ABSTRACT

NITHYA MALTI NATRAJAN
Human Disease and Gain-of-Function Mutations in the KIT/KITL Signaling Pathway
Under the Direction of MARY A. BEDELL

KIT ligand (KITL) interacts with and activates the class III receptor tyrosine kinase KIT. In vertebrates, the KIT/KITL signaling pathway is involved in the development and proliferation of germ cells, melanocytes, hematopoietic cells (mast cells and erythrocytes), and interstitial cells of the Cajal in the gastrointestinal tract. These cells express KIT on their surface and consequently respond to KITL expressed from surrounding supporting cells. Upon binding, KIT dimerizes and a reaction cascade is initiated within the cell expressing KIT. Abnormal activation of the KIT/KITL signaling pathway has been associated with several human neoplastic diseases, such as gastrointestinal stromal tumors (GISTs), systemic mastocytosis, acute myeloid leukemia, sinonasal natural killer/T-cell lymphoma, and testicular seminoma. GISTs, which are mesenchymal neoplasms in the GI tract, are the most extensively studied diseases with regards to constitutive activation of the KIT/KITL pathway. The most commonly used pharmacological intervention for treating neoplastic disorders related to the misregulation of this pathway is a small-molecule inhibitor of KIT known as Gleevec. However, this treatment is not effective against all activating mutations in KIT. Other therapeutic drugs are currently being tested and designed to target abnormal KIT expression associated with gain-of-function (GOF) mutations. The KIT/KITL pathway and the role of KIT in disease must be examined further so that more effective treatments can be designed.

INDEX WORDS: KIT, Gain-of-Function mutations, Class III Receptor Tyrosine Kinase, Cancer, Tumor, Gastrointestinal Stromal Tumors, Systemic Mastocytosis, Acute Myeloid Leukemia, Sinonasal NK/T-Cell Lymphoma, Testicular Seminoma, Rational Drug Design, Gleevec

HUMAN DISEASE AND GAIN-OF-FUNCTION MUTATIONS IN THE KIT/KITL SIGNALING PATHWAY

By

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DEDICATION

"Science does not know its debt to imagination." ~Ralph Waldo Emerson

To my family. Without their love and support, I would not have developed the imagination or determination to persevere in the sciences.

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CHAPTER 1
INTRODUCTION

Mutations resulting in germ cell, melanocyte, and hematopoietic cell migration defects in mouse embryogenesis have been associated with the *White spotting locus (W)* and the *Steel locus (Sl)*, whose gene products were identified as KIT and KIT ligand (KITL) respectively about twenty years ago (Witte 1990). KIT was first described as the transduced oncogene *v-Kit* in the Hardy-Zuckerman 4 feline sarcoma virus (HZ4-FeSV), which was isolated from a feline fibrosarcoma (Figure 1). The v-KIT amino-acid sequence showed homology to the intracellular, kinase domain of receptor tyrosine kinases. However, no transmembrane or extracellular domains were described in v-KIT from HZ4-FeSV (Besmer et. al. 1986). Further studies described the structure and function of KIT, the vertebrate receptor tyrosine kinase (RTK) from which v-KIT was probably derived (Yarden et. al. 1987). KIT was identified as the gene product for the *W* locus through genetic mapping experiments (Geissler et. al. 1988). Further studies showed that KIT is normally expressed in cells affected by mutations in *W* and that the effects of a mutant *Kit* gene product resemble the result of mutations at the *W* locus (Nocka et. al. 1989).

While mutations in *W* and *Sl* have similar phenotypes, *W* was shown to encode an intrinsic factor within the hematopoietic cell while *Sl* mutations appeared to affect an environmental stimulus that acted on the cell of interest. Since KIT was identified as an RTK at the cell surface encoded by the *W* locus, it was hypothesized that a binding partner or ligand for KIT existed and was encoded at the *Sl* locus. In 1900 three different labs identified KITL [also known as stem cell factor (SCF) and mast cell growth factor (MGF)] as the ligand for KIT and

the product of the *Sl* locus (Witte 1990). Williams et. al. identified KITL as a ligand for KIT via cross-linking and immunoprecipitation studies (Williams et. al. 1990). Copeland et. al. mapped *Kitl* near the *Sl* locus in mouse and found that the locus for *Kitl* was deleted in *Sl* mutants, which suggests that the *Sl* locus encodes for KITL (Copeland et. al. 1990). Zsebo et. al. also identified KITL as the ligand for KIT, mapped it to the *Sl* locus, and sequenced cDNAs of *Kitl* (Zsebo et. al. 1990a; Zsebo et. al. 1990b). KITL was shown to interact with KIT as a cell surface molecule (Flanagan and Leder 1990). Later studies showed that KITL exists in membrane-bound and soluble forms that are both able to stimulate cell proliferation and suppress apoptosis by binding to and activating KIT (Flanagan et. al. 1991).

