant therapies of the future in CML (**Table 3**)

Compound	Target(s)	Study
Pimozide, Pioglitazone	STAT5, PPAR-G	Pimozine + Imatinib* Pioglitazone + Imatinib*
Gliptins	CD26	Vildagliptine + Imatinib
FTY720, Forskolin (PAD)	PP2A	PAD + TKI
BP-5-087	STAT3	BP5 + TKI
Trametinib	PRKCH	Trametinib + TKI
F79 aptamer	RAD52	F79 + TKI
EW 7197	TGF-b inhibitor	N/A

Table 3: Potential targets for adjuvant therapies (Chomel and Turhan 2011)

CHAPTER 3: microRNA (miRNA, miR)

3.1 Biogenesis of miRNAs

miRNAs are single-stranded, non-coding RNAs with about 22 nucleotides (nt). They work by suppressing gene expression at the post-transcriptional stage (Bartel 2004). The interaction between miRNAs and target mRNAs either stops translation or causes mRNA degradation. Mature miRNAs bind to their target mRNAs via a particular miRNA region of roughly 6-8 nt in length (**Figure 24**). This area is known as the 'seed region', in general, the 3' untranslated regions of the mRNA (3'UTR).

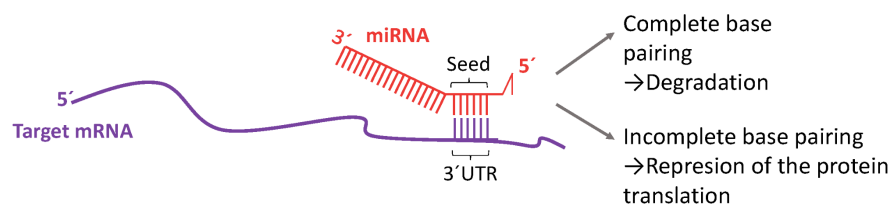


Figure 24: Gene silencing through the complementarity of miRNA/mRNA

(Carregal-Romero et al. 2020)

A group of microRNAs collectively known to have similar "seed regions" would have been considered a single miRNA family. Typically, members of a miRNA family control the same set of genes or participate in the control of related biological processes. Indeed, miRNA-mediated gene expression downregulation was linked to the phenomena of RNA-mediated interference (RNAi), implying that both pathways may overlap the same biological effectors (Doench et al. 2003). Mammal miRNAs come in a variety of families, some of which have been linked to mechanisms of differentiation and self-renewal and are thus being considered potential biomarkers for these kinds of activities. For instance, members of the let-7 or miR-30 families induce differentiated cell fates (Peck et al. 2016; Wells et al. 2017), whereas those of the miR-290 family are linked to self-renewal (Gu et al. 2016). The number of mRNAs that a single

miRNA targets might vary depending on the microenvironment and a single mRNA can be targeted by a number of distinct miRNAs.

It is worth mentioning that the study of miRNAs has taken a major step since the discovery of the first miRNA, *lin-4*, in *Caenorhabditis elegans* in 1993. Two research groups published side-by-side, describing a small non-coding RNA derived from a longer hairpin precursor that works by inhibiting the post-translational process of the protein encoded from the *lin-14* mRNA via an antisense RNA-RNA interaction (Lee et al. 1993; Wightman et al. 1993). 7 years later, *let-7* was discovered and demonstrated to control the timing of larval *C. elegans* development (Reinhart et al. 2000). *Let-7* was also the first human miRNA found in 2000, and there are presently 2,675 human mature miRNAs annotated in the miRBase miRNA database (Release March 22, 2018).

Tom Tuschl, Victor Ambros, and David Bartel coined the term "microRNA" (miRNA) to describe this family of micro post-transcriptional regulators (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001). In the next decade post the discovery of miRNAs, these tiny molecules were observed further in complex organisms such as plants, mammals and humans over ten years after their discovery (Reinhart et al. 2002; Lagos-Quintana et al. 2003; Lim et al. 2003), and they were also linked to several human disorders (Calin et al. 2002). The relevance of miRNAs in human pathophysiology has been disclosed in the recent decade by particular research that linked their regulatory functions to the genesis, development, and prognosis of various disorders such as cancer (Nucera et al. 2016; Yeh et al. 2016; Javanmardi et al. 2017), cardiovascular (Feng and Yu 2011; Lock et al. 2018), and metabolic diseases (López-Pastor et al. 2020; Sun et al. 2021).

A multi-stage process is used to produce mature miRNAs, which are encoded by host genes' introns or by their own genes (**Figure 25**, Monterisi et al. 2015). Even though the miRNA biogenesis processes are comparable across phyla, there are notable distinctions between plants and animals that necessitate a separate investigation of either biogenesis pathway (Axtell et al. 2011). This chapter will emphasize the biogenesis of miRNAs affiliated with the animal

kingdom. The majority of miRNA genes are transcribed as large polyadenylated RNA molecules named primary miRNAs (pri-miRNAs) by RNA polymerase II (Pol II), with one or a few stem-loop structures of roughly 70 nucleotides apiece. In the nucleus, pri-miRNAs are cleaved into a stem-loop structure called precursor miRNAs (pre-miRNAs) by an RNase III family nuclease, DROSHA (Lee et al. 2003; Denli et al. 2004). Pre-miRNAs are 70-nucleotide hairpin RNAs with a 2-nucleotide 3' overhang, which is characteristic of RNase III-mediated cleavage. Pre-miRNAs are transported to the cytoplasm via a RanGTP/exportin 5-dependent process (Yi et al. 2003; Lund et al. 2004; Bohnsack et al. 2004). After that, Pre-miRNAs are identified and transformed into mature miRNAs by Dicer once they are in the cytoplasm (Hutvagner et al. 2001). Because mature miRNAs enter the RNA-induced silencing complex (RISC) in an asymmetrical way, only one strand of most miRNAs is capable of detecting and suppressing the expression of target genes (Schwarz et al. 2003).

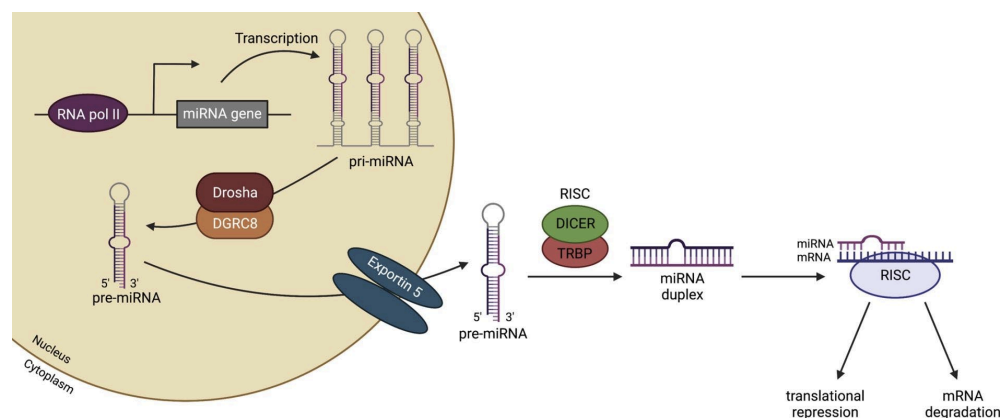


Figure 25: Biogenesis of miRNAs (Cerqueira et al. 2022)

3.2 Links between miRNAs and hematological malignancies

Growing amounts of research over the past 20 years have demonstrated that miRNA expression profiles are related to the various types and stages of cancers (Peng and Croce 2016). In fact, the discovery that about 50% of human miRNAs are found in CAGRs (cancer-associated genomic regions) raises the possibility that miRNAs play a key role in carcinogenesis (Calin et al. 2004). Depending on the tissue in which they are expressed, they can function as either

oncogenes (oncomiRs) or tumor suppressors (anti-oncomiRs) (Calin and Croce 2006). In research on CLL, Calin et al. provided the first evidence of miRNA involvement in cancer. They identified miR-15 and miR-16 as the first tumor suppressor miRNAs and often deleted anti-oncomiRs encoded in chromosomal region 13q14 (Calin et al. 2002). Interestingly, in many types of leukemia, miR-146a has both oncogenic and tumor-suppressive features (Shahid et al. 2021).

3.2.1 Expression of miRNAs in hematological malignancies

Alteration of miRNA expression levels is a hallmark of hematological malignancies, which is linked to various subtypes of acute leukemia with diverse cytogenetic and molecular genetic changes (Szczepanek 2020). This specific subject of research has been thoroughly discussed, which can be found in a review (see further; Han et al. 2020).

For instance, seven distinct subtypes of pediatric ALL have been characterized based on the expression patterns of microRNAs, such as the down-regulation of miR-127 and miR-143 in precursor B-cell ALL (Schotte et al. 2011). miR-126 was upregulated in individuals with AML who had the chromosome 16 inversion *inv(16)(p13q22)* mutation (Zhang et al. 2021), whereas miR-10 was highly expressed in the subset of Nucleophosmin-mutated (NPM1mut-AML) patients with a high rate of complete remission (CR) to induction chemotherapy (Havelange et al. 2014). Fulci et al. discovered that miR-150 levels were considerably higher in CD19+ B cells from CLL patients in comparison to healthy donors (Fulci et al. 2007). In contrast to its overexpression in CLL, miR-150 was shown to be considerably lower in CD34+ blasts or mononuclear cells derived from CML patients than in healthy donors (Srutova et al. 2018). After two weeks of imatinib therapy for CML, Flamant et al. discovered the increased expression of miR-150 and miR-146a and decreased expression of miR-142-3p and miR-199b-5p, indicating that these miRNAs may serve as a potential therapeutically valuable biomarker (Flamant et al. 2010). As a result, these tiny molecules might be used as diagnostic, prognostic, and therapeutic biomarkers at various stages of leukemia.

3.2.2 Functionality of miRNAs in hematological malignancies

For the functionality of miRNAs in hematological malignancies, it has been determined that miR-192 corresponds to the pathogenesis of CLL by controlling Bcl-2, p21, and p53 (Fathollahzadeh et al. 2016). In a mouse model, the miRs miR-128a and miR-130 are downstream targets of the MLL-AF4 fusion oncoprotein that promote the development of acute leukemia (Malouf et al. 2021). It has been determined that anti-oncomiR let-7a dysregulation is necessary for the survival of leukemic cells with MLL rearrangements (Zhu et al. 2020). In contrast, miRNAs can also act as tumor suppressor genes in several leukemias. A collection of miRNAs in acute promyelocytic leukemia (APL) showed overexpression and prospective tumor suppressor activity after treatment with the differentiation agent ATRA (all-trans-retinoic acid) (Garzon et al. 2007). Oncosuppressor miRNAs such as miR-223, miR-342, and miR-107 were included, as well as members of the let-7 family and miR-15a, miR-15b, and miR-16-1 with proven targets in CLL pathogenesis (Marton et al. 2008).

3.3 Altered expression of miRNAs in CML

miR-10a, miR-17/92, miR-150, miR-203, and miR-328 are the most commonly un-regulated miRNAs in CML (Gordon et al. 2013). When compared to normal CD34+ cells, miR-17/92 is up-regulated in early CP CML CD34+ cells but not in BP CML CD34+ cells. Furthermore, both BCR-ABL and c-Myc increased the expression of miR-17/92 in BCR-ABL-positive cell lines, indicating that it might be exploited as a therapeutic target (Venturini et al. 2007). The down-regulation of miR-328, which occurs in a BCR-ABL dose- and kinase-dependent manner, is an important step in the development into the CML-BP. Ectopic expression of miR-328 in cell lines restored leukemic blast differentiation by inducing the survival factor PIM1 and inhibiting the binding of heterogeneous ribonucleoproteins E2 with the hematopoietic transcription factor CEBPA (Eiring et al. 2010). In patients receiving a TKI (Imatinib), a sequential analysis of their blood showed increased expression of miR-150 and miR-146a and decreased expression of

miR-142-3p and miR-199b-5p, indicating that this treatment has the capacity to modify very early the miRNA profiles of CML cells (Flamant et al. 2010).

Researching novel biomarkers for CML is difficult. miRNAs, which are dynamic regulatory molecules that have been discovered to be potentially involved in pathogenesis of CML, could offer a significant potential as therapeutic targets. Specific molecular processes have been identified that explain the down-regulation of certain miRNAs in the setting of CML, such as miR-150 and the miR-29a/b cluster. These miRNAs are potential possibilities for biomarkers for prognosis and the development of novel therapeutics.

3.3.1 miRNAs as biomarkers for CML diagnosis

Patient survival rates are significantly impacted by early CML diagnosis prior to BP. Multiple studies have consistently shown that miR-150 is down-regulated, making it an effective tool for the early diagnosis of CML (Agirre et al. 2008; Machová Poláková et al. 2011; Fallah et al. 2015). Reintroducing miR-150 has been shown to improve symptoms in cell lines, and decreased expression of miR-150 is associated with a worse prognosis and a more advanced stage of CML (Agirre et al. 2008). miR-150 was shown to be down-regulated in CD34+ cells taken from six CP CML patient samples, indicating that this miRNA may contribute to the initiation of cancer (Agirre et al. 2008). Two research on newly diagnosed CP CML patient samples revealed support for miR-150 as a diagnostic biomarker of CML and confirmed significant miR-150 down-regulation (Flamant et al. 2010; Fallah et al. 2015). Importantly, Flamant et al. discovered that miR-150, which was previously only known to be down-regulated in CP, is also down-regulated in BP. This finding suggests that, although this miRNA may have the potential for the diagnosis of CML, it may not be a reliable biomarker to differentiate between CP and BP (Flamant et al. 2010).

Another example, miR-203 gene locus's methylation patterns have been proposed as possible biomarkers for the detection of CML (Bueno et al. 2008; Chim et al. 2011). Authors analyzed a number of leukemia cell lines and discovered that miR-203 was significantly down-

regulated and hypermethylated in CML cell lines that expressed the BCR::ABL1 fusion protein but not in cell lines from other related myeloproliferative disorders (Bueno et al. 2008). The transfection of miR-203 into CML cell lines lacking miR-203 expression reduced BCR::ABL1 expression significantly, which in turn reduced the rate of proliferation (Bueno et al. 2008). The team additionally verified the expected interaction of miR-203 with the 3'UTR of BCR::ABL1. According to these findings, the deregulation of this miRNA may be exploited as a biomarker for the diagnosis of CML, maybe in combination with other miRNAs.

3.3.2 miRNAs as biomarker candidates for treatment response

Several miRNAs can be used to follow up on the efficiency of TKIs by investigating their altered expressions after the treatments. For example, Dasatinib altered miR-let-7d, miR-let-7e, miR-15a, miR-16, miR-21, miR-130a, and miR-142-3p expression, whereas Imatinib was shown to alter the levels of miR-15a, miR-21, miR-122, miR-126, and miR-130a (Ferreira et al. 2014; Mirza et al. 2019; Ali Beg et al. 2021). Using a QRT-PCR technique on 85 newly diagnosed CP CML patients, down-regulation of miR-10a was detected in 71% of patients, demonstrating its clinical value as a biomarker for diagnosis (Agirre et al. 2008). Remarkably, the expression of miR-10a varies significantly among cell lines obtained from CML patients in the BP. The BP-derived cell lines had higher amounts of miR-10a, indicating that miR-10a might be a useful biomarker for differentiating between them (Agirre et al. 2008). Flamant et al. analyzed miRNAs expressed in leukemic cells as biomarkers for therapeutic response directly in the blood of patients undergoing Imatinib therapy (Flamant et al. 2010). By using microarray examination of patient samples 2 weeks after Imatinib treatment, the data revealed relatively rapid increased expressions of miR-150 and miR-146a or decreased expressions of miR-142-3p and miR-199b-5p after the initiation of treatment. Interestingly, after only two weeks, the abnormal expression of these miRNAs was returning to normal levels. Thus, this study suggests that these miRNAs could be served as biomarkers for the newly diagnosed stage.

Another gene whose expression levels can be utilized as a biomarker for treatment response is miR-150. The BCR::ABL1 transformed leukemia cell line Mo7e-p210 (megakaryoblast) was shown to have miR-150 down-regulation, which could be recovered in response to imatinib therapy (Agirre et al. 2008). Another finding showed that miR-150 might function as a biomarker for diagnosis and therapy response by using patient samples at various stages of CML (Machová Poláková et al. 2011). The study characterized miRNA expression levels and confirmed that miR-150 is down-regulated in both CP and BP. However, most notably, their findings demonstrated that individuals who developed resistance to Imatinib therapy did not have their miR-150 expression levels restored. Together, their findings support miR-150 as a possible biomarker for treatment response and imply that lower expression levels of miR-150 are suggestive of a poor prognosis for patients undergoing TKI therapy.

3.3.3 miRNAs as biomarkers of drug resistance

miR-29a/b expression is persistently down-regulated in CML, and it corresponds with therapeutic resistance to Imatinib, making it a good biomarker candidate for treatment response (San José-Enériz et al. 2009). When evaluating the miRNA expression patterns, it was shown to be down-regulated in drug-resistant patients undergoing Imatinib therapy over a 12-month period. An in-depth investigation of miR-29b has shown its possible mechanism of action. miR-29b is reported to target BCR::ABL1 transcripts, interacted directly with Abl-1's 3'UTR, and is frequently found at lower levels in patient samples upon diagnosis (Li et al. 2013). Overexpression of miR-29b in K562 cells reduces ABL-1 protein levels and triggers G1 phase cell arrest via p21 and p27 activation.

Furthermore, several more miRNAs are also associated with TKI resistance in CML. For instance, upregulation of miR-486 in CML progenitors resulted in increased imatinib resistance (Wang et al. 2015), while decreased expression of miR-142-5p, miR-199b, miR-217, miR-221, and miR-365a-3p linked with resistance to TKIs in CML (Yeh et al. 2016; Jiang et al. 2019; Klümper et al. 2020). In particular, Jiang et al. demonstrated that miR-221 restoration and

STAT5 knockdown improved the sensitivity of CML cells to Imatinib by lowering the Bcl2:Bax ratio. Their results collectively revealed that the miR-221-STAT5 axis was essential in regulating how sensitive CML cells were to Imatinib (Jiang et al. 2019). The expression of miR-146a and miR-451 in the blood may be measured to predict how well TKIs will work in a certain situation (Alves et al. 2019; Habib et al. 2021). miR-9 and miR-577 have also been shown to play an important role in the development of multidrug resistance in a resistant CML cell line and patients by inhibiting the ABCB1 transporter and by targeting the NUP160 subcomplex of the nuclear pore, respectively (Li et al. 2017; Zhang et al. 2019).

3.4 miR-495

The miR-495 gene is found on one exon of 14q32.31 on chromosome 14 (**Figure 26**). It is located in this miRNA gene cluster 14q32.31, together with genes for miR-299-5p, miR-376a, miR-376c, miR-300, miR-380, miR-494, miR-1197, and others. miRNAs in this cluster have comparable functions in both healthy and diseased tissues, involving normal tissue development and regulating proliferation, apoptosis, metastasis, and chemosensitivity in cancer cells (Vinuesa et al. 2009; Formosa et al. 2014). The 3' and 5' strands of pre-miR-495 stem-loop structure are cleaved, creating miR-495-3p and miR-495-5p, respectively (Choi et al. 2014; Guo et al. 2015).

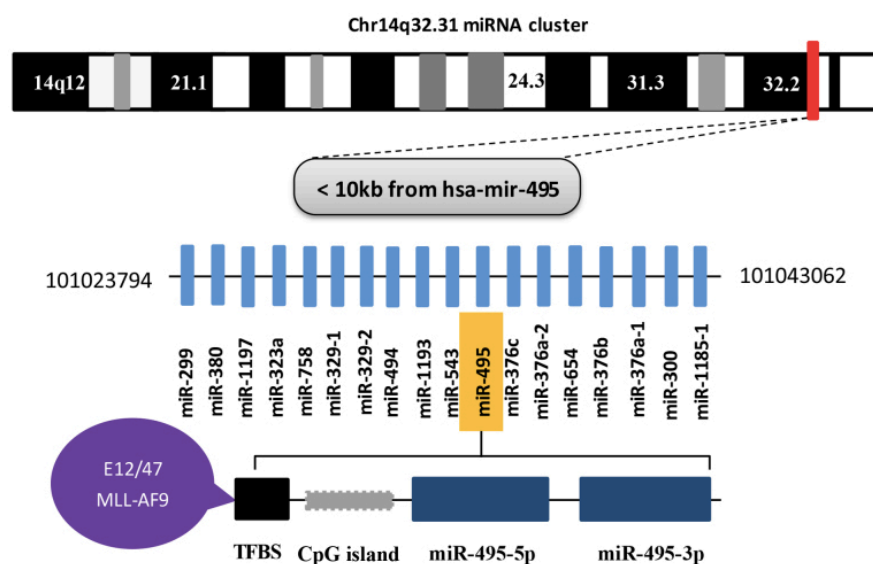


Figure 26: Location of the 14q32.31 miRNA cluster on chromosome 14 (<10 kb from hsa-miR-495), the miRNA genes it contains and the structure of the gene encoding miR-495. CpG, cytosine-phosphate-guanine; TFBS, transcription factor binding site (Chen et al. 2017).

3.4.1 miR-495 deregulation in cancer

Pioneer investigations on this miRNA have shown that miR-495 expression is linked to promoter methylation and transcriptional factors like EF12/47 (**Figure 27**, Hwang-Verslues et al. 2011). Further studies in gastric cancer (GC) found that promoter hypermethylation causes miR-495 dysregulation and treatment with 5-Aza-2' deoxycytidine, a DNA methyltransferase inhibitor, restored miR-495 expression and low-level cytosine-phosphate-guanine (CpG) methylation of the miR-495 gene (Li et al. 2015). Further research into the mechanisms that regulate miR-495 expression is needed.

Studies in certain cancers have found the decreased expression of miR-495 (Wang et al. 2008; Li et al. 2012, 2015; Chen et al. 2013; Chu et al. 2014; Formosa et al. 2014). miR-495 expression in GC tissues is linked to PRL-3 overexpression, which is correlated to GC cell invasion and lymph node metastasis (Wang et al. 2008). Genetic manipulation experiments on human GC cells found that miR-495 mimics were able to decrease aggressive behavior of these cell lines (Li et al. 2012, 2015). miR-495 is a tumor suppressor that is depleted in non-small cell lung cancer (NSCLC). miR-495 alteration potentially impacts NSCLC metastasis by targeting MTA3, a known risk factor for lymph node metastasis in NSCLC (Chu et al. 2014). Furthermore, the reduction of miR-495 in glioblastoma multiforme (GBM) U-251 cells enhances glucose absorption and lactate release, which promotes proliferation since miR-495 is no longer regulating cyclin-dependent kinase 6 for degradation (Chen et al. 2013). Eventually, miR-495 downregulation has been linked to carcinogenesis in metastatic prostate cancer (PC). These findings support the theory that miR-495 functions as a tumor suppressor (Formosa et al. 2014).

In contrast, increased miR-495 expression has been linked to a carcinogenic impact in a number of solid tumors. Overexpression of miR-495 causes E-cadherin to be downregulated,

promoting cell invasion in breast cancer (BC) stem cells (Hwang-Verslues et al. 2011). Furthermore, reduced DNA damage-inducible transcript 4 (REDD1) expression caused by miR-495 overexpression causes the release of inhibition of the molecular target of the rapamycin signaling pathway, promoting cancer cell growth under hypoxic environments (Hwang-Verslues et al. 2011). Furthermore, miR-495 affects the development, apoptosis, and metastasis of hepatocellular carcinoma (HCC) cells (Yang et al. 2013). miR-495 upregulation in HCC reduces methionine adenosyltransferase 1A (MAT1A) levels via limiting translation and enhancing MAT1A mRNA degradation. Stable overexpression of miR-495 in HCC murine models was shown to increase tumorigenesis and metastasis (Yang et al. 2013). Lastly, miR-495 has been shown to have comparable roles in gallbladder cancer (GBC) and it has been hypothesized that a miR-495/PHLPP/AKT/survivin anti-apoptosis signaling pathway exists (Qiu et al. 2015). Identifying the precise impact of miR-495 in malignancies continues to be challenging. The explanation why miR-495 is elevated in certain tumors and downregulated in others is unproven. Because miR-495 plays important role in a variety of malignancies, more research into the regulatory mechanisms behind the miR-495 expression is needed.

3.4.2 The function of miR-495 in healthy cells

3.4.2.1 miR-495 in stem cell development

miR-495 has a function in stem cell, cartilage, and pancreatic acinar cell development. miR-495 stimulates mesendoderm differentiation of mouse embryonic stem cells in its first two days of embryoid body development by selectively targeting DNA methyltransferase 3a (Yang et al. 2014). In human mesenchymal stem cells, miR-495 is also a direct regulator of chondrogenic development. miR-495 potentially increases the expression of SRY-box 9 (SOX9), which eventually contributes to chondrogenic differentiation (Lee et al. 2014). miR-495 is also involved throughout the differentiation of neovascularization in mice following a single femoral artery ligation. miR-495 inhibition significantly increased blood flow recovery following ischemia, making it a prospective strategy for the new therapeutic neovascularization (Welten et al. 2014).

Interestingly, miR-495 as well as other miRNAs in the 14q32.31 cluster also play a role in human HSC differentiation. miR-495 expression reduced throughout the early phases of normal myelopoiesis but increased significantly in a significant proportion of AML patients. This report is the first to show the presence of a miRNA signature in human cord blood stem and progenitor cells, which may play a role in hematopoietic stemness and maybe in the leukemogenesis of particular AML subtypes (Cattaneo et al. 2015).

