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MOLECULAR MECHANISMS OF IMATINIB RESISTANCE IN GASTROINTESTINAL STROMAL TUMOR WITH FOCUS ON MICRORNAS

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En erken yaşlarımdan itibaren beni bilime yönlendiren

babam ve dedeme...

ABSTRACT

Gastrointestinal stromal tumor (GIST) is mainly initialized by mutations in receptor tyrosine kinase genes *KIT* or *PDGFRA*. The development of imatinib, a small molecule inhibitor that targets these tyrosine kinase receptors, remarkably improved patient outcome. However, imatinib resistance remains a major therapeutic challenge in GIST therapy, and its underlying mechanisms are still not completely understood. This thesis work aimed to explore the role of microRNAs (miRNAs) and DOG1 in imatinib resistance of GIST.

In **Paper I**, we identified specific miRNA signatures associated with imatinib resistance, metastatic disease, *KIT* mutational status and survival in GIST patients treated with neoadjuvant imatinib. Importantly, we demonstrate that *miR-125a-5p* modulates imatinib response in the single *KIT*-mutated GIST882 cells through PTPN18 regulation. This study highlights the clinical impact of miRNAs in GIST patients treated with imatinib pre-operatively, and suggests the important role of *miR-125a-5p* and PTPN18 in imatinib resistance of GISTs.

In **Paper II**, we tested our hypothesis that *miR-125a-5p* overexpression in imatinib-resistant GISTs suppresses PTPN18 expression that subsequently leads to defective FAK dephosphorylation. Indeed, we demonstrate that silencing of PTPN18 increased FAK phosphorylation in GIST cells, and the acquired imatinib-resistant GIST882R cells exhibited higher pFAK and lower PTPN18 expressions than the imatinib-sensitive parental cells. FAK and pFAK expressions are also associated with imatinib resistance in GIST specimens. This study highlights the potential role of PTPN18 and pFAK in imatinib resistance of GIST.

In **Paper III**, we found that *miR-320a* and *miR-320b* are upregulated and their potential target MCL1 is downregulated in imatinib-treated GISTs. Imatinib treatment affects MCL1 and *miR-320* levels in GIST882 cells, and the imatinib-resistant GIST882R cells showed higher levels of the anti-apoptotic MCL1L isoform and lower expression of *miR-320a*/*b* as compared to GIST882 cells. This study suggests that *miR-320a*/*b* and MCL1 play a role in imatinib-induced cell death and resistance in GIST.

In **Paper IV**, we evaluated the functional role of DOG1 in imatinib-resistant GIST48 and –sensitive GIST882 cells using specific DOG1 activator and inhibitor. We showed that DOG1 is localized in different cellular compartments in imatinib-resistant and -sensitive GIST cells. Pharmacological modulation of DOG1 activity has subtle effect on cell viability and proliferation, but may shift early apoptotic cells to late apoptotic stages in GIST48 cells.

Overall, this thesis work describes the role of miRNAs in cell viability and resistance to imatinib treatment in GIST.

LIST OF PUBLICATIONS

The thesis is based on the following papers, which will be referred by their Roman numerals throughout the text.

I. **Akçakaya P***, Caramuta S, Åhlén J, Ghaderi M, Berglund E, Östman A, Bränström R, Larsson C and Lui WO*.
microRNA expression signatures of gastrointestinal stromal tumors: associations to imatinib resistance and patient outcome.
British Journal of Cancer 2014; 111(11):2091-2102

II. **Akçakaya P***, Gangaev A, Lee L, Zeljic K, Hajeri P, Berglund E, Ghaderi M, Åhlén J, Bränström R, Östman A, Larsson C and Lui WO*.
FAK phosphorylation is regulated by PTPN18 and associated with imatinib resistance in gastrointestinal stromal tumors.
Manuscript

III. **Akçakaya P***, Zeljic K, Åhlén J, Gangaev A, Ghaderi M, Caramuta S, Berglund E, Bränström R, Larsson C and Lui WO*.
Involvement of MCL1 and *miR-320* in imatinib-induced cell death of gastrointestinal stromal tumor cells.
Manuscript

IV. Berglund E*, **Akçakaya P**, Berglund D, Karlsson F, Vukojević V, Lee L, Bogdanović D, Lui WO, Larsson C, Zedenius J, Fröbom R, Bränström R.
Functional role of the Ca^{2+}-activated Cl^- channel DOG1/TMEM16A in gastrointestinal stromal tumor cells.
Experimental Cell Research 2014; 326(2):315-325

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LIST OF RELATED PUBLICATIONS

1. Berglund E, Ubhayasekera SJ, Karlsson F, **Akçakaya P**, Aluthgedara W, Åhlén J, Fröbom R, Nilsson IL, Lui WO, Larsson C, Zedenius J, Bergquist J, Bränström R.
   Intracellular concentration of the tyrosine kinase inhibitor imatinib in gastrointestinal stromal tumor cells.
   Anticancer Drugs 2014; 25(4):415-422.

2. Caramuta S, Lee L, Özata DM, **Akçakaya P**, Georgii-Hemming P, Xie H, Amini RM, Lawrie CH, Enblad G, Larsson C, Berglund M, Lui WO.
   Role of microRNAs and microRNA machinery in the pathogenesis of diffuse large B-cell lymphomas.
   Blood Cancer Journal 2013; 3:e152.

3. Caramuta S, Lee L, Özata DM, **Akçakaya P**, Xie H, Höög A, Zedenius J, Bäckdahl M, Larsson C, Lui WO.
   Clinical and functional impact of TRBP2 over-expression in adrenocortical carcinoma.
   Endocrine Related Cancer 2013; 20(4): 551-564.

4. Berglund E, Berglund D, **Akçakaya P**, Ghaderi M, Daré E, Berggren PO, Köhler M, Aspinwall CA, Lui WO, Zedenius J, Larsson C, Bränström R.
   Evidence for Ca(2+)-regulated ATP release in gastrointestinal stromal tumors.
   Experimental Cell Research 2013; 319(8):1229-1238.

5. Özata DM, Caramuta S, Velázquez-Fernández D, **Akçakaya P**, Xie H, Höög A, Zedenius J, Bäckdahl M, Larsson C, Lui WO.
   The role of microRNA deregulation in the pathogenesis of adrenocortical carcinoma.
   Endocrine Related Cancer 2011; 18(6):643-655.

6. **Akçakaya P**, Ekelund S, Kolosenko I, Caramuta S, Özata DM, Xie H, Lindforss U, Olivecrona H, Lui WO.
   miR-185 and *miR-133b* deregulation is associated with overall survival and metastasis in colorectal cancer.
   International Journal of Oncology 2011; 39(2):311-318.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

3`UTR	Three prime untranslated region
7-AAD	7-aminoactinomycin D
A	Adenine
ABL	Abelson murine leukemia viral oncogene homolog 1
ADAR	Adenosine deaminases that act on RNA
AGO	Argonaute
AKT	V-akt murine thymoma viral oncogene homolog 1
ANO1	Anoctamin 1
APC	Adenomatous polyposis coli
APEX1	Apurinic/apyrimidinic nuclease 1
ATG5	Autophagy related 5
ATP	Adenosine triphosphate
AXL	AXL receptor tyrosine kinase
BCL-2	B-cell CLL/lymphoma 2
B-CLL	B cell chronic lymphocytic leukemias
BCR	Breakpoint cluster region
BIM	BCL2-like 11
BRAF	B-Raf proto-oncogene, serine/threonine kinase
C	Cytosine
Ca^{2+}	Calcium ion
CCD	Charge-coupled device
CCR4	Chemokine (C-C motif) receptor 4
CD133	Prominin 1
CD90	Thy-1 cell surface antigen
CDH1	Cadherin 1
cDNA	Complementary deoxyribonucleic acid
Cl^-	Chloride ion
CML	Chronic myeloid leukemia
CSC	Cancer stem cells
CSF1R	Colony-stimulating factor-1 receptor
DAB	3, 3’-diaminobenzidine
ddNTP	Dideoxyribonucleoside triphosphate
DGCR8	DiGeorge syndrome critical region 8
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
DOG1	Discovered on GIST-1
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGIST	Extra-gastrointestinal stromal tumor
ERK	Extracellular signal-regulated kinase
ETV1	Ets translocation variant 1
EXP5	Exportin 5
FAK	Focal adhesion kinase
FDA	U.S. Food and Drug Administration
FDG	Fluorodeoxyglucose
FDR	False discovery rate
FGFR	Fibroblast growth factor receptor
FITC	Fluorescein isothiocyanate
FLT3	Fms-like tyrosine kinase 3
FSCN1	Fascin actin-bundling protein 1
G	Guanine
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GIST	Gastrointestinal stromal tumor
GW182	Glycine/trytophan repeat protein, 182 kDa
H2AX	H2A histone family, member X
HCV	Hepatitis C virus
HIF1α	Hypoxia inducible factor 1, alpha subunit
HSP90	Heat shock protein 90
ICC	Interstitial cells of Cajal
IDO	Indoleamine 2,3-dioxygenase
Ig	Immunoglobulin
IGF1R	Insulin-like growth factor 1 receptor
kb	Kilobase
kDa	Kilodalton
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
M	Molar
MAPK	Mitogen-activated protein kinase
MCL1	Myeloid cell leukemia 1
MCL1L	Myeloid cell leukemia 1, long isoform

MCL1S	Myeloid cell leukemia 1, short isoform
MEK	Mitogen-activated protein/extracellular signal-regulated kinase kinase
MET	MET proto-oncogene, receptor tyrosine kinase
MGB	Minor groove binder
miRNA	MicroRNA
mTOR	Mammalian target of rapamycin
MYC	v-myc avian myelocytomatosis viral oncogene homolog
NDRG2	N-MYC downstream-regulated gene 2
NF1	Neurofibromin 1
NFQ	Non-fluorescent quencher
nM	Nanomolar
nt	Nucleotide
p27	Cyclin-dependent kinase inhibitor 1B
p53	Tumor protein p53
PABP	Poly(A) binding protein
PACT	Protein activator of interferon-induced protein kinase
PARP	Poly (ADP-ribose) polymerase
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGFRA	Platelet-derived growth factor alpha
PDGFRB	Platelet-derived growth factor beta
PE	Phycoerythrin
pFAK	Phosphorylated focal adhesion kinase
PI	Propidium iodide
PI3K	Phosphatidylinositol-3-kinase
piRNA	PIWI-interacting RNA
pre-miRNA	Precursor microRNA
pri-miRNA	Primary microRNA
PTPN18	Protein tyrosine phosphatase, non-receptor type 18
PUMA	P53-upregulated modulator of apoptosis
RAF1	Raf-1 proto-oncogene, serine/threonine kinase
RAN-GTP	Ras-related nuclear protein guanosine triphosphatase
RAS	Rat sarcoma viral oncogene homolog
RET	Rearranged during transfection proto-oncogene
RNA	Ribonucleic acid
RT-qPCR	Reverse transcription quantitative polymerase chain reaction

SAM	Significance analysis of microarray
SCF	Stem cell factor
SDH	Succinate dehydrogenase
SHP-1	SH2 domain-containing protein tyrosine phosphatase-1
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SKP2	S-phase kinase-associated protein 2
SMARCA3	SWI/SNF-related, matrox-associated, actin-dependent regulator of chromatin, subfamily A, member 3
SNPs	Single nucleotide polymorphisms
STARD13	StAR-related lipid transfer (START) domain containing 13
STAT3	Signal transducer and activator of transcription 3
TEK	Tie2 endothelial-specific tyrosine kinase
TKI	Tyrosine kinase inhibitor
TMEM16A	Transmembrane member 16A
TARBP	TAR RNA-binding protein
Twist1	Twist family basic helix-loop-helix transcription factor 1
U	Uracil
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

1 INTRODUCTION

Discovery and development of tyrosine kinase inhibitors, such as imatinib, revolutionized the treatment approaches to cancer, by making possible to target the molecular events specific to cancer cells that are responsible for the pathogenesis. When first introduced in 2001, imatinib was welcomed with a big hope and excitement. Its application to gastrointestinal stromal tumor (GIST) together with chronic myeloid leukemia (CML) provided a clinical benefit for the majority of patients and has served as a model system for targeted therapies. The major contributors to imatinib development (Brian Druker, Nicholas Lydon and Charles Sawyers) received the Lasker-DeBakey Clinical Medical Research Award (known as the "American Nobel Prize") in 2009 for "converting a fatal cancer into a manageable chronic condition".

Despite the remarkable success of imatinib treatment, resistance is one of the main challenges. Follow-up studies revealed that the vast majority of patients eventually develop disease progression after an initial response. Although the initial events in GIST development are well characterized, the prognosis is clearly influenced by other genetic or epigenetic events that are still poorly understood. This thesis work contributes to the understanding of the molecular mechanisms underlying imatinib resistance in GIST.

1.1 GASTROINTESTINAL STROMAL TUMOR

Cancer is defined as a group of more than 100 different diseases characterized by uncontrolled cell growth with ability to invade or spread to other parts of the body, which is the second leading cause of death worldwide (Jemal *et al*, 2010). Sarcomas are rare and a heterogeneous group of malignant connective tissue tumors with mesenchymal origin, which accounts for 1-2% of all malignancies (Ferrari *et al*, 2011; Mastrangelo *et al*, 2012; Stiller *et al*, 2013). Because mesenchymal cells are present all around the body, sarcomas can arise in nearly all locations, and they are classified as skeletal and soft tissue sarcomas.

GISTs comprise one-fifth of soft tissue sarcomas, making them the most common single type of sarcoma (Ducimetiere *et al*, 2011). Population-based reports show that GISTs have an annual incidence between 11 and 19.5 per million (Chan *et al*, 2006; Goettsch *et al*, 2005; Nilsson *et al*, 2005; Tryggvason *et al*, 2005) and have a prevalence of about 130 cases per million population (Chan *et al*, 2006; Nilsson *et al*, 2005). GISTs can be found anywhere along the gastrointestinal tract, but predominantly occur in the stomach (50-60%) and the small intestine (30-35%), less frequently in the colon/rectum (5%) and esophagus (<1%) (Joensuu *et al*, 2012). In rare cases, GISTs can occur outside the gastrointestinal tract, such as in the omentum, mesentery or retroperitoneal (<5%), and they are named as extra-gastrointestinal stromal tumors (EGISTs). Conversely, EGISTs are under an ongoing debate whether they are metastasis of an undetected primary tumor (Joensuu *et al*, 2012). GISTs can arise at any age, with a median age of diagnosis at 63 years. More than 80% of the patients are older than 50 years, and only 0.4% of them are younger than 20 years

(Ducimetiere *et al*, 2011; Joensuu *et al*, 2012). The tumor size varies between 2 to 30 cm at the time of diagnosis (Corless *et al*, 2002). As an example, macroscopic and histologic images of a gastric GIST are represented in Figure 1.

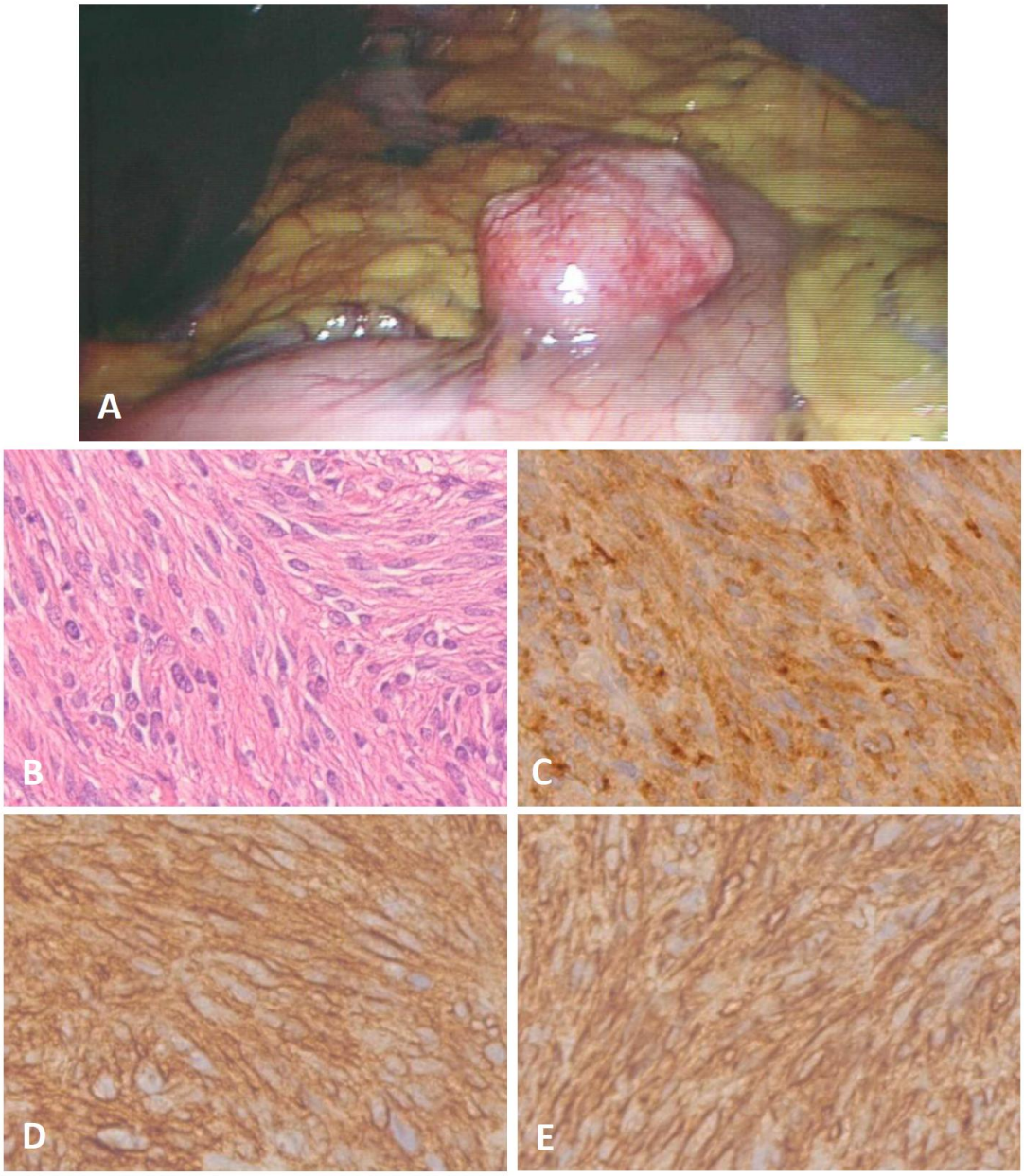

Figure 1. Macroscopic and histologic images of a gastric GIST. **A)** Intra-operative image. **B)** Hematoxylin and eosin staining showing the spindle-shaped phenotype. Immunohistochemistry images showing the positivity for **C)** CD117, **D)** CD34 and **E)** DOG1.

For many years, GISTs were considered as smooth muscle sarcomas based on their morphology, and misclassified as leiomyomas, leiomyosarcomas or leiomyoblastomas. In 1983, Mazur and Clark used the term GIST for the first time to distinguish these tumors that do not express the immunohistochemical markers of Schwann cells or do not possess the ultrastructural characteristics of smooth muscle cells (Mazur & Clark, 1983). Later, CD34 was introduced as a clinically useful marker for distinguishing GIST from leiomyomas and schwannomas (Miettinen *et al*, 1995).