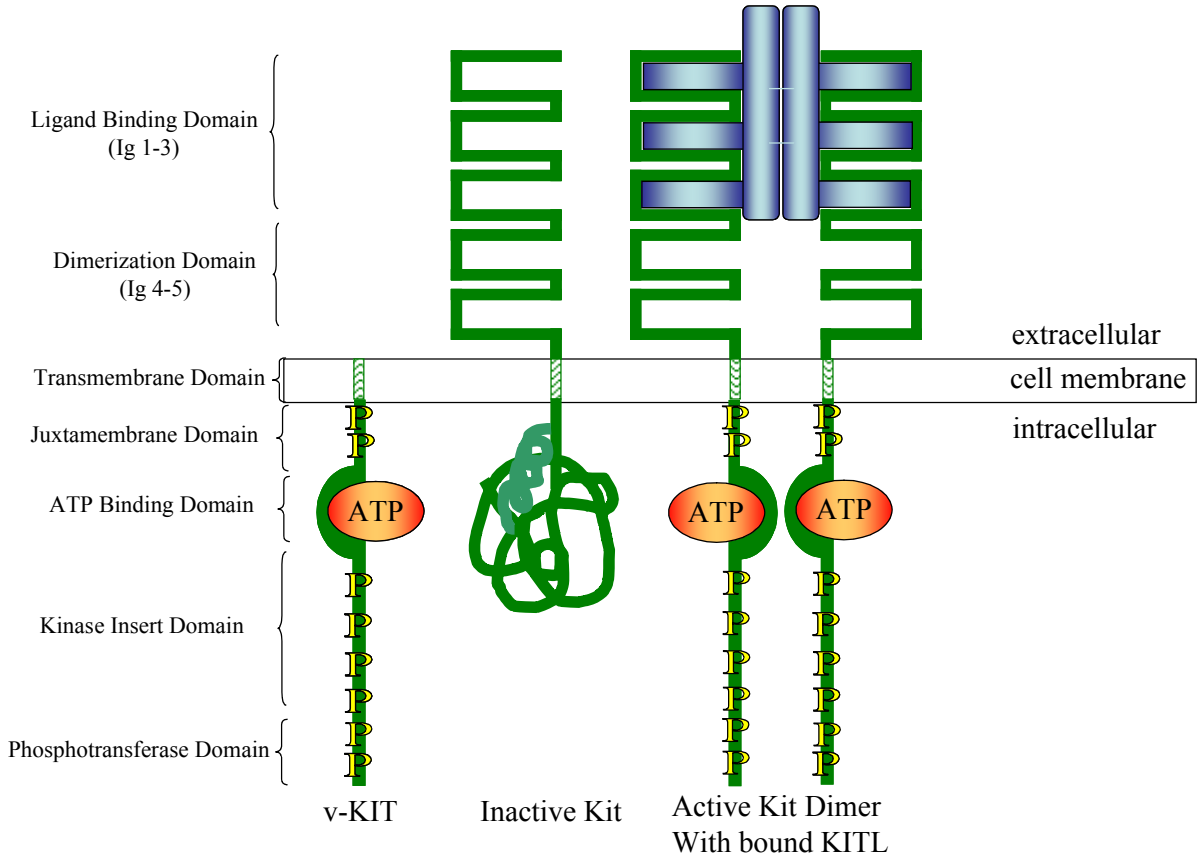

Figure 1: KIT Structure and Activation

Structure and Molecular Function of KIT

KIT is a highly conserved protein in vertebrates but does not appear in invertebrates. Structurally, KIT is a class III RTK that has an extracellular domain, a transmembrane domain, and an intracellular domain (Figure 1). When KITL is not present, KIT exists in a monomeric, inactive state (Roskoski 2005). In human and mouse, the Kit gene is located on chromosome 4q and chromosome 5, respectively (OMIM 2009). In both species, *Kit* genomic DNA spans 89 kB, contains 21 exons, and a typical *Kit* cDNA is approximately 3.5 kB. Exons 1-9 encode an extracellular domain containing five immunoglobulin (Ig)-like regions, exon 10 encodes the transmembrane domain, exon 11 encodes the juxtamembrane domain, and exons 13-21 encode the enzymatically active split tyrosine kinase (TK) domain (Miettinen and Lasota 2005). KITL binds as a dimer to Ig-like domains 1-3 of KIT (Lev et. al. 1993), causing dimerization of KIT through interactions in Ig-like domains 4-5. After dimerization, KIT becomes autophosphorylated and activates a complex set of intracellular pathways (Figure 1) (Liu et. al. 2007).

The intracellular domain of KIT contains an autoinhibitory juxtamembrane (JM) domain and a TK catalytic domain, which is divided into ATP binding and phosphotransferase portions by the kinase insert domain (Miettinen et. al. 2002). The JM domain contains an α-helix that stabilizes KIT in an inactive state by interacting with the kinase-active site and preventing the formation of the activated structure. Upon KIT dimerization, autophosphorylation of tyrosines (Tyr) 568 and 570 occurs in the JM domain. This phosphorylation (p-Tyr) induces conformational changes that shift the α-helix in the JM domain, thus activating the catalytic domains of KIT. Autophosphorylation also occurs at Tyr823 (p-Tyr823) in the phosphotransferase domain of KIT, stabilizing it in its extended, activated state.

Autophosphorylation of the JM domain at Tyr568 and Tyr570 (p-Tyr568 and p-Tyr570) occurs prior to autophosphorylation in the activation loop at Tyr823 (p-Tyr823) *in vitro* (Mol et. al. 2004).

Downstream Signaling of Activated KIT

Many intracellular cascades that promote cell growth and proliferation or suppress apoptosis are activated by phosphorylated KIT (Figure 2). In addition to Tyr568, Tyr570, and Tyr823 discussed above, five other tyrosine residues (Tyr703, Tyr721, Tyr730, Tyr900, and Tyr 936) in the intracellular domain of KIT become phosphorylated (Roskoski 2005), resulting in the docking of substrates on the phosphorylated tyrosines (Miettinen et. al. 2002) at Src-homology 2 (SH2) docking sites (Roskoski 2005).

