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RESEARCH ARTICLE

ROLE OF ANGIOGENESIS IN CHRONIC MYELOID LEUKEMIA

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ABSTRACT

Chronic Myeloid Leukemia (CML) is a myeloproliferative neoplasm of the hematopoietic system, characterized by the presence of the BCR-ABL oncoprotein due to the chromosomal translocation t (9;22). This oncoprotein has elevated tyrosine kinase activity, which leads to enhanced proliferation, reduced differentiation and apoptosis, increased angiogenesis etc. Even though several targeted tyrosine kinase inhibitors (TKIs) such as imatinib, dasatinib etc. are being employed in treating CML, a proportion of patients (25-30%) exhibit resistance to TKIs leading to treatment failure and unchecked disease progression. Progression of CML may be due to genetic instability which include chromosomal translocations, mutations, polymorphisms and gene amplification which ultimately causes up and down regulation of genes in various pathways including angiogenesis. Increased angiogenesis is associated with CML due to the up regulation of various angiogenic factors and their transcriptional regulators, which in turn has been found to lead to disease progression to advanced phases, as the protein products of these genes may act synergistically with BCR-ABL oncoprotein in advancing the disease. Single nucleotide polymorphisms (SNPs) are one of the causes for up regulation of antigenic genes and are associated with susceptibility and progression of CML by affecting therapeutic outcome. This review focuses mainly on the role of upregulated pro-angiogenic factors- VEGF, IL-8 and their transcriptional regulators HIF1α, NF-kB and also the role of SNPs in these genes in disease susceptibility, progression, drug response, prognosis and survival in CML patients. Identification of SNPs and up regulated genes of angiogenesis may serve as biomarkers for predicting disease progression, drug response, prognosis etc. Anti-angiogenic therapy is aimed at targeting the new blood vessels that supply nutrients to rapidly growing tumor cells. Combinations of targeted therapy and anti-angiogenic therapy may serve as the novel therapeutic strategies in overcoming drug resistance and thereby preventing the disease progression in CML.

INTRODUCTION

Chronic Myeloid Leukemia (CML) is a hematopoietic stem cell malignancy that affects the myeloid, erythoid and platelet cells, resulting in their uncontrolled proliferation. CML incidence is high in adults and very rare in children. Symptoms of CML include lethargy, weight loss, unusual bleeding, sweats, anemia, and splenomegaly. The main characteristics of the disease include growth factor independence, enhanced proliferative capacity, prolonged survival of hematopoietic stem cells, reduced apoptosis and modified cell adhesion properties (Di Bacco *et al*, 2000). CML is largely characterized by a genetic abnormality, Philadelphia (Ph) chromosome, formed due to the reciprocal translocation between chromosomes 9q34 and 22q11, which produces a 210-KDa fusion protein BCR-ABL, detected in more than 95% of CML patients (Rowley, 1973). The resulting oncoprotein possesses increased tyrosine kinase activity which enhances the growth and proliferation of leukemic cells. Additionally, leukemic blasts expressing this oncoprotein exhibit resistance to apoptosis, lack of cell adhesion, reduced differentiation, increased angiogenesis etc. (Ren, 2005; Lewis *et al*, 1996). Down regulation of BCR-ABL has been observed during leukemic cell differentiation, but the underlying mechanism is not known (Wetzler *et al*, 1993). The cellular event responsible for this translocation or initiation of leukemogenesis is unclear. Some studies showing that some healthy individuals contain very low level of BCR/ABL fusion gene, and suggesting that only a minor fraction of spontaneous Ph translocations leads to CML. So in addition to the fusion

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gene some cellular or molecular events are essential for leukemogenesis (Bose *et al*, 1998).

CML occurs in three different phases: chronic phase (CP), accelerated phase (AP) and blast crisis (BC). CML-CP is characterized by an increase in immature and mature myeloid cells with maintenance of hematopoietic differentiation, whereas CML-AP and BC are characterized by more number of immature cells with great reduction in hematopoietic differentiation process and are associated with increased resistance to apoptosis (Calabretta and Perrotti, 2004). More than 90% of CML patients are diagnosed when their disease is in a relatively early phase known as the chronic phase (CP) (Perrotti *et al*, 2010). CML-BC is characterized by enhanced genomic instability, dysregulated proliferation and arrested differentiation of hematopoietic progenitors.

The molecular factors that contribute to the progression of CML is still unclear. Genomic instability may play a role in the transformation of CML to advanced stage, which is characterized by greater prevalence of mutations within the genome cellular lineage. These mutations may involve chromosomal instability and alteration in the DNA sequence. Chromosomal instability is the dominant form of genomic instability and is attributed to changes in the structure and number of chromosomes observed in many cancers (Negrini *et al*, 2010).

