Expression Profiling of Gastrointestinal Stromal Tumors Biomarkers for Prognosis and Therapy

Gabriella Arne

UNIVERSITY OF GOTHENBURG

Sahlgrenska Cancer Center

Department of Pathology

Sahlgrenska Academy at the University of Gothenburg

Sweden

2012

Front cover illustrations:

Left) Gene expression microarray of GIST. *Middle)* Immunohistochemical staining of PROM1 (CD133) protein in GIST biopsy on tissue microarray (TMA) *Right)* Primary small intestinal GIST with multiple abdominal and liver metastases visualized by octreotide scintigraphy.

ISBN 978-91-628-8437-6

Printed by Ineko AB, Gothenburg
http://hdl.handle.net/2077/28261

Till min underbara familj

ABSTRACT

Expression profiling of Gastrointestinal Stromal Tumors Biomarkers for Prognosis and Therapy

Gabriella Arne

Sahlgrenska Cancer Center, Department of Pathology,
Sahlgrenska Academy at the University of Gothenburg, Gothenburg, Sweden

Gastrointestinal stromal tumor (GIST) is a mesenchymal tumor of the gastrointestinal tract with a clinical spectrum ranging from indolent tumors to tumors with aggressive behavior and poor patient survival. The established model for prediction of prognosis for GIST is the NIH risk score, which is based on tumor size and mitotic index. Even so, there are difficulties in predicting the clinical outcome for individual GIST patients, which may lead to inadequate treatment. The majority of GISTs have activating mutations in the genes encoding the tyrosine kinase receptors KIT, or PDGFRA, which are considered to be pathogenic events in tumor development. Imatinib, a tyrosine kinase inhibitor (TKI) that inhibits KIT, has become an important therapeutic option in addition to surgery.

To identify biomarkers that accurately predict clinical outcome in GIST patients, global gene expression profiling was performed based on *KIT* mutations associated with poor prognosis. Tumor material from 16 GISTs was analyzed with expression microarray for identification of multiple candidate genes with differential expression related to mutational status. *PROM1* was shown to be highly expressed in GIST with *KIT* exon 11 mutations. Detection of PROM1 protein with immunohistochemical staining of 204 GISTs arranged in a tissue microarray (TMA) showed that PROM1 expression was predominant in gastric GISTs of high-risk type. Multivariate Cox analysis showed that PROM1 expression was significantly associated with poor prognosis and short patient survival, independently of NIH risk score. To evaluate the usefulness of immunohistochemical biomarkers for prognostication of GIST, we performed a comprehensive study of 14 biomarkers in 205 GISTs in a TMA. There was a significant correlation between expression of CA2, CDKN2A, CXCL12, EPHA4, FHL1, and DPP4 protein and survival. Furthermore, survival analysis using Cox regression showed that CA2, EPHA4, and FHL1 provided prognostic information additional to that from the NIH risk score. Construction of a decision-tree model combining NIH risk and expression of biomarkers further improved the prediction of patient survival. GISTs are effectively treated with surgery and imatinib, but some patients are refractory and develop drug resistance. We have investigated the prerequisites for alternative treatment strategies with peptide receptor-mediated radiotherapy (PRRT), by analyzing the expression of somatostatin receptors (SSTRs) and uptake of radiolabeled somatostatin analogs in GIST. Analysis of 34 GISTs with pPCR and immunohistochemistry showed expression of SSTR1 and SSTR2. Primary cultures established from GIST showed specific binding and internalization of ^{177}Lu-octreotate. Diagnostic imaging with ^{111}In-octreotide showed tumor uptake of ^{111}In in 3/6 GIST patients *in vivo*. Tumor-to-blood activity ratios for ^{111}In measured in biopsies from excised tumor tissue showed ratios that may be adequate for therapy.

We conclude that the expression of PROM1 in GIST may be used as a prognosticator of patient survival and may provide a therapeutic target. Several immunohistochemical biomarkers provide additional prognostic information in addition to NIH risk score and may be useful in constructing decision-trees for improved prognostic accuracy for GIST patients. Binding and uptake of radiolabeled somatostatin analogs via SSTR enable tumor imaging and targeted therapy in selected GIST patients.

Key words: Gastrointestinal stromal tumor (GIST); KIT; Biomarker; PROM1 (CD133); Somatostatin receptor (SSTR); Peptide receptor-mediated radiotherapy (PRRT); Expression profiling; Immunohistochemistry; Tissue microarray (TMA); Survival analysis

POPULÄRVETENSKAPLIG SAMMANFATTNING

Cancer är ett globalt hälsoproblem och en ledande orsak till dödsfall i västvärlden. Cancer orsakas av att något går snett i kontrollen över kroppen egna celler. En cells förmåga att växa okontrollerat beror på genetiska förändringar i arvsmassan (DNA), vilket kan få till följd att tumörfrämjande proteiner produceras av dessa förändrade gener. Genom framsteg inom tumörbiologin har vi fått en ökad förståelse för hur cancer uppstår och utvecklas, och ett stort antal av nya biomarkörer som bidrar till att förbättra diagnos, riskbedömning och terapi av cancerpatienter.

Sarkom är relativt ovanliga tumörer som uppstår i ben, brosk och mjukdelar. GIST är det vanligaste sarkomet i mag-tarmkanalen och uppstår oftast i magsäcken eller tunntarmen. GIST är en heterogen tumörform som uppvisar ett varierat kliniskt förlopp, från långsamväxande tumörer till tumörer med aggressivt växtsätt och dålig patientöverlevnad. I denna avhandling har vi undersökt biomarkörer som har ett värde vid prognosbedömning och terapi av GIST.

Den mest etablerade modellen för att förutsäga prognos för GIST patienter är baserad på tumörstorlek och celldelningsfrekvens (kallad NIH risk). Överlevnaden för en väsentlig andel av patienterna avviker dock från angiven riskbedömning, vilket kan få till följd att de inte erbjuds optimal cancerbehandling. I delarbete I ämnade vi att identifiera nya biomarkörer som förutsäger aggressiv tumörväxt och dålig prognos av GIST. Genom att använda microarray, en avancerad DNA-teknik som studerar hela genuttrycket i samma analys, identifierade vi flera kandidatgener som var intressanta i ett prognos-sammanhang. Speciellt genen *PROM1 (*även kallad *CD133)* och dess proteinprodukt visade sig kunna ge mer information om patienters överlevnad än vad NIH riskgradering gör som ensam variabel. Vi drar därför slutsatsen att PROM1 kan användas som ett prognostiskt verktyg för GIST-patienter.

För att bedöma värdet av såväl nya som redan kända biomarkörer vid prognosbedömning av GIST utförde vi i delarbete II en jämförande studie av 14 olika proteinmarkörer. Genom statistiska undersökningar jämförde vi proteinuttrycket av dessa biomarkörer med patientöverlevnad, NIH riskgradering, och andra kliniska variabler, och fann att proteinuttrycket för 6 av dessa markörer gav information om GIST-patienters överlevnad i vårt material (CA2, CDKN2A, CXCL12, DPP4, EPHA4 och FHL1). Vi föreslår dessutom att genom att konstruera ett beslutsträd som inkluderar såväl NIH riskgradering som utvalda biomarkörer (CA2 och EPHA4) kan vi göra en mer korrekt prognosbedömning av GIST patienters överlevnad än vad den etablerade NIH riskgradering ger ensamt.

Hälften av GIST-patienterna botas med kirurgi, men somliga går dock inte att operera radikalt beroende på tumörens utbredning. För dessa patienter är läkemedlet Imatinib en viktig tilläggsbehandling genom att den dämpar tumörtillväxten. Emellertid, utvecklar flertalet patienter resistens mot Imatinib och därför finns att behov av andra behandlingsalternativ. Vi har undersökt förutsättningarna för en alternativ behandlingsmetod med radioterapi via specifika receptorer på cellytan, s.k. somatostatinreceptorer (SSTR). I delarbete III har vi studerat i vilken omfattning GIST-celler uttrycker SSTR och dessutom om det är möjligt för cellen att ta upp radionuklider via dessa receptorer. Vi kunde visa att GIST uttrycker två olika varianter av SSTR (SSTR1 & 2) både på gennivå och som protein. Genom studier både på cellkultur (*in vitro*) och på patienter (*in vivo*) kunde vi även visa att bindning och upptag av radionuklider in i GIST-celler är möjligt. Vår slutsats är därför att radioterapi via SSTR skulle kunna bli en ny möjlighet till behandling för utvalda GIST-patienter.

LIST OF PAPERS

This thesis is based on the following papers, referred to in the text by their roman numerals (I-III):

I. **Arne G**, Kristiansson E, Nerman O, Kindblom LG, Ahlman H, Nilsson B, and Nilsson O. Expression profiling of GIST: CD133 is associated with KIT exon 11 deletions, gastric location and poor prognosis. *International Journal of Cancer* 2011; 129(5): 1149-1161.

II. **Arne G**, Kristiansson E, Nilsson B, Ahlman H, and Nilsson O. Comparative analysis of biomarkers as prognosticators for survival in GIST patients. *In manuscript.*

III. **Arne G**, Nilsson B, Dalmo J, Kristiansson E, Arvidsson Y, Forssell-Aronsson E, Nilsson O, and Ahlman H. Gastrointestinal stromal tumors (GISTs) express somatostatin receptors and bind radiolabeled somatostatin analogs. *Submitted to Acta Oncologica* 2011.