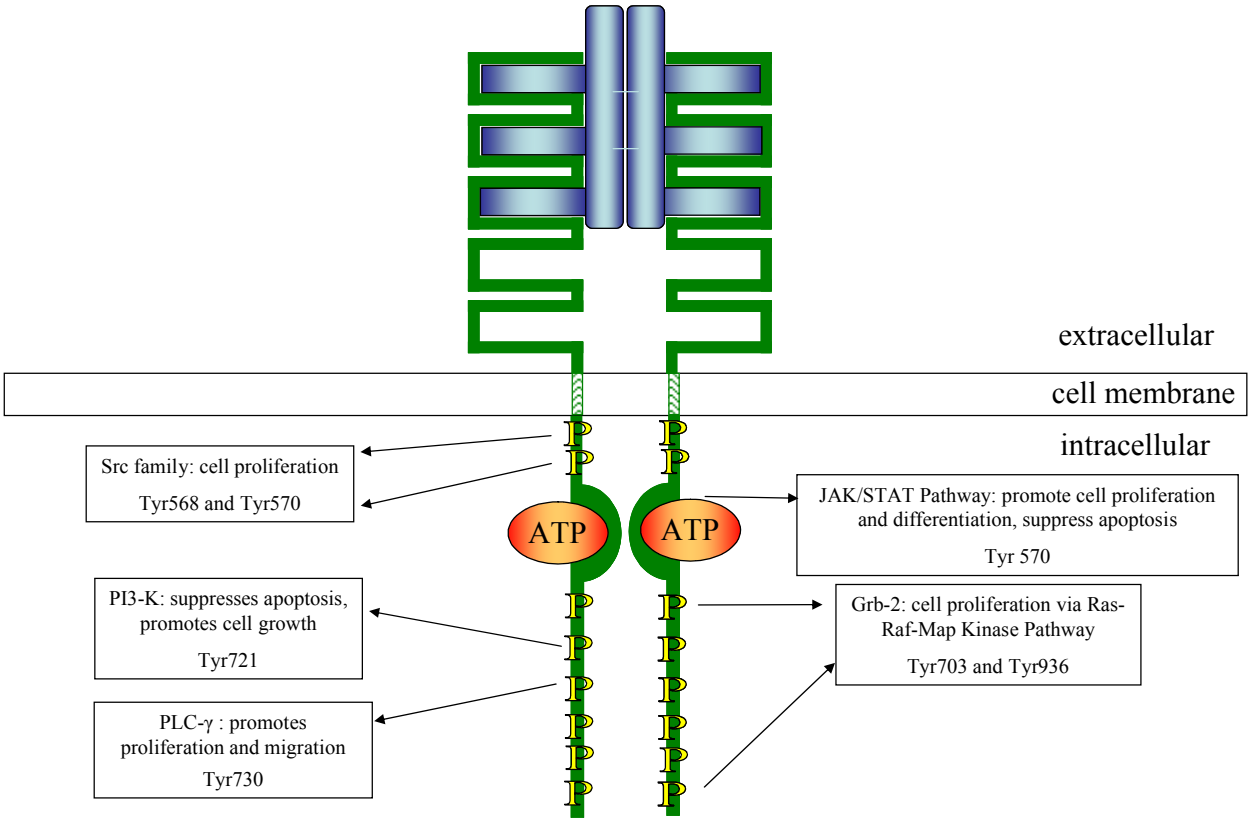

Figure 2: Downstream Signaling Pathways of KIT

Phosphatidylinositol-3-Kinase (PI3-K) signaling is activated by PI3-K docking on p-Tyr721. This pathway suppresses apoptosis and promotes cell growth. Src family members are activated when they interact with KIT at p-Tyr568 (essential to Src function) and p-Tyr570 in the JM domain. An isoform of KIT lacking an alternatively spliced four amino acid sequence in exon 9 seems to enhance Src kinase family interactions with phosphorylated JM domain tyrosines. Grb-2, which is an intermediate in the Ras-Raf-Map Kinase pathway, binds p-Tyr703 and p-Tyr936 and promotes cell proliferation by activating Ras. Shc and Shp2 interact with p-Tyr570 and p-Tyr568 respectively in the JM domain to negatively regulate the Ras-Raf-Map Kinase pathway**.** Phospholipase C-γ (PLC-γ) interacts with p-Tyr730 residue to produce a large variety of cellular effects that result in cellular proliferation and migration. Interestingly, only the membrane-bound isoform of KITL and not its soluble isoform, is able to activate this pathway . Truncated KIT did result in PLC-γ pathway activation. The JAK/STAT pathway has also been shown to be activated by phosphorylated KIT, but its specific interactions with KIT have not yet been elucidated (Ali and Ali 2007). The cascades activated by KIT promote growth and proliferation and inhibit apoptosis via many pathways. For example, PI3-K activates the Akt pathway that inhibits Bad, an apoptosis promoting factor (pathways reviewed in Linnekin 1999).

Functions of the KIT/KITL Pathway

Classical studies on *Sl* and *W* mice revealed that these mutants were anemic, sterile, and partially or fully lack coat pigmentation (Nocka et. al. 1989). Further studies revealed that the KIT/KITL pathway is required for the development and proliferation of the interstitial cells of Cajal (ICCs) of the gastrointestinal (GI) tract, germ cells, melanocytes, mast cells, and hematopoietic stem cells in a number of vertebrates (Miettinen and Lasota 2005).

ICCs function as regulators and pacemakers of intestinal peristalsis by acting as intermediaries between the autonomic nervous system and smooth muscle in the intestine. KIT has been shown to be required for the development and pacemaker activity of ICCs (Huizinga et. al. 1995; Maeda et. al. 1992), and is likely activated by expression of KITL from smooth muscle cells of the gut (Lammie et. al. 1994). The disruption of KIT has been shown to be involved in the differentiation of multipluripotent ICCs into smooth muscle cells in the GI tract (Isozaki and Hirota 2006; Torihashi et. al. 1999).

KIT is an essential part of primordial germ cell (PGC) migration and development during embryogenesis (Nocka et. al. 1989). KITL is also involved in PGC migration and can be found in the microenvironment of KIT positive cells around the migratory path of PGCs and in the developing gonads. In the postnatal ovary, KITL is expressed in granulosa cells of growing follicles and KIT is expressed in pre-ovulatory theca cells and oocytes. After ovulation, *Kit* mRNA expression in the oocyte decreases to undetectable levels but it is expressed again in the embryo before endometrial attachment where it may be stimulated by KITL expressed in the oviduct and the uterus. In testicular tissue, Leydig cells and pre-meiotic spermatagonia express KIT during many stages (Ali and Ali 2007). Sertoli cells, which act as an intermediary for and are essential to endocrine control of spermatogenesis, express KITL (Mauduit et. al. 1999) in both membrane-bound and soluble forms (Ali and Ali 2007).

The KIT/KITL pathway is important in the migration and survival of melanoblasts during embryogenesis in an organism specific manner. In mouse, KIT and KITL are both necessary for melanocyte migration and survival (Wehrle-Haller 2003). In humans, epidermal melanocytes show constitutive activation of KIT, which is important to their survival, proliferation, development, and migration (Nocka et. al. 1989). Additionally, the KIT/KITL pathway acts in

concert with other signaling pathways and environmental stimuli to cause melanocyte differentiation. KIT activity alone does not appear to be sufficient to promote survival, proliferation, and differentiation of melanocytes (Grichnik 2006). KITL acts a chemokinetic factor for KIT-positive cells and is essential for migration and survival of these cells (Wehrle-Haller et. al. 2001).

KIT shows constitutive expression in mast cells and is important for tissue mast cell development (Miettinen and Lasota 2005). Mast cells (MCs) are long-lived hematopoietic cells that are most likely derived from hematopoietic progenitors in the bone marrow. MC-committed progenitors are thought to circulate in the peripheral blood and then mature and differentiate in specific tissues. This maturation is most likely a result of activation by KITL, which is derived from stromal cells and interacts with KIT receptors on the surface of MC-committed progenitors. These cells reside in tissues within many organs in proximity to blood vessels and nerves. The metachromatic granules which can be observed within mast cells in tissue sections contain vasoactive and immunoregulatory molecules. These factors may be involved in processes such as the inflammatory response and the immune response (Valent et. al. 2005).

KIT is expressed in other hematopoietic cells, and the KIT/KITL signaling pathway is essential for erythropoiesis both during embryogenesis and postnatally (Nocka et. al. 1989).. Mutant mice completely lacking KIT or KITL die in utero or perinatally with severe macrocytic anemia (Munugalavadla and Kapur 2005). Other KIT-positive hematopoietic progenitors that have been identified include $CD34^+$ cells and a small portion of mononuclear cells. A significant number of triple-negative thymic lymphoid precursor cells and prothymocytes as well as a small percent of $CD56^+$ natural killer cells are also KIT positive (Miettinen and Lasota 2005).

CHAPTER 2
HUMAN DISEASE

Many human diseases have been causally associated with mutations in KIT. These mutations can either be loss-of-function (LOF) or gain-of-function (GOF). LOF mutations have been described in patients with Piebald trait. Piebald trait is an autosomal dominant leukoderma in which individuals have a forelock of white hair along with patches of skin lacking pigment, typically on the anterior trunk and central extremities (Fleischman et. al. 1991). GOF mutations in *Kit* have been linked to a number of proliferative disorders in humans, including many types of cancer and systemic mastocytosis. These GOF mutations result in constitutive activation of KIT in the absence of KITL. The two major types of mutations in *Kit* resulting in GOF diseases include mutations in the JM domain (exon 11) and in the TK domain (exon 17 in the phosphotransferase domain and, less commonly, exon 13 in the ATP binding domain) (Longley et. al. 2001; Miettinen and Lasota 2005). Mutations in the JM domain do not directly affect the enzymatic pocket of KIT while mutations in the TK domain directly alter the enzymatic pocket (Heinrich et. al. 2002). Additional mutations have been identified in the extracellular domain (exons 2, 8, and 9) of KIT in patients with neoplastic disease (Longley et. al. 2001; Miettinen and Lasota 2005). This paper will focus on describing diseases related to GOF mutations in *Kit*, including gastrointestinal stromal tumors (GISTs), systemic mastocytosis (SM), acute myeloid leukemia (AML), sinonasal natural killer (NK)/T-cell lymphoma, and testicular seminoma (summarized in Figure 2) (Akin and Metcalfe 2004).