Current therapeutic interventions

Tyrosine kinase (TK) inhibitors such as Imatinib, dasatinib, nilotinib, bosutinib etc, have been developed to decrease the amount of BCR-ABL transcripts or to inhibit tyrosine kinase activity. Among them Imatinib is widely used as the first line therapy, functions by preventing the binding of ATP to the activated tyrosine kinase and permit the differentiation of leukemic cells and undergoing apoptosis (Deininger *et al*, 2005). Despite the availability of targeted TK inhibitor therapy, leukemic cells develops resistance to imatinib has been a major disadvantage especially in the treatment of advanced stage CML (Talpaz *et al*, 2006). This may be attributed to the up- and down regulation of genes of various pathways such as apoptosis, angiogenesis, cell cycle, differentiation etc. that regulate myelopoiesis which has necessitated the need for identification of molecular targets that play a synergistic role along with the BCR-ABL oncoprotein, which inturn will be helpful in design and discovery of novel therapeutic strategies for anti-leukemia therapy. Further information on the mechanisms of imatinib resistance has been discussed in the following section.

Imatinib resistance

Even though several tyrosine kinase inhibitors are available, are effective for the treatment of CML patients during chronic phase (Druker *et al*, 2001), but they are incapable of killing leukemic stem cells (LSCs) effectively (Graham *et al*, 2002). 100% remission in CML patients can be achieved by the complete elimination of LSCs. LSCs utilize similar mechanisms for self-renewal and survival as Hematopoietic Stem Cells (HSCs). For ex. developmental genes required for normal HSCs such as Wnt and Hedgehog signalling pathways, Bmi-1 and p53 are involved in the regulation of both HSCs and LSCs (Zhao *et al*, 2009). LSCs also use specific pathways (Chen *et al*, 2009), contributing or supporting a novel scenario or blue print (strategy) for targeting especially LSCs without affecting normal HSCs**.**

Imatinib resistance may be explained by various mechanisms: (a) Acquisition of ABL kinase point mutations (b) BCR-ABL amplification at the genomic level (Gorre *et al*, 2001) (c) increased expression of other tyrosine kinases (ex: Src kinase family) and cytokines (Donato *et al*, 2003) (d) acquired additional genetic (chromosomal and molecular) abnormalities (e) differences in the level and function of the drug influx protein organic cation uptake transporter (OCT-1) encoded by SLC22A1 gene (Goldman *et al*, 2006). When imatinib binds to the BCR-ABL fusion oncoprotein, it inactivates signal transduction leading to apoptosis. Recent studies have shown that alternative pathways like Src kinase mediated pathway are activated when imatinib blocks the BCR/ABL mediated pathway, thereby CML cells escape apoptosis (Meyn MA *et al*, 2006).

Recent studies have elucidated that BCR-ABL alternative splicing in CML patients is one of the mechanisms for development of drug resistance (Ma *et al*, 2009). Enhanced levels of BCR-ABL expression has been demonstrated during progression from chronic phase to advanced phases (Gorre *et al*, 2001). BCR-ABL fusion proteins, with constitutive tyrosine kinase activity, stimulate several signalling molecules such as p21Ras (Pendergast *et al*, 1993), signal transducer and activator of transcription 5 (STAT5) (Sillaber *et al*, 2000), and promote the expression of pro-angiogenic cytokines such as VEGF and its transcriptional regulator HIF1α, NF-kB, etc via phosphoinositide 3-kinase (PI3-kinase) (Mayerhofer *et al*, 2002) pathway. As a result, BCR-ABL oncoprotein activity leads to enhanced proliferation, increased angiogenesis, reduced apoptosis and differentiation of progenitor cells (Puil *et al*, 1994). With the recent insights in the role played by the bone marrow microenvironment in hematolgical malignancies, there is a need for a better understanding of the bioprocesses that function in this microenvironment. One of the most important processes that have been found to aid leukemic cells is angiogenesis. Hence, this review discusses the role of angiogenesis genes in CML development, progression to advanced phases and also its influence on therapy.

Angiogenesis

Angiogenesis is the process of formation of new blood vessels from pre-existing ones and is essential for maintainance of integrity of the body (Cines *et al*, 1998). This process involves endothelial cell division, degradation of the basement membrane and the surrounding extracellular matrix, migration of endothelial cells and the establishment of a tubular structure. Once the blood vessel formation taken place, endothelial cells undergo tissue-specific changes to produce active blood vessels. During embryogenesis blood vessels are formed from endothelial cell precursors known as angioblasts through differentiation process, which are joined to form primitive vessels by a process known as vasculogenesis. Angiogenesis is mediated by a highly complex regulatory system that involves various proangiogenic and antiangiogenic factors (Ferrara *et al*, 2005). Angiogensis is controlled by a phenomenon known as “angiogenic balance”, which is a physiological balance between the stimulatory and inhibitory signals for the formation of blood vessels. In healthy status, new blood vessel formation takes place during organ degeneration, wound healing, menstruation, in the female

reproductive system during ovulation and during placenta formation (Cines *et al*, 1998). New blood vessel formation is also an important factor in diseases such as in tumor growth, solid tumors, psoriasis, diabetic retinopathy and rheumatoid arthritis (Hahahan *et al*, 1996).