TABLE OF CONTENTS

ABBREVIATIONS

ANO1	anoctamin 1 (also DOG1)
CSC	cancer stem cell
DNA	deoxyribonucleic acid
DOTA	1,4,7,10-tetraazaciclododecane- N,N′,N′′,N′′′- tetraacetic acid
DTPA	diethylene triamine pentaacetic acid
ETV1	ETS variant 1
GAPDH	glyceraldehyd-3-phosphate dehydrogenase
GIST	gastrointestinal stromal tumor
HDACI	histone deacetylase inhibitor
ICC	interstitial cells of Cajal
%IA/g	percent of injected activity per gram of tissue
^{111}In	indium-111
^{177}Lu	lutetium-177
mRNA	messenger RNA
MSC	mesenchymal stem cell
NE	neuroendocrine
NET	neuroendocrine tumor
NF1	neurofibromatosis type 1
NIH	National Institute of Health
PDGFRA	platelet-derived growth factor receptor α
PDGF	platelet-derived growth factor (PDGFRA ligand)
PROM1	prominin-1 (also CD133)
PRRT	peptide receptor-mediated radiotherapy
qPCR	quantitative real-time polymerase chain reaction
R0	totally resected tumor, no residual tumor
RFS	recurrence-free survival
RNA	ribonucleic acid
SCF	stem cell factor (KIT ligand)
SSTR	somatostatin receptor
T/B	tumor-to-blood activity concentration ratio
TK	tyrosine kinase
TKI	tyrosine kinase inhibitor
TMA	tissue microarray
wt	wild type

INTRODUCTION

CANCER

Cancer is a global health problem and a leading cause of deaths in industrialized countries. The incidence of cancer is increasing due to an aging population in the western world. Improvements in surgery and development of new cancer therapies have prolonged the survival of patients. Advances in molecular biology and genetics have given insight into basic principles of cancer initiation and development. Cancer may occur as hereditary or sporadic tumors. Genetic alterations in tumors include chromosomal alterations as well as mutations in specific genes (Weinberg, 2007). These advances in cancer genetics have provided a molecular classification of tumors that help to improve tumor diagnosis, prognostication, and therapy. Introduction of high-throughput techniques in tumor biology has increased the multitude of novel biomarkers predicting diagnosis, prognosis and therapeutic response. However, application of biomarkers remains a challenge in translational medicine (Brooks, 2012).

Cancer development and genomic instability

Uncontrolled growth is a characteristic feature of a cancer cell resulting from changes occurring in the tumor cell or in its microenvironment. The changes in the tumor cell are due to the accumulation of somatic gene aberrations, or through epigenetic alterations. The most common mechanism to induce mutations in the genome includes spontaneous errors in DNA replication and repair. The majority of mutations does not affect the function of the cell and have accordingly no consequence for tumor development. However, mutations involving genes that control growth or the integrity of the genome may give rise to transformed cells that proliferate abnormally and may have the ability to invade surrounding tissues (Vogelstein and Kinzler, 1993; Yokota, 2000; Hahn and Weinberg, 2002). The clonal multistep model for tumor development assumes a series of randomly occurring mutations and epigenetic alterations of the DNA (Nowell, 1976; Klein and Klein, 1985; Vogelstein and Kinzler, 1993). A first mutation may transform a normal cell into a new cell clone with proliferative advantage, leading to a clonal expansion at the expense of neighboring cells. A second mutation occurs in one of the clones, resulting in yet another cell clone with even greater proliferative ability and survival advantage. As this clonal expansion repeats itself, new stronger populations develop that will drive tumor progression towards a fully developed malignant phenotype.

Chromosomal aberrations, epigenetic alterations, and mutations in specific genes all cooperate in carcinogenesis and tumor development. There are three classes of genes, in which genetic alterations contribute to the pathogenesis of cancer: oncogenes, tumor suppressor genes, and DNA repair genes.

Oncogenes promote cell proliferation

Oncogenes arise by mutations in normal genes, which are known as proto-oncogenes (Bishop, 1991; Weinberg, 1994; Vogelstein and Kinzler, 2004). Proto-oncogenes are normally strictly regulated and encode a wide range of proteins including signal transducers (SRC, RAS family), transcription factors (MYC, ETV1), growth factors (SCF, PDGF, EGF), growth factor receptors (KIT, PDGFRA, RET), and inhibitors of apoptosis (MDM2, BCL2) (Croce, 2008). Proto-oncogenes can be activated into oncogenes by dominant gain-of-function mutations (Tabin *et al.*, 1982) or to increased expression by chromosomal amplification or translocation (Slamon, 1987). Activated oncogenes may lead to tumorigenesis by elevated cell proliferation and inhibition of cell death. Novel cancer drugs have been designed to target proteins encoded by oncogenes, including the tyrosine kinase inhibitor (TKI) imatinib mesylate (Glivec®) against the fusion protein BCR-ABL found in chronic myeloid leukemia (CML), as well as the receptor tyrosine kinases KIT and PDGFRA activated in gastrointestinal stromal tumors (GISTs) (Buchdunger *et al.*, 2000; Joensuu *et al.*, 2001).

Tumor suppressor genes control cell growth and apoptosis

Tumor suppressor genes have the opposite function of oncogenes, by acting as negative regulators of cell proliferation (Klein, 1987). Tumor suppressor genes encode proteins involved in many cellular functions including cell cycle inhibition, transcriptional regulation, apoptosis, and genetic stability (e.g. TP53, RB1, NF1, and CDKN2A) (Sherr, 2004). The inactivation of a tumor suppressor gene requires that both alleles are affected by chromosomal deletions, point mutations, or promoter hypermethylation (Knudson, 1971; Sherr, 2004). The loss of a tumor suppressor gene and its encoding protein may result in loss of response to external growth-inhibitory signals and thus increased likelihood of cancer development (Weinberg, 2007).

DNA repair genes protect the integrity of the genome

DNA repair genes encode proteins involved in maintaining the integrity of the genome, by participating in the cellular response to DNA damage (Peltomäki, 2001; Friedberg, 2003). DNA repair genes (e.g. *BRCA1*) are considered as caretakers of the genome since they detect DNA-damage, repair damaged DNA, and inactivate mutagenic molecules that may damage the DNA (Kastan, 2008; Negrini *et al.*, 2008). Mutations in such a gene may cause loss of DNA repair function, which results in genomic instability and an elevated mutational rate in the genome. Hence, defects in the DNA repair mechanisms allow the successive accumulation of mutations in oncogenes and tumor suppressor genes, which promote tumor development. (Kinzler and Vogelstein, 1997, Friedberg, 2003)

Epigenetic regulators of gene transcription

Unlike genetic alterations, epigenetic aberrations are chemical modifications of the DNA or chromatin proteins that may result in changes in gene expression without altering the DNA sequence (Jones and Baylin, 2002). ***DNA promoter methylation*** is known to have profound effects on gene expression. Hypermethylation of promoter regions causes gene silencing through transcriptional inactivation. In cancer, both DNA hypomethylations and hypermethylations may occur, causing inactivation of tumor suppressor genes (Herman and Baylin, 2003). Another epigenetic event regulating gene expression is ***histone modifications*****.** The genetic information is packaged as chromosomes in the cell nucleus. The chromatin is composed of DNA wrapped around histones. The chromatin may be in a transcription-competent or -incompetent state, thus controlling accessibility of the genome. The state of the chromatin is mainly controlled by post-translational modifications of histone proteins, e.g. acetylations, methylations, and phosphorylations (Sharma *et al.*, 2010). Unlike genetic changes, epigenetic changes are potentially reversible and represent promising target molecules and predictive biomarkers in tumor treatment (Sharma *et al.*, 2010). Histone deacetylase inhibitors (HDACIs), inducing cell apoptosis and/or cell cycle arrest, have already been shown to have selective toxicity against tumor cells. HDACIs (e.g. valproic acid, vorinostat) in combination with conventional therapy (e.g. chemo- or radiotherapy) have been tested with encouraging results in Phase I and II clinical trials of hematological malignancies and solid tumors (Marks *et al.*, 2001; Johnstone, 2002; Tan *et al.*, 2010).

MicroRNAs are short non-coding RNA molecules regulating gene expression of proteins involved in various biological processes, including proliferation, differentiation, and cell death (Ambros, 2004). MicroRNA inhibits translation of DNA by degrading mRNA transcripts. Aberrant microRNA expression may have profound influence on cellular consequences, since a single microRNA can bind and regulate multiple genes. MicroRNAs are often deregulated in cancer and have been shown to be involved in tumor initiation, as well as tumor progression. Increased expression of oncogenic microRNAs can repress targets such as tumor suppressor genes, whereas loss of tumor suppressive microRNAs may enhance the expression of target oncogenes (Ambros, 2004; Volinia *et al.*, 2006). Targeting microRNAs has been proposed to be a novel strategy in cancer therapy, and experimental studies have shown that inhibition of certain microRNAs (e.g. miR-21) reduces tumor growth (Negrini *et al.*, 2009; Bonci, 2010).

Cancer stem cells

According to the cancer stem cell (CSC) theory, solid tumors are composed of hierarchies of tumor cells with different functions. CSCs represent a minority of cells in the tumor and have the ability to produce large numbers of descendant tumor cells and are responsible for tumor growth and metastasis formation (Alison *et al.*, 2011). CSCs are self-renewing cells that may divide into one daughter cell that becomes a new CSC, and another cell that becomes a tumor progenitor cell (a rapid-amplifying cell). The progenitor cell may undergo a large series of cell divisions giving rise to the bulk of tumor cells (Al-Hajj and Clarke, 2004). Chemotherapy is generally effective in killing tumor cells, but CSCs are usually resistant due to expression of cytoprotective enzymes (e.g. ABC-transporters, acetyl dehydrogenase (ALDH)) (Alison *et al.*, 2011). Thus, CSCs may persist after chemotherapy (Guzman *et al.*, 2002) causing tumor relapse. Curative treatment requires elimination of all CSCs. Targeting CSCs is therefore a promising therapeutic principle. CSCs are believed to be dependent on a restricted set of signaling pathways (e.g. those associated with KIT, Wnt, sonic hedgehog, and Notch), all of which are promising candidates for CSC targeted therapy (Marotta and Polyak, 2009). Furthermore, CSCs express unique cell surface markers (e.g. CD44, CD90, and CD133 (PROM1)), which may also be used for CSC targeted therapy (Alison *et al.*, 2011). There are growing experimental evidence for the existence of CSC subpopulations in malignant tumors (e.g. in leukemia, glioma, prostate and breast cancer, and Ewing sarcoma) (Collins *et al.*, 2005; Charafe-Jauffret *et al.*, 2009; Suvà *et al.*, 2009; Alison *et al.*, 2011).