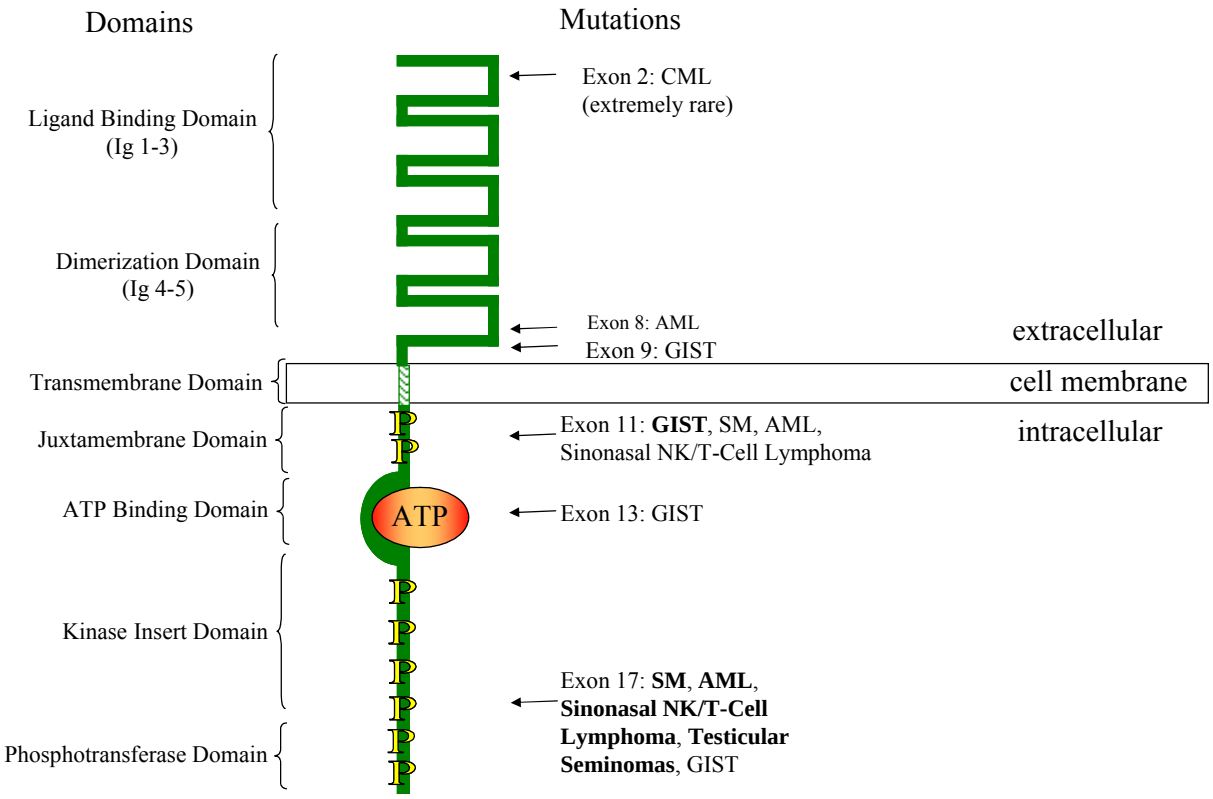

Figure 3: Activating Mutations in *Kit*: Bold disease names indicate that the majority of activating *Kit* mutations for that disease occur in that exon.

CHAPTER 3
GASTROINTESTINAL STROMAL TUMORS

Disease Pathophysiology and Epidemiology

Gastrointestinal stromal tumors (GISTs) define the largest class of mesenchymal tumors associated with the gastrointestinal (GI) tract. Disease-related upregulation of KIT signal transduction is one of the trademark features of this neoplasm (Miettinen and Lasota 2005) and mutations in KIT have been found in up to 90% of benign, malignant, and metastic GISTs (Longley et. al. 2001).. GISTs primarily affect individuals older than 40 years and are very rare in children. There is no definitive evidence regarding correlations between GISTs and gender (Miettinen et. al. 2002)

Histologically, GISTs appear to occur in spindle cells, epitheloid cells, and pleomorphic cells of the mesentery. Spindle cell GISTs tend to have large numbers of cells and appear basophilic due to the small amount of cytoplasmic area and large density of nuclei, which usually have pointed ends. Epitheloid GISTs are most common in the stomach . These tumor cells have a polygonal appearance under the microscope, with well-represented cytoplasm and rounded nuclei. Only a small percentage of GISTs appear as pleomorphic cells. GIST cells are phenotypically similar to ICCs, which are KIT positive cells in the GI tract. Recent evidence suggests that ICCs and smooth muscle cells in the GI tract have a common progenitor cell that may give rise to the KIT-positive GIST cells (Miettinen et. al. 2002).

Mesenchymal neoplasms have been identified throughout the GI tract, but are most often associated with the stomach (60-70%) or small intestine (25-35%). A small percentage of GISTs

were also identified in the colorectal area (5%), esophagus (2%), and appendix. Upon further analysis, many of the colorectal, esophageal, and appendix-related GISTs probably originated in the stomach or small intestine. Primary GISTs may also occur in the omentum, mesenteries, and retroperitoneum. In rare instances, metastases to soft tissue in peripheral locales are observed (Miettinen et. al. 2002). GISTs have also been found as part of tumor syndromes such as Carney's triad or in association with neurofibromatosis type 1 (NF1) syndrome (Miettinen and Lasota 2005).

Activating Mutations in KIT and Chromosomal Aberrations

KIT-activating mutations in GISTs usually involve the JM domain of the regulatory region, although some enzymatic site mutations and extracellular domain mutations have also been identified (Figure 2). In the regulatory region, in-frame mutations have been identified in exon 11 between Lys550 and Gly592 (Isozaki and Hirota 2006) and often involve one or more codons between Lys557 and Val560 in the proximal region of exon 11 (Miettinen and Lasota 2005). Missense mutations, insertions and deletions, and mutations in the distal part of exon 11 have also been described in GISTs (Miettinen et. al. 2002). This regulatory region contains an α-helix which inhibits KIT activity by inserting into the kinase domain and suppressing kinase activity and phosphorylation. Mutations in the regulatory helix result in functional disruption of the JM domain and constitutive activation of KIT (Longley et. al. 2001). Interestingly, some evidence suggests that GISTs with JM domain mutations in KIT are more aggressive than GISTs without these mutations (Ali and Ali 2007).