Two groundbreaking discoveries in the late 1990`s revolutionized the approach towards GIST as an entity. First, Kindblom and colleagues found that majority of GISTs (>95%) are immunohistochemically positive for the tyrosine kinase receptor KIT (v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog also known as CD117) (Kindblom *et al*, 1998). Second, Hirota and colleagues identified *KIT* mutations in GISTs (Hirota *et al*, 1998). Since then, continuous research revealed that 70–80% of GISTs harbor *KIT* gene mutations. To date, KIT immunostaining and mutation screening are used as key diagnostic markers in clinical practice for GISTs, and mutant KIT is a clinically important therapeutic target in GISTs. These findings transformed GISTs from a challenging chemotherapy-resistant disease to a model for molecular targeted therapy.

1.1.1 Origin

1.1.1.1 Interstitial cells of Cajal

In 1998, Kindblom and co-workers noted similarities between GISTs and a cell population in the gastrointestinal tract called the interstitial cells of Cajal (ICCs) which function as pacemaker cells that cause peristaltic contractions. GISTs were found positive for CD117 (KIT) and CD34 immunohistochemically, and ICCs were the only known cells in the gastrointestinal tract positive for both CD117 and CD34 (Hirota *et al*, 1998; Kindblom *et al*, 1998). This finding led to the hypothesis that GISTs originate from, or share a common origin with, ICCs. GISTs and ICCs show similar gene expression patterns, such as high levels of PKCθ, nestin and DOG1 (Gomez-Pinilla *et al*, 2009; Motegi *et al*, 2005; Poole *et al*, 2004; Sarlomo-Rikala *et al*, 2002; Southwell, 2003; Wong & Shelley-Fraser, 2010). In addition, ETV1 is highly expressed in both GISTs and the myenteric and intramuscular subpopulations of ICCs (Chi *et al*, 2010). Further evidence supporting this notion came from transgenic mice expressing KIT mutant induced diffuse ICC hyperplasia (Rubin *et al*, 2005; Sommer *et al*, 2003), expansion of ICCs (Bardsley *et al*, 2010) and GIST-like tumors (Rubin *et al*, 2005; Sommer *et al*, 2003). Notably, diffuse ICC hyperplasia has also been described in GISTs harboring heritable *KIT* mutations, and it has been associated with development of multiple GISTs (Hirota *et al*, 2002; Isozaki *et al*, 2000; Kang *et al*, 2007; O'Riain *et al*, 2005).

1.1.1.2 Micro-GISTs

Micro-GISTs are small growths of cells with less than 1 cm in size that shares similar characteristics with GISTs and ICCs. These growths are found in 2.9 - 35% of stomachs that are thoroughly examined after surgical removal or during autopsy (Corless *et al*, 2002; Kawanowa *et al*, 2006; Muenst *et al*, 2011). Their prevalence is estimated to exceed 10 million lesions in the US population (Corless, 2014). Even though they are mitotically inactive and often partially calcified, micro-GISTs harbor *KIT* mutations with the same type and frequency as in clinically relevant GISTs (Agaimy *et al*, 2007; Rossi *et al*, 2010). *PDGFRA* mutations in micro-GISTs have also been reported (Agaimy *et al*, 2007). These findings suggest that kinase mutations occur at very early stages in GIST tumorigenesis. Additional molecular events are required for progression to malignant transformation.

1.1.1.3 GIST stem cells

Cancer stem cells (CSC) have been identified in hematopoietic malignancies (Bonnet & Dick, 1997; George *et al*, 2001; Miyamoto *et al*, 2000) and solid tumors, such as melanomas (Schatton *et al*, 2008), breast (Al-Hajj *et al*, 2003; Charafe-Jauffret *et al*, 2009), brain (Corti *et al*, 2006; Salmaggi *et al*, 2006), and prostate (Tang *et al*, 2007) cancers. However, their existence and role in sarcomas remains uncertain.

The first evidence of GIST stem cells was shown in 2008 when Ördög and colleagues identified a rare population of cells that do not resemble ICC in the post-natal murine stomach (Lorincz *et al*, 2008). These $Kit^{low}Cd34^{+}Cd44^{+}Igf1r^{+}$ cells were able to differentiate into ICCs and their proliferation could be stimulated by SCF and IGF1. Two years later, the same group showed that single isolated $Kit^{low}Cd34^{+}Cd44^{+}$ cells were capable of self-renewal and differentiation into ICCs and gave rise to GIST in mice (Bardsley *et al*, 2010). Importantly, these progenitor cells were resistant to imatinib; indicating that imatinib-resistant GIST may arise from GIST stem cells.

Human GIST stem cells still remain to be determined. CD133 has been described as a specific marker for human hematopoietic stem cells (Miraglia *et al*, 1997; Yin *et al*, 1997) and CSCs of several solid tumors (Collins *et al*, 2005; Ricci-Vitiani *et al*, 2007; Singh *et al*, 2003). Lately, several studies have evaluated the significance of CD133 as a potential GIST stem cell marker (Arne *et al*, 2011; Bozzi *et al*, 2011; Bozzi *et al*, 2012; Chen *et al*, 2012). However, these studies revealed that CD133 is universally expressed in GIST (Bozzi *et al*, 2011; Bozzi *et al*, 2012; Chen *et al*, 2012). CD133 expression was detected in imatinib-sensitive, but not in the imatinib-resistant GIST cell lines. Furthermore, $CD133^{-}$ cells show more aggressive behavior than $CD133^{+}$ cells *in vitro* (Chen *et al*, 2012). Taken together, these findings suggest that CD133 represents a lineage marker, but not a CSC marker in GIST. Besides CD133, several other putative markers were also evaluated, including CD44 and CD90. The findings were similar to CD133; both markers were ubiquitously expressed in GISTs and are thus unlikely to be GIST CSC markers (Bozzi *et al*, 2011; Chen *et al*, 2012). Further investigations are still warranted to identify human GIST stem cells.

1.1.2 Molecular biology

1.1.2.1 Oncogenic mutations

KIT and PDGFRA

The main initial event in GIST tumorigenesis is gain-of-function mutations in *KIT* or *PDGFRA* (platelet-derived growth factor-α) genes. *KIT* and *PDGFRA* genes are located on the long arm of chromosome 4 (4q12), and encode transmembrane proteins that belong to the type III tyrosine kinase receptor family. The receptor family also includes three other members: platelet-derived growth factor-β (PDGFRB), colony-stimulating factor-1 receptor (CSF1R) and Fms-like tyrosine kinase 3 (FLT3).

The members of type III tyrosine kinase receptor family consist of a ligand-binding extracellular domain of five immunoglobulin (Ig) regions, an autoinhibitory intracellular juxtamembrane domain, and a kinase domain of an amino terminal ATP-binding region and a carboxy terminal phosphotransferase region (activation loop) (Figure 2).

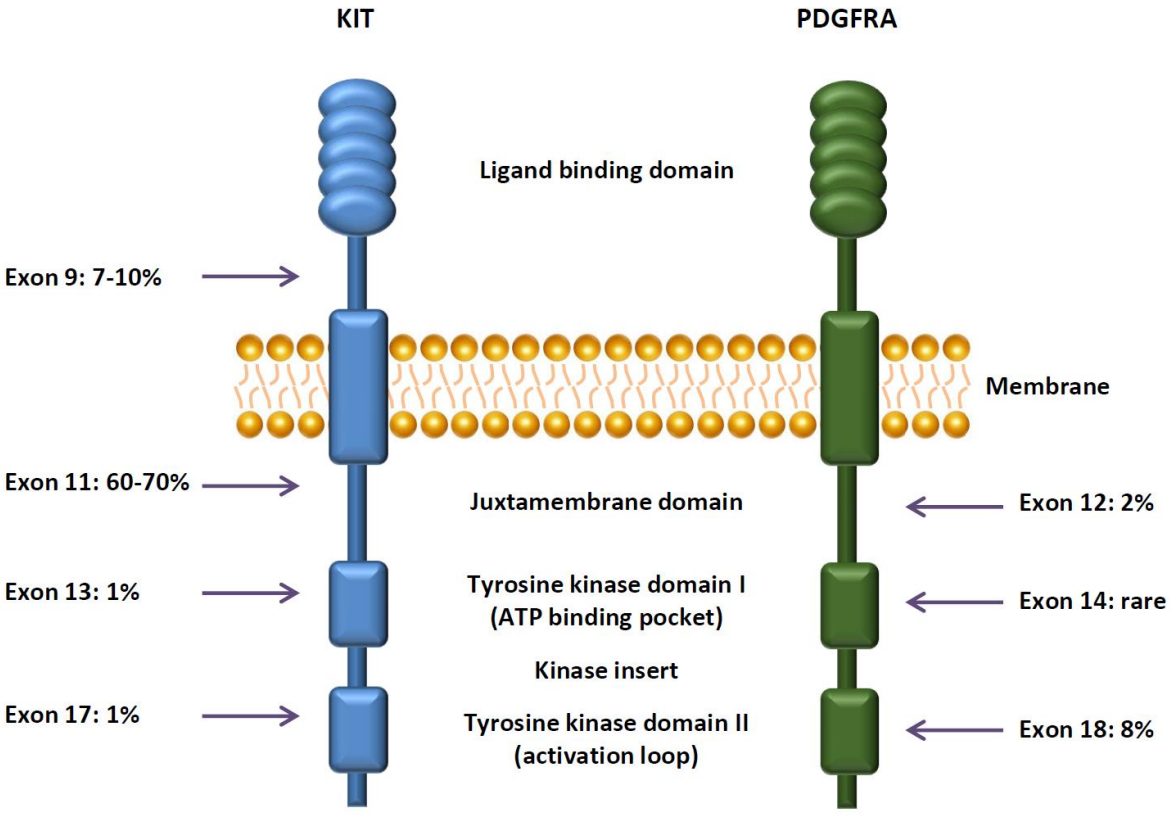

Figure 2. Location and frequency of *KIT* and *PDGFRA* mutations in GIST. Modified from (Corless, 2014).

KIT and PDGFRA serve as the receptors for stem cell factor (SCF) and platelet-derived growth factor (PDGF), respectively. Binding of these ligands to the receptors results in homodimerization, transphosphorylation of the tyrosine residues and kinase activation, initiating signal transduction cascades that promote cell proliferation, growth and survival (Heldin, 1995; Hubbard *et al,* 1998; Roskoski, 2005). In humans, KIT expression is required for cellular maintenance of germ cells, melanocytes, mast cells, hematopoietic stem cells and ICC (Huizinga *et al*, 1995).

Under normal conditions, KIT is maintained in an inactive state in the absence of SCF. The kinase activity of KIT is tightly controlled by auto-regulation mechanisms. KIT is stabilized in inactive conformation by juxtamembrane domain that physically hinders the kinase domain by inserting a hairpin directly into the cleft between amino- and carboxyl- terminal lobes (Mol *et al*, 2004). Furthermore, active KIT is inhibited by a rapid dephosphorylation through the phosphatase SHP-1 (also known as PTPN6) (Kozlowski *et al*, 1998), and by activation-induced endocytic uptake of the receptor from the cell surface, followed by proteasomal degradation (Babina *et al*, 2006).

KIT and *PDGFRA* mutations disrupt the auto-regulatory mechanisms and cause ligand-independent constitutive activation of the encoded tyrosine kinase receptors (Gajiwala *et al*, 2009), which results in aberrant cell growth and tumor formation (Corless *et al*, 2004). Several lines of evidence support the functional role of *KIT/PDGFRA* mutations in GIST tumorigenesis. For examples, KIT mutants promote constitutive kinase activity in the absence of their ligands (Hirota *et al*, 1998; Rubin *et al*, 2001) and develop GIST-like tumors in mice (Rubin *et al*, 2005; Sommer *et al*, 2003). Phosphorylated forms of these kinases are found in most human GISTs, indicating their *in vivo* activity (Rubin *et al*, 2001). Germline *KIT* mutations are reported in familial GIST cases (Hirota *et al*, 2002; Isozaki *et al*, 2000). As aforementioned, *KIT* and *PDGFRA* mutations are found even in the micro-GISTs, which are the earliest recognizable forms of GISTs (Agaimy *et al*, 2007; Rossi *et al*, 2010).

In GIST, the most common mutations are found in *KIT* exon 11 (60-70%) that affects the juxtamembrane domain (Corless *et al*, 2011). These mutations interfere with the juxtamembrane secondary structure that normally prevents the kinase activation loop going into the active conformation (Mol *et al*, 2004). In-frame deletions, insertions and substitutions, or combinations of these are seen in exon 11. Mutations in exon 9 (7-10%) affecting the extracellular domain are the second most common following the exon 11 mutations (Lux *et al*, 2000). They are thought to mimic the structure of extracellular KIT domain when SCF binds (Yuzawa *et al*, 2007), and not interfere with the kinase domain. A minority of mutations is found in exons 13 and 17 (Corless *et al*, 2011). Exon 17 mutations affect the activation loop and stabilize the active conformation (Lasota *et al*, 2008). Exon 13 mutations disrupt the ATP-binding region (encoded by exon 13), and its biological function is still unclear. It is thought that they interfere with the normal auto-inhibitory function of the juxtamembrane domain.

About 10% of GISTs harbor *PDGFRA* mutations (Heinrich *et al*, 2003b; Hirota *et al*, 2003). PDGFRA is highly expressed in GISTs with mutations in the juxtamembrane domain (encoded by exon 12), the ATP-binding domain (encoded by exon 14) or the kinase activation loop (encoded by exon 18) (Pauls *et al*, 2005; Wasag *et al*, 2004).

Besides the kinase activation caused by the mutations, mutant KIT has a longer half-life than wild-type KIT (Corless *et al*, 2011). As mentioned above, signaling from wild-type KIT upon activation by SCF is quickly controlled by endocytosis, ubiquitination and proteasome-mediated degradation, serving as a negative feedback mechanism. Long half-life of mutant KIT/PDGFRA might be due to stabilization by chaperone heat shock protein 90 (HSP90) or defective autophagy that prevent it from degradation (Bauer *et al*, 2006; Fumo *et al*, 2004; Hsueh *et al*, 2013; Matei *et al*, 2007).

"Wild-type" GISTs and other mutations

About 10-15% of GISTs do not have *KIT* or *PDGFRA* mutations. These tumors are so-called 'wild-type' GISTs have detectable levels of KIT in phosphorylated form, suggesting that KIT is still activated (Duensing *et al*, 2004). However, the mechanism of its activation is unclear. Wild-type GISTs occur anywhere in the gastrointestinal tract and show no difference in morphology, making them clinically indistinguishable from *KIT/PDGFRA*-mutant GISTs (Duensing *et al*, 2004).

A number of studies revealed that wild-type GISTs are heterogeneous and display various oncogenic mutations. The identified mutated genes are succinate dehydrogenase (*SDHA/B/C/D*) (50%) (Janeway *et al*, 2011; Pantaleo *et al*, 2011), *BRAF* V600E substitution (13%) (Hostein *et al*, 2010), neurofibromin 1 (*NF1*) (7%) (Andersson *et al*, 2005; Kinoshita *et al*, 2004) and RAS family members (Miranda *et al*, 2012). VEGF and IGF1R over-expressions were identified in wild-type GISTs (Antonescu *et al*, 2004), and are thought to correlate with SDH activity (Figure 3B).

Different from GISTs found in adults, pediatric GISTs (1–2% of all GISTs) are rarely positive for *KIT* or *PDGFRA* mutations, despite expressing KIT at similar levels as adult GISTs (Janeway *et al*, 2007). These tumors also have a different gene expression pattern than adult GISTs (Agaram *et al*, 2008; Janeway *et al*, 2007; Prakash *et al*, 2005), suggesting distinct oncogenic mechanisms of GIST in children and adults.

1.1.2.2 Downstream pathways

Activations of several downstream signaling pathways are found in KIT mutant GISTs, including mitogen-activated protein kinase (MAPK) pathway, phosphatidylinositol-3-kinase (PI3K)/AKT/mTOR pathway and signal transducer and activator of transcription 3 (STAT3) (Bauer *et al*, 2007; Duensing *et al*, 2004; Rossi *et al*, 2006) (Figure 3A). The oncogenic KIT signaling mechanisms are thought to vary depending on the location and type of the *KIT* mutation (Duensing *et al*, 2004). Additional mechanisms affecting downstream signaling include SHC adaptor protein interaction with KIT leading to

GRB2/RAS/MAPK pathway (Lennartsson *et al*, 1999) and expression of differential KIT isoforms as a result of alternative splicing (Caruana *et al*, 1999).

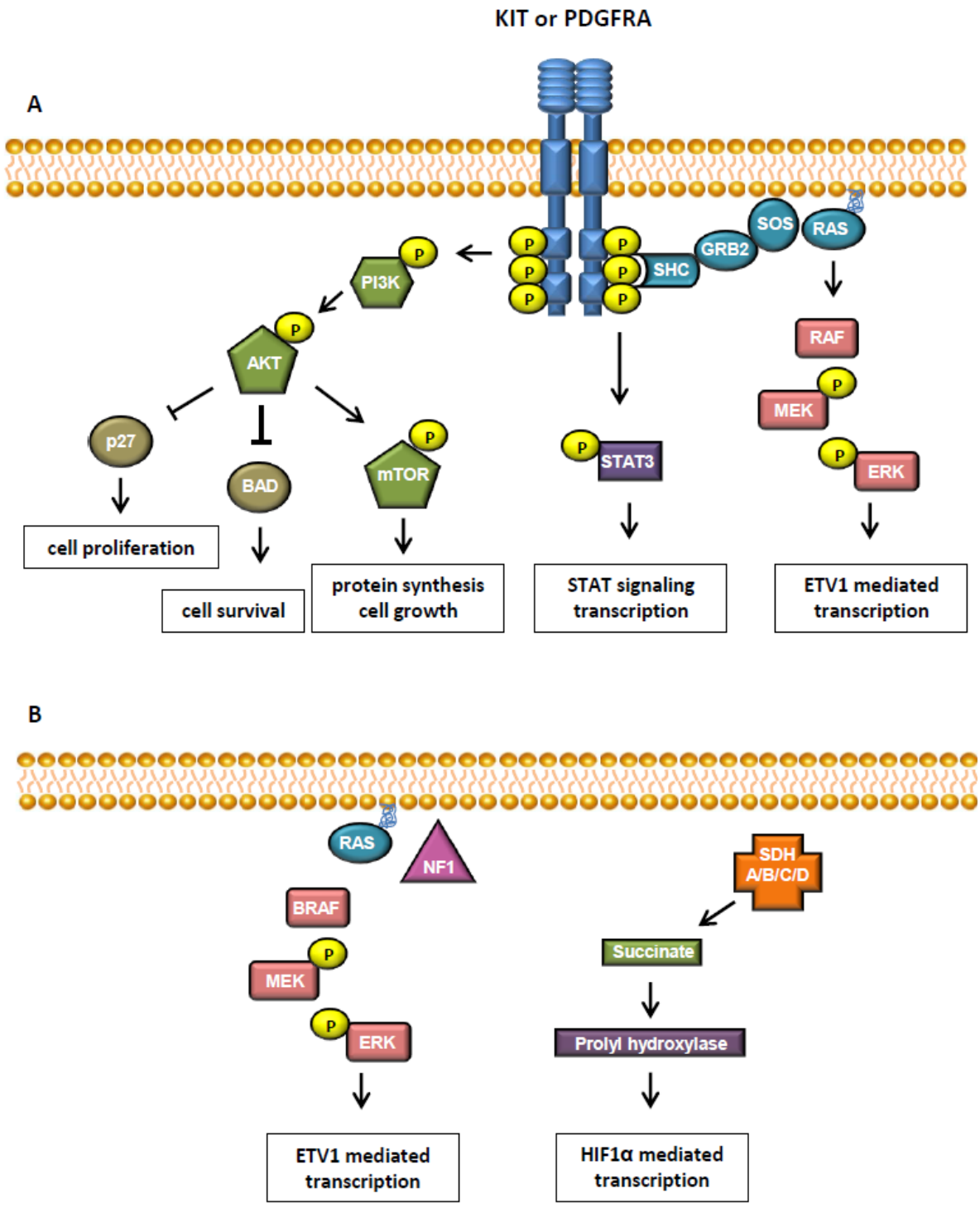

Figure 3. A) KIT and PFGFRA signaling pathways. Mutations in KIT or PDGFRA activate MAPK, PI3K/AKT/mTOR and STAT3 pathways. **B)** Signaling pathways in "wild-type" GISTs. Mutations in NF1, BRAF or RAS lead to increased MAPK signaling. Mutations in SDHA/B/C/D lead to Succinate accumulation, which inhibits prolyl hydroxylase-mediated HIF1α degradation, causing increased HIF1α-mediated transcription factor levels. Modified from (Corless, 2014). P: Phosphate group.