3.4.2.2 miR-495 in immunity

Lastly, miR-495 is involved in immunity. NOD2 influences innate immunity by being expressed in antigen-presenting cells (Hedl et al. 2014). Because ectopic miR-495 alters NOD2 expression, it is possible that miR-495 plays a role in the immune response. Choi et al. discovered a function for miRNAs in adaptive immunity by infecting mice with the extremely deadly avian H5N2 virus (Choi et al. 2014). As a result, nine miRNAs, including miR-495, were shown to be increased in the lungs. Four other overexpressed miRNAs were discovered as being involved in the immunological response, apoptosis, and viral replication. However, the function of miR-495 was not explored, and more research is needed to prove that miR-495 plays a role in an immune response.

3.4.3 The function of miR-495 in hematological malignancies

Differentially expressed miRNAs in pediatric patients with beta-thalassemia were identified using miRNA sequencing, revealing significantly higher expression of miR-495 in those patients (Wang et al. 2021a). Similarly, miR-495 expression levels in AML patients' peripheral blood were evaluated, and its diagnostic and prognostic values in AML were investigated. The miR-495 level was considerably lower in the patient group than in the control group. Patients with strong miR-495 expression had a better 5-year survival rate than those with low miR-495 expression. As a result, miR-495 are promising biomarkers for the early diagnosis and prognosis of AML (Zhang et al. 2020).

Moreover, in AML patients, mixed-lineage leukemia (MLL) rearrangements are correlated with intermediate to poor patient survival (Pigneux et al. 2015). Upregulation of the fusion protein MLL-AF9 was shown to drastically suppress miR-495 expression. Jiang et al. discovered that inducing miR-495 expression suppresses MLL-fusion-mediated cell transformation *in vitro* and leukemogenesis *in vivo*. Overexpression of miR-495 in human leukemic cells with MLL rearrangements significantly reduces cell viability and promotes apoptosis. As a result, the MLL-AF9 fusion protein may function as a miR-495 silencer (**Figure 27**), where miR-495 is considered to function as an anti-oncomiR in AML with MLL rearrangements (Jiang et al. 2012).

According to miRNA-mRNA network analysis, miR-495 is among a group of miRNAs that are thought to be important in the regulation of the cytogenetically normal AML (CN-AML) subtype because they are linked to the negative control of myeloid leukocyte differentiation, the negative control of myeloid cell differentiation, and the positive control of hematopoiesis. They discovered that interactions of miR-495 with PIK3R1 and CDK6 may control myeloid cell development in CN-AML (Esa et al. 2021).

Previous studies showed that the functionality of miR-495 in cancers and hematological malignancies is also associated with the networks with long-noncoding RNAs (lncRNAs) (Wang et al. 2019; Xia et al. 2020; Yuan et al. 2022; Zhang et al. 2022). According to Wang et al., the lncRNA MIAT can act like a sponge to inhibit miR-495, which acts as the tumor suppressor in AML cells. Collectively, their findings show that MIAT promotes AML development, at least partially by negatively regulating miR-495, and thus offers a prospective target for AML therapy (Wang et al. 2019). In addition, in an inflammation-stimulated mouse mononuclear macrophage leukemia cell line RAW264.7, up-regulation of the lncRNA NEAT1 and down-regulation of miR-495 level may worsen inflammation. The miR-495 in this study was subjected to sponging of NEAT1, which may have impacted inflammatory responses by altering STAT3 signaling (Xia et al. 2020).

3.4.4 miR-495 in chemotherapeutic resistance in cancer

Generally, miRNAs contribute to improving chemosensitivity in cancer. However, chemotherapeutic resistance in cancer is linked to the suppression of miRNAs whose encoding genes are located on chromosome 14q32.31 (Haller et al. 2010; Laddha et al. 2013; Xu et al. 2013). The fact that miR-495 is downregulated in drug-resistant cell lines and patients suggests that it predominantly works as an anti-tumor component by boosting chemosensitivity. In addition to leukemia, miR-495 and other miRNAs expressed by the 14q32.31 gene cluster may prolong survival by increasing chemosensitivity in several cancers.

In human lung adenocarcinoma (LAC) and esophageal cancer (EC), overexpression of miR-495 downregulates ATPase copper transporting (ATP7A) and increases sensitivity to cisplatin in both cancers (Song et al. 2014; Li et al. 2021). ATP7A suppression has comparable consequences. Furthermore, miR-495 may raise intracellular cisplatin levels, whereas ATP7A overexpression may reduce this impact of miR-495 (Song et al. 2014). Furthermore, miR-495, miR-134, and miR-379, whose genes are clustered on chromosome 14q32.31, also have been shown to improve susceptibility to anti-cancer medicines comprising cisplatin, etoposide, and doxorubicin in small cell lung cancer (SCLC) (Guo et al. 2010).

Because there are no confirmed therapeutic targets for triple-negative BC, the only current pharmacological treatment is chemotherapy such as Pirarubicin. Liu et al. showed that miR-495-3p expression levels are lower in a pirarubicin-resistant triple-negative BC cell line. Through the p-AKT/mTOR signaling pathway, transfecting cells with miR-495-3p mimics reduced pirarubicin chemoresistance while also inhibiting cell proliferation and migration (Liu et al. 2022).

3.4.5 miR-495 in multidrug resistance in cancer

Interestingly, multidrug resistance (MDR) is overexpressed in MDR ovarian cancer (OC), gastric cancer (GC) and metaplastic BC, leading to recurring cancers. It works by eliminating cytoplasmic therapeutic drugs from those cells, reducing drug accumulation and leaving cancer

cells prone to multidrug resistance (Zou et al. 2017; Kumar et al. 2021). According to Zou et al., miR-495 was predicted to target 3'UTR of ATP Binding Cassette Subfamily B Member 1 (ABCB1), which encodes the protein MDR1 (P-glycoprotein, p-gp). miR-495 mimics were overexpressed in OC and GC cell lines as well as *in vivo* transplanted MDR ovarian tumors in order to decrease drug efflux and overcome MDR in cancer cells (**Figure 27**, Zou et al. 2017). While miR-495-3p mimic expression decreased TGFB2 and FOXC1 expression and re-sensitized cells to doxorubicin and paclitaxel, miR-495-3p inhibition increased resistance (Kumar et al. 2021). As indicated by the findings of this study, pre-treatment with miR-495 before chemotherapy may boost the curative impact of MDR1-based MDR cancer.

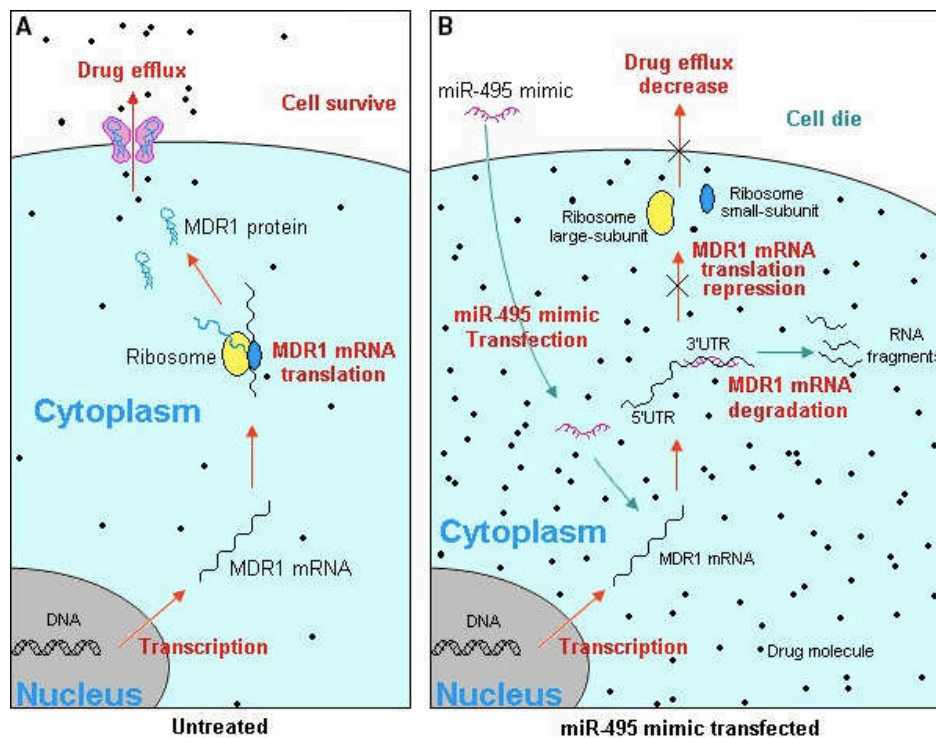


Figure 27: miR-495 overcomes drug efflux via inhibiting MDR1 in MDR cancer cells

(Zou et al. 2017)

Likewise, miR-495 also promotes chemotherapeutic sensitivity in CML via inhibiting MDR1 protein. In the stable Adriamycin (ADM)-resistant cell line K562/ADM, several 14q32.31 cluster-located miRNAs are downregulated. Transfecting K562/ADM cells with miR-495 mimics

increased ADM sensitivity by decreasing MDR1 expression (Xu et al. 2013). However, it is worthful to note that the down-regulation of miR-495 in CML in comparison with healthy controls and its effects on the efflux of BCR::ABL1-targeted drugs like TKIs remains unknown. In the most comparable study, Bruton's tyrosine kinase inhibitors (BTKi) Ibrutinib and Acalabrutinib downregulate miRNAs in the 14q32.31 miRNA cluster, including miR-495 in CLL and diffuse large B-cell lymphoma (DLBCL) (Kapoor et al. 2021). BTKi-resistant CLL and DLBCL cells displayed significant overexpression of miR-495 as well as decreased PTEN expression, indicating that the PI3K/AKT/mTOR pathway is further regulated in acquired BTKi resistance. miR-495 was also shown to be upregulated in resistant CLL patient samples (Kapoor et al. 2021).

Considering drug resistance is a critical concern in cancer treatment, patients who are unable to respond well to chemotherapy will benefit from alternative components that sensitize cancer cells to therapy. The use of miR-495 in the treatment of multidrug-resistant tumors is a potential therapeutic technique. In summary, miR-495's regulatory responsibilities in cancer are essentially dictated by its negative effect on the expression of its target mRNAs. At the moment, the expression and control of miRNA-495 have only been examined in a few malignancies (**Table 4**). More investigation into the involvement of miRNA-495 in different cancers is needed.

Type of cancer	Expression of miR-495	Target gene(s)	Functions of miR-495	Reference(s)
BC stem cells	Up	E-cadherin and REDD1	Cell invasion and hypoxia resistance	(Hwang-Verslues et al. 2011)
DLBCL	Up	n/a	n/a	(Kapoor et al. 2021)
GBC	Up	PHLPP	Apoptosis and tumor growth suppression	(Qiu et al. 2015)
HCC	Up	MAT1A	Cancer development, apoptosis, and metastasis	(Yang et al. 2013)

Resistant CLL	Up	n/a	n/a	(Kapoor et al. 2021)
AML	Down	PBX3 and MEIS1	Poor prognosis	(Jiang et al. 2012; Zhang et al. 2020)
CML	Down	MDR1	Drug resistance	(Xu et al. 2013)
CN-AML	Down	PIK3R1 and CDK 6	Myeloid cell development	(Esa et al. 2021)
EC	Down	ATP7A	Drug resistance	(Li et al. 2021)
GBM	Down	CDK6	Cell proliferation	(Chen et al. 2013)
GC	Down	MDR1	Drug resistance	(Zou et al. 2017)
NSCLC	Down	MTA3	Lymph node metastasis	(Chu et al. 2014)
OC	Down	MDR1	Drug resistance	(Zou et al. 2017)
PC	Down	n/a	Metastasis	(Formosa et al. 2014)
SCLC	Down	n/a	Drug resistance	(Guo et al. 2010)

Table 4: Expression level and function of miR-495 in different types of cancers.

CHAPTER 4: Circadian rhythm

4.1 Circadian rhythm: overview

The rotation of day and night is one of the most powerful aspects of our environment. Almost all species' behavior and/or physiology change on a daily basis, as a consequence (Pittendrigh 1993). These periodic rhythms are not purely a response to the 24-hour shifts in the physical environment dictated by the earth's rotation, but rather result from a timekeeping mechanism within all life forms. This timekeeping mechanism, or biological "clock", enables the organism to forecast and prepare for influence by the physical environment associated with day and night, ensuring that the organism "does a proper thing" at the appropriate time of day, including body temperature fluctuations (Buhr et al. 2010), circulating hormone levels (Lightman 2016), and metabolism (Green et al. 2008).

These daily cycles known as circadian rhythms also offer internal temporal structure and guarantee that internal changes occur in tandem. The word "circadian" (from the Latin words, *circa diem*, about a day) is used because the free-running period in constant conditions (independent from external time signals) might be either longer or shorter than, but not precisely, 24 hours. The preservation of rhythms self-perpetuating in the lack of a dark-light cycle or other extrinsic time signals (a *Zeitgeber*; a German word for *time-giver*, Halberg 1959) strongly suggests the presence of an internal timekeeping system or biological clock. Although the light-dark cycle is undoubtedly the primary *Zeitgeber* for all species, other variables such as social activities, physical movement or exercise, food intake, as well as temperature may all influence the phase of a cycle (**Figure 28**).

Even though observations of unicellular creatures indicate that the system creating circadian rhythms is cellular in origin, the circadian pacemaker in larger organisms is situated in cells of particular structures of the body. Certain sections of the brain (i.e., the optic and cerebral lobes) in insects; the eyes in certain invertebrates and vertebrates; and the pineal gland, which is positioned inside the brain, in nonmammalian vertebrates, are examples of these structures. The circadian clock is located in two clusters of nerve cells termed the suprachiasmatic nuclei

(SCN) in the anterior hypothalamus, which is located near the base of the brain (Moore and Eichler 1972; Stephan and Zucker 1972). The early 1970s finding that scientists could interrupt and eliminate endocrine and cognitive circadian rhythms in rats by injuring (i.e., lesioning) the SCN served as a pivotal example of the SCN's function (Moore and Eichler 1972; Stephan and Zucker 1972). Furthermore, researchers were able to partially restore the circadian rhythms by transferring SCN from other animals into the animal subjects with the lesioned SCN (Ralph et al. 1990). The ability of separated lungs, livers, and other tissues cultured in a culture plate (i.e., in vitro), which are not under the direction of the SCN, to retain circadian rhythms has recently astonished; indicating the existence of cellular “peripheral” clocks (Welsh et al. 1995; Yamazaki et al. 2000).

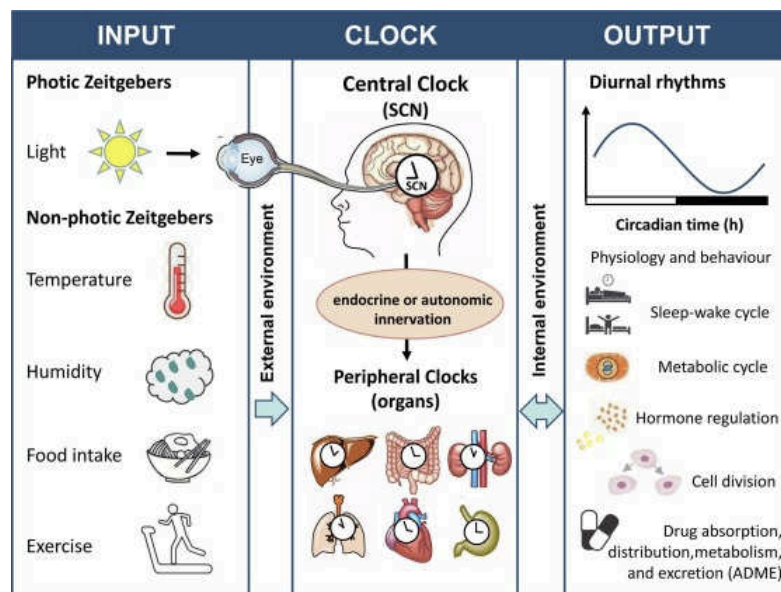


Figure 28: The physiological basis for the generation and maintenance of circadian rhythms (Yu et al. 2022)

4.2 Brief history of circadian rhythm discovery

Jean-Jacques d’Ortous de Mairan, a French multidisciplinary scientist, pioneered the foundation of chronobiology studies. He discovered in 1729 that the leaves and branches of mimosa plants open and close on a daily periodic basis, even in complete darkness (**Figure 29**, De Mairan

1729). However, De Mairan did not notice that the plants' leaves opened slightly early each day. This occurrence is now classified as a "free-running" rhythm (unrestricted by external stimuli) with a duration somewhat less than 24 hours, indicating that plant photoperiodism is governed by an endogenous clock that may be synced by external inputs. Indeed, in 1832, the Swiss botanist Augustin Pyramus de Candolle measured the free-running period of *Mimosa pudica* and discovered it to be approximately 22–23 hours, far less than 24 hours (Candolle and Todd 1832).

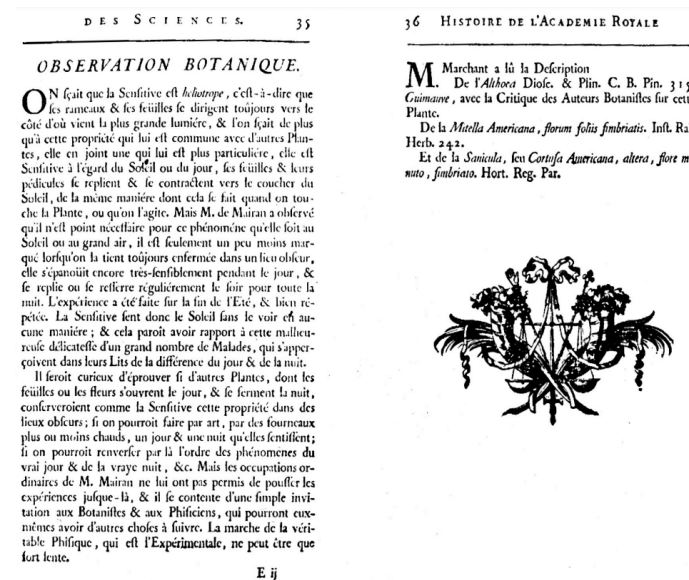


Figure 29: Observation botanique. Texts and atlas (De Mairan 1729)

It took another 200 years until Erwin Bünning provided the first support for the genetic basis of circadian rhythm production by proving that period length is heritable in bean plants (Bünning 1937). As a result, the study of circadian rhythms developed from visual examination of plants, whereas it was not until much later that endogenously driven rhythms were first observed in bacteria (Mitsui et al. 1986), single-cell eukaryotes (Sweeney and Hastings 1957), insects (Beling 1929), birds (Kramer 1957), rodents (Richter 1922), primates (Simpson and Galbraith 1906), and humans (Von Aschoff and Wever 1962). In 1971, Konopka and Benzer found three mutants by using mutagens to treat the fly and screening for anomalies in the

circadian rhythm of pupal eclosion and locomotor activity. One mutant was arrhythmic, another had a shorter period of 19 hours, and the third had a longer period of 28 hours (Konopka and Benzer 1971). This landmark study signaled the start of a continuous search for molecular processes underlying circadian rhythms.

It would not happen until the early 1980s when the fast advancement of recombinant DNA made it possible to clone a gene and subsequently identify it by genetic rescue (Rosbash 2017). Jeffery C. Hall and Michael Rosbash of Brandeis University and Michael Young of Rockefeller University separately cloned and recovered the *Drosophila period* gene in 1984, leading to the discovery of the first clock gene *period (per)* (Bargiello and Young 1984; Bargiello et al. 1984; Reddy et al. 1984; Zehring et al. 1984). In 1988, the *Drosophila single-minded* gene was discovered. This gene produces a nuclear protein that shares a sequence resemblance with the *period* gene product PER, providing the first indication of PER's potential function as a transcription factor (Crews et al. 1988; Thomas et al. 1988). Following that, Hall and Rosbash produced a number of significant discoveries, starting in 1988 when they identified a free-running circadian rhythm in the presence of PER protein in the fly visual system (Siwicki et al. 1988), for which Hall, Rosbash and Young received the Nobel Prize in Physiology or Medicine 2017. A free-running circadian rhythm in the amounts of *per* mRNA was also discovered in the fly head two years later. This rhythm has a peak in the early hours of the night, several hours before the peak PER protein abundance. Importantly, the *per* nonsense mutation eliminates the mRNA level oscillation, which is restored by the presence of wild-type PER protein. The phase of mRNA oscillation and behavioral rhythm is also equally impacted by the *per* missense mutations. A feedback concept of the PER protein directly impacting its own gene expression was put out by Hall the Rosbash in response to the findings (Hardin et al. 1990).

The mechanism by which the PER protein accesses the nucleus and functions as a transcription factor was still unknown. The answer to the query was supplied by Young's 1990s discovery of the second clock gene *timeless (tim)* (Myers et al. 1995; Sehgal et al. 1995). The combined findings support the transcriptional-translational feedback loop (TTFL) theory by

showing that cyclic expression of *tim*-encoded TIM protein controls the cyclic accumulation and nuclear localization of the PER protein. However, to maintain an autonomous oscillation continuing, a positive input is needed to drive the transcription of *tim* and *per*. The CLOCK-BMAL1 heterodimers binding to the enhancer E-box serve as the positive input component to drive individual transcriptional oscillations, according to Joseph Takahashi's discovery of the *Clock* gene in the mouse (Vitaterna et al. 1994; Antoch et al. 1997; King et al. 1997) and the subsequent identification of its companion BMAL1 (Gekakis et al. 1998). Notably, PER and TIM block Drosophila CLOCK activity, breaking the circadian feedback loop (Darlington et al. 1998).

4.3 Circadian clock genes

Over the last few decades, the molecular arrangement of the core circadian clock has been discovered (**Figure 30**). To establish circadian oscillations, the peripheral and SCN clocks rely on the same molecular mechanism, which is founded on auto-regulatory TTFL comprising positive (activating) and negative (inhibitory) components (Reppert and Weaver 2001). The TTFL causes the cyclic production of clock genes and proteins during a 24-hour period.

Firstly, Brain Muscle Arnt-Like Protein-1 (Bmal1) and Circadian Locomotor receptor Cycles Output Kaput (Clock) are the activator (positive) components of the circadian oscillator (Lowrey and Takahashi 2004). CLOCK and BMAL1 heterodimerize and drive transcriptional activation of core clock genes and additional clock-controlled genes (CCGs) (Rey et al. 2011; Koike et al. 2012) by binding to E-box sites of their target genes, make up the circadian clock's molecular machinery (Ripperger and Schibler 2006).

Within these CCGs, CLOCK and BMAL1 drive transcription over their own repressors, Period (Per1, Per2) and Cryptochrome (Cry1, Cry2) family members (Thresher et al. 1998; Reppert and Weaver 2001), resulting the negative elements of a closely self-regulated TTFL (Welsh et al. 2010). After the CLOCK/BMAL1 complex promotes transcription of the Per and Cry genes, CRY and PER proteins are produced, accumulate in the cytoplasm, and then migrate from the cytoplasm to the nucleus following heterodimerization (Yagita et al. 2002). Once

PER/CRY complexes reach sufficient amounts in the nucleus, they suppress transcription of E-box genes (including their own genes) by preventing CLOCK/BMAL1-mediated transcription.

An extra feedback loop strengthens the CLOCK/BMAL1 and PER/CRY loops. CLOCK/BMAL1 heterodimers are also responsible for the transcription of two nuclear receptors, Rev-Erb α and ROR α . REV-ERB α suppresses *Bmal1* and, to a lesser extent, *Clock* gene production by binding to ROR-responsive regions in the promoters of these two genes (Lowrey and Takahashi 2004; Takahashi 2017). Though REV-ERB α suppresses *Bmal1* transcription, ROR α increases it. As a result, *Bmal1* mRNA exhibits a high-amplitude circadian oscillation. Other CCGs that can be identified are *Per3*, *Timeless (Tim)*, *PPAR α* , *CKI ϵ* , and *SHARP1*, although their responsibilities are unclear.

The rhythmicity of the expression of multiple genes involved in diverse biological processes is provided by circadian transcription of the core clock genes. According to the most current papers, clock-controlled genes account for about 10% to almost 50% of a tissue's transcriptome (Zhang et al. 2014; Mavroudis et al. 2018). Notably, clock-controlled genes underneath circadian transcription differs in each tissue, implying that circadian modulation of cellular physiology and metabolism is tissue specific (Mohawk et al. 2012). Because of the significance of the circadian clock and the broad range of physiological activities that are controlled in a circadian manner, it is not unexpected that genetic or environmental disruptions of circadian rhythmicity (such as jet lag) provoke a variety of diseases, including metabolic syndrome, cardiovascular disease, and cancer (Arble et al. 2010; Fu and Kettner 2013; Chaves et al. 2019).