Mutations in the enzymatic domains of KIT are not common in GISTs. KIT exon 13 and exon 17 mutations (Lys642Glu and Asn822Lys/Asn822His, respectively) have been reported in

a few GIST cases. Insertions and duplications were also found in the extracellular domain in exon 9. An insertion/duplication of codons Ala502-Tyr503 was found in exon 9. While this insertion is rare, it is most commonly seen in malignant intestinal GISTs. A three codon insertion/duplication has been observed at Phe506-Ala507-Phe508 (Miettinen and Lasota 2005) (reviewed in Heinrich et. al. 2002; Miettinen et. al. 2002). The mutations found in exons 9, 13, and 17 account for the majority of KIT-positive GIST cells without mutations in the JM regulatory domain (Isozaki and Hirota 2006; Miettinen et. al. 2002)

In GIST cells lacking KIT coding mutations but with increased KIT expression levels alternative mechanisms may play a role in activation. Mutations within the noncoding introns and regulatory regions of KIT may result in the KIT GOF phenotype. Proteins, such as KITL, which interact with KIT may also play a role in increased KIT levels in GIST tumor cells. Four isoforms of KIT resulting from alternative splicing events have been observed and may affect expression of KIT-regulated pathways. Expression of these isoforms has been observed in both tumor cells and normal cells. Alternative splicing events may occur in exon 9 and KIT isoforms can either have or lack a GNNL sequence in their extracellular domain. Isoforms of KIT may also have or lack a kinase insert domain serine in exon 15 of the tyrosine kinase domain. A percentage of GISTs do not have aberrantly increased expression of KIT. In these cases, RTK oncoproteins related to KIT may have GOF mutations or increased expression in these GISTs (Heinrich et. al. 2002).

The mutation type in GISTs may affect the downstream pathways activated by KIT. For example, exon 9 GOF mutations showed weak to no activation of the MAPk pathway while exon 11 and 13 GOF mutations showed increased expression of pMAPk. However, studies have yet

to reveal which downstream paths (if any) are required for the oncogenic properties of GISTs (Heinrich et. al. 2002).

Diagnostic Tests

GISTs can present as small, benign tumors or larger sarcomas and can either be local or metastasized tumors. In general, metastases to the liver or peritoneum result in faster disease progression and death and benign GISTs are usually detected accidentally and are small in size (Miettinen et. al. 2002). The best morphological parameters to use for GIST diagnosis and staging are tumor size and mitotic activity (measured using mitotic counts). These two criteria have been well-studied and provide the most accurate form of morphological identification of the level of malignancy of a GIST (Miettinen et. al. 2002). KIT staining at the cell membrane with polyclonal antibodies is used as a diagnostic test for GISTs since abnormal KIT expression and GOF in KIT is specifically associated with GISTs in the GI tract. In this assay, spindled ICCs and mast cells in the area are used as positive controls. KIT is usually strongly and globally expressed in GIST cells (Miettinen et. al. 2002)

Many GISTs have chromosomal deletions, but they appear to be secondary events to *Kit* mutations and do not involve the *Kit*-containing region of the genome (chromosome 4q). These chromosomal aberrations are analyzed with comparative genomic hybridization (CGH), loss of heterozygosity (LOH), and fluorescent in-situ hybridization (FISH) techniques. (Debiec-Rychter et. al. 2001; Miettinen et. al. 2002). Overall, malignant GISTs tend to have more chromosomal abnormalities, but their karyotypes are not nearly as complex as other similarly-staged spindle-cell tumors. Benign GISTs usually do not have large scale deletions based on karyotype (Heinrich et. al. 2002) and have fewer large scale deletions compared to malignant GISTs. Loss

of genetic material on chromosomes 14q and 22q are equally common in benign and malignant GISTs and appear to play a role early on in GIST pathogenesis. DNA loss on chromosome 1p and complete or partial deletions of chromosome 9 have been identified with multiple malignant GISTs. Amplification of genetic material at chromosomes 5p and 20q and deletions at chromosome 9p were observed solely in malignant GISTs. Chromosome 8q and 17q amplifications were most often seen in metastic GISTs, and losses in chromosome 13q were most common in malignant and metastatic neoplasms (Debiec-Rychter et. al. 2001; Miettinen et. al. 2002)

CHAPTER 4
SYSTEMIC MASTOCYTOSIS

Disease Pathophysiology and Epidemiology

Mast cell disease can occur in the skin (cutaneous mastocytosis, CM) or in tissues throughout the body (systemic mastocytosis, SM). Accumulation of mast cells in focal points within various organs and tissues is common to all types of mastocytosis. Spontaneous regression of CM occurs in many cases, and this disease is most common in children before the onset of puberty. In CM, mast cell infiltration is limited to the skin and this disease is subcategorized into maculopapular CM (urticaria pigmentosa, UP), diffuse CM, and solitary mastocytoma of the skin. SM, which is most often seen in adults, is a long-term myeloproliferative disorder which is divided into multiple stages. The most common form of SM is indolent SM (ISM), which is a slowly progressing disease connected to UP-like lesions and is not associated with organ dysfunction. In ISM, the bone marrow usually has low grade mast cell infiltration at dense foci. Isolated bone marrow mastocytosis, which usually requires no treatment, and smoldering SM (SSM), which is more aggressive than most types of ISM, are two of the subtypes of ISM. Aggressive SM (ASM) involves the progressive penetration of multiple organs by MCs and associated functional disruption of infiltrated organs (Valent et. al. 2005).

Mast cell sarcoma (MCS) is an extremely rare form of mastocytosis that is characterized by a localized sarcoma-like neoplasm containing atypical MCs. Mast cell leukemia (MCL) is an aggressive, rare MC-related neoplasm characterized by increased MCs both in the bone marrow

and in circulation. MCL has a bad prognosis with typical survival being less than one year. SM may also be accompanied by associated hematopoietic clonal non-MC lineage diseases (AHNMD) and subtypes are categorized by the type of AHNMD. For example, SM patients with associated AML are said to have SM-AML. Mast cells are thought to be derived from a myelopoietic progenitor, which may help explain the relatively high rate of blood-related disorders, such as secondary acute myeloid leukemia (AML), that are associated with SM but do not involve mast cells (Valent et. al. 2005). Myeloproliferative disorders (MPDs) such as chronic myeloid leukemia, essential thrombocytosis, polycythemia vera, and primary myelofibrosis, occur at the myeloid stem cell level (Longley et. al. 2001).

Activating Mutations in KIT and Chromosomal Aberrations

SM is associated with KIT enzymatic domain mutations, most commonly an Asp816Val mutation in exon 17 of the phosphotransferase region of the TK domain (Figure 2). The Asp816Val mutation in KIT has been detected in greater than 80% of SM patients. This mutation is thought to contribute to SM disease development and progression, but it may not be the causative agent for increased MC proliferation in indolent SM. In more advanced mast cell proliferative disorders (such as ASM and MCL), the Asp816Val mutation in KIT may result in MC clustering and increased proliferation. KIT mutations in the JM domain and chromosomal defects may also be involved in SM and are often found in SM patients with AHNMD, most of which are myeloid neoplasms such as acute myeloid leukemia (Valent et. al. 2005). Mutations in exon 11 at V559 of KIT have also been reported in SM, but were only seen in cases that also had Asp816Val mutations (Longley et. al. 2001; Miettinen and Lasota 2005). SM-AHNMDs also commonly have codon 816 mutations in KIT (Corless et. al. 2006).