Therapeutic targeting of the downstream pathways of KIT reveals the relative importance of these pathways in GIST. Inhibition of MAPK pathway (with U0126, a MEK2 inhibitor) showed inconsistent effects on cell proliferation (5–40% inhibition) and had no effect on apoptosis (Bauer *et al*, 2007). On the other hand, PI3K inhibitor (LY294002) remarkably inhibits cell proliferation (40–75% inhibition) and induces apoptosis (three to four fold) in both imatinib-sensitive and –resistant cells (Bauer *et al*, 2007). mTOR inhibitors reduce cell proliferation and induce apoptosis but are less effective than PI3K inhibitors (Bauer *et al*, 2007). In addition, although they induce cell cycle arrest, mTOR inhibitors do not result in any histological or apoptotic response in GIST mouse models (Rossi *et al*, 2006). These findings suggest determinants of cell survival signaling in GIST are located downstream of PI3K but upstream of mTOR, and PI3K/AKT pathway seem to play a crucial role in GIST proliferation and survival.

1.1.2.3 GIST progression

Patients harboring germline *KIT* mutations do not manifest GISTs until their early adulthood (Kim *et al*, 2005). In addition, even though micro-GISTs are commonly found in the general population, most of them do not transform into malignant stage. These observations suggest that other genetic events are required for tumor progression in addition to the oncogenic kinase mutations.

Cytogenetic studies demonstrated that about 65% of GISTs have either monosomy of chromosome 14 or partial loss of 14q (Bergmann *et al*, 1998; Debiec-Rychter *et al*, 2001; Fukasawa *et al*, 2000). Loss of heterozygosity and comparative genomic hybridization studies showed two regions of this chromosome, 14q11.2 and 14q32, as hotspot regions harboring tumor suppressor genes that might be important for GIST development (Debiec-Rychter *et al*, 2001; El-Rifai *et al*, 2000b). Several candidate genes are suggested within these regions, such as *PARP2, APEX1* and *NDRG2* genes at 14q11.2, *SIVA* (Assamaki *et al*, 2007) and a miRNA cluster at 14q32 (Kelly et al., 2013). Interestingly, two members of the 14q32 miRNA cluster are associated with shorter disease-free survival/tumor progression (Choi *et al*, 2010; Haller *et al*, 2010). Loss of the long arm of chromosome 22 is observed in approximately 50% of GISTs and associated with malignant behavior (Bergmann *et al*, 1998; Fukasawa *et al*, 2000; Kim *et al*, 2000).

Losses on chromosomes 1p, 9p, 11p, 17p, 13q and 15q have also been reported in GISTs (Bergmann *et al*, 1998; Gunawan *et al*, 2007; Kim *et al*, 2000; O'Leary *et al*, 1999; Wozniak *et al*, 2007). Chromosome 9p21 deletion causes inactivation of the tumor suppressor gene *CDKN2A* and associated with malignancy (Perrone *et al*, 2005; Ricci *et al*, 2004; Sabah *et al*, 2004; Schneider-Stock *et al*, 2003). Gains on chromosomes 8q (including *MYC*), 3q (including *SMARCA3*) and 17q are associated with metastatic behavior (Debiec-Rychter *et al*, 2001; El-Rifai *et al*, 2000a; O'Leary *et al*, 1999; Ylipaa *et al*, 2011).

Gene expression profiles of high-risk tumors show significant changes in cell cycle regulator genes and genes associated with PI3K pathway (Hur *et al*, 2010). p27 is commonly downregulated in malignant GISTs, but the association with tumor progression is not very well supported (Nakamura *et al*, 2005; Pruneri *et al*, 2003). Increased expression levels of cyclin A and cyclin H are associated with high-risk GISTs (Dorn *et al*, 2010; Huang *et al*, 2009; Nakamura *et al*, 2005). *TP53* mutations and decreased p53 expression also correlate with a poor prognosis in GIST (Feakins, 2005; Panizo-Santos *et al*, 2000; Romeo *et al*, 2009).

In 2004, expression of another protein, DOG1 (Discovered on GIST-1), was discovered in ~98% of GISTs (West *et al*, 2004). DOG1 is encoded by *ANO1* (also known as *TMEM16A*), functions as calcium (Ca^{2+})-dependent chloride (Cl^-) channel. Notably, DOG1 is highly and specifically expressed in both GISTs and ICCs (Espinosa *et al*, 2008; Gomez-Pinilla *et al*, 2009), and detected in ~35% of "wild type" GISTs. However, its biological function in GISTs has not been fully characterized. Ca^{2+}-dependent Cl^- channels are involved in several physiological processes including gastrointestinal muscle contractions (Hwang *et al*, 2009), regulation of neuronal excitability, and transduction of sensory stimuli (Ferrera *et al*, 2010; Frings *et al*, 2000). Functionally, DOG1 is essential in generation of gastrointestinal muscle contractions (Hwang *et al*, 2009); and it regulates tumorigenesis and cancer progression through MAPK signaling in head and neck squamous cell carcinoma (Ayoub *et al*, 2010; Duvvuri *et al*, 2012; Ruiz *et al*, 2012). A recent functional study in GIST reported that although DOG1 does not affect GIST cell growth or KIT signaling *in vitro*, its inhibition delays the growth of GISTs in certain GIST cell-line derived xenografts *in vivo* (Simon *et al*, 2013). Further studies are warranted to illuminate the functional impact of DOG1 in GIST tumorigenesis.

1.1.3 Treatment of gastrointestinal stromal tumor

Treatment options for patients with advanced GIST were few until 2000s. Surgical resection has been the main therapy for GIST, with the main goal of complete resection and avoidance of tumor rupture (Hohenberger *et al*, 2010). The response rate to conventional chemotherapy agents was extremely low (<5%) (Dematteo *et al*, 2002). The resistance to chemotherapy in GIST might be due to the increased levels of P-glycoprotein and multidrug resistance protein (Plaat *et al*, 2000). Alternatively, oncogenic activation of tyrosine kinases might cause increased anti-apoptotic signaling and activation of other drug resistance pathways. Because of the diffuse pattern of recurrence in the liver or the peritoneum, radiation therapy was beneficial only to palliate patients with bleeding, but not for treatment. Median overall survival for patients with advanced disease was 18 months (Dematteo *et al*, 2002) until imatinib was introduced. The work-flow of GIST treatment according to the Scandinavian Sarcoma Group and the European Sarcoma Network Group guidelines is illustrated in Figure 4.

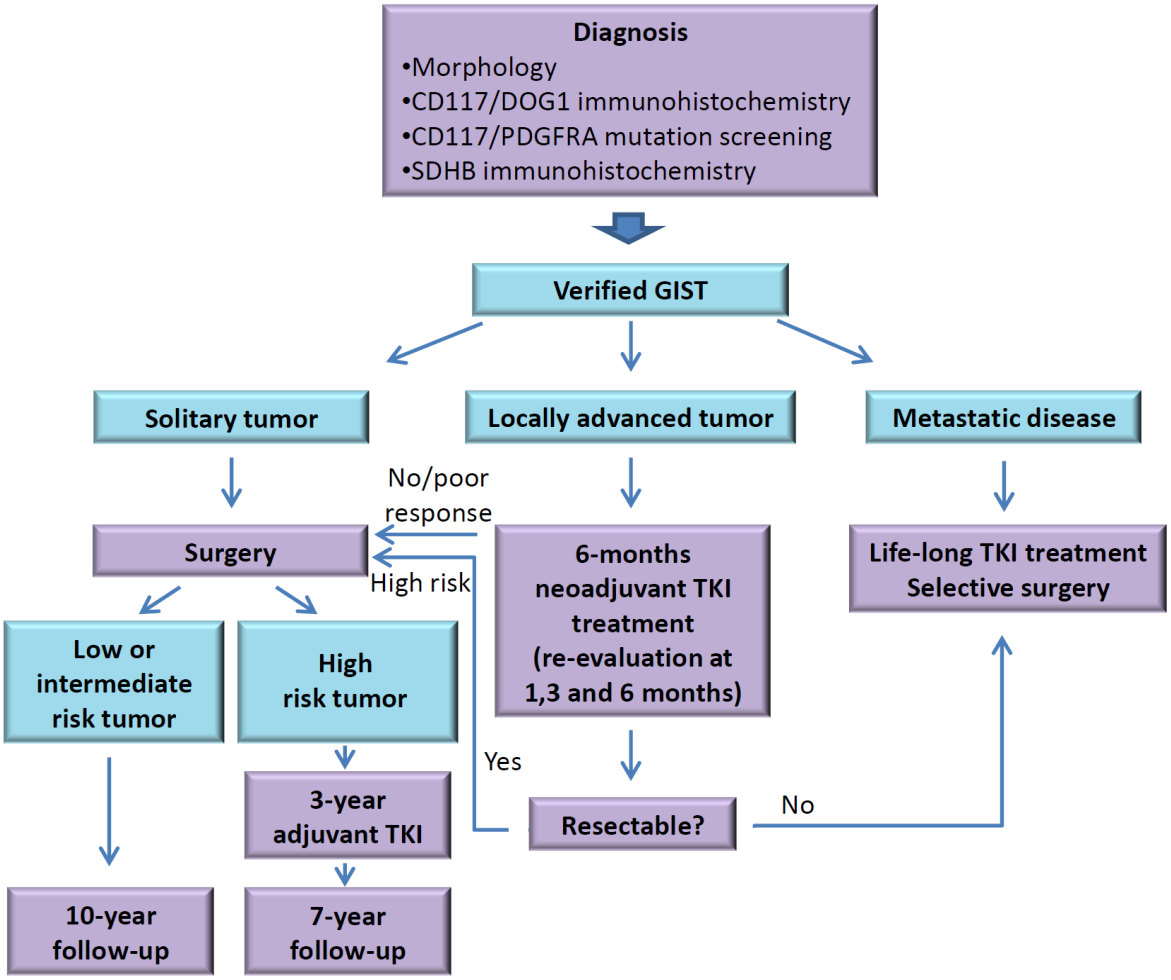

Figure 4. Clinical practice of diagnosis, treatment and follow-up of GISTs. Modified from (Berglund E, thesis for doctoral degree 2014, ISBN 978-91-7549-520-0), (ESMO, 2014).

1.1.3.1 Imatinib

1.1.3.1.1 Discovery

"There is new ammunition in the war against cancer. These are the bullets."

Time Magazine Cover, 28 May 2001

Discovery of imatinib (also known as Gleevec, Glivec, and STI-571) led to a major paradigm shift in cancer therapy towards molecular targeted therapy. Until then, most of the anti-cancer therapies have been non-specific; generally function by interfering with cellular machinery that is common for both normal and neoplastic cells (*e.g.* DNA/RNA synthesis, formation of microtubules). These therapies lack selectivity, have a narrow therapeutic index, and induce toxicity.

In 1996, Druker and colleagues have published their identification of a small molecule, now known as imatinib, that can selectively block the ABL kinase activity and induce cell death of BCR-ABL positive chronic myeloid lymphoma (CML) cells (Druker *et al*, 1996). The

finding was translated into the clinical trials rapidly. The striking results from clinical trials with CML patients came in 2001 (Druker *et al*, 2001a; Druker *et al*, 2001b), showing more than 90% complete response rate in patients with chronic-phase. Shortly after, U.S. Food and Drug Administration (FDA) approved the usage of imatinib as an efficient and safe therapy for CML patients.

Based on the discovery of *KIT* mutations in GIST by Hirota and co-workers in 1998 (Hirota *et al*, 1998), many scientists investigated KIT activation as a crucial event in GIST pathogenesis. The mutations causing constitutive kinase activation and an uncontrolled cell growth behavior in GIST, was reminiscent of the mechanism of BCR-ABL in CML. These findings led to the hypothesis of KIT inhibition might be a therapeutic strategy for GIST. Concurrently, imatinib was shown not only specific to BCR-ABL, but also blocks the enzymatic activity of the transmembrane receptor tyrosine kinases KIT, PDGFRA and PDGFRB (Buchdunger *et al*, 2000; Heinrich *et al*, 2000a). The inhibitory effect of imatinib on mutant KIT was functionally confirmed first in a mast leukemia cell line that harbors a similar mutation as clinically relevant GISTs (Heinrich *et al*, 2000a; Heinrich *et al*, 2000b), then in the GIST cell line with a mutant KIT (Tuveson *et al*, 2001). Inhibition of mutant KIT by imatinib led to GIST cell growth arrest and apoptosis (Tuveson *et al*, 2001). Thereafter, clinical development of imatinib for GIST therapy rapidly progressed, and FDA approved imatinib therapy for advanced or metastatic GIST in 2002. In 2008, FDA approved adjuvant use of imatinib for patients with high risk of recurrence.

1.1.3.1.2 Mechanism of Action

Kinases show high plasticity that allows the interplay between open (active state) or close (inactive state) conformation (Huse & Kuriyan, 2002). The binding of SCF to the extracellular Ig domain turns KIT into the active state by initiating its dimerization. Dimerization permits the kinase domains of KIT to phosphorylate each other at specific tyrosine residues located in juxtamembrane regions. Phosphorylation of the tyrosine activates the kinase domains, and initiates the downstream cascade that leads to cell growth and proliferation.

Imatinib binds to the ATP-binding site located in the amino-terminal lobe of the kinase domain that competitively blocks ATP binding and consequent phosphorylation of KIT (Figure 5). Imatinib can only bind to inactive conformation of KIT, to the amino acids Cys673, Glu640, Asp810 and Phe811, giving it a greater degree of specificity. However, this characteristic has implications for potential mechanisms of developing resistance.

1.1.3.2 Response to imatinib therapy

In 2000, imatinib treatment of the first patient with metastatic GIST started in Finland, and a dramatic response was observed (Joensuu et al, 2001). The following Phase I and II trials reported partial response rates as 54% and 68%, respectively, and majority of the remaining patients achieved a stable disease (van Oosterom et al, 2002). Promising results led to two phase III trials, comparing the dose levels of imatinib (400 mg and 800 mg per day). These

studies reported that imatinib achieved disease control in 70-85% of patients with advanced GIST, median progression-free survival was 20-24 months, and median overall survival was 50 months (Blanke et al, 2008b; MetaGIST, 2010; Verweij et al, 2004).

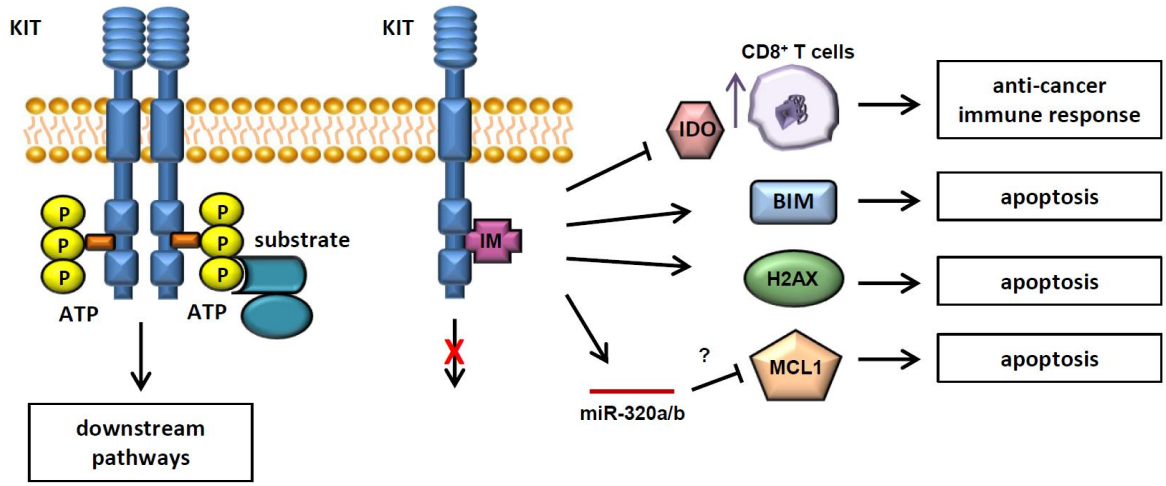

Figure 5. Mechanisms of imatinib action in GIST. Imatinib competitively binds to ATP binding pocket of the kinase domain. It inhibits downstream survival signaling pathways including MAPK, PI3K/AKT/mTOR and STAT3 (Tuveson *et al*, 2001); induces anti-cancer immune response through IDO inhibition (Balachandran *et al*, 2011); and activates apoptotic pathways through BIM (Gordon & Fisher, 2010), H2AX (Liu *et al*, 2007) and MCL1 (**Paper III**). IM: Imatinib, P: Phosphate group.

A study evaluated the effect of imatinib therapy using positron emission tomography on fluorodeoxyglucose (FDG) levels revealed that tumors had a robust response to imatinib present a significant decrease in FDG signal, even within 24 hours of the first dose (Van den Abbeele & Badawi, 2002). This result suggests that a decrease in glycolytic mechanism is one of the initial effects of kinase inhibition. At molecular level, imatinib was shown to inhibit oncogenic signaling that down-regulate downstream survival pathways such as PI3K-AKT and MAPK (Bauer *et al*, 2007), and to induce cell apoptosis through BIM (Gordon & Fisher, 2010) and soluble histone H2AX (Liu *et al*, 2007) (Figure 5). In addition, imatinib reduces the expression of indoleamine 2,3-dioxygenase (IDO), which is an enzyme produces immunosuppressive metabolites (Balachandran *et al*, 2011). Reduction of IDO causes depletion of regulatory T cells and increase of tumor-infiltrating $CD8^+$ T cells. Thus, imatinib stimulates an anti-cancer immune response by diminishing IDO-mediated immunosuppression.

Clinical observations demonstrate that long-term imatinib treatment is not sufficient to eradicate GIST cells. In order to determine the optimal duration of imatinib therapy, an

interesting clinical trial was conducted with patients who had continuous disease control after 3 years of imatinib treatment (Le Cesne *et al*, 2010). Patients were grouped as either to continue or to discontinue the treatment. The 2-year progression-free survival rates were 80% in the continuous patient cohort and only 16% in the discontinuous cohort. The relapse after the discontinuation of imatinib was due to persistent disease, showing that imatinib fails to eradicate cells although it stops their proliferation. The progression during continuous imatinib treatment was due to resistant disease.