Characteristics of the cancer cell

Tumor cells arise from normal cells by a multistep process known as tumor progression. During this process cancer cells acquire a multitude of different properties, which are common to all types of cancer. These properties are identified as the "Hallmarks of cancer" (Hanahan and Weinberg, 2011).

The proposed characteristics of a cancer cell are functions of specific control systems that govern the transformation of normal cells into cancer cells. Normal cells require growth signals from the extracellular environment to proceed into an active proliferative state in the cell cycle. Tumor cells develop the ability to ***sustain proliferative signaling*** on its own, either by self production of growth signals or by constitutive activation of growth signaling pathways. There are several cellular processes limiting proliferation to keep the delicate balance of homeostasis in a tissue. Normal cells are regulated by antigrowth signals, including tumor suppressors, which may force the cell to enter a non-proliferative state or even undergo apoptosis to maintain the balance. Tumor cells acquire the ability to ***evade growth suppressors*** and/or ***resist cell death*** in order to remain proliferative.

Several mechanisms may be involved in these circumvention strategies, including the loss of TP53 tumor suppressor function.

Telomere shortening limits the replicative potential in normal cells to a fixed number of multiplications. Tumor cells overcome this limitation which ***enables replicative immortality***. By the deregulation of telomerase, which protects the chromosome ends, the tumor cell harbors a capacity of unlimited replicative potential that progresses tumor growth.

Formation of new blood vessels is a vital process in normal tissues as well as in tumors, for the critical supply of nutrients and oxygen. Tumor cells induce an angiogenic switch to harbor unrestricted ability to ***induce angiogenesis***. Furthermore, the contact with blood and lymphatic vessels allows the tumor to enter the circulation and disseminate. All malignant tumors have the potential to ***invade and metastasize***. The invasion-metastasis cascade is a multistep process describing how a cancer cell acquires the ability to penetrate surrounding tissue and finally colonize vital organs in distant sites. The ability to invade and metastasize is a characteristic feature of malignant tumors, as opposed to benign tumors. The ability of a tumor to metastasize and invade vital organs is responsible for the vast majority of cancer deaths (Weinberg, 2007).

Another characteristic involved in the pathogenesis of cancer is the ***reprogramming of energy metabolism***. Rapid cell division and growth increase the need of energy to survive and progress. The capacity to modify the cellular metabolism allows tumor cells to adapt to both aerobic and hypoxic environments to enable effective growth. Further, the immune system may eliminate tumor cells by the action of natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) (Pagés et al., 2010). Hence, the tumor cell needs to avoid the immune surveillance and ***evade immune destruction*** in order to stay vital.

These hallmarks of cancer explain the acquired functional capabilities that drive the transformation of a normal cell into a tumor cell. Furthermore, Hanahan and Weinberg (2011) also addressed two key characteristics that are important for the initiation of tumor development. The surrounding microenvironment that nurtures the tumor with bioactive molecules is supported by ***tumor-promoting inflammation*** in the tissue. Finally, cancer has a genetic basis and is primarily induced by ***genome instability and mutations*** as described earlier.

BIOMARKERS

> *"A biomarker is defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a specified therapeutic intervention"*
>
> - Biomarker Definitions Working Group (2001)

A biomarker is a biological feature used as an indicator of a biological state. The term biomarker is used in many scientific fields, including cell biology and medicine. In medicine a biomarker can be a molecule that detects a particular cell type or a substance that correlates to a particular disease state, but biomarkers are not necessarily molecules. A biomarker can be any kind of measurable quantity which may have clinical relevance, e.g. a protein that indicate a stem cell phenotype, the presence of an antibody that indicate an infection, or a specific DNA sequence that indicates susceptibility to therapy. In oncology, detection of biomarkers may provide important information on diagnosis, tumor progression, or effects of cancer treatment (Brooks, 2012).

Diagnostic biomarkers

Diagnostic biomarkers are used as tools for the identification of patients that have a specific disease or an abnormal medical condition. To identify a specific cancer, the expression pattern of certain tumor-specific proteins is often used as diagnostic biomarkers, together with clinical information as tumor location and morphology (e.g. PSA expression to diagnose prostate cancer) (DeMatteis, 1992). The importance of a correct diagnosis is at time a matter of life and death for a patient, due to choice of and response to therapy.

Prognostic biomarkers

Prognostic biomarkers give information on disease outcome for a patient and correlates to tumor recurrence. Clinical parameters like tumor size, number of metastatic sites, or tumor risk grade can serve this purpose. A prognosticator can also be an elevated expression of a certain protein or lack of expression of the same. A genetic alteration may carry prognostic value for a patient, e.g. amplification of *MYCN* indicates poor prognosis in neuroblastoma (Schwab, 1997).

Predictive biomarkers

Predictive biomarkers can be used to characterize the patient´s disease in order to determine whether that individual is a suitable candidate for a certain treatment modality. With an increasing awareness of the heterogeneity among tumors (Reya *et al.*, 2001), the need for improved selection of patients for a given anticancer treatment is evident. Individual patients within a tumor disease may be treated with different therapies in order to obtain optimal outcome.

Predictive biomarkers, i.e. specific gene mutations or expression of certain proteins, might function as tools in the search for such tailored therapies. Furthermore, the term predictive biomarker is also used when referring to a patient´s response to a drug, and may further be used as a term for the therapy target itself. In this sense, the predictive biomarker could be referred to as a *therapeutic biomarker* (e.g. breast cancer with *ERBB2* amplifications respond to therapy with monoclonal antibodies (i.e. Herceptin®) that targets the growth factor receptor protein ERBB2 (Baselga *et al.*, 1998).

Biomarkers are evaluated in order to acquire relevant knowledge about a disease entity and translate that information into clinical practice. In the search for novel biomarkers, single factors are often found to correlate to the biological state investigated. The ultimate biomarker would be one that allows unequivocal distinction of that state. However, in tumor biology where multiple cellular processes might be essential for a certain tumor entity, it might be too simple to rely on a single biomarker to predict prognosis or response to treatment. Instead, a set of biomarkers may provide a more accurate prediction. High- throughput technologies including global genome analysis and proteomics may provide an expression profile of several factors that may be a signature for such a prediction (Oldenhuis *et al.*, 2008; Brooks, 2012).

GASTROINTESTINAL STROMAL TUMOR (GIST)

Incidence of GIST

Tumors may arise from almost any dividing cell in the body. Most human tumors (80%) originate from epithelial tissues. Sarcomas are derived from cells of connective and supporting tissues, i.e. muscle, nerves, fat, bone, cartilage, synovial tissue, or blood vessels. Sarcomas are also named mesenchymal tumors and represent 1% of all adult tumors (Weinberg, 2007). Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor in the gastrointestinal tract with an estimated incidence of approximately 10-20 cases per million inhabitants annually (Nilsson *et al.*, 2005; Tryggvason *et al.*, 2005; Tzen *et al.*, 2007). GISTs are rare compared with other tumors in the gastrointestinal tract, and account for about 2% of gastric malignancies. GIST usually affects the elderly, but they are also seen in younger age-groups, e.g. so called pediatric GIST (preferentially seen in young women) (Benesch *et al.*, 2009). The median age for sporadic GIST has been reported to be about 60-70 years and affects men and women with equal frequency (Nilsson *et al.*, 2005; Joensuu *et al.*, 2011; Rossi *et al.*, 2011).

Clinical presentation and histopathological characteristics of GIST

GIST arises in the muscular wall along the entire digestive system. The most frequent primary sites are the stomach (about 60%) and the small intestine (25-30%), followed by the colo-rectum (5%), and the esophagus (3%). On rare occasions, primary GISTs are reported in extragastrointestinal locations (e.g. omentum, mesentery, or retroperitoneum) (Corless & Heinrich, 2008; Joensuu *et al.*, 2011; Rossi *et al.,* 2011). The clinical spectrum of GIST is divergent, including indolent tumors and tumors with aggressive behavior. Tumor size may vary from small tumors less than 2 cm, to large tumors exceeding 30 cm. The diagnosis of GIST is established on their characteristic morphology and expression of the KIT protein. GISTs show variable cellularity regardless of malignancy and may be composed of spindle-shaped cells (60-70%), epitheloid cells (15%), or a mixture of both (15-20%) (Rossi *et al.*, 2011) (Figure 1).

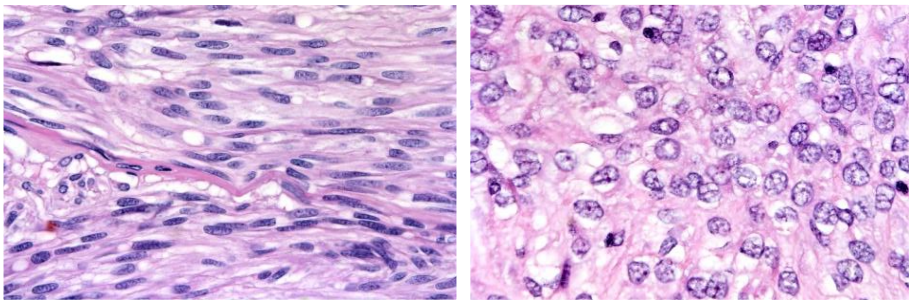

Figure 1. Hematoxylin & eosin staining of GIST.
Left) Spindle cell GIST. *Right*) Epitheloid GIST.

Previously, the majority of GISTs were regarded as benign tumors. However, most GISTs, including small incidentally detected tumors, have been shown to have metastatic capability (Corless *et al.*, 2002). In fact, up to 50% of all GISTs have shown to be metastatic at diagnosis. The most common metastatic sites for GIST are the peritoneum and the liver, and rarely the lymph nodes, lung or bone (DeMatteo *et al.*, 2000). GISTs give rise to symptoms due to local effects of the primary or its metastases. The most frequent symptoms are abdominal pain, gastrointestinal obstruction or bleeding. Since GISTs normally grow non-invasively, the tumors may become large until a palpable mass has developed.