Various growth factors are involved in the regulation of endothelial cell tube formation. These include fibroblast growth factor, epidermal growth factor and platelet derived growth factor. Disturbances in the regulation of angiogenesis factors may lead to severe consequences. Solids tumors grow rapidly by pushing the cells away from the capillaries, which generally provide nutrients and oxygen for the cells to survive. To survive in the hypoxic environment, tumor cells secrete various pro-angiogenic molecules that facilitate new blood vessel formation and proliferation, thereby suppling the necessary nutrients and oxygen for cells. The identification of new proangiogenic factors that promote angiogenesis and developing new targets to inhibit these factors in order to block angiogenesis in tumors is of great importance in anti-angiogenic therapy (Martinez *et al*, 2006).

Role of angiogenesis in normal hematopoiesis and Chronic myeloid leukemia (CML)

Angiogenesis plays an important role in the hematopoiesis, leukemogenesis and disease progression of chronic myeloid leukemia (CML). Hematopoiesis and angiogenesis are closely linked processes because hematopoietic cells (HCs) and endothelial cells (ECs) derive from common progenitor cell in the yolk sac known as 'hemangioblast'. ECs perform important activities in hematopoiesis as components of bone marrow stromal cells while HSCs promote angiogenesis in adult tissues such as pericardium, head, peritoneal wall etc. hence both are related developmentally and functionally (Li *et al*, 2003).

HCs have been reported to play a role in tumor angiogenesis, demonstrated by the fact that the pro-angiogenic cytokines and extracellular matrix metalloproteinases released by macrophages and mast cells promote the tumor angiogenesis (Coussens and Werb, 2002).

Increased micro vessel density (MVD) resulting in enhanced bone marrow angiogenesis has been reported in CML (Aguayo *et al*, 2000), which is associated with the overexpression of various pro-angiogenic cytokines; some of them such as VEGF, HIF1alpha (VEHF regulator), IL-8, NF-kB are discussed here. Moreover, endothelial cells (ECs) in CML have faster proliferation and longer survival abilities which results in apoptotic suppression (Green, 2000). Korkolopoulou P *et al*. (2003) suggested that the new blood vessel formation is a pre-requisite for the conversion of normal bone marrow to CML and ultimately to blast crisis. The initiation of leukemogenesis and progression to advanced phases despite the availability of targeted therapy is associated with the enhanced survival or proliferation of leukemic cells, which is followed by reduced differentiation, increased angiogenesis, and metastasis to other sites favoring leukemic cell growth.

Angiogenic factors in CML

Angiogenesis is regulated by angiogenic factors such as VEGF (Ferrara 1996) and its transcriptional regulator HIF1α, and IL-8, its regulator NF-kB etc. Elevated levels of these angiogenic factors associated with poor prognosis have been reported in solid tumors (Salven *et al*, 1997). The miscellaneous (diverse) interactions between leukemia cells and a highly complicated network including growth factors, other cytokines, cell-cell adhesion receptors, stromal cells and matrix molecules present in the bone marrow may play a key role in the progression of CML (Gordon *et al*, 1987). Thus, angiogenesis and angiogenic factors play an important role in the pathogenesis of CML, as evident by the significantly increased vascularity reported in CML. Increased number of blood vessels and larger vascular area were identified in CML. Elevated levels of plasma VEGF has been observed in CML, suggesting that increased vascularity and upregulation of angiogenic factors in CML may be involved in leukemogenesis and pathogenesis (Aguayo *et al*, 2000). The clinical significance of increased bone marrow vascularity and the role of angiogenic factors individually or collectively, in CML need to be further investigated as this information may be useful in designing and developing new therapies for Leukemias. In the following sections we have focussed on four of the most important genes that have been reported to be deregulated in angiogenesis in CML.

Vascular endothelial growth factor (VEGF)

VEGF, also known as vascular permeability factor (VPF), is a dimeric cytokine produced by various tissues and cells. VEGF acts as an important regulator of endothelial cell growth and angiogenesis (Ferrara *et al*, 1997). Inflammatory mediators such as prostaglandins stimulate angiogenesis via VEGF up regulation. Studies have shown that VEGF is an autocrine regulator of growth of normal murine hematopoietic stem cells (Gerber *et al*, 2003). Chemically VEGF is a glycoprotein and functionally is a mitogen in inducing vascular permeability in vascular endothelial cells (Ferrera, 2003). In normal human bone marrow VEGF is basically expressed in megakaryocytes and immature myeloid progenitors while during the differentiation and maturation of myeloid progenitors, VEGF levels in the cytoplasm decrease.

VEGF expression in induced by binding to its tyrosine kinase receptors VEGF-R1 (flt-1) and VEGF-R2 (KDR/flk-1), expressed on hematopoietic stem cells and vascular endothelial cells (Wu Y *et al*. 2006). Blood vessel formation is regulated by VEGF and its receptors (Ferrara *et al*, 2003). VEGF and VEGFR-1 are indispensable for hematopoietic (Gerber *et al*, 2003).