Another study investigated the histological responses of the tumors upon imatinib treatment (Agaram *et al*, 2007). After a range of 1 to 31 months of treatment, tumors showed a size reduction range between 10 to 90%. Overall responses did not correlate with duration of treatment or KIT and PDGFRA mutational status. The residual tumor cells in the 75% of the tumors were mitotically inactive, showing a quiescent state. These results demonstrate that GIST cells may avoid apoptosis by evading the cell cycle under imatinib exposure. Indeed, imatinib was shown to cause tumor cell quiescence through the APC/CDH1-SKP2-p27^{Kip1} signaling axis (Liu *et al*, 2008), and to induce autophagy that protects tumor cells from cell death (Gupta *et al*, 2010).

Only a small percentage of patients (3-5%) show a complete disappearance of their tumor upon imatinib treatment (Abhyankar & Nair, 2008; Choi *et al*, 2007). However, it was reported that patients with tumors that shrink or remain stable in size show a similar clinical benefit from the treatment (Blanke *et al*, 2008a).

1.1.4 Imatinib resistance

The majority of GIST patients with advanced disease achieve a clinical benefit from imatinib treatment. However, approximately 10% of patients progress within 6 months of initial therapy, which is defined as primary resistance to imatinib (Blanke *et al*, 2008a; Blanke *et al*, 2008b; van Oosterom *et al*, 2002; Verweij *et al*, 2004). Approximately 50-60% of the initially responding patients develop disease progression within two years. Such cases are regarded as secondary or acquired resistance to imatinib (Blanke *et al*, 2008a; Blanke *et al*, 2008b; van Oosterom *et al*, 2002; Verweij *et al*, 2004).

1.1.4.1 Primary imatinib resistance

Primary resistance can be observed in GISTs with all kind of known mutations, however, it shows stronger correlation with certain genotypes (Debiec-Rychter *et al*, 2006; Debiec-Rychter *et al*, 2004; Heinrich *et al*, 2003a; Heinrich *et al*, 2008b). For example, wild-type, *KIT* exon 9 mutated, and *PDGFRA* D842V mutated GISTs are more likely to show primary resistance. In experimental cell culture systems, GIST cells expressing exon 11 mutant KIT are highly sensitive to imatinib (Heinrich *et al*, 2008a). Correspondingly, patients with GISTs harboring *KIT* exon 11 mutations have a better progression-free and overall survival

compared to patients with wild-type GISTs or GISTs harboring *KIT* exon 9 mutation (Debiec-Rychter *et al*, 2006; Heinrich *et al*, 2008b; MetaGIST, 2010).

The primary resistance arises in GISTs with no identifiable *KIT* or *PDGFRA* mutations is likely due to different mechanisms causing the disease development and activation of alternative signaling pathways. Therefore, treatment of these GISTs with the targeted agents other than imatinib, such as VEGFR, BRAF or MEK inhibitors, might be a better clinical alternative (Janeway *et al*, 2009).

Mutations in exon 9 affect the extracellular *KIT* domain, mimicking the conformation change when SCF binds to the receptor, which induces higher degree of dimerization (Yuzawa *et al*, 2007). Since this mutation does not interfere with the kinase domain, exon 9 mutated *KIT* has the kinase domain same as the wild-type *KIT*, in which decreased sensitivity to imatinib was observed *in vitro* compared to exon 11 mutant KIT (Corless *et al*, 2011). Dose escalation is suggested for treatment of GISTs harboring these mutations (MetaGIST, 2010).

Both clinical and *in vitro* studies have reported that *PDGFRA* D842V mutation is strongly resistant to imatinib (Corless *et al*, 2005; Heinrich *et al*, 2008a; Weisberg *et al*, 2006). This mutation results in a change in the kinase activation loop that strongly favors the active conformation of the kinase domain, which consequently disfavors imatinib binding (Gajiwala *et al*, 2009; Heinrich *et al*, 2003a). Patients with D842V mutant GISTs show low response rates and short progression-free and overall survival during imatinib treatment (Biron *et al*, 2010).

In addition to mutations, gene amplification of *KIT* or *PDGFRA* was shown as a potential mechanism leading to either primary or secondary resistance (Debiec-Rychter *et al*, 2005; Liegl *et al*, 2008; Miselli *et al*, 2007).

1.1.4.2 Secondary imatinib resistance

Secondary mutations in the same gene is the main known mechanism for developing secondary resistance (Antonescu *et al*, 2005; Grimpen *et al*, 2005; Heinrich *et al*, 2006; Wakai *et al*, 2004). A clinical trial revealed that 67% of the patients whose tumors showed secondary resistance had a new mutation in *KIT* (Heinrich *et al*, 2006). Secondary mutations involve two regions in the *KIT* and *PDGFRA* kinase domains: (i) the ATP-binding pocket (encoded by exons 13 and 14) that directly interfere with imatinib binding, and (ii) the kinase activation loop (encoded by exons 17 and 18) that can stabilize the kinase in the active conformation and hinder imatinib binding.

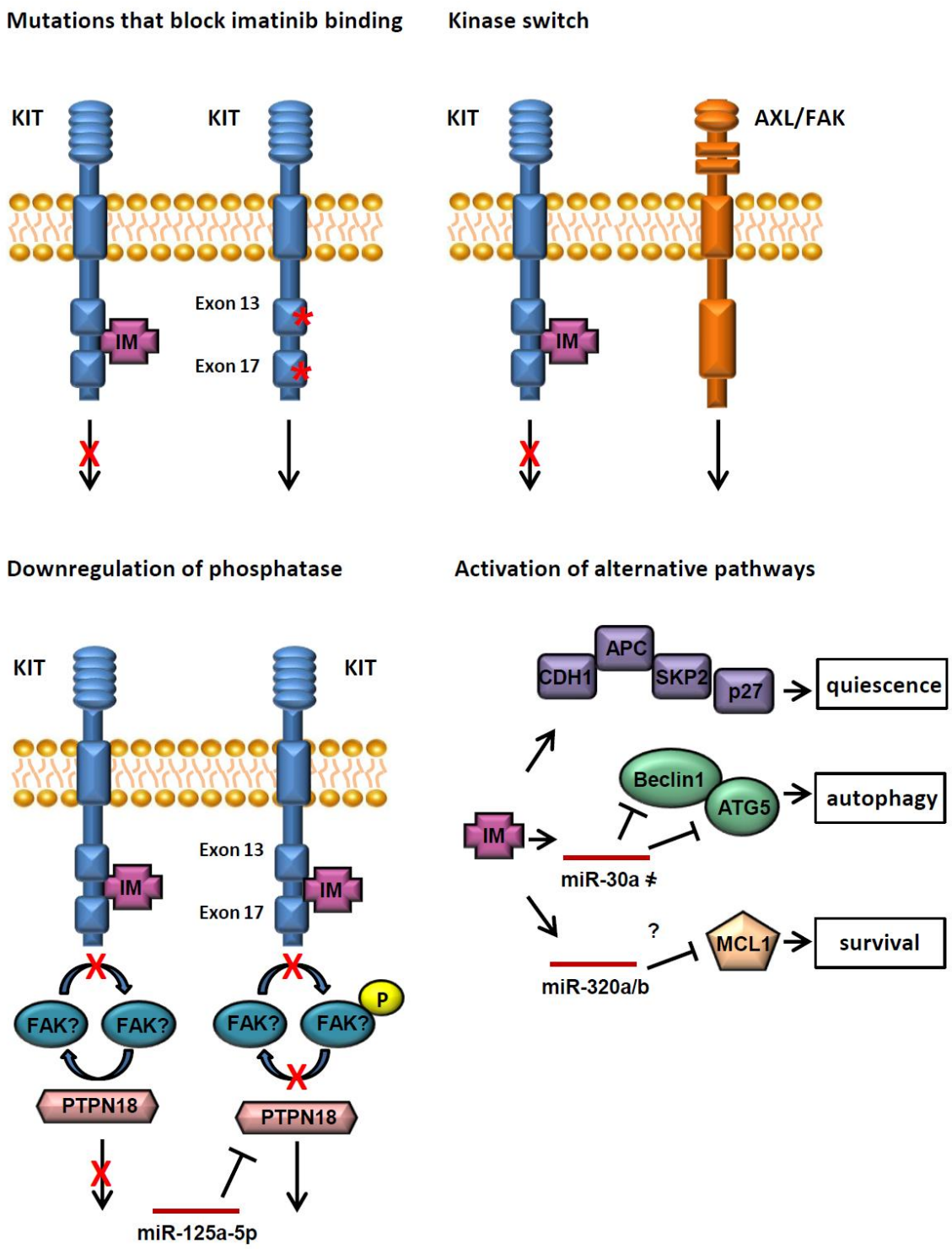

Figure 6. Mechanisms of acquired resistance to imatinib in GIST. Secondary mutations in exons 13 and 17 block imatinib binding and activity (upper left). GIST cell lines that acquire imatinib resistance activate AXL (Mahadevan *et al*, 2007) or FAK (Takahashi *et al*, 2013) instead of KIT (upper right). GIST cells downregulate phosphatase PTPN18 (by *miR-125a-5p* overexpression) (**Paper I**) that dephosphorylate either the substrates of KIT, or other kinases such as FAK (**Paper II**) (lower left). Imatinib induces pathways that protect tumor cells from cell death including quiescence (Liu *et al*, 2008), autophagy (Gupta *et al*, 2010) and pro-survival (**Paper III**) (lower right). IM: Imatinib, P: Phosphate group, ≠ Shown in CML.

It is noted that most of the imatinib-resistant tumors exhibit extensive intra- and inter-tumor heterogeneity (Liegl *et al*, 2008; Loughrey *et al*, 2006; Wardelmann *et al*, 2006). As an illustration, up to five different types of secondary mutations had been observed in different areas of the same tumor, and up to seven different secondary mutations across the multiple tumors of the same patient (Liegl *et al*, 2008). This heterogeneity has important implications in regard to the efficacy of second-line TKI therapy after the first-line imatinib treatment, because the diverse resistant subclones render the complete eradication of GIST cells by any particular TKI.

Several alternative mechanisms of imatinib resistance have been described. Kinase switching is one of them and number of kinases have been involved in such mechanism. The first one is AXL, which is an oncogenic RTK that regulates the same downstream signaling pathways as KIT. GIST cells switch from KIT to AXL during acquisition of imatinib resistance *in vitro* and *in vivo* (Mahadevan *et al*, 2007). In addition, another study reported a switch from KIT to FAK and FYN activation in GIST cells upon acquisition of imatinib resistance, and pFAK inhibition can re-sensitize the resistant cells to imatinib-induced cell death (Takahashi *et al*, 2013). FAK has also been implicated in growth and survival of imatinib-resistant GIST cells, and FAK inhibition induces apoptosis in GIST cells and decreases tumor size in mice (Sakurama *et al*, 2009). Concordant with these findings, we also observed increased expression of FAK phosphorylation in imatinib-resistant GIST cell lines and clinical samples (**Paper II**).

miRNA-mediated regulation is known to play a role in resistance mechanism of TKI (such as EGFR inihibitor) (Bryant *et al*, 2012; Garofalo *et al*, 2012; Wang *et al*, 2014; Wang *et al*, 2012; Weiss *et al*, 2008; Zhong *et al*, 2010). The role of miRNAs in imatinib resistance has been demonstrated in CML (Hershkovitz-Rokah *et al*, 2014; Joshi *et al*, 2014; Li *et al*, 2013; Liu *et al*, 2012; Lopotova *et al*, 2011; Shibuta *et al*, 2013; Venturini *et al*, 2007; Xu *et al*, 2014; Zimmerman *et al*, 2010) (see section *1.2.4.1*) and is now described in GIST in this thesis work (**Papers I-III**).

Mechanisms of secondary resistance to imatinib in GIST are summarized in Figure 6.

1.1.4.3 Strategies to overcome the resistance

In clinical guidelines, for GIST patients who progress on the standard dose of imatinib, it is recommended to increase the dose prior to change of therapy. As described above, resistance results from *KIT* exon 9 mutations or secondary mutations causing inefficient imatinib binding might be overcome by dose escalation. Even though the median time to progression after the dose escalation is only five months, 33% of the patients achieve a stable disease up to one year or more (Blanke *et al*, 2008b).

1.1.4.3.1 Alternative TKIs

Several other tyrosine kinase inhibitors can be the alternatives for those patients who do not respond to imatinib dose escalation or progress after an initial response (Figure 7). Most of

these agents also target vascular endothelial growth factor receptors (VEGFR), giving them a potential advantage to decrease the progression by inhibition of angiogenesis (Demetri, 2011).

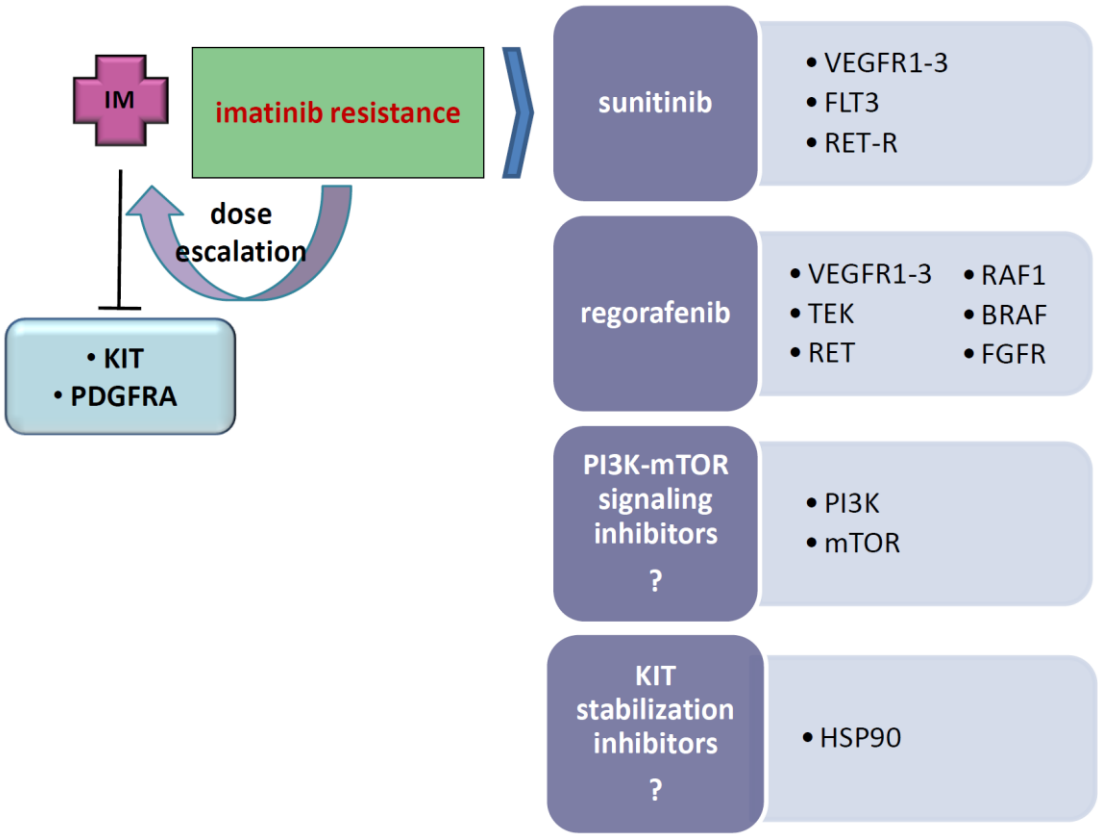

Figure 7. Strategies to overcome imatinib resistance in GIST. The dose of imatinib is increased prior to change of therapy. Sunitinib and regorafenib are current clinically approved drugs for imatinib-resistant GISTs. Clinical trials are ongoing for inhibitors of PI3K-mTOR pathway and KIT stabilizer protein HSP90. IM: Imatinib.

Sunitinib is the first FDA approved TKI inhibitor for the treatment of advanced GISTs which are not non-responsive to imatinib. It has more inhibition activity against the wild-type KIT than imatinib, and it also inhibits other targets, including VEGFR1-3, FLT3 and RET (Abrams *et al*, 2003; Broutin *et al*, 2011; Mendel *et al*, 2003; O'Farrell *et al*, 2003). However, its activity against secondary kinase mutations is not optimal. A phase III placebo control trial study with imatinib-resistant or intolerant patients reported that sunitinib prolonged the progression-free survival only 18 weeks compared to placebo (Demetri *et al*, 2006). As demonstrated by *in vitro* studies, although mutations in the ATP-binding pocket are very sensitive to sunitinib, mutations in the activation loop are strongly resistant (Heinrich *et al*, 2008a). Due to the equality in frequency of these secondary mutations within a lesion or in a patient, mixed responses are commonly observed when using sunitinib for imatinib-resistant GISTs. Similar to imatinib, sunitinib can only bind and inhibit the inactive form of KIT.

Regorafenib is the third approved therapeutic agent for GIST patients who no longer respond to imatinib and sunitinib treatments. Besides to KIT and PDGFRA, this TKI also inhibits VEGFR1–3, TEK, RET, RAF1, BRAF, and $BRAF^{V600E}$ and FGFR (Wilhelm *et al*, 2011). Similar to sunitinib, regorafenib delayed the progression of patients only 3.9 months compared to the placebo treatment (Demetri *et al*, 2013).

Accumulating evidence show that resistance to ATP-competitive inhibitors is inevitable even with newer drugs such as regorafenib, and alternative treatment strategies based on non-ATP mimetic kinase inhibitors (switch pocket kinase inhibitors, such as DP-2676) are suggested as a novel strategy for kinase inhibition (Eide *et al*, 2011; Heinrich *et al*, 2010). These inhibitors suppress the conformational switch to the active form of KIT.

1.1.4.3.2 Other agents

PI3K-mTOR signaling pathway is known as one of the crucial pathways for survival of GIST cells (Bauer *et al*, 2007), and agents targeting the components of this pathway (*e.g.* everolimus against mTOR; BYL719 and BKM120 against PI3K) are in clinical trials (clinicaltrials.gov). Using these agents in combination with KIT inhibitors might provide a successful treatment strategy.

Inhibition of HSP90 has been proven to have a dramatic inhibitory activity against KIT-positive imatinib-resistant GIST cell lines, but it was not effective to inhibit wild-type KIT (Bauer *et al*, 2006). Clinical trials are ongoing to test these inhibitors (AT-13387, AUY922) on TKI-resistant GISTs (Corless *et al*, 2011).

Combinational treatment of cancer stem cell targeting drugs together with imatinib has been proposed to eradicate the persistent GIST stem populations in tumor bulk. Salinomycin, a breast cancer stem cell inhibitor, was shown to inhibit the growth of ICC stem cells, especially with a greater degree when used together with imatinib (Bardsley *et al*, 2010).

1.2 MicroRNAs

The explanation of the genetic information flow within a biological system, *i.e.* central dogma of molecular biology, was first described by Francis Crick in 1956. It stated that genetic information is transcribed from DNA to RNA, and subsequently translated into proteins. RNA is a mediator for transmitting the message and proteins are the key regulators of the biological functions (Crick, 1970). In 2000s, development of genome-wide transcriptome technologies revealed that ~80% of the human genome is transcribed into RNAs, but only a small fraction (<2%) of the transcripts encode proteins (IHGSC, 2004). These findings opened a new era of "non-coding RNAs", which are now known as important regulatory elements of gene regulation, cellular functions and disease development.