Diagnostic Tests

SM can be clearly identified using bone marrow biopsies. After obtaining bone marrow tissue, mast cells are marked by tryptase and antibodies to mast cells are able to detect multiple abnormal aggregates of mast cells in the bone marrow in patients with SM. In advanced cases of SM with related secondary blood disorders such as AML, MC infiltrates do not form the foci seen in patients without related hematopoietic disorders. In these patients, increased MC expression in tissues are usually composed of smaller aggregates with MC infiltration of the whole cell or simply diffused MC expression within affected tissues (Valent et. al. 2005). KIT-positive SM and rare cases of KIT-positive CM show a unique pattern of KIT expression on the membrane that can be used as a diagnostic test for mastocytosis (Miettinen and Lasota 2005). DNA analysis of bone marrow cells from SM patients is routinely used to test for KIT Asp816Val mutations. In the event of a negative result, sequencing can be used to analyze KIT and determine if a KIT mutation is present. Other factors which can help confirm diagnoses include altered MC morphology, phenotypic changes such as CD2 and CD25 expression, and increased levels of tryptase in the serum (Valent et. al. 2005).

Tumor-Related Inflammatory Response

Recent findings suggested that KIT/KITL mediated migration of mast cells to the tumor site is important in modulating the tumor microenvironment. KITL helps recruit mast cells to the site of the tumor, where it stimulates the mast cells to release adenosine. Adenosine release inhibits the migration of mast cells from the site of the tumor, which may explain mast cell accumulation in blood vessels around tumors. It also inhibits $CD4^+$ T-cells from releasing cytokines such as interferon gamma and interleukin-2, which are important in controlling tumor

growth. Mast cells release many cytokines in the tumor microenvironment that are important for tumor growth, development, and metastasis. In addition to this, a siRNA KITL-knockdown H22 tumor cell line was not able to attract mast cells. The KIT/KITL pathway and KITL itself appear to be important initial factors in the recruitment of mast cells to the tumor site (Huang et. al. 1990).

CHAPTER 5
ACUTE MYELOID LEUKEMIA

Disease Pathophysiology and Epidemiology

The majority of acute myeloid leukemia (AML) cases occur in elderly patients and the risk for developing this neoplastic disease increases with age. This form of cancer is rarely seen in people younger than thirty (Buckman 1997). AML accounts for 54% of adult leukemias and is characterized by the uncontrolled growth and proliferation of abnormal, immature myeloid cells in the bone marrow. The abnormal cells, which are called myeloblast cells in myelogenous leukemia, arrest in an early stage in the bone marrow and proliferate at an increased rate. High numbers of blast cells in the bone marrow interrupt normal marrow functions. There is also an increase in the number of immature white blood cells in circulation and patients present with anemia, thrombocytopenia, and neutropenia (Morra and Potts 2003).

Activating Mutations in KIT and Chromosomal Aberrations

In AML, KIT positivity has been observed in approximately 70% of cases (Miettinen and Lasota 2005)**,** and the mutations associated with AML seem to be mostly of the enzymatic type (Figure 2) (Longley et. al. 2001)**.** The mutations in KIT include insertions and deletions in exons 8 and 11 as well as point mutations within the kinase domain (Miettinen and Lasota 2005). Asp419 was involved in the mutations observed in AML patients. In chronic myeloid leukemia, extremely rare KIT mutations are seen in exon 2 at codon 52. (Longley et. al. 2001)

Diagnostic Tests

Certain characteristic histological tests can be done to determine if a patient has AML. AML patients present with an abnormally high number of immature white blood cells and a decreased number of normal blood cells. A bone marrow smear will show characteristic blast cells, which are leukemic myeloid progenitors that produce immature white blood cells (Morra and Potts 2003). In AML, KIT detection using flow cytometry provides an excellent diagnostic tool to more specifically identify and characterize AML. KIT is found at basal levels in the serum under normal conditions and becomes elevated in patients with AML. KIT levels in the serum may be a useful tool for tracking AML disease progression (Miettinen and Lasota 2005). In cases of SM associated AML t(8, 21), del 20(q12), and +chromosome 9 can be detected using cytogenetic analyses (Longley et. al. 2001).

CHAPTER 6
SINONASAL NATURAL KILLER/T-CELL LYMPHOMA

Disease Pathophysiology and Epidemiology

Sinonasal lymphomas are most commonly seen in East Asian countries. They are characterized by lesions on the face and in the upper respiratory tract called lethal midline granulomas. Sinonasal natural killer (NK)/T-cell lymphomas are more aggressive than other sinonasal lymphomas and involve lesions in the nasal cavity (Longley et. al. 2001). This disease may have increased levels of KIT expression in certain subgroups of the population. Sinonasal NK/T-cell lymphoma patients in China, Korea, and Japan commonly show GOF in KIT expression (Miettinen and Lasota 2005)

Activating Mutations in KIT and Chromosomal Aberrations

Kit mutations in both exon 11 and exon 17 have been reported in studies on patients with sinonasal NK/T-cell lymphoma in China, Korea, and Japan. (Figure 2) (Miettinen and Lasota 2005) In one study, patients in China appeared to have a higher frequency of KIT mutations (71.4% with KIT mutations) when compared to Japanese patients (22.2% with KIT mutations). The majority of mutations appear to occur at codon 825 or 816 of KIT and affect the phosphotransferase domain in the enzymatic pocket. Mutations in the JM region have also been identified at codons 559 and 561 (Hongyo et. al. 2000; Longley et. al. 2001)

Diagnostic Tests

Along with the visual observation of the lethal midline granulomas, histological observations include polymorphous patterns. Cells involved in this disease can resemble a variety of hematopoietic cells, including B cells, NK cells, or T-cells. Since mutations in KIT and increased KIT expression have been observed in a large proportion of Chinese patients, visualizing KIT expression may be used as a confirmatory test for the Chinese population. However, KIT positivity and KIT mutation identification probably does not provide a universally practical identification test for sinonasal NK/T-cell lymphomas (Longley et. al. 2001).

CHAPTER 7
TESTICULAR SEMINOMA

Disease Pathophysiology and Epidemiology

Forty percent of testicular germ cell tumors are seminomas, which are relatively slow growing neoplasms of spermatogenic cells in the seminiferous tubules (Buckman 1997). Testicular seminomas tend to affect young adult males and have been associated with undescended testicles and viral damage to testicular tissues. Baby boys with low birth weights or with mothers who had bleeding or spotting during pregnancy tend to be at higher risk for developing this type of cancer later in life. During pregnancy, alcohol consumption and sedative use by the mother may also increase the risk for the baby developing this neoplasm during young adulthood. X-ray exposure during pregnancy also places the baby at higher risk for eventually developing a testicular seminoma (Morra and Potts 2003). Family history is linked to an individual's predisposition to developing this tumor (Chaganti and Houldsworth 2000).