The overall 5-year survival rate of GIST is about 54%, and the 5-year disease-free survival rate is about 45% after radical surgery (R0 resection) (DeMatteo *et al.*, 2000, Gold *et al.*, 2009). However, about half of all GISTs are metastatic at presentation, and the median overall survival for these patients was reported to 19 months prior to targeted therapy. With the introduction of tyrosine kinase inhibitors (TKIs), e.g. imatinib, the median overall survival for metastatic GIST patients has extended to more than 50 months (Van Glabbeke *et al.*, 2007).

Diagnostic biomarkers in GIST

The two most specific and sensitive diagnostic biomarkers for GIST are protein expression of KIT (CD177) (Hornick and Fletcher, 2002) and anoctamin 1 (ANO1) (also named DOG1) (Espinosa *et al.*, 2008), which are positive in about 95% of all GIST (Miettinen *et al.*, 2009) (Figure 2).

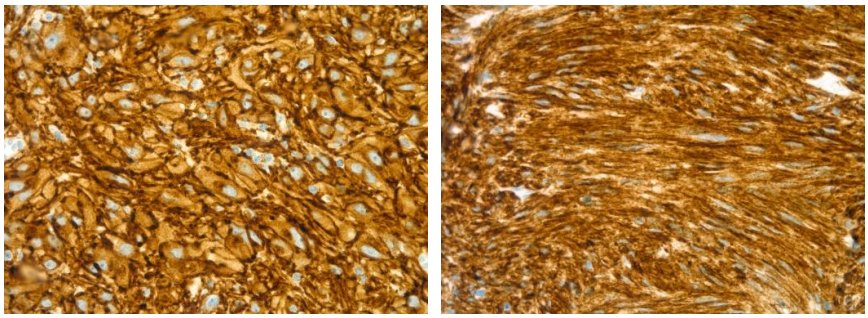

Figure 2. *Left)* KIT and *right)* ANO1 immunohistochemical staining of GIST

A small subset of GIST (less than 3% of the tumors) stain negatively for both KIT and ANO1, preferentially gastric epitheliod GISTs (Miettinen *et al.*, 2009), which makes the diagnosis more difficult for this group. Therefore, additional immunohistochemical markers to improve GIST diagnosis have been searched for. CA2 (carbonic anhydrase II) is one proposed novel marker which is expressed in 95% of GISTs (Parkkila *et al.*, 2009). CA2 expression was independent of tumor site and did not show positive staining in other tested malignancies, indicating an additional diagnostic value. PKCΘ (protein kinase C theta), is another biomarker frequently expressed in GISTs and therefore proposed as a diagnostic marker (Blay *et al.*, 2004, Duensing *et al.*, 2004). CD34 has previously been used to diagnose GIST. However, this marker has lower sensitivity and is only expressed in 70-80% of the tumors, mainly in gastric GIST (Miettinen and Lasota, 2006). GIST can be difficult to distinguish from other abdominal soft tissue tumors, which may show positive immunoreactivity for KIT and/or ANO1, including schwannomas, angiosarcomas, peritoneal leiomyomatosis, uterine type retroperitoneal leiomyomas, metastatic melanomas, and synovial sarcomas (Miettinen *et al.*, 2009). Hence, a panel of biomarkers (e.g. KIT, ANO1, S-100, SMA, desmin, and CD34) is often used to establish the final diagnosis of GIST.

GISTs show neuroendocrine phenotype and express peptide receptors

Neuroendocrine tumor (NET) cells share a set of common properties including expression of storage vesicles, which can be divided into two types: large dense-core vesicles (LDCV) and synaptic-like microvesicles (SLMV). These vesicles contain peptide hormones and biologically active amines (Rindi *et al.*, 2004). Release of hormones from NET cells is frequently regulated by G-protein coupled receptors. The diagnosis of neuroendocrine (NE) differentiation is based on

typical morphology and the expression of vesicle proteins, e.g. chromogranin A (CHGA) and synaptophysin (Feldman and Eiden, 2003). NE differentiation may also include expression of hormones and peptide receptors. Bümming *et al.* (2007) identified the expression of several synaptic vesicle proteins (e.g. SV2, synapsin 1, synaptobrevin, and amphiphysin) in GIST, indicating a possible NE phenotype in these tumors (Jakobsen *et al.*, 2001; Bümming *et al.*, 2007). The search for a hormonal activity in GIST has revealed production of the appetite-stimulating peptide hormone ghrelin (Ekeblad *et al.*, 2006). GIST has also been demonstrated to express peptide receptors including bombesin subtype 2 receptor, cholecystokinin subtype 2 receptor, vasoactive intestinal peptide subtype 2, and somatostatin receptors (SSTR) (Reubi *et al.*, 2004; Palmieri *et al.*, 2007). SSTRs are G-protein coupled membrane receptors occurring in five different subtypes (SSTR1-5) (Patel, 1999). SSTR2 is the most widely expressed SSTR subtype in certain NETs (e.g. midgut carcinoids) (Nilsson *et al.*, 1998). SSTR2 & 5 are frequently used as targets for both diagnostic and therapeutic purposes in NETs, utilizing the binding and internalization of radiolabeled somatostatin analogs (e.g. ^{111}In-DTPA-octreotide and ^{177}Lu-DOTA-octreotate) to these receptors (Kwekkeboom *et al.*, 2010; Swärd *et al.*, 2008). In GIST, SSTRs have been demonstrated with variable protein expression in subsets of tumors (Reubi *et al.*, 2004; Palmieri *et al.*, 2007). The SSTR expression pattern in GIST may enable peptide receptor-mediated radiotherapy (PRRT) as a treatment option for certain patients.

Molecular pathology in GIST

GISTs have a phenotype similar to ICC

GISTs have a phenotype similar to the interstitial cells of Cajal (ICC) (e.g. expression of KIT) and are therefore thought to be derived from ICC, or from a precursor cell (Kindblom *et al.*, 1998). ICC progenitor cells have been identified in the murine stomach and shown to be KIT^{low}, $CD44^{+}$, $CD34^{+}$ (Bardsley *et al.*, 2010). ICCs form an intimate network within the intestinal wall, transducing signals from the nervous system to muscle cells to control motility. ICCs are therefore referred to as "pacemaker cells" (Faussone-Pellegrini, 1992). ICC and GIST have a close phenotypic resemblance, e.g. strong expression of KIT receptor tyrosine kinase protein (Kindblom *et al.*, 1998; Sircar *et al.*, 1999). ICCs are dependent on a regulated *KIT* proto-oncogene expression for their normal development from a mesenchymal progenitor cell into a gastrointestinal pacemaker cell (Faussone-Pellegrini, 1992). In a pioneering publication, Hirota *et al.* (1998) showed that gain of function mutations in *KIT* was a pathogenic event in the development of GIST. Later studies have indicated that interactions between KIT and ETS variant 1 (ETV1) are necessary for both ICC and GIST development (Chi *et al.*, 2010) (Figure 3).

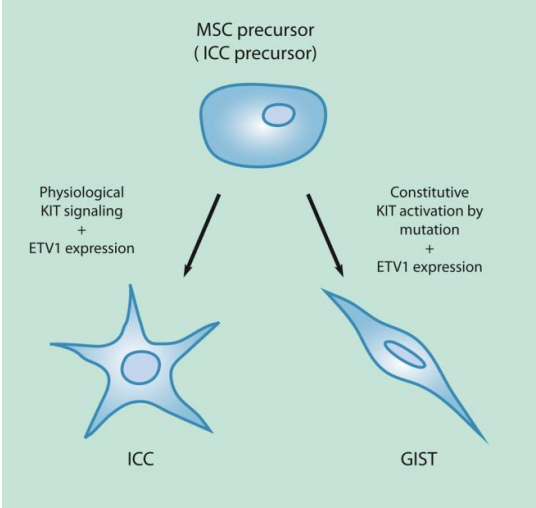

Figure 3. KIT and ETV1 cooperate in the development of ICC and GIST. ICC develop from mesenchymal stem cell (MSC) precursors (ICC precursors) as a result of physiological KIT signaling and ETV1 expression. GISTs develop from MSC precursors (ICC precursors) as a result of constitutive activation of KIT signaling and ETV1 expression.

ETV1 is a critical regulator of oncogenesis in GIST

ETV1 is a member of the *ETS* gene family acting as a transcriptional activator by binding to consensus DNA sequences. Several fusion genes have been identified with *ETV1* as one of the partners (e.g. *EWS-ETV1*). Formation of an *ETV1* fusion gene is considered to be the oncogenetic event in the development of Ewing sarcoma and prostate cancer (Im *et al.*, 2000; Tomlins *et al.*, 2007). Furthermore, full-length *ETV1* has been reported to be over expressed in GIST, as well as in melanoma and prostate cancer (Chi *et al.*, 2010; Jané-Valbuena *et al.*, 2010; Gasi *et al.*, 2011). ETV1 protein may bind enhancer elements in promoter regions of several target genes, and thus regulates biological processes such as cell proliferation, differentiation, and migration. A recent study by Sawyers and colleagues identified several genes, normally overexpressed in GIST and ICC, to be dependent on *ETV1* expression. Knockdown of *ETV1* in GIST cell lines reduced the expression of e.g. *PROM1*, *DUSP6*, and *TIMP3*, and caused reduction of cell proliferation (Chi *et al.*, 2010). ETV1 was suggested to be a key regulator in the development of ICC, as well as the formation of GIST from ICC precursor cells, by cooperating with activated KIT. Mutated KIT activates MAPK signaling and thus inhibits proteasomal degradation of ETV1, which in turn is required for GIST development (Chi *et al.*, 2010; Rubin, 2010) (Figure 3). Together with KIT, ETV1 has been proposed to be a lineage survival factor in GIST.