Non-coding RNAs are classified into two major classes: (i) small non-coding RNAs (~18-200 nt) that include microRNA (miRNA), small interfering RNA (siRNA) and PIWI-interacting RNA (piRNA); and (ii) long non-coding RNAs (>200 nt), such as pseudogenes, H19, XIST and AIR (Grosshans & Filipowicz, 2008). miRNAs constitute the most abundant class of small non-coding RNAs in human and are extensively studied. The expression and function of miRNAs have been related to many cellular functions, and their deregulation is often associated with human diseases including cancer.

1.2.1 Discovery

In 1993, a gene called *lin-4* was discovered to regulate developmental timing in *C. elegans*, which does not encode a protein but produces a pair of small RNAs with sizes 22 and 61 nt (Lee *et al*, 1993; Wightman *et al*, 1993). These small RNAs have antisense complimentary to multiple sites in the 3`UTR of the *lin-14* gene and regulate *lin-14* translation (Lee *et al*, 1993; Wightman *et al*, 1993).

Lin-4 remained to be a unique regulator until the discovery of *let-7* in 2000. *Let-7* was also discovered in *C. elegans* as a regulator of transition from late-larval to adult cell fate developmental stages (Reinhart *et al*, 2000; Slack *et al*, 2000). Unlike *lin-4*, *let-7* homologs were soon identified in human, Drosophila and 11 other animals (Pasquinelli *et al*, 2000). This finding suggested the existence of a class of small RNAs involved in gene regulation, and many scientists began to hunt for these tiny RNAs in different multicellular genomes. In 2001, three independent studies identified an extensive class of endogenous small RNAs (~22 nt) in human, flies and worms (Lagos-Quintana *et al*, 2001; Lau *et al*, 2001; Lee & Ambros, 2001). They termed these small RNAs as "miRNAs". To date, more than 2500 mature miRNAs were annotated in the human genome (miRBase release 21, June 2014), and the functional roles of most of them have yet to be determined.

1.2.2 Biogenesis and function

An illustration of miRNA biogenesis and function is shown in Figure 8. The details are briefly mentioned below:

Transcription

miRNAs are generally transcribed by RNA polymerase II into long primary transcripts with a hairpin structure called primary miRNAs (pri-miRNA) (Lee *et al*, 2004). Majority of human miRNAs are encoded by introns of coding or non-coding genes, while some miRNAs are encoded by exonic regions. The miRNAs located in the introns of protein-coding genes are generally transcribed together with the host gene by the same promoter (Bartel, 2004). However, miRNA genes commonly have multiple transcription start sites, and some of the intronic miRNAs can have independent promoters from their host genes (Monteys *et al*,

2010; Ozsolak *et al*, 2008). Around 40% of human miRNAs are located in clusters and co-transcribed from a poly-cistronic transcription unit (Altuvia *et al*, 2005). Following the transcription, pri-miRNA undergoes nuclear and cytoplasmic processing steps, as described below (Lee *et al*, 2003).

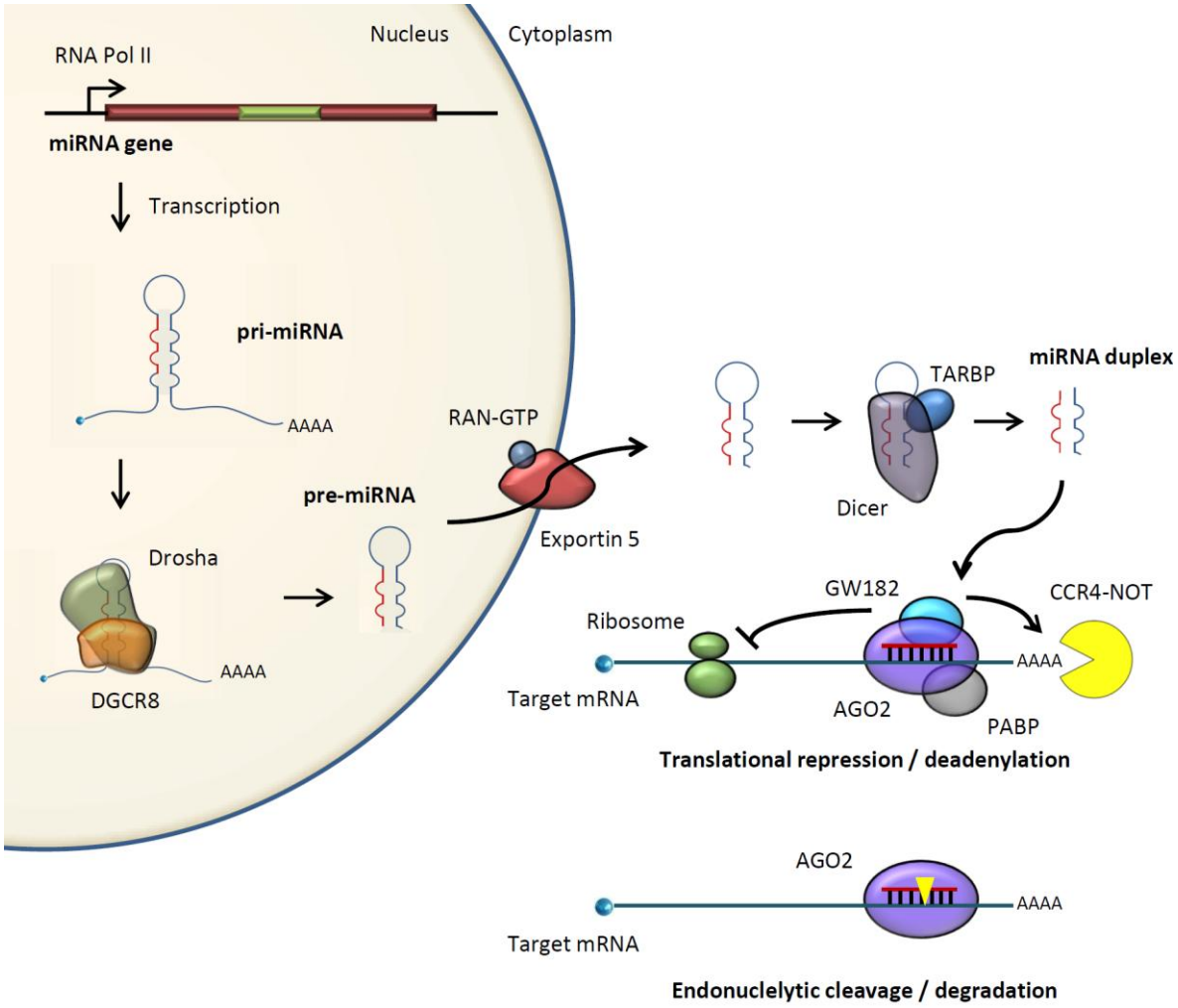

Figure 8. Canonical pathway of miRNA biogenesis and function.

Nuclear processing

Pri-miRNA is typically over 1-kb long, consisting of a stem (33-35 bp), a terminal loop and single-stranded RNA sequences at both 3` and 5` ends. In nucleus, pri-miRNA is cleaved by Drosha together with its co-factor DGCR8 (DiGeorge Syndrome critical region 8) (Lee *et al*, 2003). Drosha cleaves the stem at ~11 bp away from the basal junction (the junction between single and double stranded RNAs) and ~22 bp away from the apical junction linked to the terminal loop (Han *et al*, 2006), which yields a small hairpin of ~65 nt long with a 2-nt 3`overhang called precursor miRNA (pre-miRNA) (Lee *et al*, 2003). Following Drosha processing, pre-miRNA is exported from nucleus to cytoplasm by Exportin 5 (EXP5)

together with its co-factor GTP-binding nuclear protein (RAN-GTP) (Bohnsack *et al*, 2004; ESMO, 2014).

Cytoplasmic processing

Upon the export from nucleus to cytoplasm, Dicer continues to the maturation process with its co-factors TARBP2 (TAR RNA-binding protein 2) and PACT (protein activator of PKR) (Chendrimada *et al*, 2005; Hutvagner *et al*, 2001; Lee *et al*, 2006). Dicer binds to pre-miRNA with a preference for 2-nt 3`overhang generated earlier by Drosha (Zhang *et al*, 2004), and cleaves the pre-miRNA at a distance of 22 nt away from the 5`end of the double-stranded RNA to remove the loop of pre-miRNA and yield a miRNA duplex of ~22 bp (Macrae *et al*, 2006; Park *et al*, 2011).

miRNA-induced silencing complex (miRISC)

The miRNA duplex generated by Dicer is subsequently loaded into the AGO2 (Argonaute 2) containing complex called miRISC (Hammond *et al*, 2001; Mourelatos *et al*, 2002). Following the loading, one of the strands (*i.e.* passenger strand) is quickly degraded or discarded, while the other strand called guide strand is used for silencing activity. Selection of guide strand is determined on the basis of thermodynamic stability of the two ends of the duplex, in which relatively less stable strand at the 5`side is typically selected as guide strand (Khvorova *et al*, 2003; Schwarz *et al*, 2003). In addition, presence of U at nucleotide position 1 is favored by AGO protein for guide strand selection (Hu *et al*, 2009). Notably, even though passenger strands are less abundant than the guide strands, they can still be loaded into miRISC and active in silencing (Okamura *et al*, 2008).

Mature miRNA functions as a guide by base pairing with sequences in the 3`UTR of the target mRNA (Bartel, 2009), while the AGO2 complex recruits the necessary factors that induce translational repression, mRNA deadenylation and/or mRNA decay (Huntzinger & Izaurralde, 2011). For miRNA target recognition, it generally requires perfect base pairing at positions 2 to 7 in the 5` end of miRNA (known as "seed region"). In addition to seed region, the downstream nucleotides at positions 8 and 13-16 can also contribute for base pairing with the targets (Bartel, 2009). Given the relatively short sequence for recognition, each miRNA can have multiple targets, and each mRNA can have target sites for multiple miRNAs.

The degree of miRNA-mRNA complementary determines the subsequent silencing mechanism. Perfect base-pairing leads to cleavage by AGO followed by degradation of mRNA target (Yekta *et al*, 2004). This mechanism is rare in animals, but common in plants. Imperfect base-pairing leads to translational repression or mRNA deadenylation. Translational repression mechanism involves the disruption of the translation initiation complex assembly when miRISC recruits CCR-NOT to dissociate PABP from mRNA poly(A) tail (Zekri et al., 2013), or interruption of the translation elongation when miRISC promotes drop-off or proteolysis of the nascent peptide (Petersen et al., 2006; Nottrott et al., 2006). Deadenylation is initiated by GW182 and PABP, which recruit CCR4-NOT

deadenylation complex that removes the poly(A) tail of mRNA and causes destabilization of the target (Behm-Ansmant *et al*, 2006).

1.2.3 MicroRNAs and cancer

The first evidence of miRNA involvement in cancer was reported by Calin and colleagues in 2002 (Calin *et al*, 2002), in which they found two miRNA genes, *mir-15a* and *mir-16-1*, located at a region on chromosome 13q14, were deleted in more than half of B-cell chronic lymphocytic leukemias (B-CLL). Since this discovery, many scientists began to investigate the role of miRNAs in cancer development and progression. To date, numerous evidence support that miRNAs are involved in all hallmarks of cancer defined by Hanahan and Weinberg (Hanahan & Weinberg, 2011). Importantly, cancers have miRNA expression signatures that can distinguish them from normal tissues and among cancer types (Calin & Croce, 2006; Lu *et al*, 2005). Most of the cancers can be further subclassified according to their clinical and molecular characteristics based on their miRNA expression profiles (Dvinge *et al*, 2013; Kim *et al*, 2011a), suggesting the potential diagnostic and prognostic implications of miRNAs in cancer.

The mechanisms of miRNA deregulation are similar to other cancer-associated genes, such as genomic abnormalities (*e.g.* chromosomal amplification/deletion) (Calin *et al*, 2002), transcription factor activation (Hermeking, 2012; Jin *et al*, 2013), and epigenetic changes (*e.g.* promoter methylation and histone modifications) (Langevin *et al*, 2011; Vrba *et al*, 2013). In addition to these, there are miRNA regulation-specific mechanisms, such as single nucleotide polymorphisms (SNPs) or mutations on either miRNA or target 3`UTR sequences (Sun *et al*, 2009; Ziebarth *et al*, 2012), defects in miRNA biogenesis machinery (Hill *et al*, 2009; Kumar *et al*, 2007; Melo *et al*, 2009) and post-transcriptional editing catalyzed by ADARs (adenosine deaminases that act on RNA) (Choudhury *et al*, 2012).

miRNAs can function as oncogenes or tumor suppressor genes. However, some miRNAs can have dual function as an oncogene or tumor suppressor depending on cellular context. *miR-125* family is an example of such dual function, which is discussed in detail in **Paper I**.

1.2.3.1 MicroRNAs in gastrointestinal stromal tumor

miRNA signature of GISTs was first described by Subramanian and colleagues in 2008 (Subramanian *et al*, 2008). The study compared miRNA profiles in 27 types of sarcomas, and demonstrated that GISTs were clearly distinguished from other sarcomas based on their miRNA expressions. Shortly after, two groups characterized miRNA expression profiles of GISTs in relation to clinical and molecular features (Choi *et al*, 2010; Haller *et al*, 2010). They identified specific miRNA expression signatures associated with anatomic site, *KIT* or *PDGFRA* mutation, tumor risk and chromosome 14q loss. Both studies reported downregulation of multiple miRNA clusters (located at chromosome 14q) in GISTs. A miRNA located in this region, *i.e. miR-494*, was shown to regulate *KIT* expression and

downstream signaling, cell growth and apoptosis in GIST (Kim *et al*, 2011b). In addition to *miR-494*, several other miRNAs have been identified as regulators of *KIT*. *miR-221/222* and *miR-17-92* cluster members (*miR-17, miR-20a* and *miR-222)* were found downregulated in GISTs (Gits *et al*, 2013; Koelz *et al*, 2011). Furthermore, these miRNAs target *KIT* and *ETV1*, and reduce cell proliferation and induce apoptosis in GIST cells (Gits *et al*, 2013). Recently, *miR-218* was also found downregulated in GISTs, and it regulates *KIT*, suppresses proliferation and invasion, and induces apoptosis *in vitro* (Fan *et al*, 2014b).

Several miRNAs have been described in GIST progression. For examples, *miR-196a* overexpression is associated with high-risk, metastasis and poor survival in GISTs, and it regulates cell invasion in GIST cells (Niinuma *et al*, 2012). *miR-133b* is downregulated in high-grade GISTs and associated with *FSCN1* overexpression (Yamamoto *et al*, 2013). Down regulation of *miR-137* was reported to induce epithelial to mesenchymal transition (EMT) through regulation of *TWIST1* in GIST cells (Liu *et al*, 2014). A summary of miRNAs and their functional roles in GIST tumorigenesis are listed in Table 1.

Table 1. Functionally characterized microRNAs in GIST

miRNA	Location	Target	Functional Relevance	Reference
miR-494	14q32.31	*KIT*	Cell growth, apoptosis	Kim *et al*, 2011b
miR-221/222	Xp11.3	*KIT* and *ETV1*	Cell proliferation, apoptosis	Gits *et al*, 2013
miR-17-92	13q31.3	*KIT* and *ETV1*	Cell proliferation, apoptosis	Gits *et al*, 2013
miR-218	4p.15.31	*KIT*	Imatinib resistance	Fan *et al*, 2014a
			Cell proliferation, invasion, apoptosis	Fan *et al*, 2014b
miR-137	1p21.3	*TWIST1*	EMT	Liu *et al*, 2014
miR-125a-5p	19q13.41	*PTPN18*	Imatinib resistance	Akçakaya *et al*, 2014

EMT: Epithelial to mesenchymal transition.

1.2.4 MicroRNAs and targeted therapy resistance

Targeted therapies, include tyrosine kinase inhibitors and monoclonal antibodies, are widely used to treat cancers that are dependent on specific target proteins whose constitutive activity is crucial for survival of the tumor cells (also known as oncogene addiction). Despite the remarkable success of targeted therapy, development of acquired resistance is common and remains a major challenge in the clinic.

The most common mechanisms of targeted therapy resistance include: non-responsive primary target mutations, secondary mutations in the target gene, activations of downstream signal transduction pathways and alternative pathways. In addition to these mechanisms, miRNA-mediated regulation of target genes involved in resistance pathways has recently emerged as a resistance mechanism to targeted therapy in both hematological and solid malignancies.

The best example is the EGFR-TKI (such as gefitinib and erlotinib) resistance in lung cancer. Weiss and colleagues reported that *miR-128b* directly regulates EGFR in lung cancer cells, and loss of *miR-128b* correlates with a better gefitinib response and longer patient survival in lung cancer (Weiss *et al*, 2008). Several miRNAs were found to regulate ERK (*let-7*, *miR-126*, *miR-145*) and PTEN/AKT (*miR-214*) signaling pathways, and modulate gefitinib resistance *in vitro* (Wang et al, 2012; Zhong et al, 2010). A signature of 13 miRNAs can predict erlotinib response in lung tumors and cell lines, and one of these miRNAs (*miR-200c*) functionally regulates EMT and erlotinib sensitivity (Bryant *et al*, 2012). Furthermore, several tyrosine kinase receptors, such as EGF, MET and AXL, can alter specific miRNA expressions for regulating gefitinib-induced apoptosis and EMT *in vitro* and *in vivo* (Garofalo et al, 2012; Wang et al, 2014).

1.2.4.1 MicroRNAs in imatinib resistance

The involvement of miRNAs in imatinib resistance is well characterized in CML. Several findings that support the role of miRNAs in imatinib resistance in CML are briefly described below and summarized in Table 2.

Table 2. MicroRNAs involved in imatinib response of CML and GIST

miRNA	Location	Target	Regulated by	Cancer	Reference
miR-17~92	13q31.3		BCR-ABL, MYC	CML	Venturini *et al*, 2007
miR-144~451	17q11.2		MYC	CML	Liu *et al*, 2012
miR-181	9q33.3	*MCL1*	LYN	CML	Zimmerman *et al*, 2010
	1q32.1				
miR-199b	9q34.11		ABL	CML	Joshi *et al*, 2014
miR-203	14q32.33	*BCR-ABL*		CML	Li *et al*, 2013
miR-30e	1p34.2	*BCR-ABL*		CML	Hershkovitz-Rokah *et al*, 2014
miR-138	3p21.32	*BCR-ABL*		CML	Xu *et al*, 2014
	16q13				
miR-203	14q32.33	*BCR-ABL*		CML	Shibuta *et al*, 2013
miR-451	17q11.2	*BCR-ABL?*	BCR-ABL	CML	Lopotova *et al*, 2011
miR-30a	6q13	*Beclin1, ATG5*		CML	Yu *et al*, 2012
miR-218	4p.15.31	*KIT*		GIST	Fan *et al*, 2014a,b
miR-125a-5p	19q13.41	*PTPN18*		GIST	Paper I
miR-320a/b	8p21.3	*MCL1*		GIST	Paper III

miR-17~92 expression is downregulated by imatinib treatment, and overexpression of these miRNAs enhance imatinib sensitivity in CML cells (Venturini *et al*, 2007). Similarly, expression of *miR-144~451* cluster was found repressed by MYC upon acquiring imatinib resistance in CML cells, and their restoration reversed the resistant phenotype (Liu *et al*, 2012). Some miRNAs directly regulate *BCR-ABL* expression and modulate imatinib

response. For examples, *miR-203*, *miR-30e* and *miR-138* regulate *ABL* and *BCR-ABL* expression, and imatinib sensitivity in CML cells (Hershkovitz-Rokah *et al*, 2014; Li *et al*, 2013; Xu *et al*, 2014). Autophagy is commonly involved in TKI resistance. Interestingly, *miR-30a* has been shown to suppress autophagy by targeting *Beclin1* and *ATG5*, and its overexpression enhances imatinib sensitivity in CML cells (Yu *et al*, 2012).

miRNA-mediated regulation of imatinib resistance in GIST has not been well studied as in CML. A recent report by Fan and colleagues showed that modulation of *miR-218* affects imatinib sensitivity in GIST cell lines through regulation of PI3K/AKT pathway (Fan *et al*, 2014a). In this thesis work, we identified two miRNA networks involved in GIST cells viability and resistance upon imatinib treatment. Most importantly, we provide functional evidence that *miR-125a-5p* regulates imatinib response through PTPN18-pFAK axis. Discussion of these findings is given in **Papers I-III**.