VEGF production is in turn regulated by a complex network of signal transduction cascades and transcription factors. Hypoxia inducible factor-1α (HIF-1α) is the major transcriptional regulator of VEGF production (Forsythe *et al*, 1996). HIF-1α is involved in hypoxia stimulated VEGF production and also plays a role in oncogene dependent expression of VEGF (Blancher *et al*, 2001). Various factors such as hypoxia, inflammation, hypoglycemia are the major stimulus of VEGF production. Hypoxia is a common phenomenon observed in the microenvironment of solid tumors and is regarded as a primary stimulus of VEGF expression in neoplastic cells (Shweiki *et al*, 1992). Some recent studies have suggested that in the absence of hypoxia, oncogenes such as v-src and Ras stimulate VEGF production (Pal *et al*, 2001). VEGF levels are upregulated in a variety of solid tumors and has been involved in tumor related angiogenesis (Ferrara *et al*, 1997).

Some studies have shown that VEGF is over expressed in malignant myeloid cells and contributing to the

leukemogenesis of CML (Sillaber *et al*, 2004). The prognostic significance of VEGF in CML reported by Verstovsek *et al*. (2002), they observed no significant difference of VEGF expression in the three phases of CML, but the levels were found to be more in patients older than 60yrs, and they also reported that VEGF is a good predictor of poor survival in CML. They also reported that VEGF is a good predictor of poor survival in CML. It has been reported that in CML, increased expression of VEGF in the bone marrow, peripheral blood and elevated VEGF levels are observed in serum. Thus, leukemia cell derived VEGF plays an important role in the pathogenesis of CML. Some studies revealed that bone marrow cells showed higher expression of VEGF in CML patients than in controls. VEGF expression is induced by its receptors such as VEGFR-1 and VEGFR-2 (Lundberg *et al*, 2000). Some other studies reported the increased expression of both receptors in the bone marrow of CML patients (Santos *et al*, 2004). VEGFR-2 mRNA was also detected in chronic phase CML samples tested (Ratajczak *et al*, 1998) and high VEGFR-2 expression is associated with poor survival of CML patients (Verstovsek *et al*, 2003). VEGF levels are also up regulated in CML patients who progressed to advanced phases (Liu P *et al*., 2005). Verstovsek *et al*. (2003) reported that patients in the AP and BC phases of CML with higher VEGFR2 expression had slighlty worse survival rates and suggested that up regulated VEGFR2 may serve as prognostic marker for decrease in survival rate in CML patients. Thus, this receptor, VEGFR2, may be involved in progression to advanced stages.

Molecular mechanism of VEGF mediated angiogenesis in CML

Little information is available on the mechanism of expression of VEGF in CML cells. VEGF may promote the growth of vascular endothelium thereby supplying required blood for the growing leukemic cells.

Mayerhofer *et al*. (2002) performed studies to assess whether BCR/ABL oncoprotein induces the expression of VEGF gene and hypoxia inducible factor-1α. They have observed that BCR/ABL induced VEGF expression in growth factor dependent Ba/F3 cells, stimulated the activity of VEGF promoter and was responsible for high levels of VEGF protein in Ba/F3 cells. In CML, it has been observed that BCR-ABL induces the expression of VEGF and HIF1α through phosphoinositide-3 kinase and mammalian target of rapamycin (mTOR). BCR/ABL activates several signalling pathways such as PI-3 kinase pathway, Ras and downstream MAPKs (Raf, MEK, ERK) (Pendergast *et al*, 1993). It has been shown that oncogenic Ras induces the expression of VEGF via PI-3 dependent kinase pathway in human epithelial breast cancer cell lines (Blancher *et al*, 2001). These studies indicate that VEGF expression, induced by BCR/ABL, may be involved in the pathogenesis and increased angiogenesis in CML. The signalling pathways involved in the VEGF expression induced by oncogenes differ as per the cell type analysed. Therefore, it is essential to identify the signalling pathways devoted to VEGF expression in human BCR/ABL CML cells. BCR/ABL dependent VEGF induction also involves STAT 5 pathway (Sillaber *et al*, 2000), thus a link between STAT 5, angiogenesis and VEGF has been proposed (Niu *et al*, 2002).

A study by Aguayo *et al*. (2002) suggested that greater vasculature, higher levels of blood vessels and elevated plasma VEGF levels were observed in CML than other leuemias. It has also been observed that BCR-ABL induced VEGF binds to specific receptors on endothelial cells and promotes production of myeloid and lymphoid growth factors such as G-CSF, GM-CSF, IL-6 and SCF (Stem Cell Factor) by endothelial cells, which inturn enhances the leukemic cell proliferation leading to disease progression ultimately drug resistance, hence VEGF and its receptors are important therapeutic targets (Rafii *et al*, 2002). VEGF expression is regulated by various transcription factors such as HIF1α (Hypoxia Inducible Factor 1alpha), AP-1 (Activator protein-1), SP-1 (Stimulatory Protein-1), NF-kB etc. Once these transcription factors are activated in leukemic cells, they stimulate VEGF expression, which results in tumor progression (Shi *et al*, 2001).