Almost all testicular germ cell tumors show duplications (either tandem duplications or a duplication transposed elsewhere in the genome) of 12p. Since this chromosomal aberration is seen in nearly all testicular germ cell tumors, it may be the genetic abnormality that causes the formation of testicular germ cell tumors (Chaganti and Houldsworth 2000).

Activating Mutations in KIT and Chromosomal Aberrations

Abnormal KIT expression on the membrane has been observed in over 90% of testicular seminomas and kit mutations have been reported in about 30% of testicular seminomas. These

mutations are in exon 17 between codons 816 and 823 (Figure 2). Most of the reported mutations are in codon 816 (Miettinen and Lasota 2005). Kemmer et al found that 82% of the seminomas they studied were KIT positive. Upon screening for mutations, 23.8% of the seminomas were found to have exon 17 mutations in the enzymatic portion of KIT and 25.9% of the seminomas screened had mutations in kit. The majority of the mutations were at D816. Mutations were also observed at Tyr823 and Asn822 in exon 17 and at Tyr801 (not an activating mutation). In this study, no mutations were observed in exons 9, 11, and 13. Lymphocytic infiltration may account for the KIT-positive staining in some of the samples which did not have kit mutations (Kemmer et. al. 2004)

Diagnostic Tests

The first step in confirming the presence of testicular cancer is to remove the abnormal testicle and examine it under a microscope. A blood test can also be administered to detect alpha-fetoprotein (AFP) and beta human chorionic gonadotrophin (βHCG) levels in the blood, which can then be used to diagnose testicular cancers (Buckman 1997). Histochemical and genetic analysis for KIT is not a sound diagnostic tool because kit mutations are only found in a relatively small percentage of tumors and a large percentage of KIT positive tumors do not have mutations in the kit gene.

CHAPTER 8
RATIONAL DRUG DESIGN AND CURRENT TREATMENTS

Rational Targeted Drug Development

Rational or targeted drug design may prove an extremely efficient way to develop new therapeutic options for neoplastic diseases. This approach utilizes bioinformatics to identify genes and proteins which can be specifically targeted in a disease. After identifying the target, drugs that interact specifically with the gene or protein of interest can be developed using organic synthesis methods. The idea of rational targeted drug design is to help reduce the monetary investments and time necessary for drug development by identifying specific molecules that can be targeted and synthesizing compounds that will be able to interact with those molecules. For instance, Gleevec (Imatinib) was shown to specifically interact with the ATP binding region of class III receptor tyrosine kinases such as KIT, PDGFR, and ABL. The hybrid *Bcr-Abl* gene has been implicated in the development of chronic myeloid leukemia (CML). Since Gleevec was shown to target ABL it was examined for use in treating patients with CML. Clinical trials utilizing Gleevec to treat CML proved successful in treating the disease and extending patients' lives. In addition to utilizing knowledge of biochemistry, molecular biology, and organic chemistry to more efficiently produce therapeutic agents, rational drug design provides treatments with fewer side effects for patients because drugs will be able to target the disease with more accuracy (Redig 2008).

Treatments for KIT GOF Mutations

Gleevec is currently used for treating neoplasms with KIT mutations in the regulatory JM domain (mutations between codons 550 and 592). It can also be used to treat neoplasms with mutations in the extracellular domain (exon 9 - duplication of Ala502 and Tyr503), which have a moderate tumor response (Miettinen and Lasota 2005). Since Gleevec functions by binding to the kinase domain and acting as a JM domain inhibitory α-helix homologue, mutations affecting the kinase domain are resistant to Gleevec treatment because these mutations prevent the drug from binding the kinase domain (Table 1 and Figure 4) (Akin and Metcalfe 2004). Second site mutations, usually found in exon 17 of the kinase domain of KIT, have been shown to render GISTs that are initially sensitive to Gleevec resistant to this drug (Antonescu et. al. 2005).

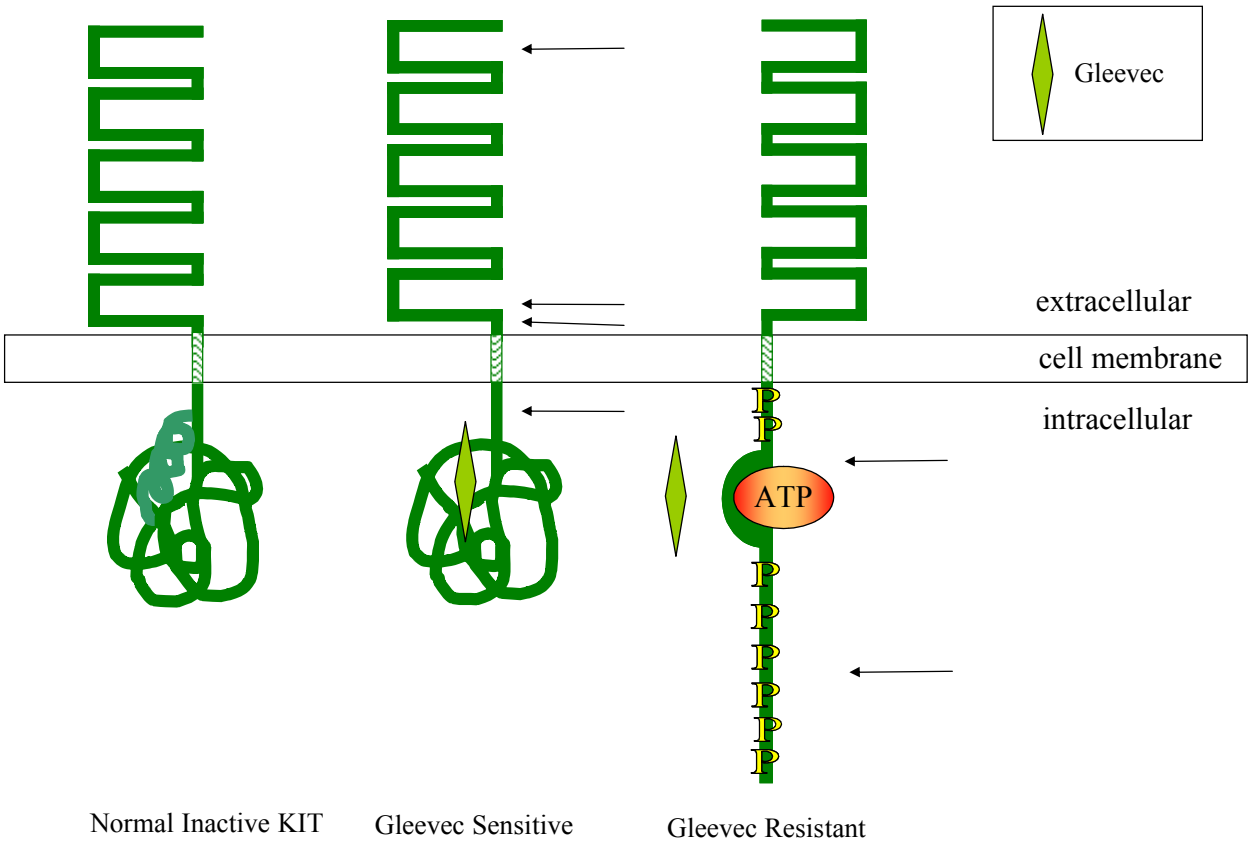

Figure 4:Gleevec Sensitivity of KIT Mutations

Work is currently being done to find a treatment that acts further down stream so that the mutations in exon 17 affecting the enzymatic function of KIT can be treated. Rossi et. al. found that RAD001 (everolimus), which is an mTOR inhibitor, may be a potential therapeutic option for patients with exon 17 mutations in KIT (Rossi et. al. 2006). 17-AAG (heat shock protein 90 or HSP90) has been shown to be effective in KIT expressing cells containing enzymatic-type mutations in KIT. Flavopiridol, a transcriptional repressor, has also shown promise as a treatment for Gleevec-resistant GISTs (Kitamura 2008). All of these therapeutic agents are being tested for use in diseases related to GOF KIT mutations in the enzymatic domain.