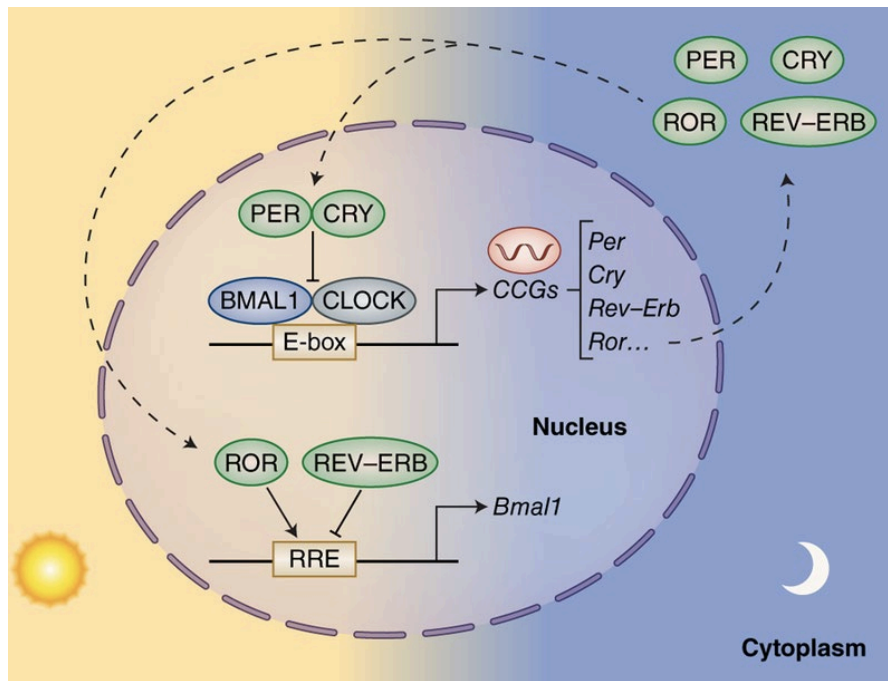


Figure 30: Mammalian Circadian Clock Molecular Components (Masri et al. 2016)

4.4 The importance of circadian clock genes on normal hematopoiesis

Having described the importance of circadian rhythms in physiology, it is not surprising that they also play important roles in the regulation of hematopoiesis. Pioneering works in the '90s demonstrated by CFC assays and DNA synthesis that erythroid and myeloid lineages exhibited distinct and different circadian rhythms (Smaaland et al. 1992; Wood et al. 1998). The expression of the circadian clock genes was further measured in BM cells and cyclic expression was demonstrated with some variations among hematopoietic cell lineages as exemplified with the expression of *Per1* and *Per2* in mouse total BM and Gr1-positive cells (Chen et al. 2000). Interestingly, Tsinkalosky et al. compared the clock gene expression between the Side Population (SP) which is enriched in LT-HSCs and BM cells and observed that *Per1* was highly expressed in SP while *Cry1* was preferentially expressed in BM cells suggesting that differences in intrinsic clock regulation exist within different hematopoietic cell populations (Tsinkalovsky et al. 2005). Further analysis revealed that *Per2* was expressed in a circadian manner in SP and BM, while *Per1* and *Rev-erba* were cyclically expressed in BM but not SP cells, suggesting that regular oscillations of the cell's intrinsic circadian rhythm are not perceived by HSCs and

hematopoietic stem and progenitor cells (HSPCs) (Tsinkalovsky et al. 2006). Even though HSPCs have little or no major cell-intrinsic clock systems, periodic cycling in regeneration still occurs in these cells. Several investigations have found circadian oscillations in the frequency of DNA synthesis and colony-forming capacity in human and mouse BM (See review: Mendez-Ferrer et al. 2009).

Particularly, circulating HSPCs exhibit cyclic fluctuation peaking twice a day 5 hours after light starting and another 5 hours after darkness commencement (Méndez-Ferrer et al. 2008; Golan et al. 2018). This daily trafficking is dependent on light-onset cues as demonstrated by the circadian alteration of HSPC behavior in mice subjected to continuous light or to a “jet lag” (defined as a shift of 12 h). Golan et al. proposed that these two peaks correspond to distinct HSPC activities which are regulated by TNF bursts mediated by neutrophil secretion. During the light-onset peak, Neutrophils increase vascular permeability, facilitating HSPCs differentiation and egression from the BM. On the opposite, the dark-onset peak is mediated by melatonin increase and triggers the renewal of BM LT-HSC reservoir, transforming HSPCs with a low metabolic state and an increased stem cell surface appearance equipped with great short and long-term repopulation capacity (**Figure 31**, Golan et al. 2018).

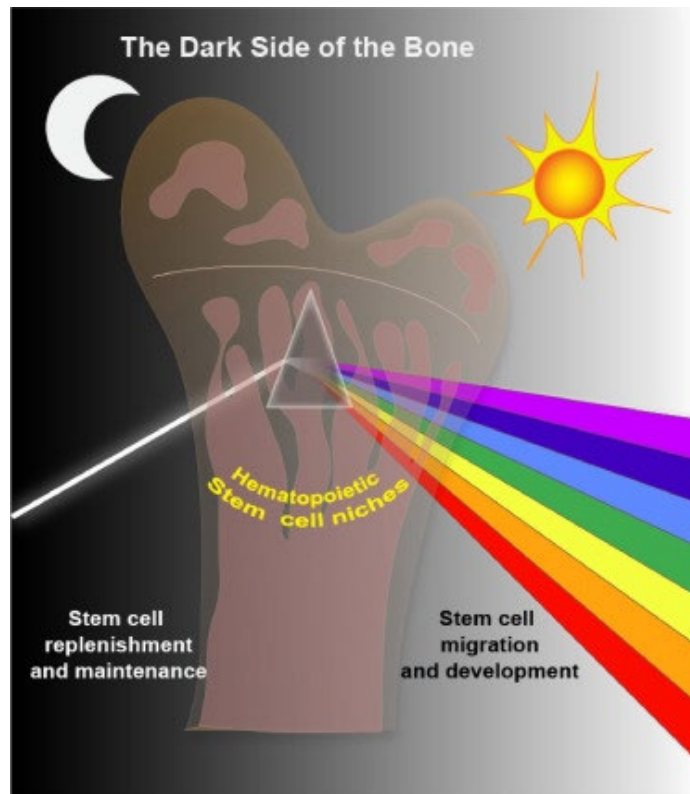


Figure 31: The dawn peak involves differentiation, migration, enhanced vascular permeability, outflow, and blood replacement. The nighttime peak is characterized by the renewal of the BM LT-HSC reservoir (Golan et al. 2018)

As mentioned previously (see chapter 1.5.3.1), the biological properties of HSCs are tightly regulated by the niche bone marrow. Particularly, HSCs are regulated by receptor-ligand interactions between CXCR4 receptors expressed by HSCs and CXCL12 chemokine released by BM stromal CXCL12-abundant reticular (CAR) cells (Nagasawa et al. 1996; Sugiyama et al. 2006). Lower CXCL12 levels following light induction result in increased HSC egress and blood cell differentiation in mice. CXCR4 receptor expression oscillations correspond with CXCL12 swings and have been linked to changes in HSC egress (Lucas et al. 2008). Mendez et al. reported a rhythmic *Cxcl12* oscillation in the bone marrow in an antiphastic manner compared to HSPC egression (Méndez-Ferrer et al. 2008) and proposed that *Cxcl12* expression is controlled by SP1 transcription factor in stromal cells by neural signals through β -adrenergic receptors (Méndez-Ferrer et al. 2008).

In addition, several studies also documented the expression of the core clock components in differentiated hematopoietic cells such as CD4+ T, B cells, macrophages and dendritic cells (Bollinger et al. 2011; Silver et al. 2012). Whether these circadian rhythms regulate their functional properties remains to be uncovered.

To conclude from this part, circadian rhythms seem to play key roles in regulating various aspects of hematopoiesis including self-renewal, differentiation and migration of HSPCs through a tight coupling between intrinsic and extrinsic clues. Importantly, it still remains to be clarified how the circadian clock affects HSC in a cell-autonomous manner as challenged by the observation that disruption of *Bmal1* in mice is dispensable for differentiation, proliferation and repopulating capacities of HSCs (Ieyasu et al. 2014) even though mutant mice completely lack the rhythmicity of circulating HSPCs (Stenzinger et al. 2019). On the contrary, in response to DNA damage and aging, *Per2* was reported to be a negative regulator of lymphoid-biased HSCs and immunological activities (Wang et al. 2016).

4.5 Clock gene dysregulation and cancer development

Normal circadian rhythms can be disrupted by genetic abnormalities that impair the operation of circadian clock components. Homologs of clock core clock components like *Per-1,-2,-3*, *Cry-1,-2*, *Rev-erb- α , - β* , *Ror- α , - β , - γ* are not expressed equally in all tissues. Instead, each homolog is expressed preferentially in certain tissues (Mure et al. 2018). As a result, mutations in any of these clock components are more likely to disturb normal circadian rhythm in specific tissues, which may explain malignancy occurrences.

Thus, disruption of circadian rhythms increases the risk of developing a number of chronic disorders (such as cancer and metabolic disorders) (Sulli et al. 2018a). The presence of a crosstalk between cancer and the circadian clock is backed up by a substantial amount of studies. In fact, tumorigenesis is a situation where the escape from circadian physiologic discipline is important. Here, the homeostasis of cells and tissues is taken advantage of hallmarks of cancer; including to fuel rapid and uncontrolled proliferation, to deal with increased

metabolic demands, to cause immune evasion, to promote resistance to apoptosis, and to create an inflammatory environment that supports tumor growth (Hanahan and Weinberg 2011). Cancer therapy may become more creative with the help of pharmacological modulation of circadian clock mechanisms.

4.5.1 Epidemiological links between the clock and cancer

Circadian disruption has been related to an increased incidence of some malignancies in several epidemiological studies, while the origins and mechanisms connected with this disruption are yet unknown. One of the well-studied aspects in this field is the correlation between cancer incidence and sleep disruption of many shift workers; it has revealed a link between chronic circadian rhythm disturbance and cancer in free-living individuals. While shift work is performed early morning, evening, or midnight hours and attempts to preserve a day-active social life on off-days, the frequent and recurring disturbance of daily patterns causes misalignment of internal and external circadian signals. Several epidemiological studies have evaluated the risk of developing cancer in shift workers, and most of these research demonstrate that a long time of shift work (more than 20 years) coincides with an increased risk of developing breast and prostate cancers (Salamanca-Fernández et al. 2018).

Correspondingly, another recent study revealed that long-term exposure to shift work increased the incidence of rectal cancer (Papantoniou et al. 2018). In response to the increased risk of cancer among shift workers, the International Agency for Research on Cancer (IARC), a division of the World Health Organization (WHO), classified circadian disruption as a possible human carcinogenic (Group 2A) in 2007 (Straif et al. 2007).

Beyond the classical shiftwork, many people in today's culture choose a lifestyle that resembles shiftwork, such as eating a massive portion of calories late at night or dividing daily caloric consumption across extended portions of the 24 h day. Epidemiological research on overnight fasting duration or supper time have discovered associations between day eating patterns and cancer risk. A prospective cohort study of 41,398 day-working people in the French

NutriNet-Sante discovered that 'late-eaters' (those who had eating sessions after 9:30PM) have a higher risk of breast and prostate cancer (Srouf et al. 2018). In the positive side, women who consume all of their calories in 11 hours or less, leaving 13 hours of nocturnal fasting, have a considerably decreased risk of breast cancer (Marinac et al. 2015). In 2018, another Spanish research discovered that eating supper before 9 p.m. linked with a considerably lower risk of prostate and breast cancer (Kogevinas et al. 2018).

In contrast, enforcing biological cycles may slow cancer growth and recurrence while improving quality of life. Cortisol rhythms, for example, are a survival predictor in lung and breast cancer (Sephton et al. 2000, 2013). Additional research indicates that individuals with metastatic colorectal cancer who have a strong rest/activity rhythm have better survival and quality of life than those who have irregular rest/activity patterns and poor sleep (Mormont et al. 2000; Innominato et al. 2018).

4.5.2 Environmental disruption of the circadian clock and cancer

Chronic jet lag has been used to replicate the environmental perturbation of the central/peripheral circadian axis in mice. For example, wild-type (WT) mice exposed to continuous jet lag have altered gene expression of the circadian repressors *Per2* and *Rev-Erba*, leading to accelerated development of Glasgow osteosarcoma (Filipski et al. 2004) along with a rising incidence of lymphoma and HCC (Lee et al. 2010). Although it remains unknown why circadian disruption affects certain cancers, comparable investigations have been carried out with a range of animals with clock gene mutations, particularly *Cry1/2^{-/-}*, *Per2^{-/-}*, or *Per1^{-/-};Per2^{m/m}*. When exposed to severe chronic jet lag, these mutant mice develop lymphoma, osteosarcoma, and HCC at a higher rate than WT mice (Lee et al. 2010). Even though these findings give experimental proof that genetic disruption of the clock causes increased carcinogenesis, there is no direct analog in human malignancies yet.

4.5.3 Circadian control of cancer metabolism

Considering that the clock is intricately engaged in modulating metabolism in peripheral tissues (Marcheva et al. 2013; Asher and Sassone-Corsi 2015) and that a large number of metabolites in the liver and serum are regulated in a cyclic pattern (Hatori et al. 2012; Abbondante et al. 2016), the convergence of cancer metabolism and its regulation by the circadian clock is an important topic of research. Ectopic expression of MYC was discovered to alter the transcription of circadian genes in an inducible system. MYC was discovered to activate the clock's negative transcriptional arm via REV-ERBa and boost metabolic sensing pathways, eventually resulting in higher consumption of glucose and glutamine (Altman et al. 2015). This particular example exemplifies another circumstance in which an oncoprotein impairs circadian rhythm, causing consequences on cellular metabolism. It's tempting to consider that changed metabolism might have an effect on cellular proliferation and clock function, contributing to tumor cells' imbalanced condition.

CLOCK, BMAL1, and MYC are indeed transcription factors with a basic helix-loop-helix protein domain in common (Ripperger and Schibler 2006). This enables these proteins to bind the same promoter element, the E-box sequence, within target gene regulatory regions. It is therefore truly astonishing that during carcinogenesis, a rewiring may occur in which the balancing of clock-controlled transcription is disrupted and compensated for by oncogenic MYC signaling. In 102 lymphoma patient samples, BMAL1 expression was discovered to be inversely associated to MYC (Shostak et al. 2016). These findings imply a possible balancing between the transcriptional pathways of the circadian clock and MYC. However, the functional importance of this possible transcriptional swing from traditional CLOCK:BMAL1 regulation to MYC-dependent signaling, and hence the scope of the prospective common gene expression network that governs metabolism in normal and malignant cells, remains unknown.

Oxygen is necessary for cellular respiration, and hypoxia has been related to cancer via metabolism and angiogenesis (Hanahan and Weinberg 2011). Furthermore, there is transcriptional interplay for both the clock and the transcription factor hypoxia-inducible factor

(HIF), which might be extended to carcinogenesis. According to recent research, the hypoxic response is regulated by the circadian clock, as the *Hif1a* promoter is strongly affected by CLOCK:BMAL1 (Wu et al. 2017). This transcriptional interaction was further confirmed by the evidence that genetic disruption of Bmal1 in C2C12 myotubes resulted in decreased HIF1a-dependent anaerobic glycolysis (Peek et al. 2017). These findings reveal an intriguing correlation between the clock system's transcriptional networks and HIF1a, and therefore this crosstalk may be implicated in the hypoxic response during cancer, an area that demands more exploration.

4.6 Roles of clock genes in Leukemia

In brief, clock genes have been shown to have a significant impact on cell cycle progression, and their dysfunctions cause abnormalities in normal cell activity, frequently driving continuous cell proliferation and blocking apoptosis. *PER1/2/3*, *BMAL1*, *CLOCK*, *REV-ERB α* , and *PPAR α* were all shown to be downregulated in all kinds of leukemia, implying involvement in leukemia formation. *NPAS2*, *CKI ϵ* , and *SHARP1* were elevated in leukemia, although their expression was restricted to one or two subtypes. Furthermore, *CRY1/2* and *TIM* genes revealed distinct levels of regulation in leukemic cells (**Figure 32**); consequently, further studies are required to demonstrate their involvement in leukemia.

As aforementioned, the *Period* genes are vital in regulating the negative expression of the circadian clock, and disruption in their expression will result in dysregulated clock-controlled gene regulation. These changes can promote malignant cell growth and survival, whereas abnormal cells do not undergo apoptosis (Borgs et al. 2009). PER1 and PER2 proteins, for example, have already been reported as tumor suppressors in several cancer cell cultures and animal studies (Gery et al. 2005, 2007; Hua et al. 2006; Liu et al. 2021). When compared with healthy controls, PERs were shown to be considerably downregulated in all AML, ALL, CML and CLL patients (Rana et al. 2014; Yang et al. 2015; Rahman et al. 2017; Wang et al. 2020). Indeed, Rana et al. discovered an increase in *Myc* and *cyclin D1* expression in CLL patients

versus controls (Rana et al. 2014). These findings are consistent with the pathophysiology of the *PER* genes that has previously been mentioned in other types of cancer, where decreased *PER* expression appears to be associated with increased aggressiveness and a worse prognosis, suggesting that the same function likely occurs in leukemia (Karantanos et al. 2014; Hasakova et al. 2018). Yang et al. found that patients with AML or ALL only exhibited increased levels of *PER1* and *PER3* during an end of their treatment, respectively (Yang et al. 2015), suggesting that this upregulation of the *Period* genes may also take an important role in tumor suppression in leukemia, as has been shown in other different cancers (Hua et al. 2006; Gery et al. 2007; Liu et al. 2021). As a result, the *PER* genes could serve as a prospective biomarker for the development of cancer when *PER* levels are low, as well as a therapeutic response in leukemia therapy when their levels are enhanced.

Cryptochrome genes, in contrast to *PER* genes, showed disparate findings. Even though *CRY1* and *CRY2* were shown to be down-regulated in the majority of leukemia patients, certain instances revealed elevated expression of these genes (Yang et al. 2015; Rahman et al. 2017; Habashy et al. 2018). Habashy et al. found variable levels of *CRY1* expression in CLL patients, with 54 showing down-regulation and 40 showing up-regulation (Habashy et al. 2018). Furthermore, Rahman et al. discovered an increase in *CRY2* expression in patients with AML relapse after 3 months of chemotherapy treatment (Rahman et al. 2017). It is crucial to note that *CRY1* and *CRY2* have independent activities outside of the circadian clock, therefore, they must be investigated separately (Papp et al. 2015). *CRY1* has notably been revealed to play a crucial role in cell cycle control via the DNA-damage response. It is plausible that dysregulation of *CRY1* expression, whether high or low, might contribute to leukemia development by many pathways, but further research is needed to confirm the role of this gene in the cell cycle. Decreased *CRY1* expression was linked to increased levels of pro-inflammatory cytokines such Interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF- α), which can regulate pro-survival or pro-death signaling. Polymorphisms in the TNF- α promoter have been identified in leukemia, with TNF- α -308 G/A polymorphism being related with a greater risk of mortality in ALL and CLL

patients (Zhou et al. 2017b; Binder et al. 2018). *CRY2* deficiency in mouse fibroblasts results in increased expression of *c-Myc*, resulting in enhanced cell proliferation, implying its role to tumor prevention via *c-Myc* turnover (Huber et al. 2016). Furthermore, Chan et al. demonstrated that *CRY2* mutations suppressed *p53* and facilitated for cell growth (Chan et al. 2021). Thus, various amounts of *CRY1* and *CRY2* expression appear to affect leukemic cells via diverse routes, indicating the need for additional research to determine their use as a leukemic biomarker.

Both the *BMAL1* and *CLOCK* genes have been found to be downregulated in all types of leukemia (Rana et al. 2014; Yang et al. 2015; Rahman et al. 2017). The transcription of the clock-controlled gene *Wee1* is activated by the increased expression of CLOCK:BMAL1, which binds to its E-box region (Borgs et al. 2009). As a result, the derived proteins would then phosphorylate CDK1/cyclin B, inhibiting the cell cycle's progression from the G2 to the M phases (Zeng et al. 2010). Due to the low levels of *BMAL1* and *CLOCK* expression identified in the studies, it is probable that this uncontrolled mechanism contributes in the accelerated cell growth of leukemic cells, as has been shown in other kinds of cancer (Taniguchi et al. 2009; Papagiannakopoulos et al. 2016). Rana et al. found lower levels of *Wee1* expression in CLL patients versus controls, lending support to this assumption (Rana et al. 2014). As a matter of fact, down-regulation of CLOCK and BMAL1 might be applied as a biomarker in leukemia to improve in cancer diagnosis; similarly, increase of its expression might be used to track therapeutic response. NPAS2, a functional analog of CLOCK, contributes in the expression of clock genes. Song et al. discovered that NPAS2 was up-regulated in AML patients, and that NPAS2 knockdown in AML cells resulted in inhibition at the G1 and G2 phases, together with apoptosis in leukemic cells, implying a role for this gene in the development of leukemia and exhibiting its potential role as a biomarker of the disease (Song et al. 2018). For the epigenetic landscape, in 126 samples of various hematologic malignancies, *Bmal1* gene silencing because of CpG promoter methylation was detected in 19.7 percent of diffuse large B-cell lymphomas, 33.3 percent of ALL, and 19.2 percent of AML (Taniguchi et al. 2009).

Only one study showed *REV-ERB α* and *PPAR α* expression levels in leukemia (Rahman et al. 2017). In contrast to the reports in the literature importance of REV-ERBs in the negative control of *BMAL1*, Rahman et al. found that both clock genes were down-regulated, indicating that *BMAL1* expression levels are not regulated by REV-ERB α and PPAR α in leukemia (Rahman et al. 2017). They have also been described as having the potential to regulate *p21* expression (Gréchez-Cassiau et al. 2008). As a consequence of the limited evidence, the relevance of *REV-ERB α* and *PPAR α* as leukemia biomarkers is unclear, and further studies are necessitated.

Yang et al. examined the levels of the TIMELESS gene in two leukemia; it was down-regulated in AML patients but up-regulated in ALL patients (Yang et al. 2015). This difference might be attributed to the unique pathophysiology of AML and ALL, where TIM may have specific mechanisms in each subtype. Downregulation of *TIM* would prevent Checkpoint kinase 1 (Chk1) and Checkpoint kinase 2 (Chk2) activation, allowing the cell to continue proliferating despite DNA damage (Engelen et al. 2013). Thus, suppressing *TIM* produced cell cycle arrest in the G0 and G1 stages of glioma cells. In any case, additional research is needed to confirm the truth of these theories before we can assess their function as leukemia biomarkers (Wang et al. 2021b).

CKI ϵ is also in charge of phosphorylating PERs in order to regulate protein levels in the cell (Eide et al. 2005). As a result, high amounts of CKI ϵ induce substantial PER degradation, which raises the levels of cyclin D1 and cyclin E in malignant cells, encouraging their proliferation (Yang et al. 2009). *CKI ϵ* was shown to be increased in AML and ALL patients compared to healthy persons (Yang et al. 2015), suggesting that it may have the same oncogenic role as other cancers.

The expression of CLOCK:BMAL1 heterodimer can be negatively modulated by SHARP1 (Nakashima et al. 2008). Numata et al. found *SHARP1* up-regulation in MLL-AF6 AML (Numata et al. 2018). Together with prior findings indicating that *CLOCK* and *BMAL1* were down-regulated, it is probable that *SHARP1* up-regulation is also implicated in this repression mechanism, implying an oncogenic involvement in leukemia.

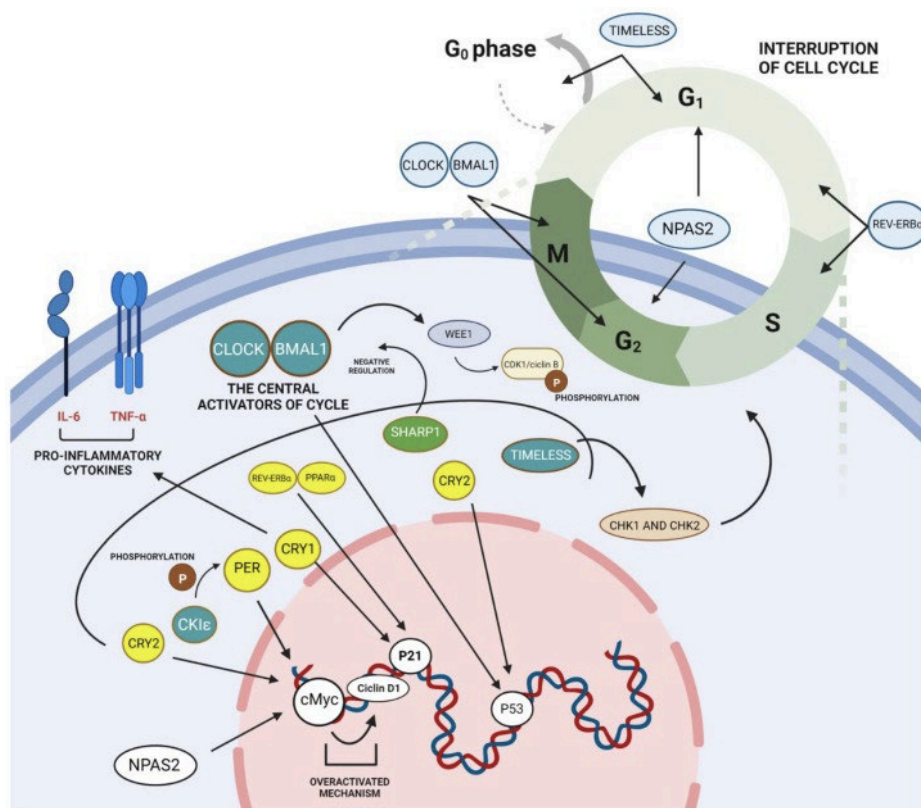


Figure 32: Role of clock genes in leukemia (Sanford et al. 2022)

4.7 Relevance of chronotherapy for cancer

Despite the rarity of clock gene mutations in malignancies, analysis of survival trends in breast cancer revealed that individuals with mutations in all of the core clock genes or all of the PERs have a worse survival rate than those with no abnormalities (Ye et al. 2018). Furthermore, gene expression dataset analyses [The Cancer Genome Atlas (TCGA) and the NCBI Gene Expression Omnibus (GEO)] demonstrate that the relative expression profile of clock genes differs considerably from the pattern found in non-tumor samples (Wang et al. 2016). Researchers discovered significant discrepancies in circadian gene expression patterns in HCC and normal liver tissues using an enhanced algorithm (CYCLOPS) that can sequentially arrange gene expression datasets. Such results demonstrate that in cancer, a constant percentage of rhythmic transcripts (almost 15 percent, including PER1 and CRY1) do not exhibit circadian oscillations (Anafi et al. 2017). Similarly, gene expression studies in metastatic melanoma indicate that circadian oscillations are reduced when compared to normal skin (de Assis et al.