Receptor tyrosine kinases - KIT and PDGFRA

Receptor tyrosine kinases (TKs) are receptors for growth factors and have the ability to induce proliferation in normal cells. KIT and platelet derived growth factor receptor alfa (PDGFRA) are evolutionary homologues of the type III receptor tyrosine kinase family. Their natural ligands are stem cell factor (SCF) and platelet-derived growth factor (PDGF), respectively. These receptor TKs are complex proteins with a similar structure, consisting of a cytoplasmic domain, a transmembrane domain, and a unique extracellular ligand-binding domain. The

intracellular part comprises two TK domains including one ATP-binding region (TK1) and one activation loop (TK2). When the receptor is bound to its ligand, two subunits of the receptor dimerize and allow TK domains to autophosphorylate. This promotes a catalytic cleft in the juxtamembrane domain (close to the plasma membrane) to open up with direct access to substrate molecules. Further phosphorylations of the TKs activate downstream signaling pathways causing cell proliferation (Lennartsson *et al.*, 2005). Activation of the KIT receptor engages downstream signaling pathways, including PI3K/AKT, RAS/MAPK, and JAK/STAT, which promotes cell cycle activation, proliferation, and inhibition of apoptosis (Lennartsson *et al.*, 2005; Corless *et al.*, 2011) (Figure 4). Normal KIT receptor function is essential for the development of ICC, melanocytes, germ cells, and hematopoetic cells (Fleischman, 1993). The PDGFRA receptor activates signaling pathways similar to those of KIT, but also phospholipase Cγ (PLCγ), which promotes cell growth and motility (Andrae *et al.*, 2008).

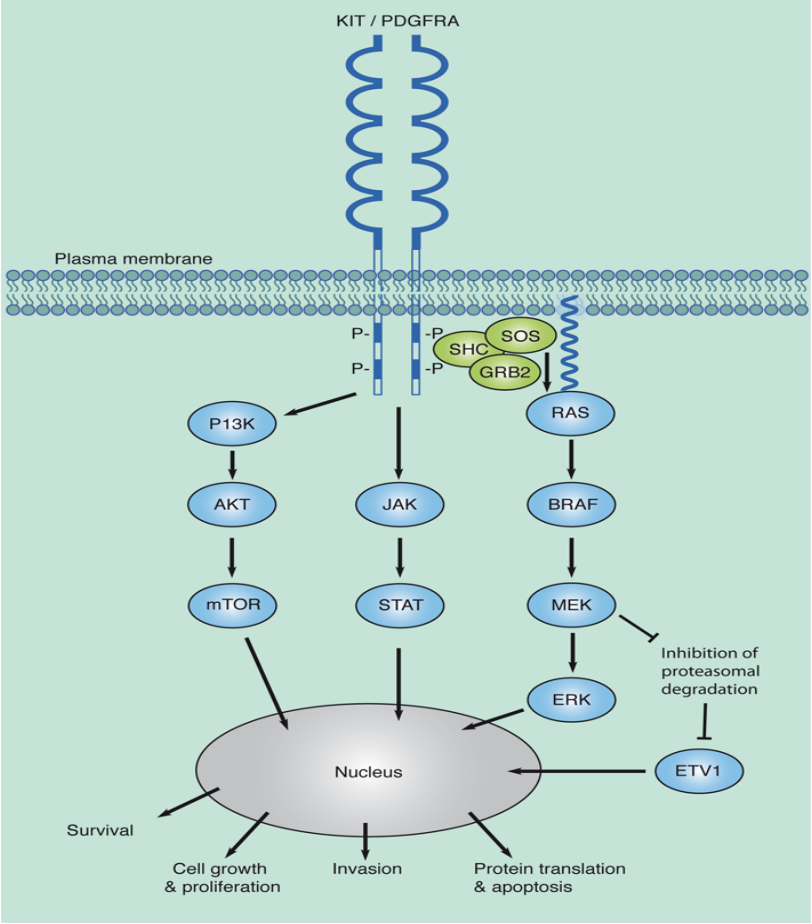

Figure 4. KIT and PDGFRA signaling pathways in GIST. Phosphorylated KIT or PDGFRA activates PI3K/AKT/mTOR, JAK/STAT, and RAS/MAPK signaling and stabilization of ETV1, causing cell cycle activation, cell proliferation, and inhibition of apoptosis.

Oncogenic mutations in sporadic GIST

Gain of function mutations in the proto-oncogenes encoding either KIT or PDGFRA may result in receptor configuration changes, which induce constitutive activation of the receptors in the absence of ligand. The ligand-independent activation initiates receptor dimerization and autophosphorylation, which activates a cascade of downstream signaling pathways promoting sustained growth in the cell. Since the demonstration that *KIT* mutations are involved in the formation of GIST (Hirota *et al.*, 1998) the genetics of this tumor have been extensively investigated. Approximately 80% of all sporadic GISTs carry activating *KIT* mutations, most frequently affecting exon 11 (68%) and exon 9 (10%), and rarely exon 13 and 17 (Corless & Heinrich, 2008; Lasota *et al.*, 2008) (Figure 5). GISTs lacking *KIT* mutations may instead carry mutations in the homologous gene, *PDGFRA* (Heinrich *et al.*, 2003a). *PDGFRA* mutations are found in 5%–8% of all GISTs, most frequently affecting exon 18 (6%), followed by mutations in exon 12 and 14 (Figure 5). Mutations in *KIT* and *PDGFRA* are mutually exclusive, and *PDGFRA* mutated GISTs often lack immunoreactivity for KIT protein (Corless *et al.*, 2011). Recent studies have shown that mutations in *KIT* (and *PDGFRA*) may not be sufficient to induce GIST, which requires transcriptional regulation by *ETV1* as well (Chi *et al.*, 2010).

In about 15% of all GISTs no mutations are detected in either *KIT* or *PDGFRA* (wt GIST). Tarn *et al.* (2008) showed that a subgroup of wt pediatric GIST instead had amplification in the insulin growth-factor receptor 1 (*IGF1R*). Mutations in B-rapidly accelerated fibrosarcoma (*BRAF*) have also been reported to occur in subsets of GIST (Agaram *et al.*, 2008). In *BRAF*-mutated GIST, ETV1 may be stabilized without constitutive activation of KIT or PDGFR.

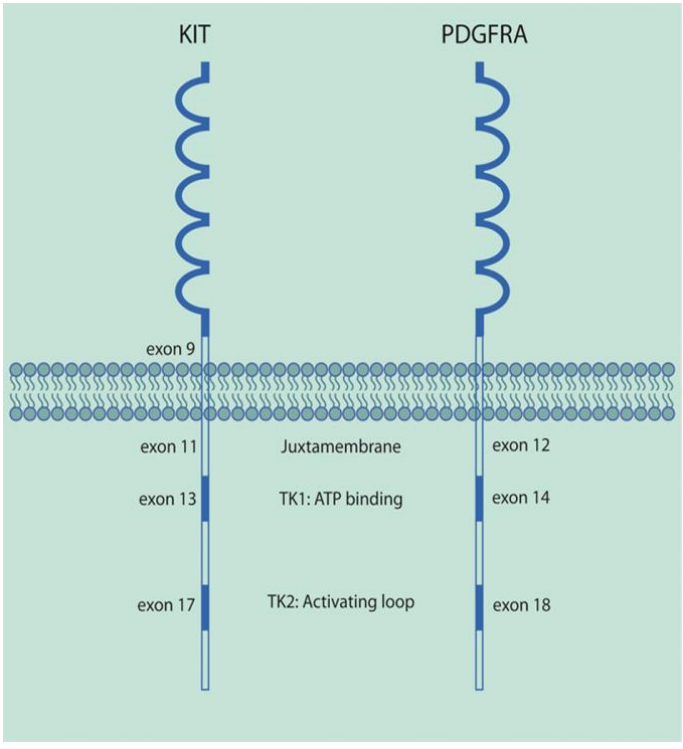

Figure 5. Localization of KIT and PDGFRA mutations in GIST. Gain of function mutations are clustered in the juxtamembrane domain and the tyrosine kinase domains (TK1 or TK2).

Although, the majority of GISTs are sporadic, GISTs may also occur in familiar settings, e.g. neurofibromatosis type 1 (NF1) or as part of Carney Triad or Carney-Stratakis Syndrome (Bümming *et al.*, 2006). In patients with Carney Triad, or Carney-Stratakis Syndrome, GIST lack *KIT* and *PDGFRA* mutations and may have reduced activity of mitochondrial complex II. In some patients this is due to germ-line mutations inactivating the succinate dehydrogenase (SDH) enzymes (Janeway *et al.*, 2011).

Prognostic biomarkers in GIST

Several prognostic factors have been proposed for GIST, e.g. morphological features such as tumor size, pleomorphism, mitotic count, high micro-vessel density, invasive growth, and tumor necrosis, but also genetic factors and molecular biomarkers (Miettinen *et al.*, 2002).

NIH risk score and tumor location

The only widely accepted system for prognostication of GIST is the NIH risk score (Fletcher *et al.*, 2002), which is based on both tumor size and mitotic index, and estimates the metastatic risk of primary R0-resected GISTs (Table 1).

Table 1. NIH risk score scheme by Fletcher *et al.*, (2002)

	Size (cm)	Mitotic count (per 50 hpf)
Very low-risk	<2	<5
Low-risk	2-5	<5
Intermediate-risk	<5	5-10
	5-10	<5
High-risk	>5	>5
	any	>10
	>10	any

The NIH risk score is used for risk assessment of GIST and serves to select patients that will be offered adjuvant therapy (e.g. patients with high-risk tumors). However, this scheme has been questioned by numerous authors suggesting that primary tumor location should be included in the model for better prognostication of GIST (Huang *et al.*, 2007; Miettinen and Lasota, 2006). GISTs located in the stomach are less aggressive compared to tumors of the small intestine, or other primary locations. A revised risk scheme that included primary tumor location was noted in the 2007 NCCN risk stratification (Demetri *et al.*,

2007). Comparing the two risk score schemes on a large set of GIST patients, Goh *et al* (2008) proved the revised risk score to predict patient outcome more effectively. However, variations were observed for the recurrence rates in the high-risk group of GIST. Another model to predict prognosis in GIST was suggested by Nilsson *et al.* (2005), which included proliferative activity (Ki67) and tumor size as prognosticators. The Ki76/size model showed distinct prognostic value.