1.2.5 Diagnostic, prognostic and therapeutic values of microRNAs

As previously described, miRNA expression profiles can distinguish cancers from normal tissues and among cancer types (*e.g.* GISTs from other sarcomas (Subramanian *et al*, 2008)), and distinct miRNA expression signatures are associated with clinical and molecular subclasses [*e.g.* imatinib resistance, anatomic site, tumor risk in GIST (Akcakaya *et al*, 2014; Choi *et al*, 2010; Haller *et al*, 2010)]. These findings suggest a promising role for miRNAs as diagnostic and prognostic indicators. Given their longer stability in clinical samples and robust expression patterns, miRNAs have been suggested to have a greater utility as biomarkers in comparison to mRNAs (Lu *et al*, 2005).

Importantly, cancer cells can release their miRNAs into the body liquids through microvesicles, which gives them a potential value as non-invasive biomarkers (Mitchell *et al*, 2008; Schwarzenbach *et al*, 2014). As an example, a recent report using 391 lung cancer patients identified serum miRNAs, which are predictive of survival for patients with advanced disease in lung cancer (Wang *et al*, 2013). However, there are some obstacles for circulating miRNAs, *e.g.* choosing an appropriate endogenous control and miRNA expression fluctuations caused by diet, infection, treatment, trauma or other factors (Jarry *et al*, 2014). Many investigations are ongoing to determine reliable miRNAs as biomarkers. Future studies evaluating the potential of circulating miRNAs as response markers for treatment would have a clinical benefit.

miRNA mimics and inhibitors are currently under investigation for their potential as therapeutic agents. miRNAs may be used directly to target tumor cells and their microenvironment, or to enhance the effect of other therapies for the purpose of overcoming resistance. Off-target effects and delivery of these molecules to specific tissues/cell types remain the biggest challenges. Several strategies have been developed for delivery, including the use of nanoparticles, liposomes, antibodies and nucleic acid structure modifications (Li & Rana, 2014).

The best example for miRNA-based therapies is the use of anti-miR-122 molecule with a locked nucleic acid modification (known as Miravirsen) for treatment of hepatitis C (Janssen *et al*, 2013). Besides mature *miR-122*, Miravirsen can also bind to *pri-* and *pre-miR-122* and inhibit its biogenesis, thus increases its effectiveness to block endogenous *miR-122* (Gebert *et al*, 2014). *miR-122* is highly abundant in the liver and it is required for hepatitis C virus (HCV) replication and HCV RNA stabilization (Jopling *et al*, 2005). Inhibition of *miR-122* by Miravirsen treatment reduces HCV RNA and viremia levels.

Another example is the use of synthetic *miR-34a* mimic loaded into liposomal nanoparticles (MRX34) for treatment of liver cancer (Bouchie, 2013). *miR-34* is a tumor suppressor, which directly regulates at least 24 known oncogenes. The investigators of this study stated that by using MRX34 they hope to overcome potential therapy resistance from the beginning by "attacking more pathways all at once".

Next question arise, what if tumor cells develop resistance also against the miRNA-based therapeutics? Future resistance problems for miRNA therapeutics may be overcome by use of agents that have a potential to target whole miRNA families instead of a sole miRNA (Obad *et al*, 2011). However, careful evaluation of potential side effects is warranted. The research on miRNA-based therapies is newly emerging. Ongoing and future studies will illuminate their effectiveness and safeness as novel agents for cancer treatment.

2 AIMS OF THE THESIS

The overall aim of this thesis was to investigate the molecular mechanisms of imatinib resistance in GIST. The specific aims were to:

- Analyze miRNA expression profiles in GISTs in relation to imatinib response, clinical features and *KIT* mutation status, and to evaluate the functional roles of *miR-125a-5p* and its potential targets in imatinib resistance (**Paper I**).
- Evaluate FAK as a candidate downstream target of PTPN18 and its role in imatinib resistance of GIST (**Paper II**).
- Identify miRNAs and their targets involved in imatinib-induced cell death of GIST (**Paper III**).
- Investigate the functional role of DOG1 in imatinib-sensitive and -resistant GIST cell lines (**Paper IV**).

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Clinical samples

In total, 63 snap-frozen GISTs were included in this thesis. The samples consisted of 30 tumors from 24 GIST patients who had received neoadjuvant imatinib treatment **(Papers I-III)**, and 33 tumors from 32 GIST patients who had not received imatinib treatment prior to operation **(Paper III)**. Among the imatinib-treated sample cohort, 14 tumors were imatinib resistant, whereas 16 tumors were imatinib sensitive. The clinical, histopathological and follow-up details of the cases are detailed in **Paper III**.

GIST6 had two tumors: GIST6a was treated with imatinib neoadjuvantly and GIST6b was not treated prior to operation. GIST9 and GIST10 had four tumors each, three of the tumors in each patient showed progressive growth (imatinib resistant) and one tumor in each patient (GIST9-3 and GIST10-3) partially responded to the treatment (imatinib sensitive). GIST25 had two recurrent tumors collected at different times, and none of them treated prior to operation.

Of the 56 patients, 26 developed tumor metastasis and the remaining 30 patients had no recurrence or metastasis during the follow-up. Thirty-nine of the tumors harbored a single non-synonymous mutation in *KIT* and 14 tumors had double *KIT* mutations. The GIST15 tumor harboring a silent mutation in *KIT* (P585P) was considered as wild-type. Five tumors had a single mutation in *PDGFRA*, whereas four were wild-type for both *KIT* and *PDGFRA*.

GISTs were diagnosed based on the routine histopathological examination and positive immunoreactivity for CD117 (KIT). Imatinib response was determined by fluorodeoxyglucose positron emission tomography and/or contrast-enhanced computed tomography based on tumor size, characteristics and metastasis.

All samples were obtained with informed consent, and Karolinska University Hospital ethical committee approved the study of the human tissue materials. The follow-up of the patients was until June 2014 or the time of death.

3.1.2 Cell lines

3.1.2.1 Established GIST cell lines

Two established human GIST cell lines were used in this thesis: Imatinib-sensitive GIST882 harboring a homozygous missense mutation in *KIT* exon 13 (K642E) **(Papers I-IV)**; and imatinib-resistant GIST48 harboring a primary homozygous *KIT* exon 11 missense mutation (V560D) and a secondary heterozygous *KIT* exon 17 (D820A) mutation **(Papers I and IV)**. The cell lines were kindly provided by Dr. Jonathan Fletcher (Brigham and Women's

Hospital, Boston, MA, USA). The authenticity of the cell lines was confirmed by short tandem repeat (STR) genotyping, as described in **Paper I**.

3.1.2.2 Imatinib resistant subclones of GIST882

Imatinib resistant subclones (GIST882R) were generated from GIST882 cell line and used in **Papers I-III**. The GIST882 cells were exposed to 1 μM of imatinib for 7 days, leading to cell death of 60–70% of cells. The remaining cells were harvested and continuously grown in growth media containing 1 μM of imatinib for more than one month prior to further experiments. Morphologies of imatinib-resistant subline GIST882R and its parental cell line GIST882 are shown in Figure 9.

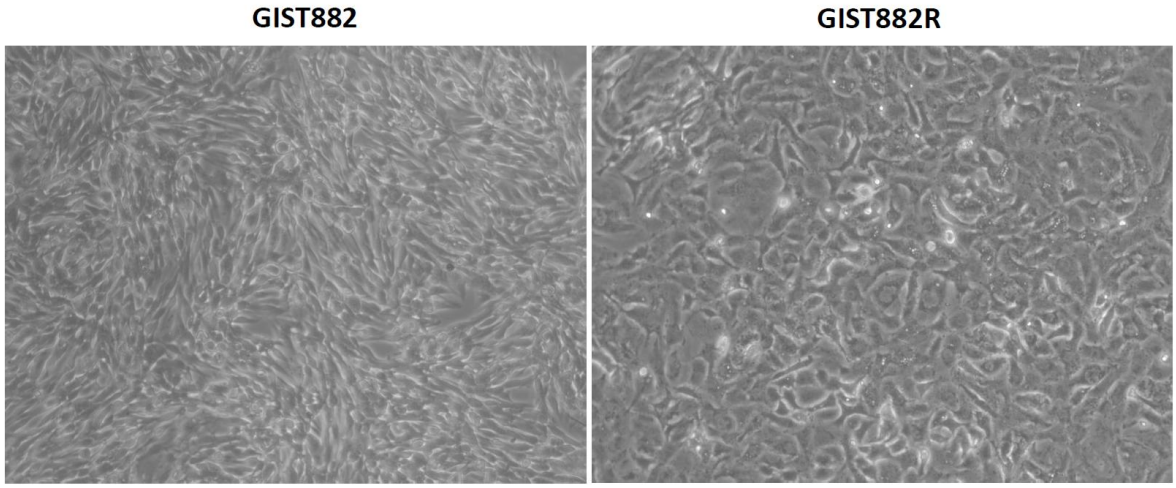

Figure 9. Morphologies of GIST882 and its imatinib-resistant subline GIST882R.

3.1.3 Pharmaceutical agents

3.1.3.1 Imatinib

Imatinib mesylate (also known as Gleevec, Glivec or STI-571, $C_{30}H_{35}N_7SO_4$) is a small molecule compound that binds to an intracellular pocket of the tyrosine kinases and blocks ATP binding, thereby preventing activation of the kinase signaling pathways. The inhibitor was kindly provided by Novartis Pharma (Basel, Switzerland). Imatinib was dissolved in sterile distilled water at 10 mM and stored at -20°C.

3.1.3.2 DOG1 activator and inhibitor

In **Paper IV**, N-aroylaminothiazole (Eact) and aminophenylthiazole ($T16A_{inh}$-A01) were used to activate and inhibit DOG1, respectively. Eact has been demonstrated to activate DOG1 and produce sustained DOG1 Cl^- currents independent of intracellular calcium levels (Namkung *et al*, 2011b). $T16A_{inh}$-A01 has been shown to completely block calcium-activated chloride channel current produced by DOG1 in salivary gland cells without interfering with

calcium signaling (Namkung *et al*, 2011a). Both compounds were purchased from Merck Millipore (Billerica, MA, USA).

3.2 METHODS

3.2.1 GIST characterization

3.2.1.1 Genomic DNA Sequencing for Mutation Screening

Sanger sequencing (or chain-termination method) was used for mutation screening of *KIT* and *PDGFRA* in **Papers I-III**. This method uses a single-stranded DNA template, primer, DNA polymerase, deoxyribonucleotide triphosphate (dNTPs) and modified dideoxynucleotides (ddNTPs). ddNTPs lack a 3'-OH group required for the formation of a phosphodiester bond between two nucleotides, causing DNA polymerase to terminate extension of DNA when a modified ddNTP is incorporated. Each of the four dideoxynucleotides (ddATP, ddCTP, ddTTP and ddGTP) is labeled with fluorescent dyes, which emit light at different wavelengths. These labeled DNA sequence fragments are separated by size as they travel through the polymer-filled capillary array. As they reach the detection window, the laser beam excites the dye molecules and causes them to fluoresce. The fluorescence emissions from samples are collected simultaneously and spectrally separated by a spectrograph, detected by a CCD camera, and displayed as chromatograms.

Mutation analyses of 23 samples had been performed in the routine pathology diagnostic laboratory at Karolinska University Hospital, while 41 samples were analyzed in this thesis work (**Papers I** and **III)**. GIST specimens were first analyzed for *KIT* mutations (exons 9, 11, 13 and 17) and the ones that were negative for *KIT* mutations were further analyzed for *PDGFRA* mutations (exons 12 and 18). The relationship between GIST882 and the three generated imatinib-resistant GIST882R subclones were demonstrated by verifying the presence of the K642E mutation in *KIT* exon 13 (**Paper II**).

3.2.1.2 Immunocytochemistry

Immunocytochemistry (ICC) is a technique that is commonly used to detect and localize specific proteins in cells using a specific primary antibody that binds to it. The primary antibody-antigen interaction is detected by use of a secondary antibody conjugated with an enzyme (*e.g*. horseradish peroxidase or alkaline phosphatase) that stained positive cells brown with the substrate 3, 3’-diaminobenzidine (DAB) or with a fluorophore that emits light at a specific wavelength.

ICC was performed for characterization of CD117, DOG1 and CD34 expressions in the three imatinib-resistant GIST882R subclones and their parental cell line in **Paper II**, and CD117 and DOG1 expression in GIST48 and GIST882 cell lines in **Paper IV**.

3.2.2 MicroRNA expression analyses

In this thesis, microarray and reverse-transcription quantitative-PCR (RT-qPCR) methods were used for detection and quantification of miRNA expression levels.

3.2.2.1 Microarray

Global miRNA expression profiling in **Papers I** and **III** were performed using the Agilent human miRNA microarray platform (Agilent, Santa Clara, CA, USA). This platform is a robust hybridization-based high-throughput method without size fractionation or amplification steps, and requires only a small amount of total RNA (≈100 ng). An advantage of this platform is the unique design of the probes, which provides optimal sensitivity and specificity for both sequence and size discrimination that can distinguish between closely-related miRNA family members. Design of the probes is illustrated in Figure 10.

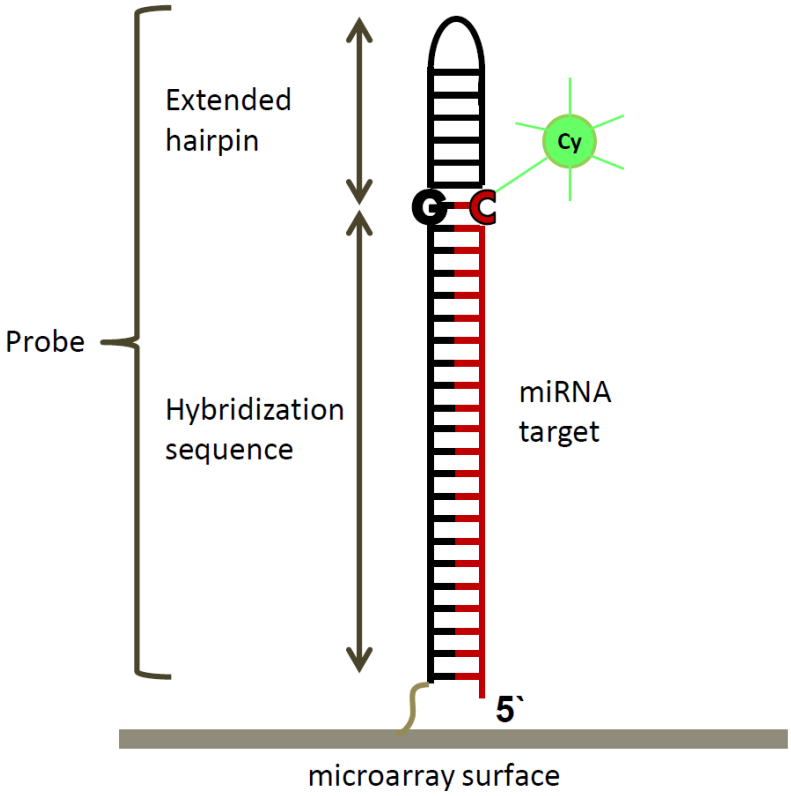

Figure 10. Agilent miRNA microarray probe design. Modified from (Wang *et al*, 2007).

Several important features of this platform are briefly mentioned here. miRNAs are first labeled by T4 RNA ligase at their 3` end with a single fluorophore-labeled cytosine (C) residue (pCp-Cy3). The probes are characterized with a guanosine (G) residue at the 5' end that is complementary to the cytosine (C) added to each miRNA during labeling, which increases the stability of hybridization to a labeled target miRNA. The differential length of the probes is also used for stabilization purpose and normalization of the melting

temperatures to hybridize optimally at 55°C for almost all miRNAs. Another unique design of this platform is the incorporation of 5`end hairpin structure that eliminates the binding of large non-target RNAs (including precursor miRNAs), which increases the target miRNA specificity.

3.2.2.2 Reverse Transcription quantitative-PCR (RT-qPCR)

Although microarray provides a high-throughput screening, it is relatively limited in terms of sensitivity and specificity. RT-qPCR is a quantification method for more accurate and sensitive detection of RNAs through a PCR reaction. The RNA template is first converted into a complementary DNA (cDNA) using a reverse transcriptase enzyme. This first-strand cDNA synthesis reaction can be primed using random primers, oligo(dT), gene-specific primers for mRNAs or stem-loop primers for miRNAs. The cDNA is then used as a template for exponential amplification using PCR. Quantification is done real-time by measuring the amount of amplified product at each PCR cycle. If a particular RNA is abundant in the sample, amplification is observed in earlier cycles; whereas amplification is observed in later cycles if the input RNA is low. Quantitative measurement of the amplified product is obtained using specific fluorescent probes (TaqMan method) or an unspecific fluorescent DNA-binding dye that binds to newly synthesized double strand DNA (SYBR Green method), and a real-time PCR instrument that measures fluorescence signal while performing the PCR reaction.

The TaqMan probes contain a reporter dye (FAM™) linked to the 5`end, a non-fluorescent quencher (NFQ) linked to the 3`end and a minor groove binder (MGB) linked to NFQ at the 3`end. MGB is a modification increases the melting temperature (T_m) without increasing probe length (Afonina *et al*, 1997; Kutyavin *et al*, 1997), which allows the design of shorter probes. MGB probe anneals specifically to a complementary sequence between the forward and reverse primer binding sites. During the amplification, DNA polymerase cleaves the probe, and separates the reporter dye from the quencher, resulting in increased fluorescence by the reporter (Figure 11).

In **Papers I** and **III**, expression levels of miRNAs were quantified using TaqMan RT-qPCR method for validation of miRNA microarray results, and evaluation of relative miRNA levels in the cell lines. In addition, mRNA expression levels of putative miRNA target genes (*PTPN18* and *STARD13*) were quantified in both clinical samples and cell lines using TaqMan RT-qPCR method (**Paper I**).

3.2.3 Short hairpin RNA (shRNA)

RNA interference (RNAi) is a widely used method for silencing gene of interest by introducing a double-stranded RNA that is complementary to the target sequence. This method can be achieved in two ways: chemically synthesized double-stranded small interfering RNAs (siRNAs) and vector-based shRNAs. While both siRNAs and shRNAs

can be used for gene knockdown, there are differences between them. siRNAs are synthetic oligonucleotides that are directly loaded into the RNA-induced silencing complex (RISC) for action. The vector-based shRNAs are synthesized in the nucleus of transfected cells and then converted into siRNA duplexes by Dicer and integrated into RISC. siRNAs provide a fast and efficient silencing of target gene expression, however the effect is short-term. On the other hand, shRNAs enable long-term and stable gene silencing.