2018). These findings suggest that changes in circadian gene expression are common in several tumor types. Such variations in clock gene expression in malignancies could be functional. In summary, genetic and genomic investigations of cancers have revealed that alterations in clock gene expression are more common than clock gene mutations. This led to the concept that oncogenic programs might disturb circadian control, hence fueling tumor development.

4.7.1 Timing of therapy

The numerous links between clock dysregulation and cancer presented here raise the topic of whether the circadian therapeutic intervention (such as time of day) should be investigated. Chronotherapy is an approach that is administered at a specified time to enhance therapeutic response while minimizing negative effects. The majority of cancer chemotherapies target rapidly reproducing cellular populations. Given the link between the circadian clock, cell proliferation, and DNA damage repair, it was predicted that timing the administration of chemotherapeutic drugs to coincide with circadian rhythms would enhance therapeutic efficacy while minimizing toxicities to non-cancerous cells. This 'chronotherapy' has lately been thoroughly studied, and it strongly supports the hypothesis that metabolic and xenobiotic detoxification enzymes display temporal peaks in activity and could thus be pharmacologically targeted depending on the ideal time of day (Dallmann et al. 2016). Indeed, multiple randomized controlled trials investigating the precise timing of therapy for breast, colorectal, and endometrial cancer found fewer side effects such as mucositis, stomatitis, and leukopenia (Lévi et al. 1997; Gallion et al. 2003; Coudert et al. 2008). Two randomized controlled studies for colorectal cancer found that the chronotherapy group had a higher response rate and less toxicity (Lévi et al. 1994, 1997). However, in 2016, just 0.16 percent of clinical studies considered the time of medication delivery (Selfridge et al. 2016). The recent developments in "-omics" technology, machine learning algorithms, programmable pumps for drug delivery, wearable devices, and

blood tests to easily assess real-time circadian rhythms may present new opportunities for the integration of chronotherapy into precision medicine.

4.7.2 Clock drugs

Maintaining a healthy circadian rhythm through regular daily behavioral patterns in sleep and eating may lower the risk of cancer considerably (Erren et al. 2016; Sulli et al. 2018a). The timing of medications in relation to the patient's circadian cycle has the ability to lessen unpleasant side effects and expedite prognosis (Ballesta et al. 2017). There is indeed a developing strategy for treating cancer with medications that directly or indirectly target circadian clock components. In brief, they are called as "drugging the clock" method and the substances that target clock components are "clock drugs". Clock drugs, which directly target circadian clock components such as ROR γ , REV-ERBs, and CRYs, have recently been generated. The discovery that metastatic castrate-resistant PC exhibit high amounts of ROR γ and that ROR γ stimulates androgen receptor (AR) expression encouraged researchers to investigate if ROR γ inhibition might influence prostate cancer (Wang et al. 2016). Notably, *in vivo* injection of ROR γ antagonists inhibits the development of AR-expressing tumors and restores resistance to enzalutamide (an androgen signaling inhibitor presently used to treat prostate cancer) (Wang et al. 2016). Moreover, in numerous pancreatic cancer *in vivo* models, pharmacological suppression of ROR γ also reduces tumor development and improves survival (Lytle et al. 2019).

Several REV-ERBs agonists have been explored in numerous studies to have anticancer action against various tumor types (Grant et al. 2010; Solt et al. 2012; Puram et al. 2016; Sulli et al. 2018b; Wang et al. 2018). Pharmacological stimulation of REV-ERBs suppresses autophagy and lipogenesis in cancer. Importantly, the anticancer effect of REV-ERB agonists is reduced when Rev-erb is downregulated, indicating that REV-ERBs are prospective targets for cancer therapy. These findings show how targeting circadian core clock genes might lead to combination treatment. Another well-studied family of molecules is CRY protein modulators. *In vitro* studies suggest that pharmacological suppression of CRY

decreases cancer cell growth while having little effect on normal epithelial breast lines (Chun et al. 2015).

For immunotherapy, the use of clock-drugs on IL17A-expressing T-cells to boost CAR T-cell treatment is one promising strategy. ROR γ t functions as a master transcription factor in Type 17 T-cells. The addition of a ROR γ t agonist during ex-vivo growth improves the anticancer efficacy of CAR-engineered Th17 cells, and mice with ROR γ t-primed T-cells are guarded against cancer when cancerous cells are reintroduced into the animal (Hu et al. 2016, 2018). In vivo, ROR γ agonists can also be used as monotherapy. Interestingly, ROR γ agonists have anticancer effects due to the activation of numerous tumor suppressive pathways, including increasing Th17 cell activity and inhibiting Treg-driven immunosuppression (Hu et al. 2016). ROR γ treatment increases the synthesis of IL-17A, GM-CSF, and the co-stimulatory receptors CD137 and CD226, increasing T-cell proliferation and survival. Furthermore, ROR γ agonists influence immunosuppression by suppressing the expression of co-inhibitory receptors PD-1 and TIGIT, as well as by reducing Treg activity via downregulation of CD39 and CD73 expression (Hu et al. 2016). An improved version of ROR γ agonist is currently in clinical trials as a single agent (clinical trial NCT0292862) or in pairing with pembrolizumab (anti PD-1, clinical trial NCT03396497) for the therapy in patients with advanced, relapsed, or refractory solid tumors, with no major negative clinical manifestations in Phase I.

The increasing link between both the circadian clock and cancer provides a distinctive opportunity for cancer prevention and opens up new avenues for advancements in lifestyle intervention, genetic testing, diagnostic markers, chronotherapy, and, lastly, novel therapies targeting clock components. However, further research is required to address a number of critical concerns; including *are changes in core clock genes cause cancer due to their influence on circadian rhythms? is there a tumor kind or stage where disturbance of the circadian rhythm is no longer a major bottleneck? what are the unforeseen consequences of current tool chemicals that target particular circadian clock components?*

OBJECTIVES OF THE THESIS

The objectives of the work presented in this thesis are as follows:

1. To evaluate the expression of *miR-495-3p* in CML and to study its role in leukemic cell growth
2. To investigate the potential role of miR-495-3p in CML TKI chemo-sensitivity and to determine its underlying effect on MDR1-mediated drug efflux
3. To analyze the impact of BCR::ABL1 on the deregulation of core clock genes in CML

PAPER 1 – Summary

MIR-495-3P SENSITIZES BCR::ABL1 EXPRESSING LEUKEMIC CELLS TO TYROSINE KINASE INHIBITORS BY TARGETING MULTIDRUG RESISTANCE 1 GENE INCLUDING IN T315I MUTATED CELLS

Since 2002, the introduction of Imatinib to the clinical practice changed the natural history of the disease. The development of Imatinib and following tyrosine kinase inhibitors (TKIs) has effectively improved the survival of CML patients, resulting in a normal life expectancy in patients responding to these therapies. Nonetheless, despite significant advances in CML therapy over the last decades with the advent of TKIs, one patient out of four (20 to 30 percent) still develops resistance over the course of the treatment due to either intolerance or resistance. Several mechanisms leading to these biological processes have been reported, including amplification or mutations of *BCR::ABL1* oncogene. One of the major concerns is T315I mutation since it confers resistance to all TKI approved to frontline while being only sensitive to Ponatinib. Another key mechanism for TKI resistance is highly dependent on the accessibility of the drug to reach BCR::ABL1 and the balance between drug influx and drug efflux. The alteration of MDR1 protein (P-gp, or ABCB1), an efflux pump of all TKIs approved for CML, results in an initiator process that favors the development of another BCR::ABL1-independent resistant mechanism.

Recent experimental data suggest an involvement of the *miR-495-3p* in chemo-resistance in several cancers. It was shown to directly targets 3'UTR of *ABCB1/MDR1 gene* and downregulated in drug-resistant cell lines and patients. In CML, Xu et al. discovered that drug-resistant CML K562 cell line expressed lower levels of *miR-495-3p* and that transfecting those cells with *miR-495-3p* mimics restores their susceptibility to Adriamycin chemotherapeutic agent. However, it is unknown how *miR-495-3p* expression varies between CML and healthy controls and how this varies affects MDR1-mediated drug efflux, which results in TKI resistance in CML.

In this work, our study was aimed at investigating the potential role of miR-495-3p in CML TKI chemo-sensitivity and determining the underlying molecular circuitry involved. We first used various in vitro CML cellular models to address a possible link between the expression of *miR-495-3p*, *MDR1* and *BCR::ABL1*. Gene expression levels were quantified by quantitative real-time PCR (RT-qPCR) in UT7, UT-7/11 and KCL22 cell lines. We next addressed the role of miR-495-3p in TKI resistance using complementary gain- and loss-of-function experiments in UT-7/11 cell line. The phenotypic consequences were then analyzed according to several parameters including cell proliferation by trypan blue exclusion, cell cycle by Hoechst 33342 staining and cell viability using XTT assay. The drug efflux activity of MDR1 was assessed by Rhodamine 6G efflux assay.

In order to further investigate a clinically relevant role of miR-495-3p in CML patients, we first determined using bioinformatics tools a set of putative miR-495-3p target genes and then compared their expression using transcriptome data obtained from CML patients at various phases of the disease (GSE4170) and from TCGA AML cohort.

We first noticed that *miR-495-3p* expression was down-regulated in *BCR::ABL1* expressing cellular models *in vitro*. Loss-of-function experiments revealed that the inhibition of miR-495-3p stimulated cell proliferation associated with a decreased number of non-dividing cells (G0/G1) and increased the resistance to Imatinib. Conversely, overexpression of *miR-495-3p* impairs leukemic cell growth and Imatinib resistance in leukemic cells expressing *BCR::ABL1*. This effect was demonstrated even in Ponatinib resistance in the Imatinib-resistant T315I-mutant variant. At the functional level, we further demonstrated that miR-495-3p targets MDR1 which ultimately modulates drug efflux activity.

Based on patient data analysis, we found that predicted miR-495-3p targets encode proteins were upregulated in patients in BP, involved in protein phosphorylation and might have important clinical relevance in leukemic pathogenesis and drug resistance mechanism. We also found that the up-regulation of miRNA-495-3p target gene expression is associated with a worse prognosis in the TCGA AML patient cohort. Overall, our results demonstrate that the down-

regulation of *miR-495-3p* expression is involved in the malignant phenotype of BCR::ABL1 cells. Its up-regulation could overcome TKI resistance of CML by targeting drug efflux activity of MDR1 and drug resistance mechanism. We propose that miR-495-3p could be used as a useful biomarker and a prospective therapeutic target for CML chemoresistance.

IN SUBMISSION EXPERIMENTAL HEMATOLOGY

miR-495-3p Sensitizes BCR::ABL1 Expressing Leukemic cells to Tyrosine Kinase Inhibitors by Targeting Multidrug Resistance 1 Gene including in T315I Mutated cells

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HIGHLIGHTS

- miR-495-3p inhibits leukemic cell growth and is downregulated in BCR::ABL1 expressing cell lines
- miR-495-3p modulates response to TKI treatment including in UT7 cells expressing T315I
- Overexpression of miR-495-3p leads to a decrease of *MDR1* and drug efflux activity
- Bioinformatics analyses reveal that MiR-495-3p target genes are upregulated in blast crisis

ABSTRACT

Chronic myeloid leukemia (CML) is a clonal hematopoietic malignancy driven by the BCR::ABL1 fusion oncoprotein. The development of tyrosine kinase inhibitors (TKIs) has deeply increased long-term survival of CML patients. Nonetheless, one patient out of four will switch TKI off owing either to drug intolerance or resistance partly due to amplification or mutations of *BCR::ABL1* oncogene and alteration of ATP-binding cassette (ABC) transporters. Increasing evidence suggests an involvement of the microRNA miR-495-3p in cancer-associated chemo-resistance through *multidrug resistance 1 (MDR1)* gene which encodes an ATP-dependent efflux pump. Our study aimed at investigating the potential role of miR-495-3p in CML TKI chemo-sensitivity and determining the underlying molecular circuitry involved. We first observed that *miR-495-3p* expression was lower in BCR::ABL1 expressing cellular models *in vitro*. Notably, loss-of-function experiments showed increased proliferation associated with a decreased number of non-dividing cells (G0/G1) and resistance to Imatinib. Conversely, our data showed that *miR-495-3p* overexpression hindered leukemic cell growth and TKI resistance even in Imatinib-resistant T315I-mutant cells as well as drug efflux activity through *MDR1* regulation. To further investigate the role of miR-495-3p in CML patients, we found that predicted miR-495-3p targets were upregulated in patients in blast crisis involved in protein phosphorylation and associated with the worst prognosis. Taken together, our results demonstrate that down-regulation of *miR-495-3p* expression is important in the malignant phenotype of CML and TKI resistance mechanisms, which could be a useful biomarker and a potential therapeutic target to eradicate CML.

MeSH terms:

- ATP Binding Cassette Transporter, Subfamily B
- ATP Binding Cassette Transporter, Subfamily B, Member 1 / drug effects
- ATP Binding Cassette Transporter, Subfamily B, Member 1 / metabolism*
- Blast Crisis / pathology
- Cell Line, Tumor
- Cell Proliferation / drug effects*
- Cell Survival / drug effects
- Drug Resistance
- Genes, MDR
- Imatinib Mesylate
- Leukemia, Myelogenous, Chronic, BCR-ABL Positive
- MicroRNAs / genetics
- MicroRNAs / physiology*

INTRODUCTION

Chronic myeloid leukemia (CML) is a clonal malignant myeloproliferative neoplasm characterized by the uncontrolled proliferation of myeloid cells. Primarily, BCR::ABL1 acts as a constitutively active tyrosine kinase that alters cell proliferation, differentiation, survival, and genetic stability in hematopoietic cells in which it is expressed [1,2].

CML therapy has greatly benefited from the fact that BCR::ABL1 expressing cells exhibit the oncogene-dependence phenomenon such that BCR::ABL1 inhibition has been found to lead to the inhibition of leukemic cell growth [3]. Imatinib (IM) treatment has been the first TKI licensed for clinical use after its validation in preclinical experimental models and the first clinical trial IRIS comparing IM to the standard therapy IFN-ARA-C [4]. It has improved deep molecular responses in more than 20-30% of newly diagnosed chronic CML patients [5]. However, several works have now shown that CML stem cells are resistant to TKI, due to their quiescence and their oncogene independence [6–8]. Moreover, responses to TKI are altered by either intolerance or by the occurrence of several resistance phenomena which have been explored in detail, including decreased OCT1 expression responsible for the entry of IM into the cells and increased expression of MDR1 [9]. The resistance is also due in 10-20% of cases to mutations in the ABL-kinase domain which impair the accessibility of TKI drugs to the ATP pocket either by direct interaction or by conformational changes. The development of resistance to TKI treatment which occurs in CML patients severely impairs the clinical application but led to the development of novel TKI of the second generation [10–12] and more recently, the development of the Asciminib, able to inhibit BCR::ABL1 TKI activity by binding to the myristoyl binding pocket [13].

The 'gatekeeper mutation' T315I mutation in the Imatinib-binding site is the most common mutation amongst BCR::ABL1-dependent resistance, identified in 4–15 percent of patients with IM resistance, and is a major concern because it renders leukemic cells resistant to all licensed TKI except Ponatinib [14]. Meanwhile, TKI resistance can also occur independently of BCR::ABL1. The activity of drug transporters is one of the mechanisms that

has been identified as mediating TKI resistance [15,16]. The P-glycoprotein (P-gp or MDR1 protein), an ATP-binding Cassette (ABC) efflux transporter encoded by the human multidrug-resistant *ABCB1/MDR1* gene [17], encodes key IM efflux pumps and has been proposed as the main drug resistance mediator in CML [9,18]. Failure modes of TKI monotherapies have prompted the exploration of the mechanisms regulating drug resistance. On that account, multiple microRNAs have been implicated in the drug resistance of CML [19,20].

MicroRNAs are noncoding, single-stranded RNAs with 18 to 25 nucleotides that act as epigenetic negative regulators of specific gene targets at the post-transcriptional and translational levels by binding to the 3'-untranslated region (3'-UTR) of targeted mRNAs; causing in translational repression and/or mRNA degradation. Over recent years, the dysregulation of microRNA expression has been increasingly considered a regulator involved in the progression and the chemoresistance of various cancer types, including CML [21,22]. *microRNA-495-3p* (*miR-495-3p*) is a member of the 14q32.31 microRNA cluster. Accumulating evidence has linked *miR-495-3p* deregulation to cancer progression [23]. *miR-495-3p* was shown to directly regulate MDR1 translation, which reduces drug efflux from cancer cells [24]. Notably, Xu et al. discovered that drug-resistant CML K562 cell line expressed lower levels of *miR-495-3p* and that transfecting those cells with *miR-495-3p* mimics restores their susceptibility to Adriamycin chemotherapeutic agent [25]. Still, the exact role of *miR-495-3p* in drug resistance to TKIs in CML remains unknown.

In this study, we evaluated the expression of *miR-495-3p* expression in leukemic cell models expressing BCR::ABL1 either constitutively or in a DOX-inducible context. We have found that there is a BCR::ABL1-induced down-regulation of *miR-495-3p* allowing us to investigate the role of this phenomenon in TKI resistance including in cells expressing T3151.

MATERIAL AND METHODS

Cell culture and generation of stable cell lines

The UT-7 which is a GM-CSF dependent human hematopoietic cell line [26] and the KCL22 Ph1 chromosome-positive CML cell line [27] were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Rockville, MD, USA) [containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 10 ng/mL granulocyte/macrophage colony-stimulating factor (GM-CSF) for UT-7] under an atmospheric condition of 5% CO₂ at 37 °C. BCR::ABL1 positive variants of UT-7 cell line were obtained from previous studies including UT7-11 expressing wild-type BCR::ABL1 [28], UT-7 BCR::ABL1^{ind} expressing TET-OFF-inducible BCR::ABL1 [29] and UT7-T315I expressing T315I-mutated version of BCR::ABL1 [30]; which were cultured under the same conditions as mentioned above. The medium was subsequently replaced with fresh medium every 2 days.

For gain- and loss-of function studies, we used the following vectors: miR-495-3p (HmiR0226-MR03), AntagomiR-495-3p (MmiR-AN0537-AM04), and control vectors (CmiR0001-MR03 and CmiR-AN0001-AM04, respectively) all from GeneCopoeia (Rockville, MD, USA). Lentiviral particles were produced and used for transduction at a MOI of 1. Stable cells expressing fluorescent reporter proteins (mCherry or eGFP) were sorted using BD FACSAria Flow Cytometer (BD Bioscience, Mountain View, CA, USA).

RNA extraction and quantitative real-time PCR

Total RNAs were extracted using the PureLink® RNA Mini Kit (Invitrogen, Carlsbad, CA, USA) and used for reverse transcription using SuperScript™ III Reverse Transcriptase (Invitrogen) with random primers or a universal stem-loop primer for microRNA detection as described in [31]. Real-time PCR was carried out on Agilent Mx3005P QPCR System (Agilent Technologies, Santa Clara, CA, USA) with the FastStart Universal SYBR Green Master (Roche, Mannheim, Germany). Primers sequences were: *miR-495-3p*, F: 5'-GCCAAACAAACATGGTGCCTTC-3', R: 5'-CGAGGAAGAAGACGGAAGAAT-3'; *U6*, F: 5'- GCTTCGGCAGCACATATACTAAAT-3',

R: 5'-CGAGGAAGAAGACGGAAGAAT-3'; *MDR1*, F: 5'-CCCATCATTGCAATAGCAGG-3', R: 5'-GTTCAAACCTTCTGCTCCTGA-3'; *B2M*, F:5'-TGCTGTCTCCATGTTTGATGTATCT-3', R: 5'-TCTCTGCTCCCCACCTCTAAGT-3'.

Cell proliferation, colony formation (CFC), cell cycle, and cell viability assays

For cell proliferation assays, 5×10^3 UT7 cells were plated per well of a 96-well plate and counted every two days (in three replicates) using trypan blue exclusion.

For clonogenic assays of UT7 cells and their BCR::ABL expressing counterparts, 500 cells were seeded in 35-mm dish in methylcellulose media (H4435, Stem Cell Technologies, Vancouver, Canada). Colonies were photographed and scored 14 days later using ImageJ software (LOCI, University of Wisconsin, Madison, USA).

Cell cycle analysis was performed using Hoechst 33342 and BD LSR Fortessa Flow Cytometer (BD Bioscience). Data were analyzed using FlowJo v10.1 software (FlowJo LLC, Ashland, OR, USA).

Cell viability was determined using CyQUANT™ XTT Cell Viability Assay (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. 5×10^3 cells were plated per well of a 96-well and cultured with or without 1 μ M Imatinib or 100 nM Ponatinib for 48 h. After 4h staining with XTT mixture, absorbances were measured using a Modulus™ II Microplate Reader (Turner BioSystems, Sunnyvale, CA, USA) at 450 and 600 nm.

MDR1-mediated drug efflux activity assay

Drug efflux activity was determined by intracellular accumulation of Rhodamine 6G (Rho6G) (Sigma-Aldrich, France). Cells were cultured with 10 μ M Rho6G for 30 min at 37 °C in the dark, washed 3 times with ice-cold media, transferred to fresh pre-warmed Rho6G-free media, and subsequently cultured at 37 °C. A similar amount of cells was collected in ice-cold media every 5 min for time-course experiments. Cells were maintained on ice and fluorescence imaging and intensity measurements were performed using a Modulus™ II Microplate Reader at 450 nm. The interference of GFP expression was normalized by unstained samples.

Statistical analysis

All the experiments were repeated three times independently. All data were analyzed with GraphPad Prism 8.4.3 (GraphPad Software, San Diego, CA, USA) and represented as the mean \pm standard deviation (SD). The differences between two groups were evaluated using unpaired two-tailed Student's t-tests. Differences for multiple comparisons were subjected to one-way analysis of variance followed by Dunnett's test. A p-value of less than 0.05 was considered to be statistically significant.

CML transcriptome dataset

Normalized matrix of Transcriptome dataset GSE4170 [32] was downloaded at the following address: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE4170> (accessed on 2022, April 22nd) and annotated with GPL2029 Rosetta/Merck Human 25k v2.2.1 microarray technology platform available at the address: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL2029> (accessed on 2022, April 22nd). The resulting gene annotated matrix was used for downstream bioinformatics analyses.

RNA-sequencing TCGA AML cohort

Through the Cbioportal web application tool [33], clinical and RNA-sequencing Z-score V2 data were aggregated for all diploid samples inside the TCGA AML cohort [34]. These data concerning experiments were performed on 173 *de novo* AML samples. Among this AML cohort, 46 percent of patients present a normal karyotype, and 12 percent of them present a complex karyotype. Age median diagnosis was 57 years old for this AML cohort.

Transcriptome analyses

Prediction of mRNA targets of hsa-miRNA-495-3p was done in two distinct databases: miRDB [35] and targetscan [36]. Inner join between was done to build a Venn diagram and common target lists obtained from the two databases were retained to perform transcriptome analyses in the GSE4710 dataset. Bioinformatics analyses were performed in R software environment

version 4.1.3. Unsupervised principal component analysis was done with “prcomp” base function and output plot was drawn with “autoplot function” of ggfortify R-package version 0.4.14. Differential expressed gene analysis was done with a linear model for microarray algorithm implemented in limma R-package version 3.48.3 [37]. Expression heatmap with unsupervised hierarchical clustering was done with pheatmap R-package version 1.0.12 based on Euclidean distances and Ward.D2 method. Protein-protein interaction network was built with online proteomic tool NetworkAnalyst [38] with an inference of Gene Ontology Biological Process (GO-BP) database [39].

RESULTS

***miR-495-3p* is downregulated in BCR::ABL-expressing cell lines and regulates cell proliferation and IM resistance**

To investigate a possible role of *miR-495-3p* in CML, we first analyzed *miR-495-3p* expression in various cell lines including KCL22, BCR::ABL1-expressing UT-7/11 and the parental UT-7. Interestingly, compared to UT-7, *miR-495-3p* expression was decreased in KCL22 and in UT-7/11 (Figure 1A). To confirm this phenomenon, we used our TET-OFF inducible system UT/7 cell line where BCR::ABL1 is expressed in the absence of doxycycline. In these cells, *miR-495-3p* expression was also decreased in the absence of doxycycline and so when BCR::ABL1 was expressed (Figure 1B). Taken together, *miR-495-3p* expression appears to be inversely correlated to BCR::ABL1 expression at the transcriptional level, suggesting a possible role of *miR-495-3p* in BCR::ABL-induced leukemogenesis.

To test this hypothesis, we developed a loss-of-function strategy using an AntagomiR-495-3p. We first validated its functionality by confirming that AntagomiR-495 reduces *miR-495-3p* expression contrary to the control Antagomir-NC (Figure 1C). Strikingly, we observed that *miR-495-3p* inhibition markedly promoted UT7/11 cell proliferation compared to Antagomir-NC (Figure 1D) which was associated with a decrease in the proportion of cells in G0/G1 phase but an increase in G2/M phase (Figure 1E, 1F). We next tested whether *miR-495-3p* inhibition could also affect the sensitivity of CML cells to TKI treatment. To do this, we measured the ability of UT7/11 cells expressing AntagomiR-NC or AntagomiR-495 to form colonies in the presence of Imatinib. We first observed that IM treatment impaired colony formation in all cell lines (Figure 1G, 1H). Surprisingly, its toxic effect was strongly reduced in the presence of AntagomiR-495. Collectively, these results demonstrate that *miR-495-3p* knockdown promotes cell proliferation and IM resistance in BCR::ABL1 expressing cells.