Cytogenetic factors

Besides clinical and morphological biomarkers, genetic abnormalities have shown to provide prognostic information for GIST patients. A limited number of chromosomal abnormalities are observed in GIST tumors, including monosomy of chromosome 14, partial losses of 14q or 22q are the most frequent cytogenetic findings (Yang *et al.*, 2008). Gunawan *et al* (2007) found that loss of 14q characterized gastric tumors with stable karyotypes and favorable clinical course. In contrast, loss on chromosome 1p characterized small intestinal GISTs with a more aggressive course. Loss of heterozygocity (LOH) on chromosome 9p has also been shown to associate with a malignant phenotype, possibly due to loss of the tumor suppressor gene *CDKN2A* (Sabah *et al.*, 2004; Wozniac *et al.*, 2007; Corless *et al.*, 2011).

KIT and PDGFRA mutations

Mutational status of *KIT* and *PDGFRA* has been shown to influence patient survival, although several research groups report conflicting results. Furthermore, the correlation between type of mutation and patient outcome is influenced by the introduction of tyrosine kinase inhibitors (TKIs) in the treatment of advanced GIST. Several groups have observed a correlation between poor prognosis and *KIT* exon 11 mutations. Especially tumors with *KIT* exon 11 deletions (primarily involving codons Trp557 and/or Lys558) have been reported to associate with poor prognosis (Andersson *et al.*, 2006, Martin *et al.*, 2005, Singer *et al.*, 2002). Other studies failed to confirm such results (DeMatteo *et al.*, 2008). Poor prognosis has also been demonstrated for tumors with *KIT* exon 9 and *KIT* exon 13 mutations (Lasota *et al.*, 2008, Antonescu *et al.*, 2003), whereas *PDGFRA* mutant GISTs have been reported to be less aggressive (Lasota *et al.*, 2006). Although mutational analysis may have prognostic impact, the importance of *KIT* and *PDGFRA* mutations as prognostic indicators remains to be determined. On the other hand, mutational status has been shown to be useful as a predictive biomarker for the response to TKI (Corless *et al.*, 2011).

Table 2. Molecular biomarkers with prognostic information in GIST

Marker *(Alias)*	Cellular and physiological functions	Role in tumor disease	Prognostic implications in GIST	Key reference for GIST
CA2	Carbonic anhydrase II. Catalyzes the hydration of carbon dioxide and maintains pH homeostasis in the body	Influences invasive growth and is up-regulated in the endothelium of tumor vessels. High expression correlates with poor prognosis (leukemia, gliomas), but also to benign behaviour (pancreatic and colorectal cancers)	Expressed in the majority of GISTs (proposed as diagnostic marker). High CA2 expression correlates with longer RFS. Analyzed on 159 GISTs. TKI status not reported	Parkkila *et al* , 2009
CDKN2A *(p16 ^{INK4A} p16)*	Cyclin-dependent kinase inhibitor 2A. Tumor suppressor. Inhibits RB1 via CDK4-complex. Controls G1-transition in the cell cycle	Inactivated in the majority of cancers by LOH or promoter methylations. Contradictory, elevated protein expression is involved in cellular senescence and cancer progression (breast cancer)	Low expression of nuclear and high expression of cytoplasmic CDKN2A is associated with short RFS. Analyzed on 120 GISTs. No TKI treatment	Haller *et al.,* 2010
DPP4 *(CD26)*	Dipeptide-peptidase IV. Membrane associated glycoprotein. Regulates cytokine and chemokine activity by cleaving N-terminal dipeptides	Regulates the invasiveness of tumor cells by interacting with extracellular matrix (ECM). Expressed in colorectal CSCs and associated with metastatic capacity	High expression is correlated with short RFS in gastric GIST. Analyzed on 152 TKI naïve GISTs, except for seven patients which recieved adjuvant TKI	Yamaguchi *et al.,* 2008
EZR *(VIL2 villin 2)*	Ezrin. Linker between plasma membrane and actin cytoskeleton. Involved in cell adhesion, migration, and cell surface organization	Regulates cell migration, invasiveness and metastasis. Associated with poor prognosis (breast cancer and rhabdomyosarcoma)	High expression is associated with non-gastric location and short RFS. Analyzed by IHC on 347 GISTs. No TKI treatment	Wei *et al.,* 2009
KCTD12 *(pfetin)*	Potassium channel tetra domain 12. Regulator of the pharmacology and kinetics of the GABA-B receptor response	Not reported	High protein expression is correlated with long RFS. Analyzed on 299 GISTs. No TKI treatment	Kubota *et al.,* 2011
OPN *(BSP-1)*	Osteopontin. Glycophosphoprotein. Binds to several integrin receptors. Acts as an immune modulator	Cytikine regulating tumorigenicity, tumor progression, and metastasis. Overexpressed in several malignancies. Associated with poor prognosis (lung and ovarian cancers)	High protein expression is correlated with short RFS. Analyzed on 99 GISTs. No TKI treatment	Hsu *et al.,* 2010
RKIP *(PEBP1)*	Raf Kinase inhibitory protein. Regulates several intracellular signaling cascades e.g. RAF/MAPK	Inhibits tumor cell migration and invasion through regulation of the extracellular matrix (human prostate cancer cells, glioma)	Loss of RKIP expression is associated with poor RFS. Analyzed on 70 GISTs. Three patients were treated with TKI	Martinho *et al.,* 2009

Molecular biomarkers for prognosis

A number of molecular biomarkers have been shown to provide information regarding GIST patient survival. Reported markers with prognostic relevance in GIST include CA2, CDKN1B, CDKN2A, DPP4, EZR, HIF1A, KCTD12, NES, PTGS2, RKIP, SKP2, and VEGF. A summary of biomarkers with survival data in GIST is presented in Table 2. Comparative studies on biomarker performance in GIST have not been carried out and influence of TK inhibition on the usefulness has not been evaluated. None of these proposed molecular biomarkers have to date been widely introduced into clinical practice.

Treatment of GIST

Surgery is the primary treatment of GIST and approximately half of all patients are cured with surgery only. Since radiation and chemotherapy are largely ineffective in GIST, other treatment options must be explored for patients with unresectable or metastatic tumors. Today, many of these patients are treated with imatinib as first-line therapy resulting in prolonged patient survival. However, resistance to TKI is an increasing clinical problem, as well as non-responsive tumors, which urges the development of novel treatment.

Imatinib and sunitinib

Inhibition of activated KIT, or PDGFRA, by palliative therapy with TKIs has dramatically improved the survival of patients with high-risk GIST. Imatinib (Gleevec ™) is the first-line option for unresectable GIST (Bümming *et al.*, 2003; Van Glabbeke *et al.*, 2007). Imatinib binds to the ATP binding site of the intracellular tyrosine kinase domain of KIT (or PGFRA). This prevents the kinase from transferring phosphate from ATP to tyrosine residues of the substrate complex (e.g. SHC, GRB2, SOS), which leads to the inactivation of downstream signaling pathways (Lennartsson *et al.*, 2005). Approximately 80% of all GISTs show primary response to imatinib treatment. However, response rates relate to mutational status. Tumors with *KIT* exon 11 mutations are most responsive to imatinib (70–85% response rate) due to favorable conformation changes of the juxtamembrane part of the receptor (Corless et al., 2011). Tumors with *KIT* exon 9 mutations demonstrate an intermediate responsiveness (25–48% response rate), while tumors with *KIT* exon 13 or 17 mutations or no mutations respond poorly. Consequently, the prognosis is better for imatinib-treated patients with *KIT* exon 11 mutations than for patients with *KIT* exon 9, or wt GIST (Heinrich, 2003b; Corless *et al.*, 2011). *Primary resistance* to imatinib (i.e. resistance within 6 months of treatment) is seen in 10–15% of all GISTs, including wt tumors and tumors with *PDGFRA* exon 18 (D842V) mutations. *Secondary resistance* to imatinib (i.e. resistance 6 months after initial response to treatment) develops in 40% of the patients. The most important mechanism for secondary resistance to imatinib

involves acquired mutations in *KIT* and *PDGFRA* (Faivre *et al.*, 2007; Corless & Heinrich, 2008; Liegl *et al.*, 2008; Wang WL *et al.*, 2011).

Sunitinib (Sutent™) was introduced as second-line therapy (Younus *et al.*, 2010). Sunitinib has broader activity profile than imatinib (KIT and PDGFRA), and inhibits other receptor TKs such as VEGFR1-3, RET, and FLT3. Inactivation of these pathways leads to inhibition of cell proliferation and angiogenesis (Chow and Eckardt, 2007). However, the duration of response is often limited for sunitinib-treated patients (approximately one year) (Wang WL *et al.*, 2011). Resistance to imatinib and sunitinib emphasizes the need for alternative therapeutic strategies.

Therapeutic biomarkers

Novel TKIs have been developed and investigated as treatment of patients that develop resistance to imatinib and sunitinib. Dasatinib, nilotinib, and sorafenib have shown advantageous activity profiles related to a *PDGFRA* mutant (D842V) and wt GIST (Kim and Zalupski, 2011). Several other therapeutic biomarkers have been investigated for GIST, including molecules belonging to the downstream signaling pathways that are activated by KIT and PDGFRA. Drugs targeting these pathways have been evaluated with promising results in experimental studies. Several inhibitors of the PI3K/AKT/mTOR signaling cascade have been investigated, with the most promising effects observed for mammalian target of rapamycin (mTOR) inhibitors (everolimus) (Bauer *et al.*, 2007; Schöffski *et al.*, 2010).

Insulin-like growth factor 1 receptor (IGF1R), like KIT and PDGFRA, activates signaling pathways as RAS/MAPK and PI3K/AKT/mTOR. IGF1R has been demonstrated to have a significant potential as a therapeutic target for IGF1R-driven tumors, which has been investigated experimentally with promising results (Tognon and Sorensen, 2011). IGF1R has been suggested as a treatment option in wt GIST (Tarn et al., 2008, Braconi *et al.*, 2008). However, the pathogenetic role of IGF1R in GIST remains to be elucidated.