Influence of first line Imatinib therapy on VEGF

Imatinib Mesylate (IM) is capable of reversing bone marrow angiogenesis thereby decreasing the plasma VEGF levels in CML patients (Legros *et al*, 2004). BCR-ABL stimulated angiogenesis by induced VEGF expression can be suppressed by treatment with TKIs such as Imatinib, it mimics ATP for binding with ATP binding domain of VEGF and VEGFRs and results in blockade of intracellular signalling thus preventing the activation of downstream effectors involved in tumor progression. Leukemic cells which are TKI resistant due to BCR-ABL amplification/mutation, other genetic abnormalities (cytogenetic and molecular) exhibit high VEGF expression (John Ebos *et al*, 2002). Advanced CML phases are resistant to Imatinib treatment (Kantarjian *et al*, 2006), hence it is important to study in detail the underlying factors of resistance, and is critical to develop novel anti-angiogenic therapeutic strategies for advanced phases.

Influence of SNPs on VEGF expression and function

Apart from VEGF upregulation mediated by its transcriptional factors such as HIF1α, polymorphisms in VEGF and its receptors (VEGFR 1 and 2) may also influence its function. SNPs in VEGF and VEGF receptors may play a role in the CML pathogenesis, progression and also influence the prognosis by affecting the Imatinib therapy. The chromosomal location of VEGFA gene is on 6p21 and spans eight exons and seven introns. Some studies suggested that polymorphisms in VEGFA conferred interindividual differences in VEGF expression. (Vincenti *et al*, 1996). The polymorphisms in the promoter region -2578C>A (rs699947) and -460T>C (rs833061), 5'-untranslated region [+405C>G (rs2010963)] or 3'-untranslated region [+936 C>T (rs3025039)] have been associated with varying levels of VEGF expression (Koukourakis *et al*, 2004; Prior *et al*, 2006). VEGF is a soluble heparin binding glycoprotein dimer has the molecular weight of 34-46 Kda (Thomas, 1996).

VEGFR2 gene is situated on the chromosome 4q11-q12 consists of 26 exons. The protein (VEGFR2 or kinase insert domain containing receptor) encoded by this gene possess 1356 amino acids. VEGFR2 play a key role in leukemia-associated angiogenesis (Padro *et al*, 2002) and transduces important signals for angiogenesis through its tyrosine kinase activity (Shibuya, 2006).

Kim DH *et al*. (2009) analysed the effect of four VEGFA gene polymorphisms, -2578C>A (rs699947), -460T>C (rs833061), _405G>C (rs2010963) and +936C>T (rs3025039), and three

VEGFR2 gene polymorphisms (rs1531289, rs1870377 and rs2305948) on the treatment outcome of 228 Canadian CML patients undergoing imatinib therapy.

Two VEGFR2 SNPs were identified as important prognostic markers. The SNP for rs1531289 is located on intron and SNP for rs187037 is a non-synonymous mutation results in the substitution of glycine with histidine (Q472H) (Park *et al*, 2006). VEGFR2 SNP rs1531289 is associated with complete cytogentic response (CcyR), rs1870377 is correlated with CcyR, major cytogentic response (McyR) and treatment failure. Whereas VEGFA SNPs rs699947 and rs833061 are strongly associated with progression to CML AP and BC.

Hypoxia inducible factor 1 (HIF1α)

HIFα occurs in 3 isoforms, HIF1α, HIF2α and HIF3α. HIF-1α, HIF-2α exhibit structural similarity to a certain extent (Wiesener *et al*, 2003). HIF2α expression is observed to be less in tissues whereas 3α function is less studied (Makino *et al*, 2001).

HIF1α in normal hematopoietic

In mammals BM provides hypoxic culture required for self-renewal, differentiation and survival of hematopoietic stem cells. It has been shown that hypoxic culture is essential for BM function, because under hypoxic conditions hematopoietic progenitors exhibited reduced cell proliferation and were associated with the accumulation of cells at the quiescent phase of the cell cycle (G0). The mechanism of hypoxia regulation in HSCs is not clearly known; some studies suggest that HIF1 plays a key role in mediating the effect of hypoxia on HSCs. It is evident that inhibitors of cyclin-dependent kinases that are regulated by HIF 1α are correlated with the function of hypoxia in HSCs (Eliasson and Jonsson, 2010). Studies in animal models reveal that regulation of HIF 1α is required for HSC survival. Normal HSCs maintain intracellular hypoxia and stabilize HIF 1α protein and HSCs maintain cell cycle in quiescent phase through the regulation of HIF 1α level (Takubo *et al*, 2010). Deficiency of HIF 1α lead to the loss of cell cycle quiescence in a p16 Ink4a/p19Arf dependent manner and resulted in the inability of HSCs to undergo self renewal and differentiation during exposure to stresses like myelosuppression, transplantation and aging in a study by Zhang *et al.* (2012). This study has shown that hypoxic culture is essential to maintain complete pluripotency of human embryonic stem (hES) cells, as evident by the reduced production of human chorionic gonadotropin and progesterone in hypoxic conditions (Ezashi *et al*, 2005).