Overexpression of *miR-495-3p* decreases leukemia cell proliferation but increases IM sensitivity

To further characterize its role, we undertook gain-of-function experiments in which *miR-495-3p* was overexpressed in UT-7/11 following lentiviral transduction (Figure 2A). We noticed that miR-495-UT-7/11 cells exhibited a reduced proliferation capacity associated with an increase in the proportion of cells in G0/G1 and a reduction in G2/M phase (Figure 2B, 2C). Importantly, these phenotypes were not observed in NC-UT-7/11 cells expressing a control scrambled sequence. We next tested the impact of *miR-495-3p* overexpression on IM sensitivity. As expected, IM strongly decreased UT-7/11 and NC-UT-7/11 cell viability (Figure 2D). Cell viability was even more reduced in miR-495-UT-7/11 cells indicating that miR-495-3p overexpression enhances cell sensitivity to TKI treatment. To further validate this observation, we analyzed the effect of IM treatment in clonogenic assays using UT/7 cells. In all cases, IM treatment reduced colony formation but this reduction was exacerbated in miR-495-UT-7/11 compared to UT-7/11 and NC-UT-7/11 cells (Figure 2E, 2F). Taken together, complementary gain- and loss-of-function experiments demonstrate that *miR-495-3p* is a critical negative regulator of cell cycle progression and IM resistance.

***miR-495-3p* negatively regulates the expression and efflux activity of MDR1**

miR-495-3p plays multiple roles in physiological and pathological situations including cell proliferation and drug resistance in multiple cancers [24]. Particularly, according to the DIANA-microT database [40], *miR-495-3p* is predicted to bind to 2 binding sites localized in the 3'-UTR of *MDR1* with a high microRNA targeted genes (miTG) score of 0.7528 (Figure 3A). To test whether the regulation of IM resistance by *miR-495-3p* is mediated by MDR1, we first quantified *MDR1* expression and observed a significantly lower expression in miR-495-3p overexpressing cells compared to UT-7/11 cells (Figure 3B). These data suggested that miR-495-3p might regulate *MDR1* expression in BCR::ABL1 expressing cells.

Next, we analyzed MDR1 activity using a Rhodamine 6G (Rho6G) efflux assay. After a pulse of Rho6G, which is a substrate of MDR1 transporter, we monitored the dye fluorescence

dynamics as a measure of the efflux activity in miR-495-3p-overexpressed UT-7/11 cells. We observed that the fluorescence activity was reduced by almost 90% after a chase of 15 to 20 minutes in UT-7/11 and NC-UT-7/11 but remains significantly higher after 15 minutes in *miR-495-3p* overexpressing cells (Figure 3C, 3D). These results suggested that *MDR1* expression might be linked to BCR::ABL1-associated leukemogenesis and TKI resistance as this has previously been reported in primary CML [9,16]. Thus, we analyzed *MDR1* expression in bone marrow samples of patients in chronic (CP), accelerated (AP), and blast crisis (BC) phases (Figure 3E). Interestingly, *MDR1* expression was upregulated in BC compared to CP and AP. Altogether, we propose that miR-495-3p is a negative regulator of IM resistance by suppressing *MDR1* expression.

Overexpression of *miR-495-3p* suppresses the drug resistance phenotype in Imatinib-resistant CML cell line

Having established the role of miR-495-3p in the regulation of IM resistance, we next tested whether this role could be extended to other TKIs in a drug resistance context. We used the UT7-T315I cell line which expresses the T315I-mutated version of BCR::ABL1 and is resistant to most of the TKIs except Ponatinib [30]. We generated UT7-T315I overexpressing miR-495-3p (Figure 4A) and measured cell viability following IM and Ponatinib treatments. As expected, we observed that IM did not alter cell viability and *miR-495-3p* overexpression had no effect on IM resistance (Figure 4B). However, Ponatinib treatment significantly reduced cell viability of UT7-T315I and NC-T315I UT7 and its effect was even more pronounced in miR-495-T315I cells (Figure 4C). Similarly, we analyzed the effect of Ponatinib in CFC assays. These experiments showed a statistically significant reduction of clonogenic activity in miR-495-expressing UT7-T315I cells compared to UT7 T315I and NC-T315I UT7 cells (Figure 4D). Therefore, these results strongly suggest that miR-495-3p also modulates Ponatinib sensitivity in Imatinib-resistant UT7-T315I cells.

Bioinformatics analyses of the miR-495-3p targets during CML progression and identification of molecular partners

To investigate whether *miR-495-3p* could play a role during CML progression, we analyzed the expression of its target genes in CD34+ cells from CP and BC patients using bioinformatics tools. Putative targets were predicted through miRDB and Targetscan human databases with a common overlap comprising 466 transcripts, including 366 that could be analyzed in GSE4170 dataset comprising transcriptome from patients at different CML stages (Figure 5A). Interestingly, unsupervised principal component analysis based on the expression of the 366 spotted mRNA targets allowed a clear separation between CML samples from blast crisis and chronic phase (Figure 5B). Strikingly, differential expressed gene analysis between CP *versus* BC identified a set of *miR-495-3p* target genes up-regulated during the advanced phase of the disease (Figure 5C). Indeed, the up-regulation of these target genes allowed to stratify hematopoietic cells from BC and CP (Figure 5D). Among these target genes, several have already been described as upregulated during BC such as *TM4SF1*, *ZNF827*, *KLHL5*, and *CTNND2* [32,41], or implicated in leukemia pathophysiology *such as* *GAB1*, *LHX2*, *THRB*, and *CDH2* [42–45] (Figure 5D). Next, we built a protein-protein interaction network using this gene set. Based on blood differentiation gene interaction, this protein-protein interaction network collected 973 edges between 872 nodes from 32 target gene seeds (Figure 5E). Functional inference of the Gene Ontology Biological Process database on the network highlighted a significant implication of 211 molecular partners implicated in protein phosphorylation (Figure 5E). Altogether, these results suggest that during BC, a set of *miR-495-3p* target genes are up-regulated in hematopoietic progenitors and recruit partners that are implicated in the protein phosphorylation cell functionality.

Evaluation of the expression of miR-495-3p target genes in AML

We next analyzed the expression status of *miR-495-3p* target genes up-regulated in BC using RNA-sequencing transcriptome from AML TCGA cohort. Among these target genes, 8 were found strictly overexpressed in the blasts of AML patients, including *BMI1*, *SH2D2A*, *INSIG1*,

HSP90AA1, *LHX2*, *NRXN3*, *SH3PXD2A*, and *GAB1* (Figure 6A). The combined alterations affecting these 8 target genes in blast AML allowed to stratify two groups of AML patients herein referred as altered (associated with the overexpression of *miR-495-3p* target genes) and unaltered survivals. Overall survival analysis performed on this patient stratification identified a significantly worse prognosis in the altered group as compared to the unaltered group (Figure 6B). Interestingly, this stratification also identified that the altered group of patients exhibits a higher level of genomic instability and a reduced number of platelets (Figure 6C, 6D). Taken together, these results suggest that overexpression of *miR-495-3p* target genes is also associated with a dismal prognosis in AML as compared to the group with low expression of *miR-495-3p*.

DISCUSSION

Over the last two decades, microRNAs have emerged as important regulators of cancer progression and drug resistance. In CML, microRNAs can exert a tumor suppressor activity by directly targeting *BCR::ABL1*, cell cycle regulators, and hematopoiesis regulators. Alternatively, they can also exhibit oncogenic properties by blocking for example myeloid differentiation or targeting tumor suppressor genes [21].

In this study, we uncovered for the first time a role of *miR-495-3p* in *BCR::ABL1* expressing leukemic cells. We first observed a reduced expression in *BCR::ABL1* expressing cell lines. Interestingly, using a *BCR::ABL1* inducible UT-7 cell line, we observed a downregulation of *miR-495-3p* expression when *BCR::ABL1* expression was induced, suggesting that *BCR::ABL1*-mediated signaling pathways might regulate microRNA expression (Figure 1B). This could potentially be associated with the results of our bioinformatics analyses showing upregulation of *miR495-3p* putative target genes in patients in BC compared to the ones in CP, suggesting that *miR-495-3p* activity is reduced during CML progression. These findings however remain to be validated in independent cohorts of patients. Similarly, the regulation of the expression of *miR-495-3p* from a mechanistic view will require further investigation. Particularly, *miR-495-3p* expression has been linked to DNA methylation [46], the expression of E12/E47 transcription factors [47], or oncogenic fusion protein such as MLL-AF9 [48]. As an alternative but not exclusive hypothesis, the downregulation of *miR-495-3p* expression in the bone marrow of the patients could reflect a change in the cellular composition of the bone marrow with the dramatic increase of immature blast cells at advanced stages of the disease. Particularly, it is tempting to speculate that *miR-495-3p* expression reflects the number of immature blastic cells which could be used as a prognosis marker.

In previous studies, *miR-495-3p* was found to be downregulated in AML and a higher expression was associated with a worse prognosis [49,50]. Similarly, *miR-495-3p* expression is downregulated and acts as a tumor suppressor in MLL-rearranged leukemia [48]. These observations suggest that *miR-495-3p could* act as a tumor suppressor. To gain more insights

into the function of this microRNA, we undertook the analysis of the phenotypes resulting from both complementary gain- and loss-of-function strategies in BCR::ABL1-expressing cell lines.

Our first observation is that miR-495-3p expression is low in cells expressing BCR::ABL1. This phenomenon seems to be dependent on the expression of BCR::ABL1 as shown in the DOX-inducible BCR::ABL1 cell in which DOX induces the transcriptional activation of BCR::ABL1. On the other hand, AntagomiR-495 stimulates cell proliferation while miR-495-3p mimics induced a reverse phenotype associated with an increase of cells in G0/G1 phase. We propose a model in which *miR-495-3p* downregulation during CML progression might contribute to the expansion of immature blast cells. It has to be stressed that the cell lines in which these results have been obtained are essentially close to BC samples. Interestingly, *miR-495-3p* was previously described as a negative regulator of cell proliferation in several solid tumors by targeting *CDK6* in glioblastoma multiforme [51]. Inversely, *miR-495-3p* promotes cell proliferation in breast cancer presumably through the release of mTOR activity [47]. Further experiments will be required to clarify how *miR-495-3p* regulates cell proliferation in CML cells.

Our second observation is that miR-495-3p promotes TKI resistance. Indeed, UT-7/11 cells expressing AntagomiR-495 exhibit an increased clonogenic survival in presence of IM (Figure 1H) while the phenotype is reversed when *miR-495-3p* is overexpressed (Figure 2F). We proposed that these phenotypes are linked to *MDR1* expression which encodes for a major actor of multidrug resistance. Interestingly, *miR-495-3p* was previously identified as a direct negative regulator of *MDR1* expression in a cellular model of multidrug resistance induced in CML K562 cells by Adriamycin or Vinblastin [25]. We thus extend this important role of miR-495-3p in drug resistance to IM which is a major TKI used in clinics. Strikingly, in a model of TKI resistance induced by T315I mutation of BCR::ABL1, *miR-495-3p* overexpression did not alter their sensitivity to IM but to Ponatinib. Whether similar mechanisms involving *MDR1* are at play remains to be determined in the context of Ponatinib. Indeed, Ponatinib was previously shown to be transported independently from *MDR1* in K562 [52]. *MDR1* overexpression in K562 cells confers resistance to IM but to a weaker extent to Ponatinib [53]. Alternatively, miR-495-3p could

regulate drug resistance through other mechanisms. For example, miR-495-3p targets *ATP7A* and *UCA1/NRF2* in non-small cell lung cancer [54,55], and *TSPAN12* in small cell lung cancer [56].

Taken together, our results strongly suggest that miR-495-3p plays a role in the proliferation and drug resistance of BCR::ABL1-expressing leukemic cells. The expression of miR-495-3p targets is associated with CML progression. These findings open new avenues in the development of treatment and the identification of biomarkers that could be used to better stage CML and predict responses to TKI treatment. Indeed, *miR-495-3p* could be used as a biological marker for prognosis and/or pharmaceutical target.

CONFLICT OF INTEREST DISCLOSURE

The authors have no competing interests to declare in relation to this work.

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FIGURE LEGENDS

Figure 1: Expression of *miR-495-3p* and effects of *miR-495-3p* knock-down in CML cell lines. (A-C) Relative quantification of *miR-495-3p* expression by real-time PCR normalized to *U6* expression. **(A)** *miR-495-3p* expression in KCL22 (fold change (FC) = -3.01 ± 0.18 , $p = 0.0185$) and UT-7/11 (FC = -2.17 ± 0.10 , $p = 0.0329$) normalized to UT7p. **(B)** *miR-495-3p* expression in BCR::ABL1-inducible UT7 cell lines in the presence (DOX+) or absence (DOX-) of doxycycline (FC = -1.77 ± 0.05 , $p = 0.044$). **(C)** *miR-495-3p* expression in UT-7/11 cells stably expressing AntagomiR-495-3p or its control vector (antagomiR-NC) (FC = -3.38 ± 0.09 , $p = 0.0076$). **(D)** Cell proliferation of UT-7/11 cells expressing the empty vector AntagomiR-NC or AntagomiR-495-3p relative to UT-7/11 ($p = 0.0054$ on day 4, $p = 0.0030$ on day 6, and $p = 0.0008$ on day 8). Cells were plated at equal density and cultured for 10 days. Cell numbers were determined using trypan blue exclusion. **(E)** Cell cycle analysis using flow cytometry quantification of DNA content with Hoechst 33342 dye (G0/G1 phase; 34.98% versus 41.63% in control; $p = 0.0345$, G2/M phase; 43.87% versus 37.47% in control; $p = 0.0425$). **(F)** Quantitative analysis of cell cycle phase distribution of UT-7/11 expressing the AntagomiR-NC or AntagomiR-495-3p. **(G)** Effect of *miR-495-3p* overexpression in CFC assays. Representative pictures of cell culture dishes after 2 weeks in culture of UT-7/11 cells expressing AntagomiR-NC or AntagomiR-495 in the absence (0 μ M) or presence (1 μ M) of IM. **(H)** Quantitative analysis of CFC assays. The bar chart shows the ratio between colony numbers with IM and without. Each data represents mean \pm SD from three independent experiments (mean ratio = 0.76 ± 0.03 versus 0.65 ± 0.03 in UT7/11 cells, $p=0.0096$). The One-way or Two-way ANOVA approach was used to assess the statistical significance. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 2: Effect of *miR-495-3p* overexpression on UT-7/11 cell proliferation and IM resistance. (A) Relative quantification of *miR-495-3p* expression by real-time PCR in UT-7/11 cells stably expressing *miR-495-3p* (*miR-495-UT-7/11*) or its control vector (NC-UT-7/11) (FC = 2.32 ± 0.95 , $p = 0.0472$). *miR-495-3p* expression was normalized to *U6* expression. **(B)** Cell

proliferation of miR-UT7/11 and NC-UT-7/11 relative to UT-7/11. Cells were plated at equal density and cultured for 10 days ($p = 0.0072$ on day 4, $p = 0.0298$ on day 6, $p = 0.0176$ on day 8, and $p = 0.0380$ on day 10). Cell numbers were established using trypan blue exclusion assay. **(C)** Quantitative analysis of the cell cycle phase distribution (G0/G1 phase; 41.94% versus 36.24% in UT-7/11; $p = 0.0286$, G2/M phase; 30.47% versus 39.19% in UT-7/11; $p = 0.0013$). **(D)** Cytotoxic effects of IM were measured using XTT assay (40.58% reduction in UT-7/11, 39.04% in NC-UT-7/11, and 66.77% in miR-495-UT-7/11, $p = 0.0008$ compared to UT-7/11 and $p = 0.0005$ compared to NC-UT-7/11). **(E)** Effect of *miR-495-3p* overexpression in CFC assays. Representative pictures of cell culture dishes after 2 weeks in culture of UT-7/11, NC-UT7/11, and miR-495-UT-7/11 cells in the absence (0 μM) or presence (1 μM) of IM. **(F)** Quantitative analysis of CFC assays. The bar chart shows the ratio between colony numbers obtained in the presence and absence of IM (mean ratio = 0.45 ± 0.03 versus 0.59 ± 0.05 in UT7/11 cells, $p = 0.0072$). Each data represents mean \pm SD from three independent experiments. The One-way or Two-way ANOVA approach was used to assess the statistical significance. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Figure 3: Effect of miR-495-3p on *MDR1* gene expression and efflux activity. **(A)** Alignment between the miRANDA-predicted miR-495-3p sequence and the *MDR1* 3'-UTR WT target sequence. **(B)** Relative quantification of *MDR1* expression by real-time PCR in UT-7/11, NC-UT-7/11, and miR-495-UT-7/11 cells ($FC = -2.27 \pm 0.09$, $p = 0.0440$). *MDR1* expression was normalized to *B2M* expression. **(C)** Representative images of Rhodamine 6G accumulation in UT-7/11, NC-UT-7/11, and miR-495-UT-7/11 cells. Cells were treated with 1h pulse of 10 μM Rho6G and chased in Rho6G-free media for the indicated periods of time. **(D)** Relative Rho6G fluorescent intensity. Results were normalized to the time point of 0 minutes after washing. Each data represents mean \pm SD from three independent experiments. **(E)** *MDR1* mRNA expression profiling by array in patients at different stages of disease, on the basis of RNA-seq data from the GSE4170 transcriptome dataset (0.6429 in BC versus 0.1591 in CP, $p < 0.0001$; versus 0.2122 in AP, $p = 0.0024$). CP: $n = 57$, AP: $n = 17$, BC: $n = 37$ CML patients. The One-way or

Two-way ANOVA approach was used to assess the significance. *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$.

Figure 4: Overexpression of *miR-495-3p* in TKI-resistant CML cells. (A) Relative quantification of *miR-495-3p* expression by real-time PCR of UT-7/11 T315I cells stably expressing *miR-495-3p* (miR-495-T315I) or its control vector (NC-T315I) (FC = 2.19 ± 0.41 , $p = 0.0210$). *miR-495-3p* expression was normalized to *U6* expression. (B-C) Cytotoxic effects of IM (B) or Ponatinib (C) were measured using XTT assay (22.39% reduction in UT7-T315I, 26.31% in NC-T315I UT7, and 63.00% in miR-495-T315I, $p = 0.0003$ compared to UT7-T315I and $p = 0.0008$ compared to NC-T315I). (D) Cytotoxic effect of Ponatinib on colony formation of miR-NC or miR-495-3p overexpressing cells. CFC assays were performed in the absence [0 μ M] or presence of 100 nM Ponatinib (mean ratio = 0.70 ± 0.03 versus 0.78 ± 0.03 in UT7-T315I cells, $p = 0.0372$). The bar chart shows the difference in the ratio of colony intensity as a quantitative parameter of IM treatment efficiency. Each data represents mean \pm SD from three independent experiments. The One-way or Two-way ANOVA approach was used to assess the significance. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Figure 5: *miR-495-3p* regulates targets affecting protein phosphorylation during CML blast crisis. (A) Venn diagram of *miR-495-3p* target prediction overlap and quantified in GSE4170 transcriptome dataset. (B) Unsupervised principal component analysis of *miR-495-3p* targets in CD34+ hematopoietic progenitors from CML patients in chronic *versus* blast crisis phase. (C) Volcano-plot representing the differential expression of predicted *miR-495-3p* target genes between CML patients in blast crisis *versus* chronic phase chronic. (D) Heatmap representing *miR-495-3p* targets significantly upregulated in CD34+ hematopoietic progenitors obtained from CML patients in blast crisis *versus* chronic phase (E) Schematic representation of protein-protein interaction network based on *miR-495-3p* targets found up-regulated during CML blast crisis ($p = 3.69e-42$). Functional inference on the network revealed in blue a main implication of protein phosphorylation.

Figure 6: Up-regulation of miR-495-3p targets defined a worse prognosis in AML.

Analyses were performed on omics experiments of the TCGA AML cohort. **(A)** Oncoprint visual output of the expression of miR-495-3p target genes in AML leukemic cells (red square, high expression; grey square, no alteration). Percentages indicate the proportion of the patients. **(B)** Overall survival Kaplan-Meier curves stratified on combined transcriptional alteration of miR-495-3p target genes in AML leukemic cells (log rank $p = 0.0139$). **(C)** Quantification of the genomic instability stratified on combined transcriptional alterations of miR-495-3p targets in AML leukemic cells ($p = 8.417e-3$). **(D)** Quantification of the platelet number in AML patients stratified on combined transcriptional alterations of miR-495-3p targets in AML leukemic cells ($p = 0.0246$).

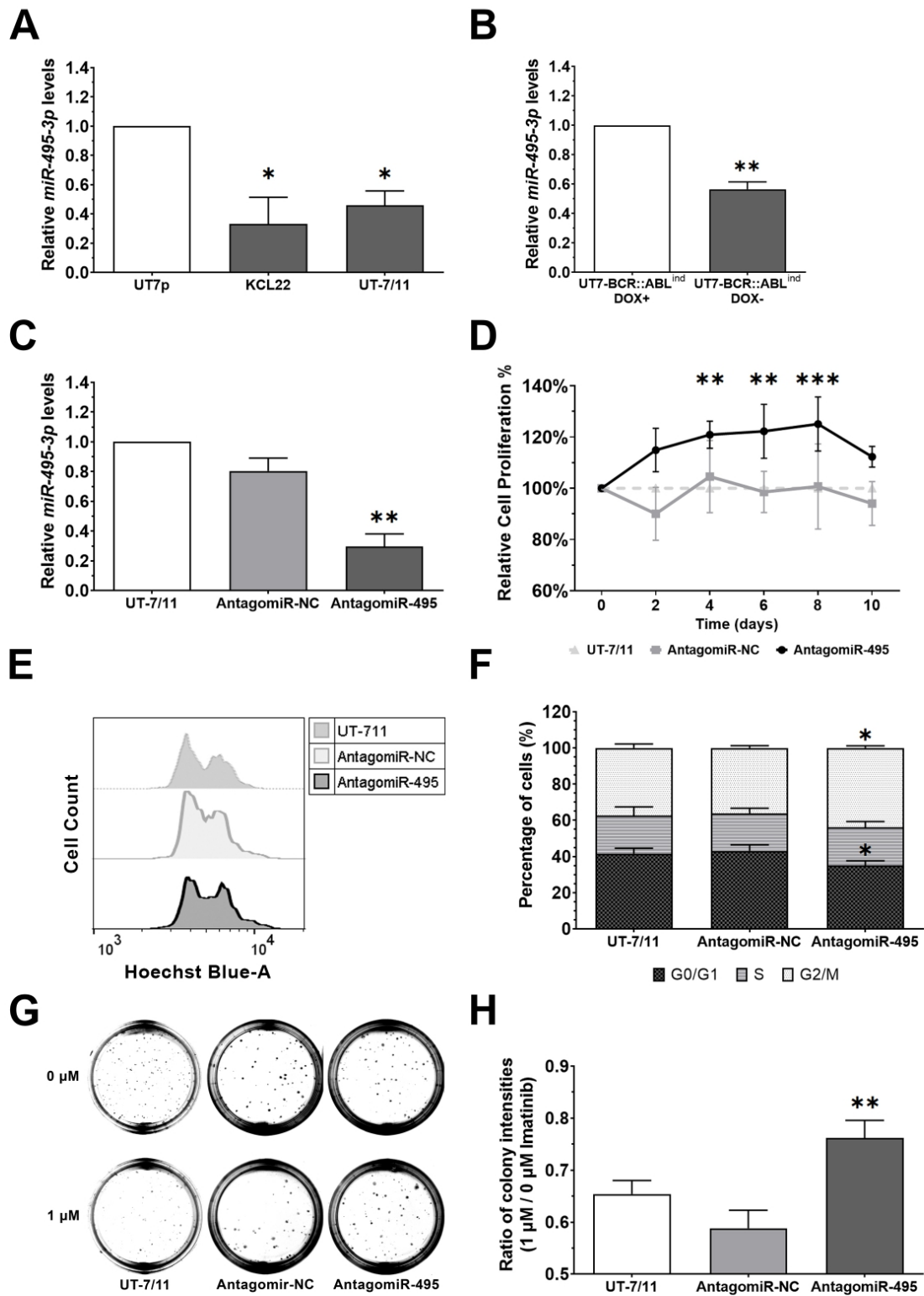


Figure 1: Rittavee et al.