The conformation of a constitutively activated KIT is stabilized by a chaperon molecule, heat shock protein 90 (HSP90), which have been suggested as a therapeutic target in GIST and other tumors (Bauer *et al.*, 2006). Several HSP90 inhibitors have been developed and proved to have anti-tumor effects in experimental studies. By combining the HSP90 inhibitor retaspimycin hydrochloride (IPI-504) and imatinib/sunitinib in xenograft GIST, treatment effects were shown to be enhanced (Floris et al., 2011). However, IPI-504 was recently suspended from phase III trials for to safety reasons.

Combinations of different TKIs, or TKIs together with other drugs have been suggested to be advantageous treatment option for specific GIST mutants (e.g. mTOR inhibitors combined with imatinib) (Nilsson *et al.*, 2009).

GIST may show a NE phenotype including expression of microvesicle proteins and peptide hormone receptors (Reubi *et al.*, 2004; Bümming *et al.*, 2007). NETs can be treated with radiolabeled somatostatin analogs (e.g. ^{177}Lu-DOTA-octreotate and ^{90}Y-DOTA-octreotide) targeting SSTRs (Kwekkeboom *et al.*, 2010; Swärd *et al.*, 2010). Expression of SSTR in GIST suggests that peptide receptor-mediated radiotherapy (PRRT) may be a future treatment alternative for these tumors (Figure 6).

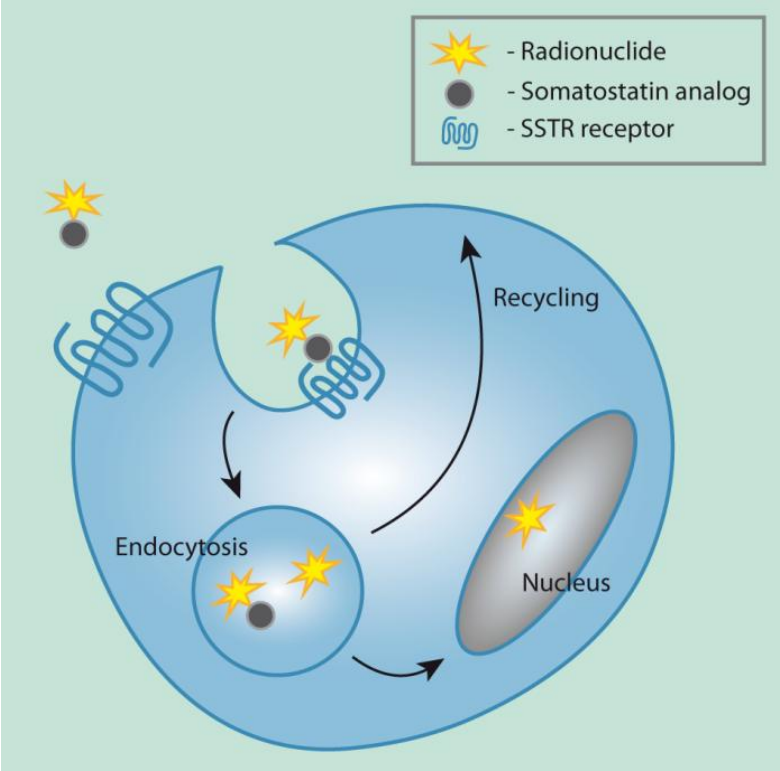

Figure 6. Peptide receptor-mediated internalization of radiolabeled somatostatin analogs, e.g. binding of radiolabeled somatostatin analog to SSTR, internalization via receptor-mediated endocytosis, and accumulation in cellular organelles.

OBJECTIVES OF THE THESIS

The general aim of this study was to characterize expression profiles for GIST in order to identify novel biomarkers for prognosis and therapy.

The specific aims were:

- to characterize the gene expression profiles of GISTs in relation to mutational status in *KIT* and *PDGFRA* in order to identify genes involved in tumor progression and aggressive behavior.

- to evaluate the usefulness and prognostic power of immuno-histochemical biomarkers as predictors of survival in patients with GIST.

- to analyze the expression of somatostatin receptors (SSTRs) in GIST, and evaluate SSTR as a therapeutic target for peptide receptor-mediated radiotherapy (PRRT).

MATERIALS AND METHODS

Tumor material

Paraffin embedded tumor material of GIST (Paper I, II, III). A total of 263 well characterized GISTs were used in immunohistochemical analyses in the three studies included in this thesis. In Paper I we used tumor biopsies from 204 patients with mutational status on *KIT* and *PDGFRA*, including 180 patients with R0-resected tumors and complete survival data and follow-up. In Paper II, tumor biopsies from 205 patients with R0-resected tumors and complete survival data and follow-up were used. The paraffin-embedded tumor material in Paper I and II, were arranged in a tissue microarray (TMA). These patients were only treated surgically for their tumor disease. The TMA is based on the population-based study of GISTs (1983–2001) by Nilsson *et al.* (2005). Mutational status and survival data have been published previously by Andersson *et al.* (2006). In Paper III we used paraffin-embedded tumor material from 34 patients that underwent resection of GIST at the Sahlgrenska University Hospital, Gothenburg, Sweden (1997–2008). Some of these patients were given TKI therapy.

Frozen tumor biopsies from GIST (Paper I, II). In Paper I, tumor biopsies from 16 patients (7 with gastric, 7 with small intestinal, and 2 with rectal GIST) were used for both expression microarray and quantitative real-time PCR (qPCR). These patients did not receive imatinib treatment before surgery (imatinib naïve patients). In Paper III, tumor biopsies from 34 patients (16 with gastric, 15 with small intestinal, and 3 with rectal GIST) were analyzed with qPCR.

Primary cell culture of GIST (Paper III). Tumor tissues from two patients were used to establish primary cell culture for radionuclide uptake studies in Paper III, including one gastric and one small intestinal GIST. Cultured tumor cells were characterized and found to express KIT and DOG-1 by immunofluorescence.

GIST patients for diagnostic imaging and activity ratios of ^{111}In (Paper III). Seven GIST patients received 170–240 MBq ^{111}In-DTPA-D-Phe^{1}-octreotide (^{111}In-octreotide) by intravenous injection (Paper III). Diagnostic imaging (scintigraphy) was performed on 6 patients within 24 h after injection. Tumor samples together with blood samples drawn during surgery were collected from five patients and tumor-to-blood ^{111}In activity concentration ratio (T/B) was measured 2–22 days after injection of ^{111}In-octreotide.

Methods

Methods used in this thesis are well established, including gene expression analyses by microarray and qPCR, immunohistochemistry, tumor cell culture, radioactivity measurements and scintigraphy, and a short summary follows below:

Gene expression analysis (Paper I, III). In Paper I, gene expression analysis was performed on 55k whole genome oligonucleotide microarrays (Swegene DNA Microarray Resource Center, Lund, Sweden). In order to extend the patient material, these data were combined with two other published gene expression datasets in a meta-analysis. The probes in all three datasets were processed to share a common annotation and further analyzed for their gene expression fold change. In Paper I and III, qPCR assays were performed in 96-well optical plates using TaqMan® Reverse Transcription Reagents (Applied Biosystems, CA, USA) and analyzed with an ABI Prism® 7500 Fast System SDS.

Immunohistochemical analysis (Paper I, II, III). Immunohistochemical analyses were performed on paraffin embedded tumors. Bound antibodies were visualized using Dako EnVision+ detection systems (DakoCytomation, Denmark) with HRP-labeled polymer and DAB substrate. For the immunohistochemical scoring of biomarkers, a dilution series of each antibody was evaluated on TMA sections. The dilution that resulted in the greatest discrimination in staining pattern between tumor biopsies was chosen for further analysis. In Paper II, each biopsy was scored according to the following criteria: 0, when <10% of tumor cells were labeled; 1+, when 10%-90% of tumor cells were labeled; and 2+, when >90% of tumor cells were labeled.

Cell culture and confocal microscopy (Paper III). Unlike a cell line, a primary cell culture consists of a mixed population of cell types and cells with advantageous growth properties will increase more rapidly *in vitro*. The primary cell cultures of GIST (Paper III) were set on collagen-coated Biocoat® Multiwell Plates (BD Biosciences, MA, USA) with RPMI 1640 medium supplemented with 10% fetal calf serum, L-glutamine, and PEST. Uptake experiments were performed within 4 days in culture and cell quality were again characterized by KIT and ANO1 expression and SSTR1-5 expression by immunofluorescence detection of Alexa Flour conjugated antibodies (Molecular Probes Inc., OR, USA) using confocal microscopy (Zeiss LSM 510 META system).

Binding and internalization of radiolabeled somatostatin analogs (Paper III). The evaluation of binding and internalization (uptake) of the radiolabeled somatostatin analogs ^{177}Lu-octreotate and ^{111}In-octreotide was performed by three different methods. Binding and internalization of ^{177}Lu was investigated in primary cell culture of GIST, where the cells were incubated with ^{177}Lu-octreotate for 48 hours (control cultures were also supplemented with unlabeled octreotide). Amount of surface-bound and internalized ^{177}Lu was measured in a gamma counter (Wallac 1480 WIZARD™; Wallac Oy, Finland). Scintigraphy of ^{111}In-octreotide in GIST patients was performed by a gamma camera (General Electric 400 AC/T; General Electric, London, UK). ^{111}In activity in tumor biopsies and blood samples was measured in the gamma counter, and tumor-to-blood ^{111}In activity concentration ratios were determined.

Statistics (Paper I, II, III). Statistical analyses used in this thesis were performed in the statistical language R (www.r-project.org) or in SPSS (IBM company, NY, USA). Gene expression microarray data was normalized by lowess normalization, and ranked according to average log-fold change and the moderated t-statistic (Paper I). Meta-analysis, combining gene expression profiles from three different data sets, was performed by re-annotating all probes to a common annotation and compare transcripts according to the average fold change (Paper I). Regression based survival analysis was performed using Cox proportional hazards model (Paper I, II). The decision-tree model was calculated using cross-validation (Paper II). Binding efficiencies in studies on cultured cells were evaluated by linear regression (Paper III).