HIF1, a basic helix-loop-helix (bHLH) transcription factors, is a heterodimer consisting of two subunits: HIF1α and constitutively expressed HIF1ß (Semenza *et al*, 2003). HIF1α expression is regulated by cytokines, growth factors and signalling pathways such as PI3K and MAPK pathways (Zhong *et al*, 2000). Expression of HIF1 α is also regulated by ubiquitination under normoxic conditions. This occurs via the hydroxylation of proline residues 402 and 564 in HIF1α by the enzyme proline hydroxylase (PHD). The hydroxylated HIF1α is recognised by the protein Von Hippel-Lindau (pVHL), a recognition constituent of E3 ubiquitin protein ligase, and is degraded (Simon and Keith, 2008). Some studies suggests that during hypoxic conditions HIF1α activates transcriptional programs in order to maintain self-renewal capacity and multipotency of cancer stem cells, thereby playing a crucial role in cancer progression (Culver *et al*, 2011).

Role of HIF1α in CML

A recent study on mice has shown that upregulation of HIF1α is observed in CML and is essential for survival of LSCs in CML and is also involved in the pathogenesis of CML. LSCs are more dependent on HIF1α than normal HSCs, and HIF1α deletion prevents the propagation of CML by inducing cell cycle arrest and apoptosis (Zhang *et al*, 2012).

HIF has been shown to be involved in the evolution of cancer (Soeda *et al*, 2009). Deletion of HIF 1α causes the increased expression of tumor suppressor genes p16 Ink4a, p19Arf and p53 both in HSCs and LSCs. Deficiency of HIF1α results in increased expression of cell cycle inhibitors p16 Ink4a, p19Arf and p57 and apoptotic gene p53 in LSCs. Enhanced expression of p16 Ink4a, p19Arf causes cell cycle arrest, p53 dependent apoptosis and results in the consumption (exhaustion) of HSCs (Park *et al*, 2003) and LSCs. Suppression of HIF1α is effective in the elimination of LSCs, providing a platfrom for developing anti-HIF1α therapy in the treatment of CML. HIF1α deletion results in the enhanced expression of homing related genes such as MMP-14, CXCL10 and CCL19. The mechanisms involved in the expression of these genes needs to be explored (Zhang *et al*, 2012). HIF1α and its target genes are overexpressed in LSCs expressing BCR/ABL oncoprotein in a leukemia cell line. BCR/ABL dependent HIF1α expression in Ba/F3 cells are mediated by PI3K and mTOR pathways (Mayerhofer *et al*, 2002).

As HIF1α is an important transcriptional regulator of VEGF, HIF1α upregulation results in the production of elevated levels of VEGF in the plasma and serum of CML patients (Aguayo *et al*, 2000). HIF 1α is required for cell cycle arrest during hypoxia (Goda N *et al*., 2003). A study by Takubo *et al*. (2010) has shown that HIF1α plays a defensive (protective) role in against senescence, , but recent studies have shown that lack of HIF1α results in the reduced proliferation of LSCs demonstrating a different mechanism (Zhang *et al*, 2012).

HIF1α plays an important role in the development, progression and metastasis of cancer by activating various genes involved in key aspects of cancer biology, including angiogenesis, erythropoiesis, cell survival and energy metabolism (Smaldone *et al*, 2009). BCR-ABL induced HIF1α is associated with Imatinib resistance.

Thorough knowledge on the significant oncogenic pathways regulated by HIFs might help in designing the novel therapeutic agents that act on the genes corresponding to hypoxia.

HIF1α SNPs

HIF1α gene is located on chromosome 14q23-q24 (Secades *et al*, 2009). Polymorphisms in HIF1 gene have been examined for a hypothetical role of genetic predisposition to cancer (Munoz-Guerra *et al*, 2009). Two SNPs of human HIF1 gene, HIF-1 1772 C/T (rs11549465, P582S) result in proline to serine amino acid substitution and 1790 G/A (rs11549467, A588T) results in alanine to threonine amino acid substitution have been identified. Both the SNPs are located in the exon 12 of the HIF-1gene (Smaldone *et al*, 2009). HIF1α polymorphisms- C > T substitution in intron 8 (rs10873142),

T418I (rs41508050) in exon 10, P564P (rs41492849), L580L (rs34005929), and dinucleotide GT repeats in intron 13 (rs10645014) have been identified. These polymorphisms have been studied in various solid tumors but no studies are available in CML, hence studies on the role of HIF1α polymorphisms in the pathogenesis, progression and influence on therapy are needed, because this information may provide path for establishing novel and alternate therapeutic strategies.