Animal Models

Animal models with activating mutations in kit have been developed to study the pathology of disease and drug development. Kit mutations in the JM domain have been identified in canine cutaneous mast cell tumors (MCT). This model is useful for studying activating mutations in KIT related to neoplasms, especially with regards to GISTs and human mast cell leukemia because these two diseases have been linked with JM domain mutations. The canine MCT model provides an excellent naturally occurring in vivo system to test chemotherapeutic agents targeting KIT (Webster et. al. 2006). Rossi et al. produced a mouse model with an activating mutation in KIT at position 558 in exon 11 of the JM domain of KIT. Heterozygous mice with this mutation develop the symptoms of GISTs and die from GI-related pathology. Upon studying neoplastic lesions in the mice GI tracts, KIT-positive cell hyperplasia was observed and histologically, the cells were indistinguishable from human GIST cells (Rossi et. al. 2006).

While these two models are excellent for developing drugs for JM domain regulatory mutations in KIT, they do not address the lack of known effective treatments for diseases that have enzymatic pocket mutations in exon 17. An animal model must first be developed for enzymatic-type mutations in KIT. A transgenic mouse model for Asp814 mutations created by Katayama et. al. may be used to develop and test drugs that are able to inhibit KIT GOF mutations affecting exon 17 (Kitayama et. al. 1996). However, this mouse model has not been used extensively as of yet and its efficacy as a model for human neoplastic disease involving KIT's enzymatic domains has not been well established.

CHAPTER 9
CONCLUSIONS AND FUTURE DIRECTIONS

Table 1. *Kit* Activating Mutations in Neoplastic Disease and Gleevec Resistance

Disease	Mutation Domain	Exons Affected	Codons Affected	Gleevec
GIST	**Juxtamembrane**	**Exon 11**	**Lys550-Gly592**	**Sensitive**
	Dimerization	Exon 9	Ala502/Tyr503 duplication	Sensitive
	ATP Binding	Exon 13	Lys642Glu	Resistant
	Phosphotransferase	Exon 17	Asn822Lys/Asn822His	Resistant
SM	**Phosphotransferase**	**Exon 17**	**Asp816Val**	**Resistant**
	Juxtamembrane	Exon 11	V559G	Sensitive
MPD	Ligand Binding	Exon 2	D52N	
AML	**Phosphotransferase**	**Exon 17**	**Asp816Val**	**Resistant**
	Juxtamembrane	Exon 11	Lys550-Gly592	Sensitive
	Dimerization	Exon 8	417-421	Sensitive
Sinonasal NK/T-Cell Lympoma	**Phosphotransferase**	**Exon 17**	**Val825Ala**	**Resistant**
	Juxtamembrane	Exon 11	Lys550-Gly592	Sensitive
Testicular Seminoma	**Phosphotransferase**	**Exon 17**	**Asp816His/Asp826Val** Tyr823Asp/Tyr823Cys	**Resistant**

It has been demonstrated that GISTs, SM, SM-AML, AML, Sinonasal NK/T-cell lymphoma, and testicular seminomas have associated activating mutations in KIT. The majority of KIT mutations have been shown to occur in the extracellular domain (exon 8 & 9), the JM domain (exon 11), and the enzymatic site of KIT in the kinase domain (exon 17). The extracellular mutations occur most often in AML. JM domain mutations in exon 11 are most closely associated with GISTs, but they have been described in AML and sinonasal NK/T-cell lymphoma. GOF mutations in both the extracellular domain and the JM domain of KIT are

sensitive to inhibition by Gleevec. Enzymatic kinase domain mutations are associated with SM, testicular seminoma, and sinonasal NK/T-cell lymphoma and are resistant to Gleevec. Second site mutations in the kinase domain resulting in Gleevec resistance in GISTs have also been observed and present an issue with regards to treatment for patients with JM domain and extracellular domain mutations (summarized in Table 1).

RNAi as a Therapeutic Tool for GOF KIT Expression

RNA interference (RNAi) has great potential to be an alternative strategy for decreasing endogenous KIT in tumors. RNAi a process by which small double-stranded RNAs (dsRNAs) knock down gene expression by inducing the degradation of specific endogenous mRNAs. Fire et. al. discovered that dsRNA with homology to an mRNA is able to stimulate degradation of the mRNA (Fire et. al. 1998). RNAi may prove a useful tool in treating diseases that have associated upregulation of certain gene products because it would be able to specifically target the mutant mRNA of interest (Arenz and Schepers 2003). With regards to KIT mutations, RNAi silencing of Kit mRNA may be an excellent way to downregulate aberrant KIT expression on the cell surface. This strategy would be able to overcome the problem of second site point mutations in KIT at exon 17 resulting in drug resistance to Gleevec.

Diseases Being Studied for KIT GOF Mutations

Many neoplastic diseases are being studied for GOF mutations in KIT. A number of neoplastic diseases related to epithelial cells have been shown to be KIT positive. There is some evidence that small cell lung carcinomas may have mutations in KIT. Studies have shown that the pulmonary epithelium of neoplastic tissue from small cell lung cancer patients is aberrantly

KIT positive. Further experiments must be done to determine whether KIT mutations may play a role in small cell lung cancer development or proliferation. Large cell neuroendocrine carcinomas, adenocarcinomas, ductal carcinomas of the breast, ovarian epithelial cancers, colon cancers, renal carcinomas, adenoid cystic carcinomas, Merkel cell carcinomas, thymic carcinomas, and neuroblastomas have also been shown to be KIT positive. All of these neoplasms can be further studied to determine if KIT mutations exist in them. If KIT mutations are present, then their role in disease progression and susceptibility to KIT inhibitors must be elucidated (Miettinen and Lasota 2005).

The KIT/KITL pathway and its role in disease have been extensively studied, but much work remains to be done. Further studies can be done on KIT expression in neoplastic diseases associated with KIT expressing systems to provide new targets for KIT inhibitors and insights into disease pathology of the neoplasms that are studied. Future drug development and testing should focus on creating treatments for mutations in the enzymatic domain of KIT that cause neoplastic disease.

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