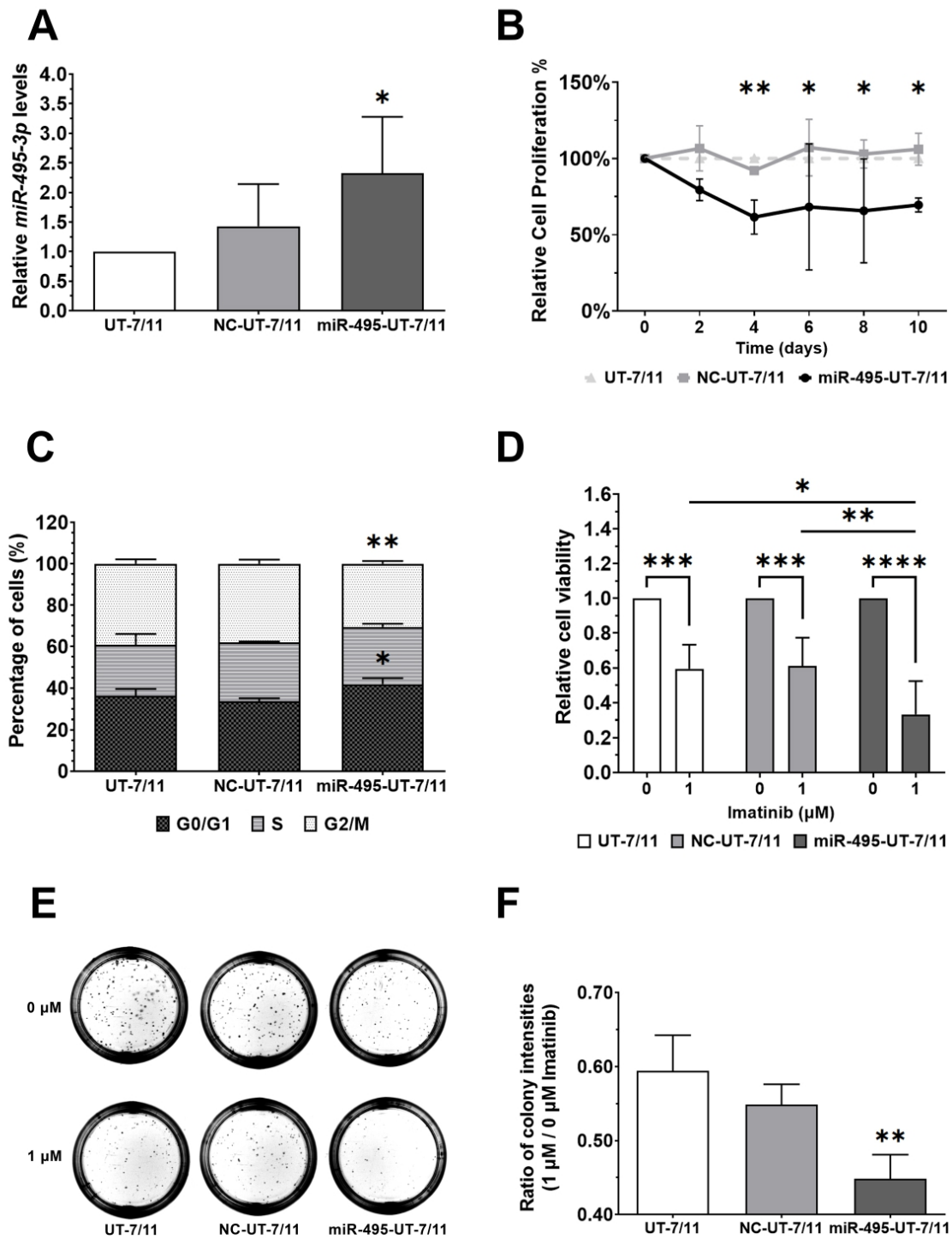


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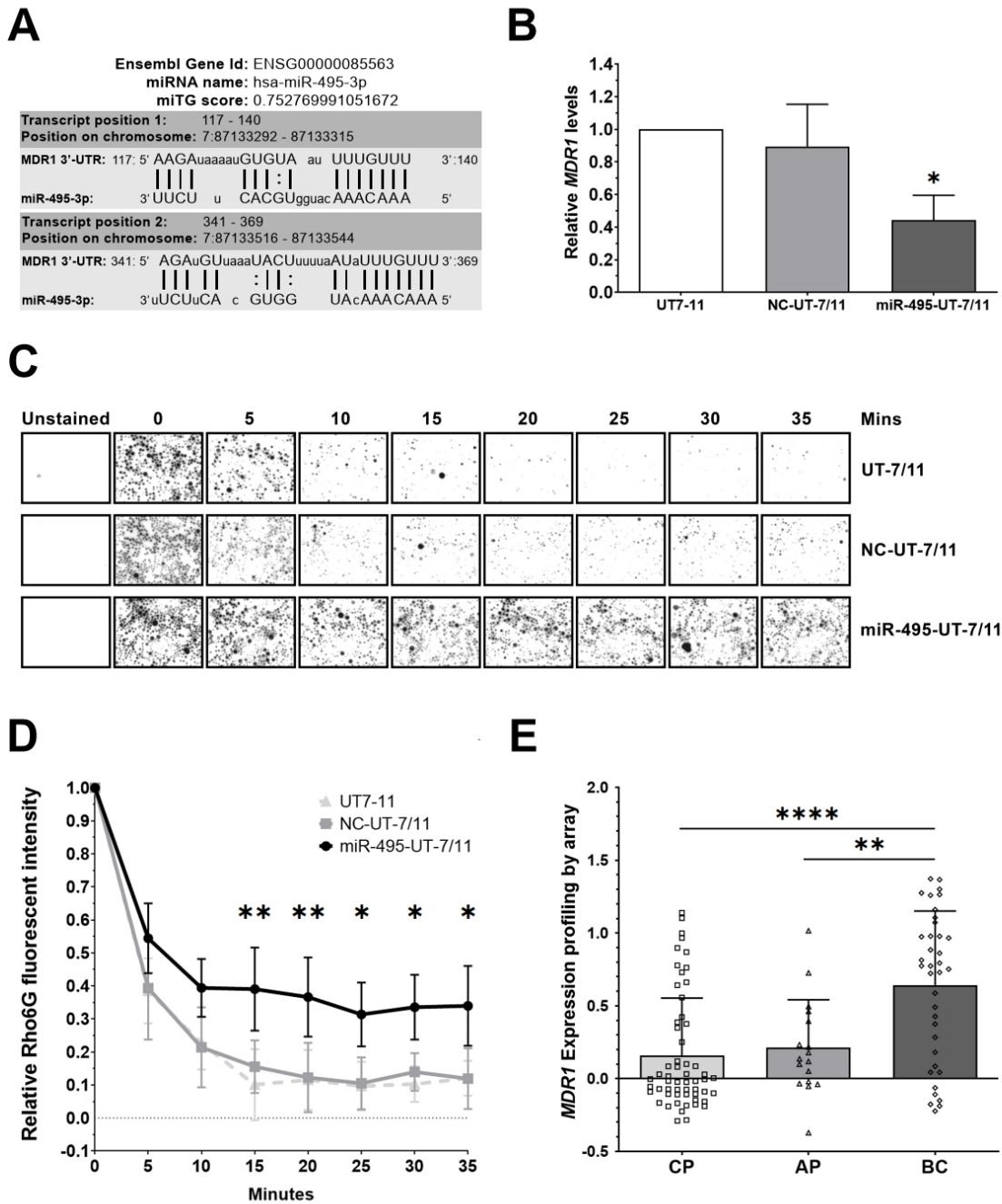


Figure 3: Rittavee et al.

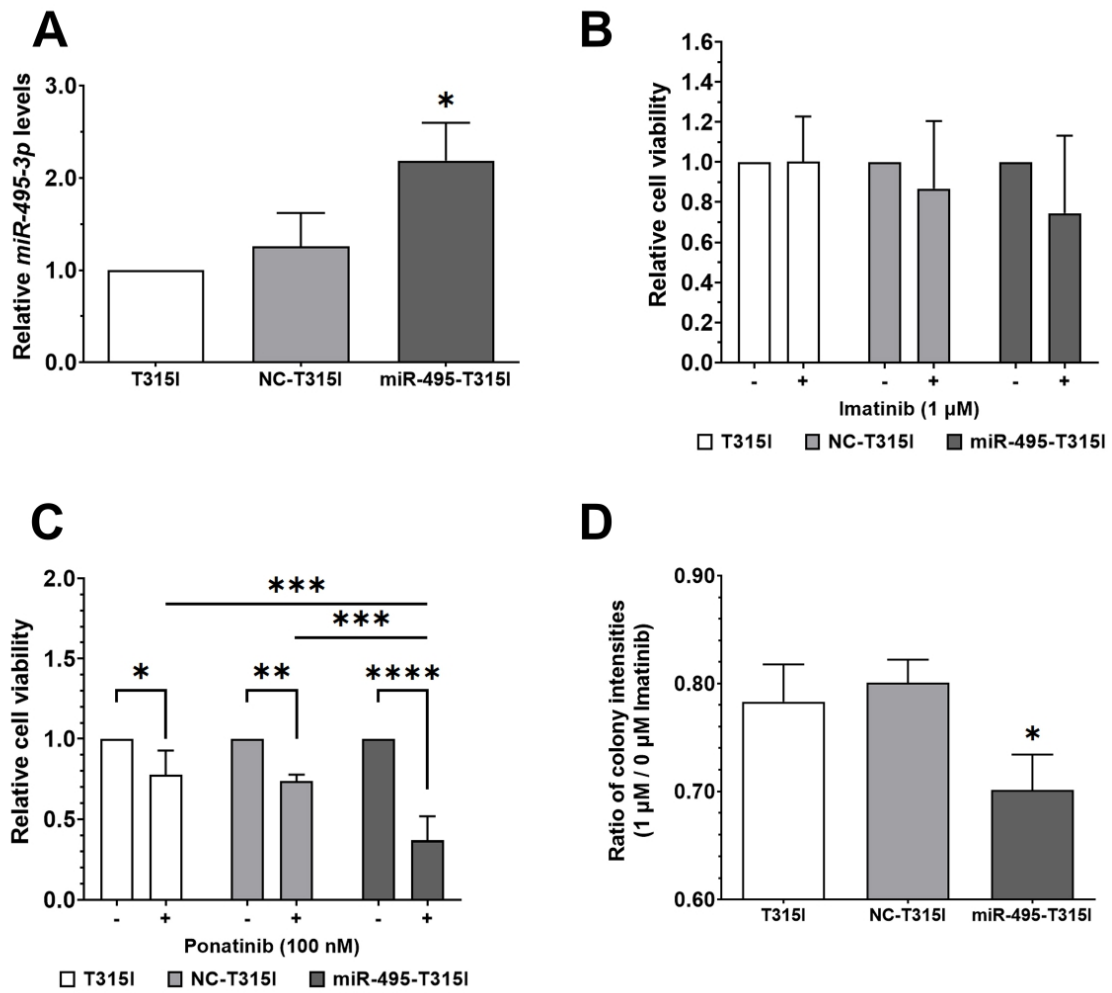
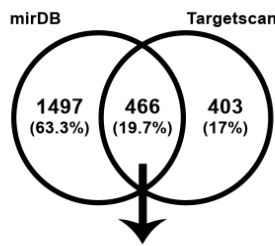


Figure 4: Rittavee et al.

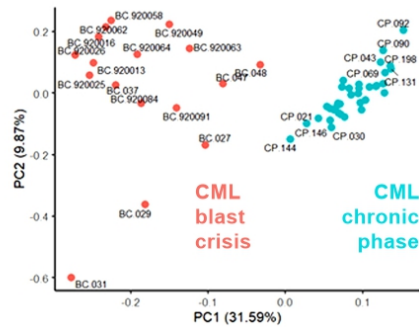
A

Prediction of *miR-495-3p* targets

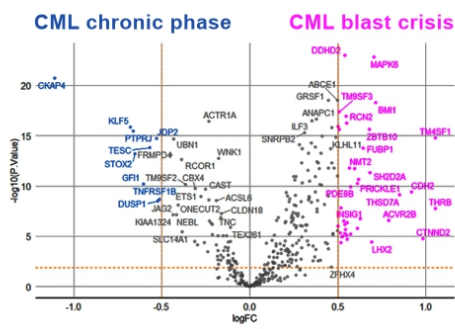


366 spotted in GSE4170 with complete cases 56 patients CML

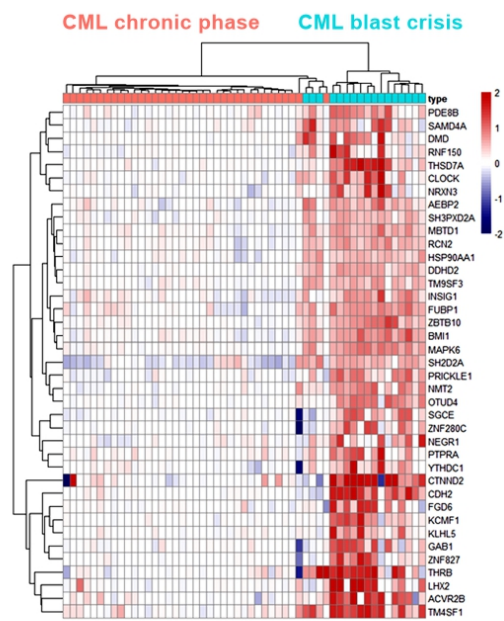
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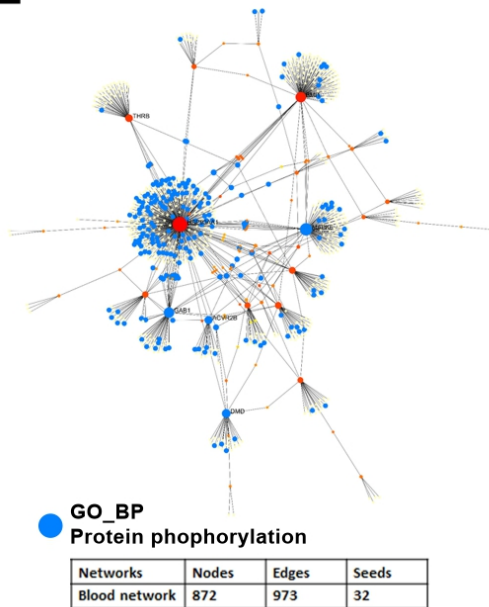


Figure 5: Rittavee et al.

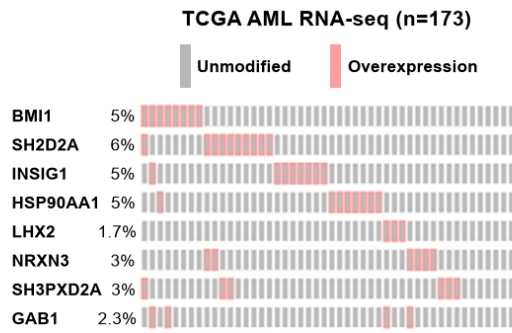
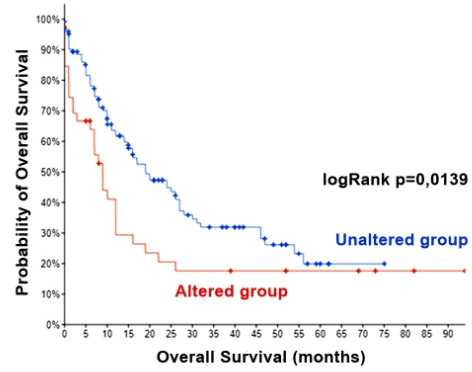
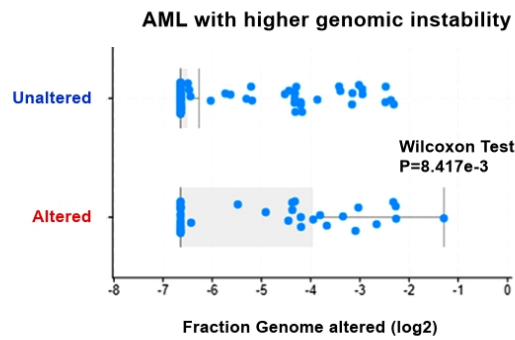
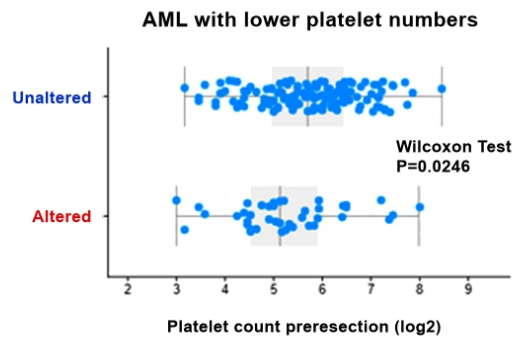
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Figure 6: Rittavee et al.

PAPER 2 – Summary

DEREGULATION OF CORE CLOCK GENES BY BCR::ABL1 IN BCR::ABL1-EXPRESSING LEUKEMIC CELLS VIA THE MAPK/ERK PATHWAY

Hematopoiesis is a continuous process generating functional blood cells during the lifetime of an individual. This is a tightly regulated process under the influence of both intrinsic and extrinsic cues, allowing not only homeostasis but also the possibility to respond to external and internal requirements by modifying the production of mature blood cells. It has also been shown the circadian rhythms can play a role on hematopoiesis. Disrupting circadian rhythms raises the risk of acquiring a variety of chronic diseases, including hematological malignancies. One of the well-studied examples in this subject is the link between cancer incidence and sleep disturbance in several shift workers. In leukemia, each individual component of core clock genes was studied separately in CML samples and demonstrated the potential role to regulate the induction of leukemogenesis. The alteration of core clock gene expressions was shown to be induced by an oncoprotein like MLL-AF9. However, the effect of BCR::ABL1 on the deregulation of core clock genes in CML and the impact of circadian rhythm disruption in CML pathogenesis remain unknown.

In this work, we hypothesized that the expression of BCR::ABL1 oncoprotein would impact the disruption of the circadian clock in CML. We first analyzed by qPCR and western blot the expression of several clock genes, including *BMAL1*, *PER1/2* and *CRY1/2*, and found an inverse correlation with the expression of *BCR::ABL1* oncogene in BCR::ABL1-expressing cell lines at both transcriptional and protein levels. Accordingly, the core clock genes *BMAL1*, *CLOCK*, *NR1D1* and *RORC* were also all downregulated in peripheral blood mononuclear cells (PBMCs) from CML patients at diagnosis.

Additionally, we exhibited that controlled expression of *BCR::ABL1* using a tetracycline-inducible system could restore the expression of clock genes. Treatment of UT7-P210^{ind} cells with Dox repressed the expression of the BCR::ABL1 oncoprotein and restored the transcription

of several core clock genes, as well as the upregulation of BMAL1 protein levels. Going a step further, in the Dox pulse-chase experiment, cells were treated with Dox for 10 days (pulse period) and another 10 days without Dox treatment (chase period). Interestingly, this recovery of core clock genes upon BCR::ABL1 repression was once again suppressed when the treatment of Dox was removed in the chase period. This data showed that the expression of core clock genes could be modulated by the expression of *BCR::ABL1* oncogene. Correspondingly, To verify if the downregulation of clock gene expression is reliant on BCR::ABL1 kinase activity, UT7-P210^{ind} cells were treated with various dosages of Imatinib and the expression of core clock genes was assessed. As expected, treatment with increasing concentrations of Imatinib resulted in decreasing levels of phosphorylated BCR::ABL1 in UT7-P210^{ind} cells. The reduction of BCR::ABL1 phosphorylation was accompanied by a gradual recovery of BMAL1 protein in UT7-P210^{ind} cells. Strikingly, treatment with 1 μ M Imatinib recovered mRNA levels of all core clock genes.

To determine the downstream pathway of BCR::ABL1 that plays a role to regulate BMAL1 expression, inhibitors of three major signaling pathways were treated in UT7-P210^{ind} cells for 72h, including the selective inhibitors of MEK PD0325901, JAK inhibitor I and PI3K LY294002. Notably, MEK inhibitor was the sole treatment that could significantly restore the expression of BMAL1 protein expression. Consequently, the expression of phospho-p44/42 MAPK Erk1/2 was also deregulated following MEK inhibition. This result indicated that the regulatory mechanism of BCR::ABL1 on *BMAL1* expression occurs through the MAPK/ERK signaling pathway. This deregulation of core clock genes by BCR::ABL1 could rule over *BMAL1* overexpression. BMAL1-overexpressed UT7-11 cells acquired higher expression of *BMAL1* expression in both transcriptional and protein levels and *clock*, a heterodimeric component of the CLOCK/BMAL1 complex. Remarkably, *BMAL1* overexpression resulted in decreasing levels of phosphorylated BCR::ABL1 in UT7-11 cells. However, the molecular mechanism underlying this retaliation remains to be elucidated.

This *paper in preparation* is the work at the current point where my doctoral study has been done. Next, we are currently planning to analyze the effects resulting from the modulation of *BMAL1* expression at the transcriptomic level by the transcriptomic platform GENOM'IC at Institut Cochin, Paris. We plan to identify the differentially expressed genes and uncover specific molecular mechanisms. We will also use this molecular signature on CML patient cohorts to determine whether their expression is correlated with TKI responders and non-responders. Taken together, this study will be the first to show the effect of BCR::ABL1 on the deregulation of core clock genes via the MAPK/ERK signaling pathway. Future work may be fulfilled to answer the pathophysiological functions of disrupting circadian rhythms in CML, especially in an aspect of TKI chemoresistance.

IN PREPARATION

BCR::ABL1 Regulates Circadian Clock Machinery Through MAPK/ERK Pathway

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ABSTRACT

BCR::ABL1 fusion oncoprotein is the main driver of the pathogenesis of chronic myeloid leukemia (CML) and activates several circadian clock-related pathways involved in malignant cell survival, cell cycle, drug resistance and disease progression. Even though clock gene expression has been found to be disrupted in CML, the impact of BCR::ABL1 in circadian clock disruption is still not fully elucidated. In this report, we confirm the downregulation of circadian clock genes in leukemic cell lines and in CML patients at diagnosis using RT-qPCR and western blot analysis and show that the expression of BCR::ABL1 oncoprotein is directly involved in the deregulation of core clock gene expression. We demonstrate that core clock genes were reversibly modulated by manipulation of BCR::ABL1 expression in DOX-inducible BCR::ABL1-expressing cell lines. In addition, pharmacological inhibition of BCR::ABL1 kinase activity with Imatinib restored molecular clock gene expression. MEK inhibitor treatment in our leukemic cellular model restored BMAL1 protein expression, indicating that MAPK/ERK signaling is most likely involved. Interestingly, *BMAL1* overexpression lowered the levels of pBCR::ABL1 and BCR::BAL1, suggesting the existence of a negative feedback loop. Our findings thus pave the way for further investigations into the role of several circadian clock-related cancer during CML pathogenesis.

INTRODUCTION

Because of adaptation to the Earth's rotation, humans and all eukaryotes' behavior and physiological function pursue a daily oscillatory circadian rhythm. This endogenous circadian clock regulates multiple important life activities in the body, including body temperature, hormone secretion, eating, and sleeping [1]. At the molecular level, the circadian clock relies on the oscillation of the expression of clock genes over a 24 hours period through a complex network of positive and negative transcription-translation autoregulatory feedback loops [2,3]. Circadian regulation is estimated to affect the expression of up to 50% of the genome, including genes related to metabolism, proliferation, survival and DNA repair [4–6]. Accumulating studies have revealed that the expression of circadian clock genes is altered in various cancer types [7] and a body of evidence converges on a link between the circadian clock disruption and cancer development as exemplified in breast cancer and colon cancer [8,9].

In hematopoiesis, circadian rhythms regulate various physiological aspects of the bone marrow functions including the proliferation, maintenance and migrations of HSCs [10–13]. In the context of leukemia, altered expression of clock genes has been reported in several types of leukemia, including chronic myeloid leukemia (CML) [14–16]. Further investigations suggested that the circadian clock plays a role in leukemogenesis. In AML leukemic cells, experimental perturbation of the circadian clock impairs leukemic stem cell maintenance and induces cell differentiation [17]. In CML patients, the daily pattern expression of *Bmal1* was disturbed raising the question of whether the circadian clock is functional [15,18]. Ectopic expression of PER2 in K562 or KCL22 CML cells or inhibition of SIRT1 to restore functional clock alters leukemic cell properties [18–20]. Thus, the deregulation of core clock genes may be crucial for CML leukemogenesis.

Interestingly, various tyrosine protein kinases including ABL1 and BCR have been proposed to modulate the circadian clock [21]. If the major interaction region of ABL1 and BCR is retained in the BCR::ABL1 fusion protein, core clock genes may physically interact with BCR::ABL1, the primary oncogenic driver of CML. However, the effect of BCR::ABL1 on the

deregulation of core clock genes in CML and the impact of circadian rhythm disruption in CML pathogenesis remains unknown.

To address this question, we hypothesized that the expression of BCR::ABL1 oncoprotein would impact the disruption of the circadian clock in CML. In the present study, we analyzed the expression of several clock genes in BCR::ABL1-expressing cell lines and peripheral blood mononuclear cells (PBMCs) from CML patients at diagnosis. To verify if the downregulation of clock gene expression is reliant on BCR::ABL1 and its kinase activity, we conducted a controlled expression of *BCR::ABL1* using a tetracycline-inducible system and various dosages of Imatinib treatment approaches. We further investigated the downstream pathway of BCR::ABL1 that plays a role to regulate *BMAL1* expression. Taken together, our results provide a unique basis to better understand the role of circadian rhythms in CML.

MATERIAL AND METHODS

Cell culture

Six different human myeloid cell lines were used in this study: Ph1 chromosome-positive CML cell lines K562 [22] and KCL22 [23]; GM-CSF dependent human hematopoietic cell line UT7-P [24], BCR::ABL1 positive variants of UT7 cell line UT7-11 [25], UT7-GFP^{ind} and BCR::ABL1^{ind} in which GFP and BCR::ABL1 are expressed in an inducible manner using a TET-OFF system [26]. Cells were routinely maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin under an atmospheric condition of 5% CO₂ at 37 °C. UT7-P cells were cultured in presence of 10 ng/mL granulocyte/macrophage colony-stimulating factor (GM-CSF). UT7-GFP^{ind} and UT7-P210^{ind} cells were cultured with 1 µg/mL of Doxycycline (Dox). Media were changed every 2 days. GFP fluorescent levels were analyzed in viable cells (DAPI negative) using a BD LSRII Fortessa flow cytometer (488 nm laser, GFP filter, BD Bioscience, Mountain View, CA, USA).

Patients-derived PBMC cells

Peripheral blood was taken from CML patients at diagnosis before therapy with informed consent from all patients in accordance with the principles of the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using the Ficoll technique. Cells were either used immediately for RNA isolation or stored at –80 °C.

RNA extraction and quantitative real-time PCR

RNA isolation was done using the PureLink® RNA Mini Kit (Invitrogen, Carlsbad, CA, USA). cDNAs were obtained using SuperScript™ III Reverse Transcriptase (Invitrogen) with random primers. Real-time PCR was performed on Agilent Mx3005P QPCR System (Agilent Technologies, Santa Clara, CA, USA) with the FastStart Universal SYBR Green Master (Roche, Mannheim, Germany). The amount of mRNA of the genes of interest was normalized to the reference gene *B2M*. The primers used are listed in Table 1.