In **Papers I and III**, shRNA targeting to human *PTPN18* gene was used for suppressing the expression of *PTPN18* in GIST882 cells.

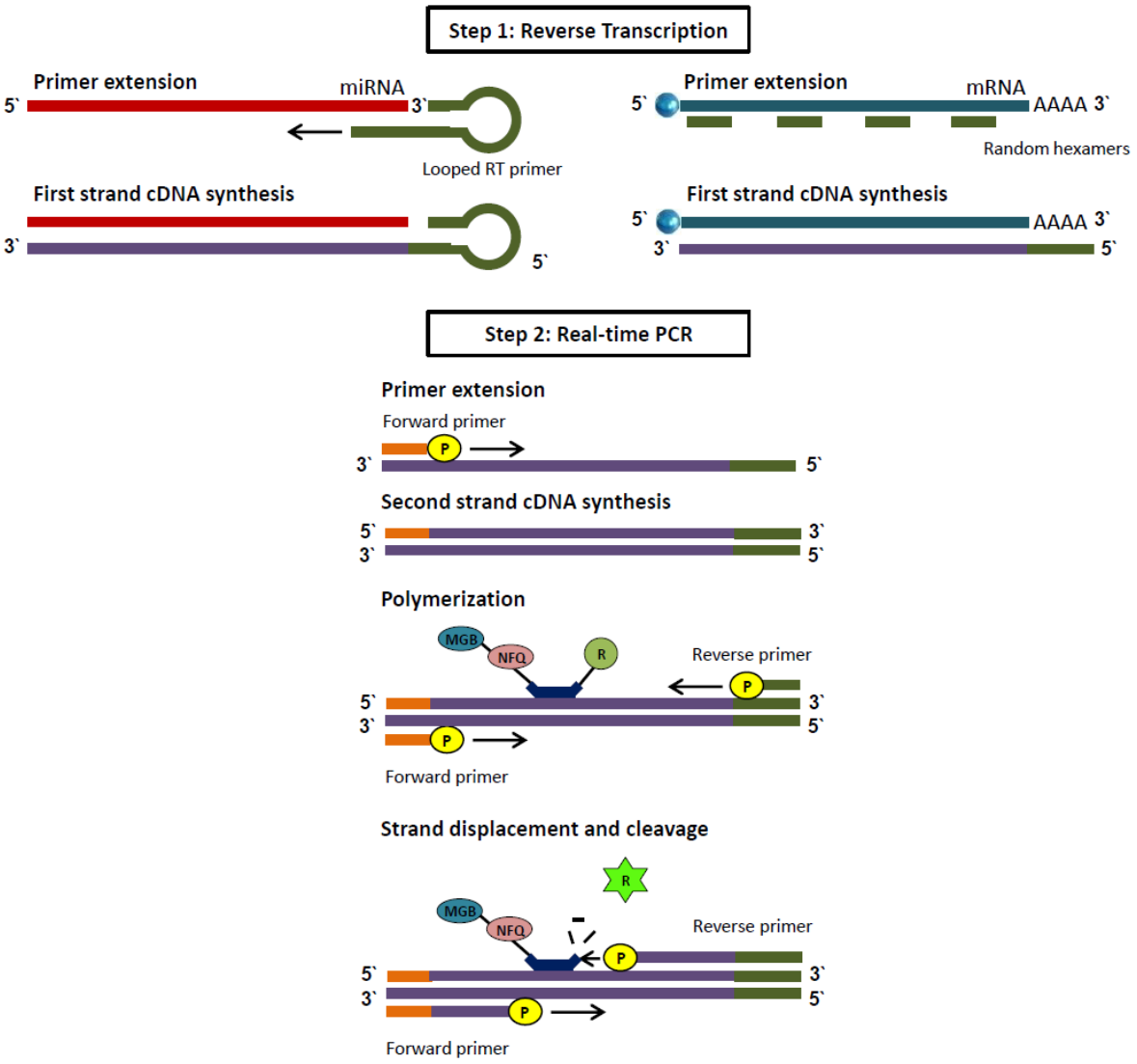

Figure 11. Chemistry of TaqMan RT-qPCR technology.

3.2.4 MicroRNA mimics/ inhibitors

miRNA mimics and inhibitors are commonly used tools for miRNA gain- and loss-of-function experiments, respectively. miRNA mimics are chemically modified double-stranded RNA molecules that mimic endogenous mature miRNAs. The chemical modifications in miRNA mimics are used to ensure that the guide strand is loaded into RISC, but not the passenger strand. miRNA inhibitors are chemically modified single-stranded oligonucleotides that are designed to bind to and inhibit endogenous miRNAs.

miRNA mimic/ inhibitor negative controls are random sequences (*i.e.* non-targeting sequences to human transcripts) that have similar chemical modifications as their miRNA mimics/ inhibitors and no identifiable effects on known miRNA functions.

In **Paper I**, miRNA mimics/ inhibitors (*miR-125a-5p*, *miR-211* and *miR-944*) and their respective negative controls were transfected into GIST882 or GIST48 cells for functional studies and target validations.

3.2.5 Transfection

Transfection is a method of delivering foreign nucleic acids into the cells. For animal cells, it typically involves opening transient pores on the cell membrane to allow the uptake of the nucleic acids. Transfection can be carried out through biological (viral or bacterial), chemical (*e.g.* liposomes, diethylaminoethyl-dextran, calcium-phosphate, nanoparticles) or physical (*e.g.* microinjection, biolistic particle delivery, electroporation) methods.

In this thesis, *miR-125a-5p* mimics/ inhibitors (**Paper I)** and shRNA against *PTPN18* (**Papers I** and **II**) were transfected into GIST882 and GIST48 cell lines using Amaxa Nucleofector™ Technology. This system is an electroporation-based physical transfection method, which provides high efficiency for the difficult-to-transfect cell lines. The DNA is directly transported into the nucleus of the target cell using a high voltage electrical pulse, which induces the formation of temporary membrane pores that enables entry of nucleic acid into the cells. The drawbacks of the conventional electroporation method, such as high cell mortality and low efficacy, are overcome by validated cell-type specific solutions and electrical pulse settings in this method.

3.2.6 Evaluation of cell viability and apoptosis

Four types of cell viability and apoptosis assays were used in this thesis, and they are briefly described below:

3.2.6.1 Trypan Blue Exclusion Assay

Trypan blue ($C_{34}H_{28}N_6O_{14}S_4$) is an organic compound derived from toluidine, and traditionally used to evaluate cell viability. It is a vital stain that is taken up by non-viable cells (that lose their intact and functional membrane) and stains the cells blue. The viable cells remain unstained. This method was used to determine the effect of imatinib on GIST882 cells in **Paper III**.

3.2.6.2 WST-1 Colorimetric Assay

This method is based on measuring the metabolic activity of viable cells. WST-1 is a tetrazolium salt, which is cleaved to soluable formazan by the mitochondrial succinate dehydrogenase enzyme in viable cells. More metabolically active cells will produce more formazan dye. Therefore, the level of formazan dye detected correlates with the number of viable cells in the culture. Other tetrazolium salt, such as MTT, is cleaved to unsoluble formazan crystal that has to be solubilized using DMSO or isopropanol. However, WST-1 yields water-soluble cleavage products that can be directly measured without a solubilization step, which makes it a more preferable method.

This assay was used for determining the effect of imatinib on cell viability upon modulation of *miR-125a-5p* and *PTPN18* expression levels in GIST cell lines (**Paper I)**, and the effect of DOG1 activator or inhibitor on GIST cell viability (**Paper IV)**.

3.2.6.3 Detection of PARP Cleavage by Western blot analysis

Poly (ADP-ribose) polymerase (PARP) is a family of zinc-finger DNA-binding proteins involved in the maintenance of genomic stability and DNA damage-triggered signaling events, such as apoptosis and necrosis. During apoptosis, the full length PARP (116 kDa) is cleaved by caspase-3 or caspase-7 into 89 kDa and 24 kDa fragments and become incapable of responding to DNA damage. Since PARP is one of the downstream targets of caspases, the PARP cleavage has been regarded as an evidence of caspase activation and widely used as a hallmark of cell apoptosis. In **Paper III**, western blot analyses of PARP cleavage products were used to evaluate the effect of imatinib exposure on cell apoptosis in GIST882 cells.

3.2.6.4 Annexin V Affinity Assay

Annexin V is a Ca^{2+}-dependent phospholipid-binding protein that has a high affinity to phosphatidylserine (PS). PS is a membrane phospholipid located in the inner membrane leaflet of viable cells. During apoptosis, the apoptotic cells lose their membrane asymmetry that causes translocation of PS from the inner side of the plasma membrane to the surface. The exposed PS on apoptotic cell surface can be detected by fluorochrome-labeled Annexin V [*e.g.* Phycoerythrin (PE) or fluorescein isothiocyanate (FITC)] using flow cytometry or fluorescence microscopy. This assay is typically performed in combination with a nuclear

dye such as propidium iodide (PI) or 7-aminoactinomycin D (7-AAD) for identification of early and late apoptotic cells.

In **Paper IV**, PE-labeled Annexin V and 7-AAD staining were used to determine the effects of DOG1 activator/ inhibitor on cell apoptosis of GIST cell lines using flow cytometry.

3.2.7 MicroRNA target prediction and validation

3.2.7.1 Computational target prediction

Identification of miRNA targets is crucial for unrevealing the role of miRNAs in regulatory networks governing biological processes. There are generally two approaches to search for miRNA targets: computational and experimental. The experimental approach is laborious and time consuming. Therefore, computational approach is more commonly used for searching candidate miRNA targets and narrowing down the potential miRNA targets for further experimental validation. An extensive number of prediction tools are available, and each of them has different sets of criteria for prediction.

There are five most commonly used features for miRNA target prediction: Seed match, conservation, free energy, site accessibility and target-site abundance (Peterson *et al*, 2014). A seed match is a Watson-Crick match between the seed region of miRNA and its mRNA target. The type of the seed match varies depending on the algorithm [*e.g.* 6mer: match for six nucleotides; 7mer-m8: match from nucleotides 2-8 of the seed; 7mer-A1: match from nucleotides 2-7 of the seed in addition to an A at miRNA nucleotide 1; 8mer: match from nucleotides 2-8 of the seed in addition to an A at miRNA nucleotide 1] (Brennecke *et al*, 2005; Krek *et al*, 2005; Lewis *et al*, 2005; Lewis *et al*, 2003). Conservation analysis evaluates the sequence conservation in the 3`UTR and miRNA across the species (Lewis *et al*, 2003). It assumes that conserved interactions may have functional importance. Free energy (Gibbs free energy, G) is a measure of the structural stability. The more stable interaction between miRNA and target mRNA is considered as being more likely a real target (Yue *et al*, 2009), which is measured by the change in free energy (ΔG) during the binding reaction. A negative ΔG means less energy to react and more stable. mRNAs may have a secondary structure which can interfere with miRNA binding (Long *et al*, 2007). Site accessibility measures the ease of miRNA can reach and hybridize with an mRNA target. Target-site abundance is a measure of number of target sites located in a 3`UTR (Garcia *et al*, 2011). A relatively new machine learning method that directly constructs the predictions from validated miRNA datasets. Less commonly used miRNA target features include: local AU content, GU wobble in the seed match, 3`compensatory pairing and position contribution analyses.

In **Papers I** and **III**, miRecords was used for miRNA target prediction. miRecords is an integrated resource for miRNA targets (Xiao *et al*, 2009). It has two components: The “Validated Targets” component is a database of experimentally validated miRNA targets

and the "Predicted Targets" component is an integrated tool for predicted targets of 11 established miRNA target prediction programs. These programs include: TargetScan, miRanda, PicTar, PITA, miTarget, RNA22, RNAhybrid, NBmiRTar, DIANA-microT, MicroInspector, MirTarget2. A summary of the features used in these prediction tools is presented in Table 3. In this thesis work, miRNA targets predicted by at least six prediction tools were chosen for subsequent analyzes.

Table 3. Summary of the features used in different miRNA prediction tools

Tool	Seed Match	Conservation	Free energy	Site accessibilty	Target-site abundance	Machine learning
TargetScan	√	√	√			
miRanda	√	√	√	√		√
PicTar	√	√	√		√	
PITA	√	√	√	√	√	
MiTarget	√		√			√
RNA22	√	√				
RNAhybrid	√		√		√	
NBmiRTar	√	√	√	√		√
DIANA-microT	√	√	√	√	√	√
MicroInspector	√	√	√			
MirTarget2	√	√	√	√		√

3.2.7.2 Evaluation of microRNA targets by western blot analysis

Expression levels of putative miRNA targets were evaluated by western blot analysis. This method is widely used to detect and quantify specific proteins in tissue or cell lysates. In this method, proteins are separated according to their size by gel electrophoresis. The proteins are then transferred from the gel to a solid surface (the membrane), where protein of interest can be detected using a specific primary antibody. After removing the unbound primary antibodies, the membrane is incubated with a secondary antibody that specifically recognizes and binds to the primary antibody. The secondary antibody is usually conjugated with a biotin, fluorescent probe or reporter enzyme (*e.g.* alkaline phosphatase or horseradish peroxidase) for signal detection using colorimetric, chemiluminescent, radioactive or fluorescent. The most commonly used methods are chemiluminescent and fluorescent detections. In chemiluminescent detection, a horseradish peroxidase-conjugated secondary antibody cleaves a chemiluminescent agent that produces luminescence detected by a CCD camera. In fluorescent detection, fluorescently labeled secondary antibodies can be directly visualized by use of appropriate emission filters with an imaging system.

In **Papers I** and **III**, Western blot analysis was performed to evaluate the expression levels of PTPN18 and STARD13 (candidate targets for *miR-125a-5p*), MCL1 (for *miR-320*) and PUMA (for *miR-483-3p*).

In addition to evaluation of miRNA target expression levels, western blot analysis was also performed to detect cleaved PARP in GIST cells treated with imatinib (**Paper III**), and to evaluate pFAK and FAK expression levels in GIST clinical samples and cell lines (**Paper II**). GAPDH was used as an endogenous normalization control.

3.2.8 Patch-clamp

In **Paper IV**, whole-cell patch was used to evaluate Cl^- currents of DOG1 in GIST882 cells upon treatment with DOG1 activator Eact or inhibitor $T16A_{inh}$-A01.

Patch-clamp is an electrophysiological technique that allows the study of ion channels in single cells. The method records ion currents across the membrane using an electrical tight seal between the electrode and cell membrane that contains one or few ion channel molecules.

Patch-clamp uses a glass microelectrode, which is pulled from borosilicate glass with a tip around one micrometer diameter. The microelectrode is positioned next to a cell, and mild suction is applied through the microelectrode to attach the cell membrane (the 'patch') into the microelectrode tip. Between the glass and the membrane a high resistance 'seal' is formed. This configuration is the "**cell-attached**" mode, and it can be used for studying the activity of the ion channels that are present in the patch of membrane.

If more suction is applied during the "cell-attached" mode, it causes rupture of the membrane patch, thus providing the microelectrode an access to the intracellular space of the cell. This "**whole-cell**" mode allows recording currents through multiple channels at once, over the membrane of the entire cell. However, the intracellular components of the cells will slowly be diluted by the solution in the microelectrode; therefore, the experiments using this mode must be completed in a short time (approximately 10 minutes).

3.2.9 Statistical analyses

All statistical tests in this thesis were performed in Statistica 8.0 (StatSoft Inc., Tulsa, OK, USA) or Microsoft Office Excel (Albuquerque, NM, USA), unless otherwise stated.

Significance Analysis of Microarray (SAM) (http://statweb.stanford.edu/~tibs/SAM/) is a statistical method to determine the most significant differentially expressed miRNAs between the sample groups in a given dataset. In our studies, microarray data were analyzed using SAM to determine the most significantly deregulated miRNAs in relation to imatinib

resistance and metastasis in **Paper I**, and imatinib-treatment in **Paper III**. The strength of significance for each gene is given in a score called false discovery rate (FDR).

Unpaired student's *t*-test was conducted to compare miRNA expressions in different patient groups (**Papers I** and **III**) or different cell lines (**Papers I-III**), while paired student's *t*-test was performed to analyze transfection (**Papers I** and **II**), imatinib treatment (**Paper III**) and ion channel currents (**Paper IV**) in cell culture experiments. Association of clinico-pathological features with gene expression levels was evaluated using Fisher's exact test (**Papers I** and **III**). Correlations between expression levels of miRNAs and their potential targets were assessed by Pearson`s correlation analyses and *p*-values were estimated by permuting the samples (**Papers I** and **III**). The protein expression levels were compared among or between different tumor groups using one-way ANOVA or Mann Whitney *U*-test (**Paper II**). The interrelationship of gene expression with survival was studied using Kaplan-Meier plots, and significant differences between curves were evaluated using log-rank test (**Papers I** and **II**). All *p*-values obtained in this study were two-tailed, and *p*-values ≤ 0.05 were considered as significant.

4 RESULTS AND DISCUSSION

4.1 PAPER I

"microRNA expression signatures of gastrointestinal stromal tumors: associations with imatinib resistance and patient outcome"

The aim of this study was to evaluate the clinical impact of miRNAs in imatinib-treated GISTs. We started by profiling global miRNA expressions in imatinib-resistant and imatinib-sensitive GISTs using microarray. Unsupervised clustering analysis classified the samples into two main subgroups: All imatinib-resistant tumors, except one, were grouped in cluster 1; while 7 out of 10 imatinib-sensitive GISTs were found in cluster 2. The result suggested that imatinib-resistant and –sensitive GISTs have distinct miRNA profiles. SAM analysis on the microarray data revealed 27 over-expressed and 17 under-expressed miRNAs with a FDR <15% in the imatinib-resistant GISTs compared to imatinib-sensitive ones.

Ten of these differentially expressed miRNAs (five up-regulated and five down-regulated in imatinib-resistant group) were chosen for validation by RT-qPCR in a larger group of samples. We found that *miR-125a-5p* and *miR-107* were significantly overexpressed in the imatinib-resistant tumors. Notably, the expression levels of these two miRNAs were not obviously different between resistant and sensitive tumor pairs in the two patients with multiple tumors harboring double mutations in *KIT*.

Tumors with double *KIT* mutations or wild-type *KIT* and *PDGFRA* are known as less responsive to imatinib treatment (Debiec-Rychter *et al*, 2006; Debiec-Rychter *et al*, 2004; Heinrich *et al*, 2003a; Heinrich *et al*, 2008b). Therefore, we performed an independent evaluation of the samples harboring only a single *KIT* mutation, and showed that expressions of *miR-125a-5p*, *miR-107*, *miR-134*, *miR-301a-3p* and *miR-365* were significantly higher in the imatinib-resistant tumors among the GISTs with a single *KIT* mutation.

miR-125a-5p was the most significant miRNA among the up-regulated miRNAs in imatinib-resistant GISTs in both analyses. In addition, *miR-125* family members have been shown to confer drug resistance (Zhou *et al*, 2010) and to regulate cell death pathways in cancer cells (Kim *et al*, 2012). Therefore, we further investigated the functional role of *miR-125a-5p* in GIST cells with a single (GIST882) and double (GIST48) *KIT* mutations. Overexpression of *miR-125a-5p* in GIST882 cells resulted in a higher (up to 26 %) cell viability, while its suppression reduced cell viability (up to 9 %). However, overexpression or suppression of *miR-125a-5p* in GIST48 cells did not affect cell viability significantly. These results suggested that *miR-125a-5p* could modulate imatinib response of GISTs harboring a single *KIT* mutation, but not in GISTs harboring *KIT* double mutations.