Interleukin-8

IL-8 is a proinflammatory chemokine, otherwise called as CXCL8 or neutrophil activating protein (NAP), produced in response to a wide variety of proinflammatory stimuli by immune cells including neutrophils, monocytes/macrophages, and also by non-immune cells such as keratinocytes, hepatocytes, chondrocytes etc. IL-8 is endoded by IL-8 gene, it is transcribed and translated to produce protein with 72 amino acids in monocytes and macrophages, whereas 77 amino acids in nonimmune cells. IL-8 expression is principally regulated by the cytokines NF-kB and secondly by TNF-α, IL-1ß (Brat *et al*, 2005). IL-8 exerts its functions by binding to two cell surface G-protein coupled receptors designated as CXCR1 and CXCR2 (Holmes *et al*, 1991). Myeloid ELF-1 like factor (MEF) expressed myeloid leukemia cells could upregulate IL-8 expression, promoting bone marrow angiogenesis, thereby stimulating the proliferation and survival of leukemia cells (Fukushima *et al*, 2003).

Some studies suggested that IL-8 and its associated cytokines cumulatively stimulated the growth of BCR-ABL expressing cells and also protects leukemia cells against Imatinib therapy (Weisberg *et al*, 2008).

Role of IL-8 in hematopoiesis

Generally hematopoietic stem cells (HSCs) are located in the bone marrow, where they undergo differentiation and proliferation resulting in the production of mature leukocytes, erythrocytes, which are released into the blood to perform their functions (Orkin and Zon, 2008). In addition to mature leukocytes some of the hematopoietic stem and progenitor cells (HSPCs) are also released into peripheral blood, where they are involved in the regeneration of damaged BM regions (Lapidot and Petit, 2002). IL-8 is involved in hematopoiesis by functioning as a chemoattractant of neutrophils (which mediate the mobilization of hematopoitic progenitors into the blood), HSPC mobilization and angiogenesis (Laterveer *et al*, 1995). Corre *et al*. (1999) reported that IL-8 enhanced the growth and differentiation of monocytes/macrophages in combination with colony stimulating factor-1 indicating its function as an autocrine growth factor for human hematopoitic progenitors. In humans circulated/mobilized HSPCs are the principal source of HSC/HPC for transplantation and the mobilization is brought by the administration of growth factors such as IL-8 and granulocyte colony stimulating factor (G-CSF).

In hematopoietic cells IL-8 expression is strongly promoted by the myeloid ELF1-like factor (MEF)/ELF4, belongs to ETS family of transcriptional regulators and MEF upregulation enhances IL-8 levels. Several myeloid leukemia cell lines expressed IL-8 after induction with 12-O-tetradecanoylphorbol-13-acetate stimulation (Steube *et al*, 2000).

IL-8 induced in response to stimuli binds to CXCR1/2 receptors, which then stimulate many signaling pathways such as PI3K, AKT, MAPK, ERK, JAK2, Src, resulting in the expression of transcriptional regulators such as HIF-1, NF-kB (role in angiogenesis, survival and invasion), AP-1 and AR (involved in cell survival, proliferation), STAT3 (cell proliferation, cell survival, angiogenesis, invasion) (David J.J. Waugh and Catherine Wilson, 2008). Up regulated IL-8, apart from BCR-ABL, may activate many downstream signaling pathways such as PI3K, MAPK, STAT3 which enhances leukemic cell proliferation, survival and thus may play a role in leukemogenesis and progression of CML.

IL-8 as a therapeutic target in CML

By suppressing the IL-8 signalling, tumor cells can be made susceptible to apoptosis and also arrest tumor progression suggests that IL-8 is an important therapeutic target (David J.J. Waugh and Catherine Wilson, 2008). IL-8 over expression is observed in peripheral blood samples of CML-BC patients (Nowicki *et al*, 2003).

Ciarcia *et al* (2012) reported that treatment of CML patients with Imatinib causes IL-8 down regulation in comparison to untreated patients and they suggested that estimation of IL-8 levels in newly diagnosed and Imatinib treated CML patients may provide insights related to progression and diagnosis to the hematologist. Another study suggested that IL-8 over expression was observed in BCR-ABL cells, which suggests its role in the pathophysiology of CML. Treatment of these cells with TKIs such as dasatinib and nilotinib down regulate IL-8 expression indicating that IL-8 may serve as an important predictor for the monitoring of TKI efficacy (Hantschel *et al*, 2008).

Nuclear Factor-kappaB (NF-kB)

Nuclear Factor K B (NF-kB) is a transcription factor, play a key role in regulating the expression of over 150 genes that control cell differentiation, proliferation (regulates cyclin-D proteins), apoptosis (regulating the transcription of anti-apoptotic genes such as Bcl-2, Bcl-XL, FLIP etc.) angiogenesis, inflammation (cytokines), tumor metastasis, cell adhecion etc (Aggarwall, 2004). NF-kB family contain five members: c-Rel (expression is restricted to erythrocytes, granulocytes, monocytes and lymphocytes), p65/RelA (ubiquitously expressed), RelB (expressed in lymphocytes and dendritic cells), p50 (ubiquitously expressed) and p52 (expressed in lymphocytes, macrophages and stomach epithelium) (Ghosh *et al*, 1998, Bottero *et al*, 2006).