Protein extraction and immunoblot

Western blots were performed using whole-cell lysates. Cells were lysed in RIPA buffer containing cOmplete™ Mini EDTA-free Protease Inhibitor Cocktail (Roche). Lysates were sonicated for 5 min in an ultrasonic bath (FB15048; Fisherbrand, Loughborough, UK) and centrifuged at 15,000× g for 25 min at 4 °C. Protein concentration of the samples was determined using the Bradford method and Bovine Serum Albumin (BSA) as standard according to the manufacturer's manual (B6916; Sigma-Aldrich, St Louis, MO, USA). Samples were separated on 4–12% NuPAGE Bis-Tris gel (Invitrogen) and transferred on Novex 0.2 µm PVDF membrane (Invitrogen). Membranes were blocked for 30 minutes with 5% milk–tris-buffered saline 0.1% Tween® 20 (TBST) or 5% BSA-TBST for phosphorylated proteins. Primary antibodies were incubated on a shaker overnight at 4°C. The list of antibodies used in this study is shown in Table 2. HRP secondary antibodies were incubated for 1 hour at room temperature. Horseradish peroxidase activity was detected with Supersignal West Dura ECL substrates (Pierce, Rockford, IL, USA) and imaged on a G:BOX image analyzer and GeneSnap v. 7.08 software (Syngene, Cambridge, UK). Normalized pixel density was analyzed using ImageJ v1.52a software (FlowJo LLC, Ashland, OR, USA).

Statistical analysis

Each experiment was done at least in duplicates. When possible, statistical significance between data groups was determined by unpaired two-tailed Student's t-tests. Differences for multiple comparisons were subjected to one-way analysis of variance followed by Tukey's post hoc test. Statistical analysis was performed using GraphPad Prism 8.4.3 (GraphPad Software, San Diego, CA, USA) and represented as the mean ± standard deviation (SD). p-values < 0.05 were considered statistically significant, with * = p < 0.05, ** = p < 0.01, *** = p < 0.001 and **** = p < 0.0001.

RESULTS

Core clock genes are downregulated in *BCR::ABL1*-expressing cell lines and in primary CML patient samples

To investigate a possible link between the circadian clock and CML, we first quantified the transcriptional activity of several clock genes in two CML blast crisis cell lines (K562 and KCL22) and in the acute megakaryoblastic leukemia cell line UT7-11, which ectopically express *BCR::ABL1* [25]. When compared to the *BCR::ABL1*-negative UT7-P cell line [24], all *BCR::ABL1*-expressing cell lines displayed lower mRNA levels for most of the core clock genes tested (Figure 1A). Noteworthy, the expression of *BMAL1*, a major component of the positive feedback loop of the molecular circadian clock, was consistently repressed in all *BCR::ABL1*-positive cell lines. The downregulation of *BMAL1* was further confirmed at the protein level (Figure 1B).

We next thought to determine whether the transcriptional activity of core clock genes was also altered in CML patients. We quantified their transcriptional levels in peripheral blood mononuclear cells (PBMCs) from CML patients at diagnosis (n = 9) and compared them with those from healthy donors (n = 10). In accordance with the data obtained from *BCR::ABL1*-positive cell lines, the core clock genes *BMAL1*, *CLOCK*, *NR1D1* and *RORC* were all downregulated in CML patients (Figure 1C).

Expression of *BCR::ABL1* is associated with the downregulation of core clock genes in an inducible model

Repression of the molecular circadian clock is commonly associated with an increased risk of cancer development [3]. However, it is unknown whether oncogenes themselves and/or their downstream effectors can disrupt the molecular circadian clock. In order to study the role of *BCR::ABL1* on clock gene repression, UT7 cells were engineered to allow a controlled expression of *BCR::ABL1* [26]. A construct containing a tetracycline response element driving the expression of *BCR::ABL1* and GFP was introduced into UT7 cells together with a second

construct coding for a tetracycline repressor. To get a better understanding of the kinetics of this TET-OFF system, we monitored by flow cytometry the levels of GFP and the percentage of GFP-positive cells following the addition of doxycycline (Dox). We first observed that by day 3 the levels of GFP and the percentage of GFP-positive cells were already markedly reduced (Figure 2A). By day 11, the levels of GFP in UT7-P210^{ind} cells were almost absent, similar to GFP-negative UT7 cells (Figure 2A) and BCR::ABL1 protein was no longer detected by immunoblot (Figure 2D).

To evaluate whether downregulation of *BCR::ABL1* would restore the expression of clock genes, UT7-P210^{ind} cells were cultured under Dox or DMSO (vehicle) for 11 days and the expression of core clock genes was evaluated by qPCR. To facilitate data visualization, qPCR results were used to build unsupervised clusters based on Euclidean distances (Figure 2B). As expected, UT7-P210^{ind} cells cultured in DMSO expressed the BCR::ABL1 oncoprotein (Figure 2D, second lane) and displayed lower mRNA levels of clock genes when compared to UT7 cells transduced with empty vector (UT7-GFP^{ind} cells) (Figure 2B-C). Reciprocally, by day 11, Dox-treated UT7-P210^{ind} cells were no longer expressing BCR::ABL1 oncoprotein (Figure 2D, third lane) and the transcriptional levels of several clock genes were fully (*BMAL1*, *CLOCK*) or partially (*CRY1*, *CRY2*, *NR1D1*, *NR1D2*) restored (Figure 2B-C). Remarkably, UT7-P210^{ind} cells acquired a core clock gene transcriptional profile similar to that of UT7-GFP^{ind} controls to an extent that both cell lines formed a unique cluster (Figure 2B). Accordingly, repression of *BCR::ABL1* in UT7-P210^{ind} cells treated with Dox was also accompanied by the upregulation of BMAL1 protein levels (Figure 2D). Of note, UT7-11 cells, which constitutively express BCR::ABL1 [25], did not display reduced mRNA levels of core clock genes nor reduced levels of BMAL1 protein upon treatment with Dox (Figure 2B-D).

Inhibition of BCR::ABL1 kinase domain with Imatinib restores the expression of core clock genes in UT7-P210^{ind} cells

Despite being a constitutively active tyrosine kinase, it is known that the BCR::ABL1 oncoprotein can also trigger signal transduction through kinase-independent mechanisms. For example,

activation of the Src family kinase HCK occurs independently of BCR::ABL1 kinase activity [27]. Upon binding to BCR::ABL1, HCK gets activated and phosphorylates STAT5, a protein that is crucial for the transforming abilities of BCR::ABL1 [28]. To test whether the alteration of clock gene expression is dependent on BCR::ABL1 kinase activity, we analyzed their expression in UT7-P210^{ind} cells treated with various concentrations of Imatinib for three consecutive days. As expected, increasing concentrations of Imatinib reduced the levels of phosphorylated BCR::ABL1 in UT7-P210^{ind} cells (Figure 3A, left). Interestingly, the reduction of BCR::ABL1 activity was accompanied by a gradual recovery of BMAL1 protein in UT7-P210^{ind} cells (Figure 3A, left), while UT7-GFP^{ind} control cells did not change BMAL1 expression upon Imatinib treatment in a dose-dependent manner (Figure 3A, right). We quantified BMAL1 normalized pixel intensities from western blot membranes and plotted their values according to Imatinib concentrations. We observed that the recovery of BMAL1 followed an inverse exponential curve with a fast increase in BMAL1 protein abundance as the concentration of Imatinib increased from 0 to 1 μ M followed by a plateau (Figure 3B). We next extended our observations to additional core clock genes and quantified their transcriptional levels in UT7-P210^{ind} cells treated with 1 μ M Imatinib. Strikingly, upon Imatinib treatment the expression of core clock genes was restored and was similar to those found in UT7-GFP^{ind} control cells (Figure 3C).

Core clock gene expression is tightly controlled by *BCR::ABL1* levels in UT7-P210^{ind} cells

Having established that the core clock gene expression is largely downregulated in CML and is dependent on BCR::ABL1 tyrosine kinase activity, we next further investigated whether these changes in the circadian clock circuitry were irreversible or still under the control of the oncogene activity. To do this, we performed a Dox pulse-chase experiment in UT7-P210^{ind} cells. In brief, UT7-P210^{ind} or UT7-GFP^{ind} cells were first treated with Dox or DMSO for 10 days (pulse period), then subsequently cultured for 10 additional days without Dox or DMSO (chase period) (Figure 4A). The expression of core clock genes and proteins during both pulse and chase periods was evaluated by qPCR and western blot.

As expected, after the 10 days pulse of Dox, UT7-P210^{ind} cells exhibited expression levels of *BMAL1*, *CLOCK*, *CRY1*, *CRY2*, *NR1D1*, *PER1* and *PER2* similar to UT7-GFP^{ind} cells treated with DMSO or Dox (Figure 4B). Strikingly, after the 10 days chase when Dox was removed, almost all core clock genes exhibited reduced expression levels similar to UT7-P210^{ind} cells treated with DMSO (Figure 4C). To confirm these observations, we also analyzed the levels of BMAL1 by immunoblot. Following the pulse in UT7-P210^{ind} cells, BCR::ABL1 levels were barely detectable while BMAL1 levels were increased (Figure. 4D, left). After the chase period, the levels of BCR::ABL1 were restored while the levels of BMAL1 were reduced similar to UT7-P210^{ind} treated with DMSO compared to UT7-GFP^{ind} cells treated with Dox or DMSO (Figure. 4D, right). Taken together, our data showed that the expression of the core clock genes is tightly modulated by BCR::ABL1 in a reversible manner.

MEK inhibition or *BMAL1* overexpression restores BCR::ABL1-modulated *BMAL1* expression in BCR::ABL1-expressing cells.

BCR::ABL1 regulates multiple downstream signaling pathways that contribute to the growth and survival of the leukemic cells including MAPK/ERK, JAK/STAT and PI3K/AKT signaling pathways [29]. To better understand the molecular mechanisms by which BCR::ABL1 subverts *BMAL1* expression, we used pharmacological inhibitors to selectively inhibit MEK (PD0325901), JAK (JAK inhibitor I) and PI3K (LY294002) activities in UT7-GFP^{ind} and UT7-P210^{ind} cells. Strikingly, in UT7-P210^{ind} cells, MEK inhibition largely restored the levels of BMAL1 while JAK or PI3K inhibition had little effect (Figure 5A-B). We further verified that MEK inhibition did not alter the levels of BCR::ABL1 and pBCR::ABL1 but reduced the levels of pERK1/2 and BMAL1 (Figure 5C). Based on these observations, we proposed that the regulation of *BMAL1* expression by BCR::ABL1 is mediated by MAPK/ERK signaling pathway.

We next thought to determine the effect of reintroducing *BMAL1* expression in BCR::ABL1-expressing cells. To do this, we transduced UT7-11 cells with lentivirus encoding to *BMAL1*. First, we confirmed by western blot and RT-qPCR the overexpression of *BMAL1*

(Figure 5D-E). We next analyzed the effect on clock core gene expression and observed a trend of an upregulation of *CLOCK* and a downregulation of *CRY1* and *CRY2* gene expression suggesting that the clock molecular circuitry is not completely altered by *BMAL1* overexpression. Strikingly, *BMAL1* overexpression also decreased the levels of pBCR::ABL1 and BCR::BAL1 (Figure 5D) suggesting some negative feedback loop on BCR::ABL1 activity. However, the molecular mechanism underlying this retaliation remains to be elucidated.

DISCUSSION

The hallmark of CML is the BCR::ABL1 fusion oncoprotein, which is the result of the t(9;22)(q34;q11.2) chromosomal translocation and is found in more than 95% of CML patients [30]. This translocation has made provision for how a single genetic aberration can drive malignant transformation and, more critically, how a targeted therapy changes the disease course and drastically improves patient outcomes. Nonetheless, this translocation also occurs in 2–5% of children and 10–20% of adults with ALL, as well as in highly uncommon (1% or so) cases of AML [31]. Although it is known that the expression of several circadian genes is altered in CML and other kinds of leukemia, a possible role of BCR::ABL1 in the regulation of core clock gene expression remains unknown.

In this study, we first confirmed the deregulation of core clock genes in BCR::ABL1-expressing leukemic cell lines and in PBMCs from CML patients. Among the panel of genes we analyzed, four genes (*BMAL1*, *CLOCK*, *NR1D1* and *RORC*) were found to be expressed at significantly lower levels and another four (*PER1*, *CRY2*, *NR1D2* and *RORA*) with slightly lower expressions in CML patients' cells compared to healthy persons. Our observations are consistent with what was previously described [14–16]. Strikingly and contrary to previous studies, we found a higher expression of *PER2* in CML patients at diagnosis. *PER2* function has been extensively studied and exerts tumor suppressor activity by interacting with *c-Myc* and *cyclin D1* [16]. Yang et al. observed that the perturbation of *PER2* expression correlates with the clinical stages of CML patients (chronic *versus* blastic phase) [14]. Therefore, our results could reflect the early clinical stage of the subjects.

Having confirmed the disruption of circadian gene expression, we further explored whether BCR::ABL1 could play a role in the deregulation. Interestingly, we found that experimental modulation of *BCR::ABL1* expression in inducible UT7-P210^{ind} cells intensively modulated the core clock gene expression in a reversible manner. Additionally, Imatinib could recover molecular clock gene expression by decreasing BCR::ABL1 kinase activity. Certain tyrosine kinase inhibitors including Imatinib were reported to impact the circadian period and

revealed the involvement of ABL1 and BCR protein kinases in mammalian circadian clock control [21]. Our finding uncovered an unexpected role of BCR::ABL1 tyrosine kinase activity in the regulation of core clock gene expression. Interestingly, other oncogenes have been also shown to functionally alter the circadian clock machinery such as *Ras*, *c-Myc* and acute myeloid leukemia (AML)-associated *MLL-AF9* fusion gene [17,32]. Our study opens new avenues to further understand, in the context of CML, the emergence of several circadian clock-related cancer hallmarks like cell cycle, cell metabolism, cell death and drug resistance [7]. Remarkably, *BMAL1* overexpression reduced pBCR::ABL1 and BCR::BAL1 levels, unraveling the existence of a negative feedback loop.

We also investigated downstream effectors of BCR::ABL1 that could regulate *BMAL1* expression using pharmacological inhibitors and found that MAPK/ERK signaling is likely to be involved. Indeed, MEK inhibition restores *BMAL1* expression. Among all the downstream signaling pathways induced by BCR::ABL1 tyrosine kinase, aberrant activation of the MAPK/ERK pathway is critical in the control of cell survival, cell cycle progression, TKI resistance and the initiation of CML leukemogenesis [33,34]. Our work suggests that BCR::ABL1-modulated disruption of the circadian clock may be entangled with the regulation of those MAPK/ERK pathway-involved pathophysiologicals.

In conclusion, we uncovered a novel effect of BCR::ABL1 on the deregulation of core clock genes via the MAPK/ERK signaling pathway in CML. Additional studies should examine the specific downstream molecular mechanisms and pathophysiological functions of disrupting circadian rhythms in CML, especially in the context of TKI chemoresistance which is a major barrier to CML therapy.

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FIGURE LEGENDS

Figure 1: Core clock genes are downregulated in CML. (A) Relative quantifications of core clock gene expression in BCR::ABL1-positive cell lines were determined by real-time quantitative PCR and normalized to UT7-P cell line (BCR::ABL1-negative). Experiments were performed on three independent biological replicates **(B)** Western blot analysis of BCR::ABL1, ABL1, and BMAL1 on protein extracts from UT7-P, UT7-11, K562 and KCL22 cell lines. **(C)** Relative quantifications of core clock gene expression in in PBMCs from CML patients at diagnosis (n = 9) and healthy donors (n = 10). (A, C) Statistical significance was determined using a Student's t-test. *, p<0.05; **, p<0.01; ***, p<0.001.

Figure 2: Downregulation of BCR::ABL1 expression restores the expression of core clock genes. (A) GFP levels of UT7-P210^{ind} cells during culture with doxycycline (Dox). The addition of 1 µM of Dox to cell cultures repressed the expression of the tetracycline responsive cassette, as indicated by the gradual loss of GFP expression over time. Gray histogram: GFP-negative control (non-transduced UT7 cells); green histogram: UT7-P210^{ind} cells cultured with Dox. **(B)** Euclidean clustering of UT7-11, UT7-GFP^{ind} and UT7-P210^{ind} (cultured 11 days with DMSO or DOX) samples (n=3) according to the expression of core clock genes. **(C)** The relative expression of core clock genes in UT7-P210^{ind} cells cultured 11 days with DMSO or DOX was determined by qPCR and normalized to UT7-GFP^{ind}. Data are represented as mean ± SD from three independent experiments. One-way ANOVA + Tukey's *post hoc* test was used to assess the statistical significance. *, p<0.05; **, p<0.01; ***, p<0.001. **(D)** BCR::ABL1 and BMAL1 protein levels were determined by Western blot analysis using protein extracts from UT7-11, UT7-GFP^{ind} and UT7-P210^{ind} cultured for 11 days with DMSO or DOX.

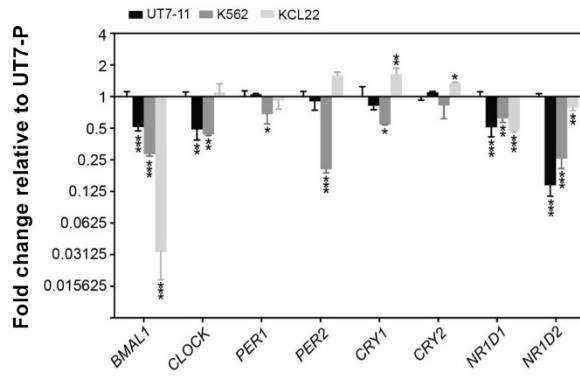
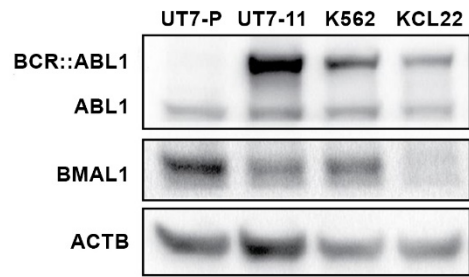
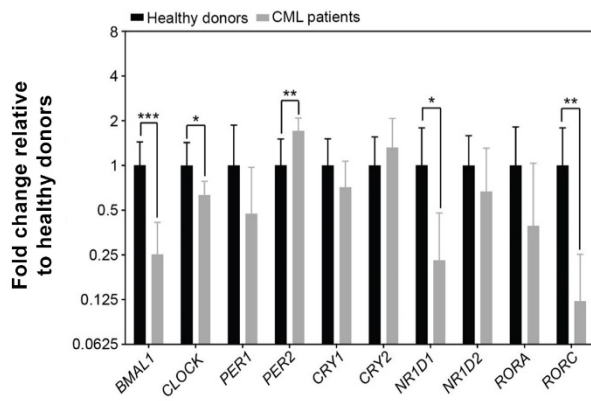
Figure 3: Inhibition of BCR::ABL1 kinase domain with Imatinib restores the expression of core clock genes. (A) Western blot of BCR::ABL1, pBCR::ABL1, ABL1, pABL1 and BMAL1 using a cellular extract from UT7-P210^{ind} and UT7-GFP^{ind} cells treated with various

concentrations of Imatinib for 72h. **(B)** Plot showing the correlation between BMAL1 pixel intensity from western blot membranes and Imatinib concentration. Using non-linear fit of inhibitor vs. response with three parameters model from GraphPad Prism 6. **(C)** Relative quantitative expression of core clock genes in UT7-GFP^{ind} and UT7-P210^{ind} cells cultured in the absence or presence of 1 μ M imatinib for 72h. Data are represented as mean \pm SD from three independent experiments. One-way ANOVA + Tukey's *post hoc* test was used to assess the statistical significance. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 4: Pulse-chase experiment using the doxycycline-inducible BCR::ABL1-GFP system modulates the expression of core clock genes. **(A)** Schematic representation of the pulse-chase experiment using UT7-P210^{ind} cells. *BCR::ABL1-GFP* expression was turned off following a pulse of Dox for 10 consecutive days and then restored following a chase of 10 days during which Dox was removed. **(B-C)** Relative quantitative expression of core clock genes in UT7-GFP^{ind} and UT7-P210^{ind} cells after a pulse (B) and a chase (C) of either DMSO or Dox cells. Data are represented as mean \pm SD from three independent experiments. One-way ANOVA + Tukey's *post hoc* test was used to assess the statistical significance. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; n.s, not significant. **(D)** Immunoblot of BCR::ABL1, ABL1, BMAL1 in UT7-GFP^{ind} and UT7-P210^{ind} cells after the pulse (left blots) and the chase (right blots). Semi-quantitative analysis of BMAL1 protein was normalized to ACTB using ImageJ.

Figure 5: MEK inhibition or BMAL1 overexpression restores BCR::ABL1-modulated BMAL1 expression. **(A)** Western blot analysis of BCR::ABL1 and BMAL1 in UT7-P210^{ind} and UT7-GFP^{ind} cells treated with inhibitors of MEK (PD0325901, 0.5 μ M), JAK (JAK inhibitor I, 1 μ M) and PI3K (LY294002, 20 μ M) for 72h. **(B)** Semi-quantification of BMAL1 protein expression following MEK, JAK and PI3K inhibitions, normalized to ACTB. Data are represented as mean \pm SD from three independent experiments. One-way ANOVA + Tukey's *post hoc* test was used to assess the statistical significance. *, $p < 0.05$; **, $p < 0.01$. **(C)** Western blot analysis of

pBCR::ABL1, BCR::ABL1, pERK1/2, ERK1/2, BMAL1 and β -actin in UT7-P210^{ind} cells treated with MEK inhibitor for 72h. **(D)** p-BCR::ABL1, BCR::AL1 and BMAL1 proteins were analyzed by immunoblot in UT-7/11 cells and UT-7/11 transduced with an empty PHAGE or overexpressing BMAL1. **(E)** Quantitative expression of core clock genes in BMAL1-overexpressed UT-7/11 cells. BMAL1 Data are represented as mean \pm SD from two independent experiments.

A**B****C****Figure 1: Rittavee et al.**

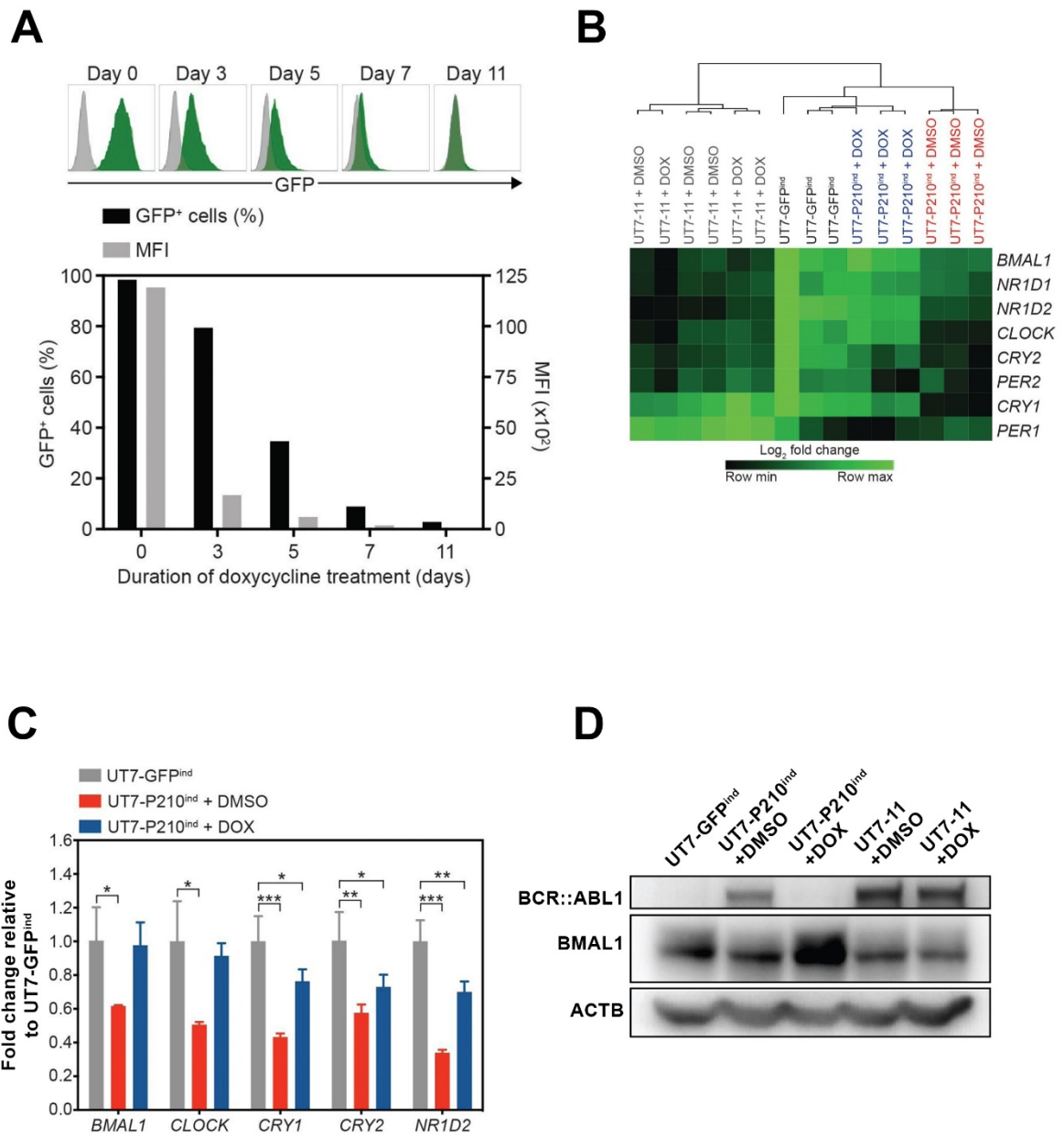


Figure 2: Rittavee et al.