To reveal the biological function of *miR-125a-5p*, we used a computational tool (miRecords) to identify its candidate targets. Among the predicted targets, we selected PTPN18 and

STARD13 for further validations because of their involvement in KIT or tyrosine kinase signaling and their regulations by *miR-125* family members (Guo *et al*, 2012; Tang *et al*, 2012). We demonstrated that overexpression of *miR-125a-5p* results in decreased protein levels of both PTPN18 and STARD13 in GIST882 cells, suggesting these genes are potential targets of *miR-125a-5p* in GIST.

To strengthen the functional evidence of *miR-125a-5p* in imatinib resistance, we generated imatinib-resistant subclones of GIST882 cells (GIST882R) and evaluated the expression levels of *miR-125a-5p* and its potential targets in GIST882R compared with its imatinib-sensitive parental cell line. The GIST882R cells showed increased *miR-125a-5p* expression together with decreased protein expression levels of PTPN18 and STARD13 compared to its parental cell line.

To evaluate the clinical significance of these findings, we next determined the protein expressions of PTPN18 and STARD13 in imatinib-resistant and –sensitive GISTs. We observed that PTPN18 levels were significantly lower in imatinib-resistant GISTs as compared to imatinib-sensitive samples, and inversely correlated with *miR-125a-5p* expressions. However, STARD13 was not significantly different between imatinib-resistant and –sensitive tumors and no correlation was found with *miR-125a-5p* expression. These results led us to investigate the direct functional role of PTPN18 on imatinib resistance in GIST cells. We showed that suppression of *PTPN18* reduced imatinib sensitivity in GIST882 cells, suggesting the direct involvement of PTPN18 in imatinib resistance in GISTs.

We also evaluated the clinical impact of miRNA expressions in relation to metastasis, *KIT* mutational status and survival in our sample cohort. We identified specific miRNAs associated with metastasis (*e.g. miR-150-3p* and *miR-301a-3p*), *KIT* mutation (*e.g. miR-150-3p*) and disease-free/ overall survival (*e.g. miR-1915*) in GIST.

This study presents the first functional evidence for involvement of miRNAs in imatinib resistance in GISTs, and highlights the role of *miR-125a-5p*-PTPN18 regulation in imatinib resistance as an alternative mechanism to secondary kinase mutations. Our results also highlight several prognostic miRNAs for GIST patients treated with neoadjuvant imatinib.

In summary, the main findings of this paper are:

- GISTs have distinct miRNA expression profiles in relation to imatinib response.
- *miR-125a-5p* is associated with imatinib resistance in GIST, and modulates imatinib response in GIST cells with a single *KIT* mutation (GIST882), but not in GIST cells with secondary *KIT* mutation (GIST48).
- *miR-125a-5p* regulates PTPN18 and STARD13 in GIST882 cells.
- Imatinib-resistant subclone cells (GIST882R) show increased expression of *miR-125a-5p* and decreased PTPN18 and STARD13 protein levels, as compared to the parental cells (GIST882).

- PTPN18 modulates imatinib response in GIST882 cells. PTPN18 expression is downregulated in imatinib-resistant GIST samples as compared to –sensitive GIST samples; and inversely correlated with *miR-125a-5p* expression.
- Several miRNA expressions are associated with metastasis, *KIT* mutational status and survival in GIST.

4.2 PAPER II

"FAK phosphorylation is regulated by PTPN18 and associated with imatinib resistance in gastrointestinal stromal tumors"

In **Paper I**, we identified *miR-125a-5p* modulates imatinib response in GIST cells through regulation of PTPN18, a member of the PEST domain containing protein tyrosine phosphatase superfamily. The subsequent question raised was how PTPN18 regulates the imatinib response in GIST, which led us to **Paper II**.

Given that higher activation of FAK was found in the imatinib-resistant GIST-T1-R subclones compared to imatinib-sensitive parental cells and inhibition of pFAK re-sensitized the resistant cells to imatinib-induced cell death (Takahashi *et al*, 2013), we hypothesized that pFAK might be the downstream target of PTPN18 and involved in imatinib resistance.

We first generated imatinib-resistant subclones of GIST882 cells in addition to the ones in **Paper I**, and characterized their relationship with their parental cell line. We showed that GIST882 and all three GIST882R subclones carried the *KIT* K642E mutation. In addition, most of the GIST882R cells showed decreased CD117 expression in line with a previous report by Mahadevan *et al*. (Mahadevan *et al*, 2007), while DOG1 and CD34 expressions remained unchanged. Notably, some GIST882R cells still expressed CD117 at very high levels, demonstrating the heterogeneous population of imatinib-resistant GIST cells.

Next, we quantified the protein expression levels of PTPN18, pFAK and FAK in GIST882 and GIST882R cells. The GIST882R subclones showed a significant decrease of PTPN18 and FAK levels in comparison to GIST882. On the other hand, pFAK levels were dramatically increased in the imatinib-resistant cells compared to the parental cells. In concordance with our results, same finding was also observed in GIST-T1 cell line (Takahashi *et al*, 2013); supporting that pFAK plays an important role in imatinib resistance in GIST.

To evaluate the direct regulation of PTPN18 on FAK phosphorylation, we silenced PTPN18 in GIST882 cells and evaluated its effect on pFAK levels. Indeed, silencing of PTPN18 expression increased the pFAK level, whereas the FAK level remained unchanged; indicating that the phosphorylation level of FAK is regulated by PTPN18.

To further investigate the clinical significance of the findings, we quantified pFAK and FAK protein levels in imatinib-resistant and imatinib-sensitive GIST specimens. We observed that pFAK expression was only significantly up-regulated in the imatinib-resistant GISTs with a single *KIT* mutation, but not in those with double *KIT* mutations, compared to the imatinib-sensitive group. This is consistent with our findings in **Paper I** that *miR-125a-5p* modulates imatinib sensitivity only in GIST882 cell line harboring a single *KIT* mutation, but not in the double *KIT* mutated GIST48 cells.

On the other hand, FAK expression was significantly higher in the imatinib-resistant GISTs with double *KIT* mutant compared to the imatinib-sensitive tumors, however its level was not significantly different between the resistant GISTs with a single *KIT* mutation and the sensitive group.

FAK expression had been associated with tumor progression and overall survival (Kamo *et al*, 2009; Koon *et al*, 2004). We therefore sought to determine whether FAK and pFAK expressions are associated with clinical outcome in our GIST cohort. In concordance with previous findings (Kamo *et al*, 2009; Koon *et al*, 2004), high FAK expression, but not pFAK expression, was significantly associated with metastatic GISTs. Together, these findings suggest that FAK may play an important role in tumor progression independent of FAK activation.

Here, we propose a novel imatinib resistance mechanism in GIST, in which *miR-125a-5p* overexpression in imatinib-resistant tumors suppresses PTPN18 expression that subsequently leads to defective FAK dephosphorylation.

To summarize, the main findings of this paper include:

- PTPN18 silencing increases phosphorylation of FAK, but not FAK levels.
- High pFAK levels are found in imatinib-resistant GIST882R cell lines and GIST specimens with a single *KIT* mutation.
- High FAK levels are associated with tumor progression in GIST.

4.3 PAPER III

"Involvement of the MCL1 and *miR-320* in imatinib-induced cell death of gastrointestinal stromal tumors"

Even though imatinib exhibits remarkable anti-tumor responses, the molecular mechanisms of imatinib-induced cell death in GIST are not completely understood. In this study, we aimed to reveal the role of miRNAs in imatinib-induced cell death mechanisms in GIST.

To evaluate the effects of imatinib on global miRNA expressions, we first profiled miRNA expression levels of imatinib-treated and non-treated GIST samples using microarray. We

found 12 up-regulated and 2 down-regulated miRNAs in the imatinib-treated GISTs compared to non-treated GISTs with a FDR < 20%. Clustering analysis using these significant differentially expressed miRNAs classified the samples into two main subgroups: All imatinib-treated samples except two were grouped together, while the majority of the non-treated samples (9/15) were clustered in a separate group. We speculated that these deregulated miRNAs and their target(s) may have important roles in regulation of cell viability in response to imatinib treatment. Notably, *MCL1* was a predicted target of the top five up-regulated miRNAs (*miR-320a, miR-193a-3p, miR-320d, miR320c* and *miR-320b*) in imatinib-treated GISTs. MCL1 is an apoptotic regulator, and its inhibition was shown to induce apoptosis in CML cells (Aichberger *et al*, 2005). In addition, one of the down-regulated miRNAs, *miR-483-3p*, is known to target PUMA, which is a pro-apoptotic member of the BCL2 family (Ozata *et al*, 2011). Therefore, we selected four miRNAs (*miR-320a, miR-320b, miR-193a-3p* and *miR-483-3p*) to verify the array results with RT-qPCR in a larger cohort of 62 GIST samples. We found significantly higher expression of *miR-320a* and *miR-320b*, and lower expression of *miR-483-3p* in imatinib-treated GISTs vs. non-treated GISTs.

We next quantified the expression levels of the two potential targets of *miR-320a*/*b* and *miR-483-3p* (MCL1 and PUMA, respectively) in GIST samples by western blot analysis. We observed that MCL1 protein levels were significantly lower in imatinib-treated GISTs vs. non-treated GISTs. In addition, among the treated samples, the imatinib-sensitive tumors also showed significantly lower MCL1 expression than the imatinib-resistant tumors. Notably, the MCL1 expression was not different between the imatinib-resistant tumors and the non-treated tumors. Furthermore, MCL1 expression was inversely correlated with *miR-320a* and *miR-320b* levels. PUMA expression was significantly lower in imatinib-treated samples compared to non-treated samples, and higher in non-treated samples vs. imatinib-resistant or –sensitive groups. However, its expression did not differ between resistant and sensitive groups. Surprisingly, PUMA expression was positively correlated with *miR-483-3p* expression. These results suggest the possible role of MCL1 and *miR320* in imatinib response in GIST. Therefore, we further evaluated the effect of imatinib exposure on MCL1 and *miR320* expressions in cell culture experiments.

We observed that the expression of the anti-apoptotic MCL1 isoform (MCL1L) was markedly induced in the adherent cells (mostly alive cells) upon imatinib treatment, while it was barely detectable in the floating cells (mostly dead cells). Furthermore, the floating cells showed clearly induced expression of the pro-apoptotic MCL1 isoform (MCL1S) after imatinib exposure, however MCL1S levels were very low in untreated control cells and in adherent cells. Similarly, *miR-320b* level was increased in floating cells; however the change was not statistically significant. Notably, *miR-320a/b* showed an inverse expression pattern compared to MCL1L, and a similar expression pattern as cleaved PARP.

To further strengthen the involvement of MCL1 and *miR-320a*/*b* in imatinib resistance, we evaluated their expressions in three independent imatinib-resistant subclones of GIST882

cells and the imatinib-sensitive parental cell lines. MCL1L levels were significantly increased and MCL1S levels were markedly decreased in imatinib-resistant GIST882R subclones compared to its parental cell line. Similarly, *miR-320a* and *miR-320b* levels were significantly decreased in GIST882R subclones. These results demonstrate that anti-apoptotic MCL1L was upregulated during acquisition of imatinib resistance, while *miR-320a/b* and the proapoptotic MCL1S was downregulated. Our findings suggest the involvement of *miR-320*-MCL1 regulation in imatinib resistance in GIST.

This paper provides evidence that *miR-320a/b* and MCL1 expressions are associated with imatinib-induced cell death and imatinib resistance in GIST, and suggests the potential of *miR-320* and MCL1 as novel targets for innovative treatment strategies for GIST patients.

As a summary, the main findings are:

- Imatinib-treated and non-treated GISTs have distinct miRNA expression patterns, including *miR-320a, miR-320b* and *miR-483a-3p*.
- *miR-320a/b* is upregulated while its potential target MCL1 is downregulated in imatinib-treated GISTs as compared to non-treated GISTs. MCL1 expression was inversely correlated with *miR-320a* and *miR-320b* levels.
- Higher level of anti-apoptotic MCL1 isoform (MCL1L) is associated with cell viability upon imatinib exposure in GIST882 cells. Oppositely, increased level of the pro-apoptotic MCL1 isoform (MCL1S) is associated with cell death.
- The anti-apoptotic MCL1L is upregulated while *miR-320a/b* and the proapoptotic MCL1S are downregulated during acquisition of imatinib resistance in GIST882 cells.

4.4 PAPER IV

"Functional role of the Ca^{2+}-activated Cl^- channel DOG1/TMEM16A in gastrointestinal stromal tumor cells"

DOG1 is a Ca^{2+}-activated Cl^- channel, and it is expressed at high levels in most of GISTs (97%). However, its biological function in GIST is not fully characterized. Recent reports showed that DOG1 facilitates cell proliferation in primary cultures of ICC (Stanich *et al*, 2011) and head and neck squamous carcinoma (Duvvuri *et al*, 2012). These findings raised the possibility for DOG1 function as a regulator of cell proliferation or apoptosis in GISTs. In **Paper IV**, we evaluated the functional role of DOG1 in GIST cells using specific DOG1 activator Eact and inhibitor $T16A_{inh}$-A01.

We first determined the subcellular localization of DOG1 in imatinib-sensitive GIST882 and imatinib-resistant GIST48 cell lines, using immunocytochemistry and confocal microscopy.

DOG1 was predominantly located at cell periphery including the plasma membrane of GIST882 cells, whereas it was found located in the perinucleus of GIST48 cells. Since DOG1 was mainly localized in the plasma membrane of GIST882 cells, we chose this cell line for further characterization of DOG1-generated currents using patch-clamp method. To verify that DOG1 is functional in GIST882 cells, we recorded the whole-cell Cl^- currents at either inhibitory (90 nM) or activating (305 nM) $[Ca^{2+}]_{pip}$ levels. The Cl^- currents increased from 3.8±0.6 pA/pF to 13.5±3.2 Pa/pF upon increasing $[Ca^{2+}]_{pip}$ to activating levels, verifying that DOG1 is functional and indeed Ca^{2+} dependent in GIST882 cells.

We next determined the effects of DOG1 inhibitor $T16A_{inh}$-A01 and activator Eact on DOG1 in GIST882 cells, by measuring whole-cell Ca^{2+}-activated Cl^- currents. We analyzed the inhibition by keeping the $[Ca^{2+}]_{pip}$ levels only at the activating levels (305 nM), while for activation both activating (305 nM) and inhibiting (90 nM) $[Ca^{2+}]_{pip}$ levels were used. Treatment with DOG1 inhibitor $T16A_{inh}$-A01 resulted in 60% decrease in the Cl^- currents in GIST882 cells, which is relatively low compared to other cells that was previously shown to be almost completely blocked by the $T16A_{inh}$-A01 (Namkung *et al*, 2011a). The difference might be due to the varying presence of Cl^- channels with different pharmacological properties other than DOG1 in these different types of cells. In addition, DOG1 activator Eact caused 70% increase in the Cl^- currents at activating $[Ca^{2+}]_{pip}$ levels, but did not affect the currents at inhibiting levels, showing the Ca^{2+}-dependent specific activation effect of Eact.

Next, we evaluated the effects of DOG1 inhibition and activation on apoptosis in both GIST882 and GIST48 cells using Annexin V / 7-AAD flow cytometric analysis. Even though we observed fewer early apoptotic cells upon DOG1 inhibition and more viable cells upon DOG1 activation in GIST882 cells, the differences were subtle. DOG1 modulation did not affect apoptosis rate in GIST48; however, its inhibition induced the apoptotic cells from early to late apoptotic stage.

We lastly evaluated the cell proliferation of GIST882 and GIS48 cells upon DOG1 modulation. In GIST882, even though we observed some minor changes in proliferation after 72 hours, these changes were subtle especially compared to the effect of imatinib. In GIST48, we observed significantly increased proliferation both upon activation and inhibition of DOG1 after 72 hours. However, similar to GIST882, the differences were small, and the results were not meaningful since both DOG1 activation and inhibition are not expected to have a pro-proliferative effect. Therefore, these findings are in line with recent report by Simon and colleagues (Simon *et al*, 2013), showing that DOG1 inhibition did not alter tumor cell growth *in vitro*; however, it delayed the growth of xenografts of GIST-T1 and GIST430 but not GIST882 *in vivo*.

In summary, the main findings of this paper include:

- DOG1 is localized in different subcellular compartments in imatinib-resistant and imatinib-sensitive GIST cells.

- DOG1 is functional and Ca^{2+}-dependent in GIST882 cells.
- DOG1 modulation does not have obvious effects on apoptosis or proliferation in imatinib-sensitive GIST882 and imatinib-resistant GIST48 cell lines; however, its activation led the early apoptotic cells to undergo late apoptotic stage in GIST48 cells.

5 CONCLUDING REMARKS AND FUTURE DIRECTIONS

In the last twenty years, growing knowledge of molecular biology in GISTs has led to remarkable achievements in its clinical management. From a treatment-resistant uncontrolled disease, GIST has become an example for development of targeted therapies against oncogene-addicted cancers. However, even though tyrosine kinase inhibitors (TKI) improve the outcome of the majority of patients, they fail to provide a permanent cure and TKI-resistant clones are observed in most of the initially responding tumors. Even the cases treated with new inhibitors, such as regorafenib (a multi-kinase inhibitor), eventually develop acquired resistance (Demetri *et al*, 2013). Despite several resistance mechanisms to imatinib have been described, a complete understanding of resistance mechanisms is needed for developing effective combinational therapies.

This thesis work contributes an effort to understand the molecular mechanisms of imatinib resistance in GIST. Here, we identified two miRNA networks involved in imatinib resistance of GIST, *i.e. miR-125a-5p*-PTPN18-FAK (**Papers I** and **II**) and *miR-320a/b*-MCL1 (**Paper III**). Further studies are needed to provide complete understanding of these miRNA regulatory networks. For examples, what causes the dysregulation of these miRNAs in imatinib-resistant tumors/cells; whether *miR-125a-5p* directly regulates FAK phosphorylation levels, and PTPN18 overexpression can re-sensitize GIST cells to imatinib; functional studies of *miR-320* modulation on MCL1 levels and imatinib response; and evaluation of the findings in additional GIST cell lines and subsequently in animal models.

DOG1 is highly and specifically expressed in most of the GISTs, and used as an established biomarker in clinical settings (Espinosa *et al*, 2008). However, its biological function in GIST remains unknown. In this thesis, we identified that DOG1 is localized in different cellular compartments, but not an effective regulator of cell proliferation or apoptosis in imatinib-resistant and –sensitive GIST cells (**Paper IV**). Future studies investigating its functional impact on other hallmarks of cancer may facilitate our understanding of its involvement in GIST progression.

Complete understanding of molecular biology in GIST development, progression and treatment response will establish a ground for innovative therapeutic strategies with a goal of not only to temporarily control the disease, but also to permanently eradicate all tumor cells. This will hopefully turn GIST from a model of targeted therapies that control the disease progression, to a model of complete cancer cure.

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