Role of NFkB in hematopoiesis

NF-kB regulates hematopoiesis by regulating the differentiation of granulocytes that takes place in the bone marrow, where the promyelocytes are differentiated to form metamyelocytes, which are further differentiated into bone marrow polymorpho nuclear neutrophilic granulocytes (BM-PMNs) which then immigrate to blood to become peripheral blood PMNs (Theilgaard-Monch *et al*, 2005). Activation of NF-kB occurs in two ways: (i) in response to pro-inflammatory cytokines (ii) triggered by the activation of p52 and RelB via exposure to certain members of the TNF cytokine (Senftleben *et al*, 2001). The ability of NF-kB in regulating the expression of proteins which promote cell proliferation, survival and

apoptotic inhibition implicates its role in oncogenesis (Karin *et al*, 2002).

Role of NF-kB in CML

NF-kB has been reported to play a role in the pathogenesis and progression of CML. In CML, BCR-ABL tyrosine kinase stimulates various signaling pathways including NF-kB (Danial *et al*, 2000). BCR-ABL mediated activation of NF-kB leads to increased translocation of NF-kB into the nucleus (Reuther *et al*, 1998) and induces the expression of genes such as c-myc that mediate leukemogenesis. NF-kB may be a key mediator of BCR/ABL signaling and play an important role in the progression/transformation to advanced phases mediated by BCR/ABL (Daniella Cilloni *et al*, 2007).

Up regulation of NF-kB was also reported to play a role in Imatinib resistance. Imatinib, the BCR-ABL TK inhibitor is capable of inhibiting NF-kB and its associated signaling pathways such as phosphatidylinositoll 3-kinase (PI3K), mitogen activated protein kinase (MAPK) etc (Appel *et al*, 2005).

Inhibitors of NF-kB

Combination therapy of imatinib and TZD18 has also been observed to decrease the NF-kB DNA binding activity (Zang *et al*, 2006). It was reported that PS-1145, an inhibitor of IKKß (Inhibitor of Nuclear Factor Kappa-B Kinase Subunit Beta) acts synergistically with imatinib to suppress the proliferation and survival in cell lines and cells from bone marrow of CML patients, and is also capable of overcoming Imatinib resistance by enhancing apoptosis, reducing proliferation and survival of leukemic cells (Cilloni *et al*, 2006). This information provided the path for the drug discovery scientists for the invention of NF-kB inhibitors and their administration along with the existing therapies to overcome Imatinib resistance.

Bortezomib (PS341), a specific proteasomal inhibitor, has been reported to overcome Imatinib resistance by apoptotic induction and inhibition of proliferation. As the deregulated proteasomes are involved in the activation of NF-kB, this suggested a novel CML therapeutic strategy in patients with Imatinib resistance (Gatto *et al*, 2003). Recent studies on CML cell lines suggest that the combination therapy involving Bortezomib (proteasome inhibitor) and Flavopiridol (Cyclin-D kinase inhibitor) greatly promoted apoptosis by inhibiting the DNA binding function of NF-kB, down regulation of BCR-ABL, anti-apoptotic proteins such as BclxL, reduced phosphorylation of signalling pathways such as Akt etc. (Dai Y *et al*. 2004). Another anti-leukemic approach developed by Yu *et al*. (2003) suggested the significance of Histone deacetylase inhibitors (HDI) in combination with the proteasomal inhibitor Bortezomib could enhance caspase activation and apoptosis in both Imatinib resistant and sensitive CML cells. Lu *et al*. (2010) investigated the role of a terpenoid, pristimerin, in growth suppression and apoptotic induction in imatinib resistant BCR-ABL T315I mutation harboring CML cells by inhibiting BCR-ABL TK and NF-kB proteins, which suggested that NF-kB is one of the key targets for CML molecular therapies.

The four angiogenesis related genes reviewed above thus serve as important therapeutic targets against CML, especially in patients with imatinib resistance. Advances in our understanding of other genes that are deregulated in CML angiogenesis could help in further our understanding of disease pathobiology and in developing more effective treatment strategies.

CONCLUSION

Estimation of VEGF, HIF1α, IL-8 and NF-kB levels in CML patients may be helpful in evaluating pathogenesis, progression and prognosis. Imatinib resistance (IR) in CML can be overcome by combining imatinib with either conventional or new therapeutic targets developed against these factors. To overcome IR recent efforts have led to the identification of novel ABL kinase inhibitors capable of suppressing the activities of even mutant ABL kinases. Combination therapy with imatinib and other anti-leukemic agents is an important strategy to enhance the sensitivity and overcome the resistance of BCR/ABL positive leukemia to imatinib. Further insights on the molecular mechanisms active in CML angiogenesis could help in understanding the processes active in the tumor microenvironment and in identifying drug targets which may aid treatment success.

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