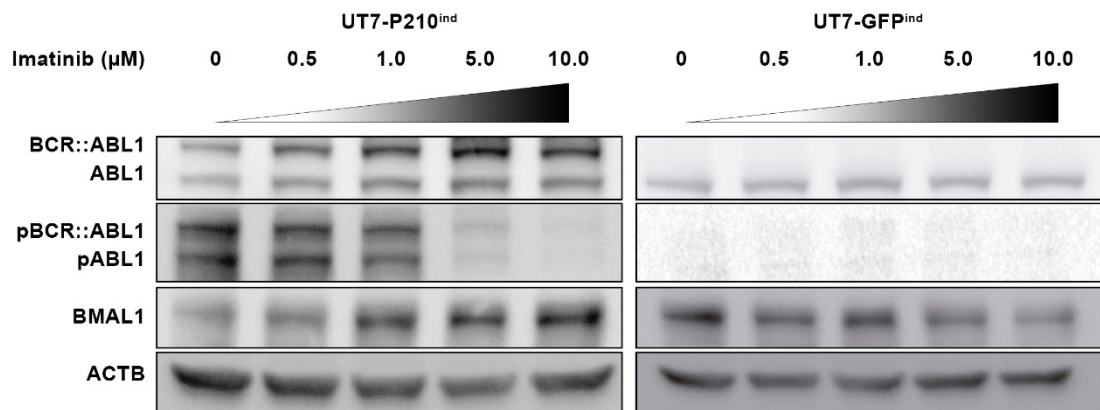
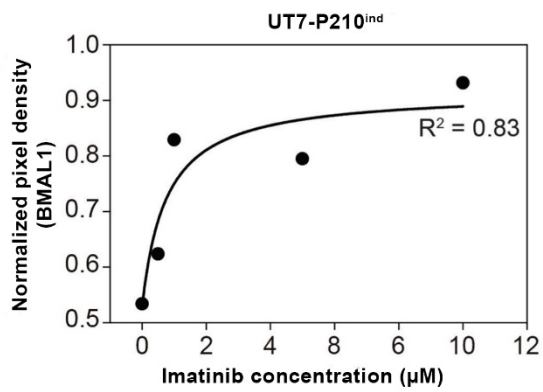
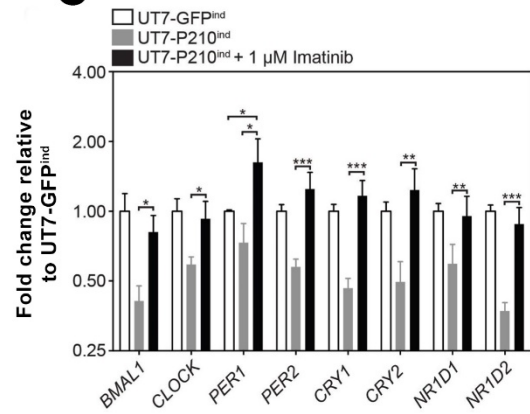
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Figure 3: Rittavee et al.

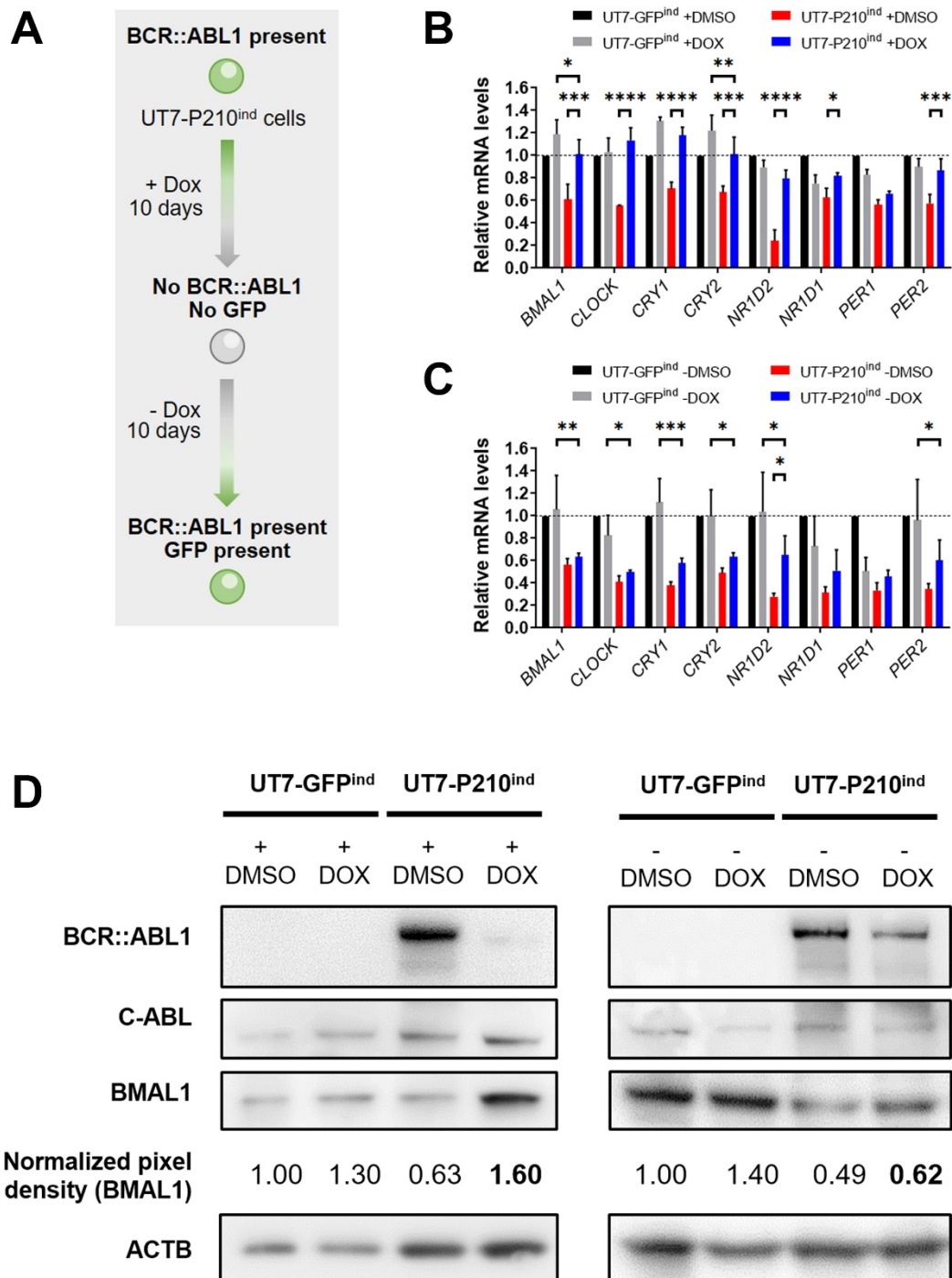


Figure 4: Rittavee et al.

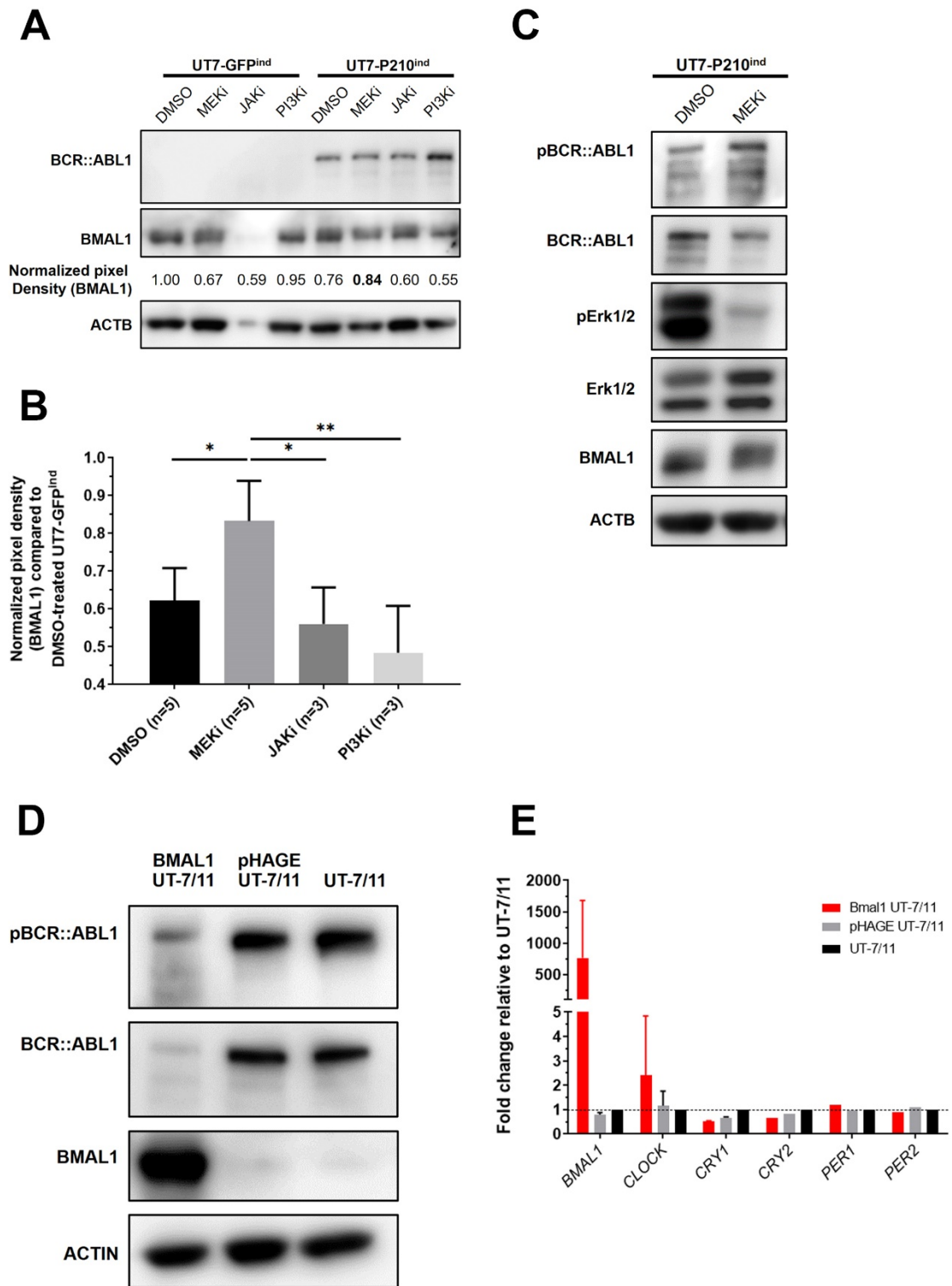


Figure 5: Rittavee et al.

SUPPLEMENTARY TABLES

Table 1: List of primers used for quantitative real-time PCR analysis.

Gene	Forward primer	Reverse primer
<i>B2M</i>	5'-TGCTGTCTCCATGTTTGATGTATCT-3'	5'-TCTCTGCTCCCCACCTCTAAGT-3'
<i>BMAL1</i>	5'-TGGAGAAGGTGGCCCAAAGA-3'	5'-TCCTCAGCAATCATTCCGGCCTA-3'
<i>CLOCK</i>	5'-AAGTTAGGGCTGAAAGACGACGA-3'	5'-GAACTCCGAGAAGAGGCAGAAG-3'
<i>CRY1</i>	5'-TAATGGGAATGGAGGCTTCATGG-3'	5'-TCCCTTGAGAGCAACTTCCACT-3'
<i>CRY2</i>	5'-GTCAACCATGCCGAGACCAG-3'	5'-GGACAGATGCCAGTAGACAGAGT-3'
<i>NR1D1</i>	5'-GAGCACCAGCAACATCACCAAG-3'	5'-TCTTGAAGCGACATTGCTGGCA-3'
<i>NR1D2</i>	5'-TAAAGTCTGTGGGGATGTGG-3'	5'-CAGTTTTTCATTCTTCAGGCACT-3'
<i>PER1</i>	5'-GCTCTCCACTCCAGCTCAATCT-3'	5'-TCAGTGACCTCCGCCAGTCT-3'
<i>PER2</i>	5'-GTCCACCTCCCTGCAGACAA-3'	5'-CTGGTAATACTCTTCATTGGCTTTCA-3'
<i>RORA</i>	5'-TGTGGAGACAAATCATCAGGAA-3'	5'-GACGAGGACAGGAGTAGGTG-3'
<i>RORC</i>	5'-TTTGA CTCTCCCACTCCCTAA-3'	5'-TGTTCTACTTTCCTTTTCTCTTGGA-3'

Table 2: List of antibodies used for immunoblot.

Antibody	Dilution	Supplier	Product number
ACTB	1:30000	Sigma-Aldrich	a3854
BCR::ABL1, ABL1	1:500	Santa Cruz Biotechnology, Dallas, TX, USA	SC-56887
pBCR::ABL1, pABL1 (Tyr245)	1:1000	Cell Signaling Technology, Danvers, MA, USA	2861S
BMAL1	1:1000	Abcam, Amsterdam, Netherlands	ab3350
ERK1/2	1:1000	Cell Signaling Technology	9102
pERK1/2 (Thr202/Tyr204)	1:1000	Cell Signaling Technology	9101
Anti-rabbit IgG, HRP-linked Antibody	1:2000	Cell Signaling Technology	7074
Anti-mouse IgG, HRP-linked Antibody	1:2000	Cell Signaling Technology	7076

GENERAL DISCUSSION AND PERSPECTIVES

The development of targeting therapies using TKIs resulted in a paradigm change in the therapy of CML, with the disease becoming a chronic condition in patients responding to therapies (Druker et al. 1996). Since then, despite remarkable advancements in determining the efficacy and processes that cause the loss of response to a specific TKI, as well as the severity of the disease from chronic to advanced phase CML, we are now confronted with the issues of TKI intolerance or resistance. TKI resistance usually involves both BCR::ABL1-dependent and BCR::ABL1-independent mechanisms and continues to be a key problem in CML therapy (Talati and Pinilla-Ibarz 2018). In this regard, my PhD project was centered on the role of *miR-495-3p* and the circadian clock in CML pathophysiology and led to identifying new levels of regulation of resistance mechanisms through post-transcriptional regulation of ABC transporter P-glycoprotein (P-gp, MDR1, ABCB1) and the 'gatekeeper' mutation T315I of *BCR::ABL1* oncogene.

One important result of my work is the identification for the first time of the down-expression of *miR-495-3p* in BCR::ABL1-expressing cellular models compared to non-leukemic control. This downregulated expression in CML is consistent with other cancers and leukemias where miR-495-3p exerts an oncosuppressor function and its downregulation is implicated as a potential mechanism of anti-cancer drug resistance (Guo et al. 2010; Xu et al. 2013; Zou et al. 2017; Li et al. 2021). Furthermore, we have revealed the function of miR-495-3p on the regulation of MDR1 activity in BCR::ABL1-expressing cells. Our results confirmed what was described previously where overexpression of *miR-495-3p* was able to suppress the efflux of chemotherapeutic drugs like Adriamycin in CML cells (Xu et al. 2013). Nonetheless, since all TKIs authorized for the treatment of CML are acknowledged MDR1 substrates (Eadie et al. 2014), we have reported for the first time that MDR1-mediated TKI efflux activity in leukemic cells was regulated by miR-495-3p. These findings contribute to a better understanding of the

progression of TKI resistance, which is mediated by epigenetic changes based on noncoding RNAs and the subsequent control of drug-transport systems.

The enhancement of chemotherapeutic sensitivity via miR-495-3p overexpression did not only occur for Imatinib treatment to WT BCR::ABL1 but also the sensitivity of T315I mutant to Ponatinib. Hence, targeting this microRNA in combination with TKI therapy could provide better therapeutic management and help to achieve a sustained DMR. These findings need to be confirmed *in vivo* as well as in clinical samples since we interestingly found that predicted miR-495-3p targets encode proteins in CML-BP patients involved in protein phosphorylation and are associated with a worse prognosis in the leukemic patient cohort. Even though several miRNAs were discovered as biomarkers for diagnosis and treatment response (as described in Chapter 3.3), currently only a few miRNAs could be used as an indicator for CML prognosis (Kotagama et al. 2015; Alves et al. 2019). Recent work from the Terry Fox laboratory demonstrated the proposed to use of a combination of several miRNAs as a prognostic tool to identify Nilotinib responders (Chen Leukemia 2021). Therefore, the up-regulation of miR-495-3p could be taken into account in the combined profile along with other well-studied prognosis makers and could serve as a novel strategy to overcome the TKI resistance of CML by targeting the TKI efflux activity through MDR1 and the resistance mechanism.

It is also worth noting that our unpublished preliminary work showed that the expression of miR-495-3p was remarkably up-regulated 40 times in proliferative HSCs (CD41⁺) (**Figure 33**) compared to quiescent ones. This result opens up the remaining field for future investigation of the role of miR-495-3p in LSC-quiescence resistance mechanisms which may have effects on various survival and tumorigenesis signaling comparable to those previously studied in cancer stem cells (Hwang-Verslues et al. 2011; Jiang et al. 2012; Cattaneo et al. 2015).

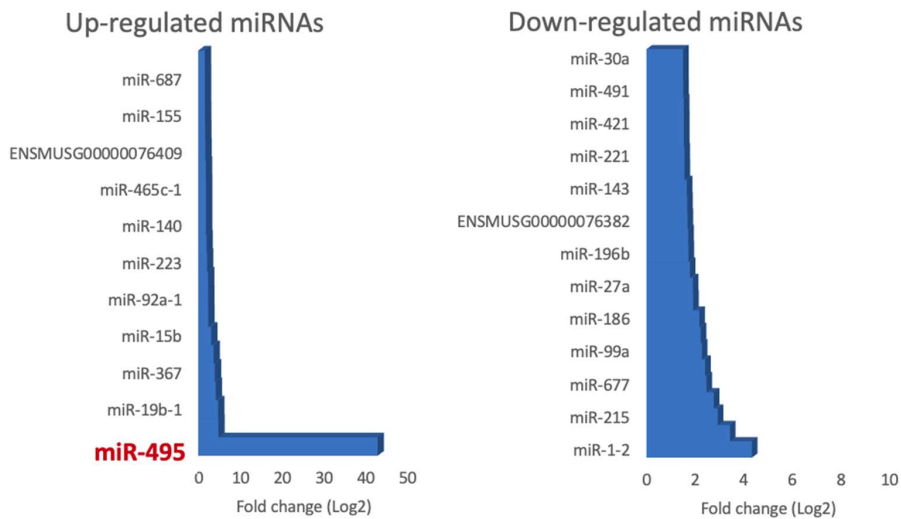


Figure 33: The miRNome analysis of up- and down-regulated miRNAs in proliferative HSCs (CD41⁺) compared to quiescent HSCs (CD41⁻) (Unpublished)

The second important result was the identification of the deregulation of core clock genes by BCR::ABL1. This area of research has received attention because altering circadian rhythms increases the risk of developing a range of cancers, including hematological malignancies. It has been shown previously that the disruption of core clock gene expressions and circadian rhythm was induced by an oncoprotein MLL-AF9 in AML (Puram et al. 2016). In CML cell line models and patient samples, in a similar sense, we found an inverse correlation of core clock gene expressions with *BCR::ABL1* oncogene. This modulation by BCR::ABL1 was also interestingly restorable via regulating inducible Tet-Off system *BCR::ABL1* expression or increasing concentrations of Imatinib treatment. At the work at the current point where my doctoral study has been finished, we found some evidence that indicated the BCR::ABL1-mediated mechanism on deregulation of core clock genes via MAPK/ERK signaling pathway. It shed the light on future works of validation further considering this signaling pathway is highly essential for CML cell lethality (Chu et al. 2004). One important finding of this work was the implication that deregulation of circadian rhythm might be an additional important BCR::ABL1-dependent mechanism that governs the pathogenesis and homeostasis of CML rather than each component of the molecular clock circuit working individually as transcription factors as

previously studied (as described in Chapter 4.5). To further test this hypothesis, we should analyze the phenotypical consequences of circadian clock disruption in BCR:ABL1-positive cells. One of our future plans to tackle this question is to perform the reversible BCR:ABL1 modulation on our *Bmal1*-promoter-driven luciferase reporter (*Bmal1:luc*) model to visualize the disrupted oscillation. This may provide that novel information on BCR::ABL1-regulated dysfunctional circadian rhythms in CML.

Moreover, further studies are required to determine the role of core clock genes, especially BMAL1, in inhibiting the kinase activity of BCR::ABL1 and CML pathogenesis as we found the overexpression of BMAL1 could lessen phosphorylated BCR::ABL1 in UT7-11 cells. It is important to point out that according to our unpublished early research, we induced CML in one cohort of *Per2* KO mice. The survival of mice receiving *Per2*^{-/-} cells was significantly lower (**Figure 34A**). Therefore, our preliminary works suggest that complete loss of circadian function (*Per2* KO) apparently increases CML aggressiveness while overexpression of circadian function (BMAL1) decreases CML-driven BCR:ABL1 phosphorylation activity. Interestingly, after leukemic initiation via retroviral transduction of *BCR::ABL1*, one-third of mice in the cohort tended to develop B-ALL instead of CML (**Figure 34B**). These results showed the unrevealed importance of circadian clock genes on leukemogenesis and the molecular mechanism underlying this retaliation to BCR::ABL1 remains to be elucidated.

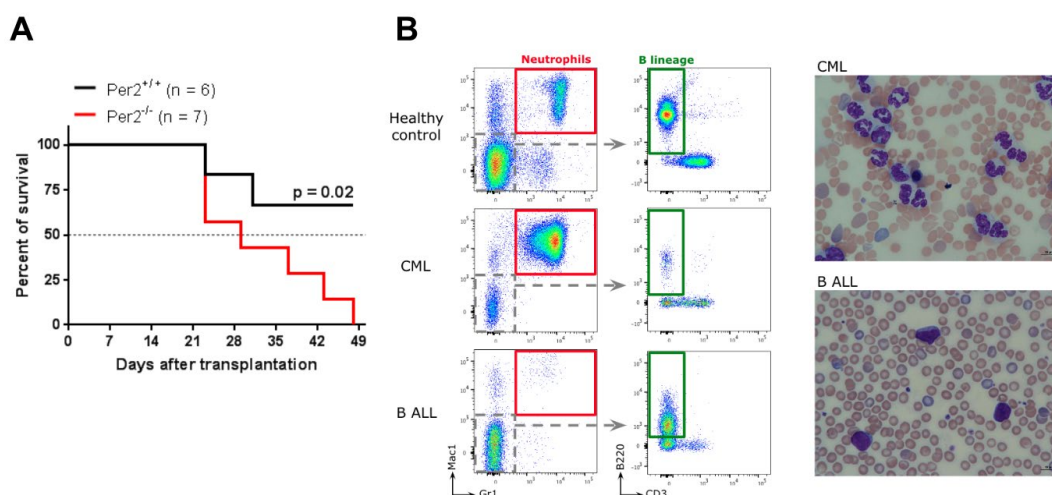


Figure 34: Effect of *Per2* loss in a mouse model of BCR::ABL1+ leukemia. (A) Loss of *Per2* facilitates the onset of leukemia (B) Loss of *Per2* induces the development of B-ALL (30% of cases) (Unpublished)

Overall, with the discovery of better treatments and the continuous focus on TKI withdrawal, attention is turning to achieving the deepest possible molecular remission by eradicating CML LSCs. As a result, new techniques are required, which will result in the removal of TKI resistance and LSC persistence. In summary, our results open new avenues to novel approaches to counteract challenges from the emergence of the TKI resistance by miR-495-3p-targeted TKI efflux activity of MDR1. A better understanding of CML homeostasis by BCR::ABL1-regulated molecular circadian circuitry will undoubtedly lead to significant progress to find a cure for CML. To achieve this goal, we are currently testing the effect of pharmacological alteration of circadian clock machinery, by modulating ROR activity, a direct transcriptional activator of *Bmal1* (**Figure 30**). By using clonogenic assay, its agonist SR1078 treatment is selectively toxic against CML progenitor cells and spares healthy HSPCs (**Figure 35**). In the same way, another ROR agonist from Lycera (LYC-55716) is currently in clinical trials for NSCLC patients (Camidge et al. 2018), hence, our findings might open up for likewise therapeutic efficacy of circadian clock gene agonist treatment strategy in mouse models of CML and in human CML CD34+ cells.

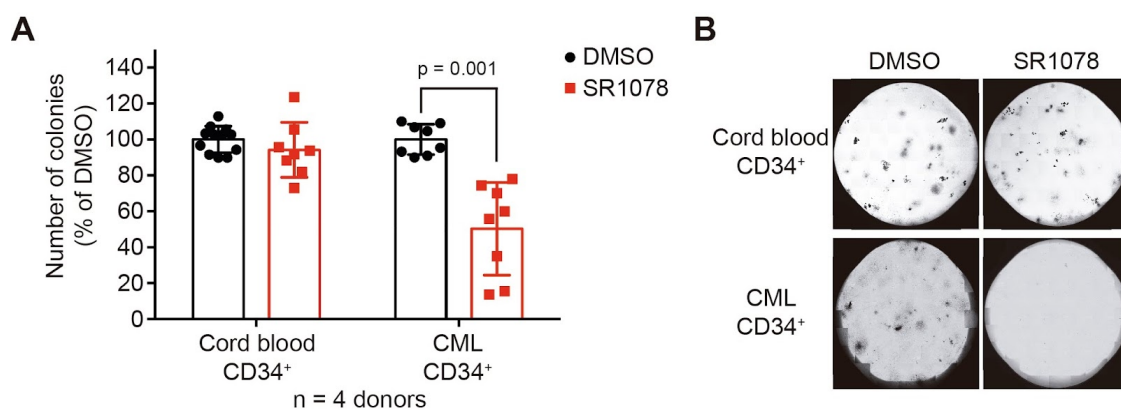


Figure 35: The ROR agonist SR1078 kills CML CD34+ hematopoietic precursors while sparing hematopoietic precursors from cord blood (Unpublished)

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