Ethical approval (Paper I, II, III). For the use of clinical materials in Paper I-III, we obtained consent from the patients and approval from the Regional Ethical Review board in Gothenburg, Sweden.

RESULTS AND DISCUSSION

GIST with different KIT and PDGFRA mutations are associated with specific gene expression profiles (Paper I)

Previous studies have shown that mutational status of *KIT* and *PDGFRA* influences prognosis for GIST patients. Notably GISTs with *KIT* exon 11 deletions were associated with short patient survival (Singer *et al.*, 2002; Martin *et al.*, 2005; Andersson *et al.*, 2006). In Paper I, we characterized the gene expression profiles of GISTs with *KIT* exon 11 deletions *vs.* GISTs without *KIT* exon 11 mutations (e.g. wt GIST, as well as GIST with *KIT* exon 9 or *PDGFRA* mutations). Global gene expression analysis using oligonucleotide microarray on a set of 16 tumors demonstrated a large number of genes differentially expressed between the two tumor groups. Meta-analysis of our tumor material including data from two previously published studies (Subramanian *et al.*, 2004; Kang *et al.*, 2005) confirmed our findings. Based on all three data sets (comprising in total 23 GISTs with *KIT* exon 11 deletions, and 28 GISTs with no mutations in *KIT* exon 11), we identified a specific gene expression profile in GISTs carrying *KIT* exon 11 deletions, including up-regulation of *PROM1*, *MMP2*, *FHL1*, *CD34*, *KCTD12*, *CXCL12*, and *EPHA4*, as well as down-regulation of *TGFBI*, *IGFBP5*, *CA2*, and *DLK1*. Differentially expressed genes represented several biological processes including genes related to cell adhesion, cell motility, and cell proliferation. qPCR analysis verified differential expression of the five most up-regulated genes (e.g. *PROM1*, *CD34*, *KIT*, *ANO1*, *MMP2*) and five down-regulated genes (e.g. *DLK1*, *CA2*, *SERPINF1*, *IGFBP5*, *TGFBI*).The most highly up-regulated genes in tumors with *KIT* exon 11 deletions was *PROM1* (*CD133*) as seen in Figure 7. The role of PROM1 in GIST is not known. However, PROM1 has recently been implied as a marker of CSCs in a number of malignancies (e.g. glioblastoma, Ewing sarcoma, prostate and colorectal cancer) (Collins *et al.*, 2005; Suvà *et al.*, 2009; Alison *et al.*, 2011).

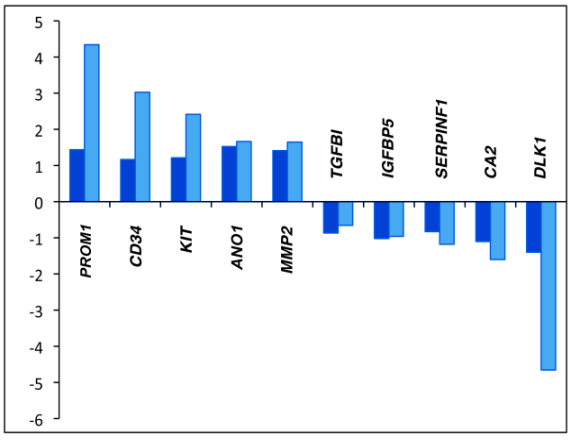

Figure 7. Gene expression analysis of GIST with *KIT* exon 11 deletions compared to GIST without *KIT* exon 11 mutations. Analysis by microarray (dark) and qPCR (light) confirms *PROM1* to be the most highly up-regulated gene in GISTs with *KIT* exon 11 deletions among the verified genes. *PROM1* demonstrated a four-fold increase of expression ($p < 0.05$) between tumor groups in qPCR analysis. The y-axis represents $\log_2$ fold change.

PROM1 ***protein is predominantly expressed in GIST with KIT exon 11 mutations, gastric location, and poor patient survival (Paper I)***

In order to further characterize the expression of PROM1 in GIST, we evaluated tumor biopsies from 204 GIST patients by TMA and immunohistochemistry using monoclonal antibodies against PROM1 (clone AC133). Out of 195 tumor biopsies evaluated for PROM1 staining, 55 showed positive labeling (28%). Comparing PROM1 protein expression and mutational status, we found the highest proportion of PROM1-positive tumors in GISTs with *KIT* exon 11 mutations (41%), with significant correlation between positive PROM1 and *KIT* exon 11 deletions ($p = 1.6 \times 10^{-5}$). Tumors with other *KIT* or *PDGFRA* mutations, or wt tumors, had lower proportion of PROM1-positive tumors (0–17%). Comparing PROM1 protein expression and tumor location, we found higher frequency of positive tumors in stomach (correlation $p = 3.1 \times 10^{-11}$), as compared to small intestine, colon, and rectum. In total, 51/106 (48%) patients with gastric GISTs were positive for PROM1. No association between PROM1-labeling and NIH risk score, tumor size, mitotic count, Ki67-labeling, or histopathological growth patterns was observed. There was a positive correlation between PROM1- and CD34-labeling, possibly due to higher expression of both PROM1 and CD34 in gastric GISTs, compared to GISTs in other locations.

We further evaluated PROM1 as a biomarker to predict survival in GIST patients. Patients with R0-resected tumors and clinical follow-up (n=180) were included in our survival analysis using Cox regression model. Univariate analysis including PROM1 labeling, NIH risk score, and mutational status in *KIT* and *PDGFRA* showed that patients with PROM1-positive GIST had shorter survival compared to patients with PROM1-negative tumors (Figure 8). Patients with high-risk GIST and patients with *KIT* exon 11 deletions also showed shorter overall and recurrence-free survival at univariate analysis. Multivariate analysis including the variables age, sex, NIH risk group, mutational status, CD34, and PROM1 labeling, demonstrated that PROM1 expression provided additional information regarding patient survival compared to all other variables.

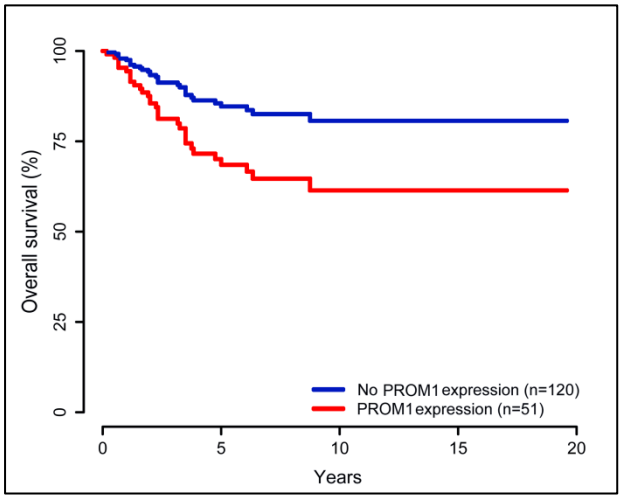

Figure 8. PROM1 protein expression is associated with shorter overall patient survival. Survival curves are calculated from survival data on GIST patients with R0 surgery and estimated by Cox regression (adjusted for age and sex) ($p<0.05$).

Survival analysis was also performed on gastric GISTs alone. Patients with PROM1 expression had significantly shorter survival in the univariate survival analysis. Multivariate Cox analysis demonstrated PROM1 expression and NIH risk score as the only significant variables in the gastric GISTs. Thus, PROM1 labeling provided additional information regarding patient survival compared with age, sex, NIH risk group, and mutational status in all GISTs, as well as in gastric GISTs. Expression of PROM1 in GIST may have therapeutic implications. Patients with PROM1 positive tumors and TKI resistance may be subjected to targeted therapies as indicated by experimental studies using anti-PROM1 (anti-CD133) monoclonal antibodies (Smith *et al.*, 2008; Wang CH *et al.*, 2011).

We showed that the PROM1 protein was associated with shorter patient survival. The fact that PROM1 is a marker of CSCs in a number of solid tumors raised the question whether PROM1 also was a marker for CSCs in GIST. Two studies have addressed this question (Bozzi *et al.*, 2011; Chen *et al.*, 2011). Both studies confirmed high expression of PROM1 in GIST as compared to other sarcomas. However, PROM1 and CD44 were shown to be universally expressed in a majority of GIST with higher mRNA value in gastric tumors compared to small intestinal tumors (Chen *et al.*, 2011). Therefore, expression of PROM1 was suggested to be a lineage marker in GIST rather than a marker for CSCs. This hypothesis was further supported by studies on the transcriptional regulator *ETV1*, which acts as a lineage survival factor in GIST. *ETV* knockdown experiments in GIST cell lines demonstrated down-regulation of lineage markers including PROM1 (Chi *et al.*, 2010). Characterization of sorted GIST cells showed enrichment of $PROM1^{-}$ cells in side populations, most likely representing CSCs. Also, $PROM1^{-}$ cells were shown to form more colonies and were more invasive than $PROM1^{+}$ cells in matrigel assays (Bozzi *et al.*, 2011; Chen *et al.*, 2011). These data suggest that PROM1 is not a CSC marker of GIST.

Evaluation of immunohistochemical biomarkers with prognostic relevance in GIST (Paper II)

The selection of biomarkers investigated for their prognostic value in Paper II was based on previously published studies on prognostic biomarkers in GIST as well as differentially expressed genes provided by the gene expression profile in Paper I. We designed a comprehensive study on protein labeling of these biomarkers in 205 GIST tumors arranged in a TMA. All immunohistochemical stainings were evaluated according to one single scoring system on tumor biopsies incubated at antibody-dilutions that discriminated negative and positive tumors on the TMA. We evaluated a series of biomarkers for their prognostic relevance in GIST patients: CA2, CDKN2A (p16), CXCL12, DPP4 (CD26), EPHA4, EZR, FHL1, KCTD12 (pfetin), MMP2, and PROM1 (CD133). Univariate analysis showed that expression of CA2, CDKN2A, CXCL12, DPP4, EPHA4, and FHL1 correlated with recurrence-free survival (RFS) in GIST patients. Expression of PROM1 only showed marginally statistic significance at univariate analysis in this series. PROM1 and the other biomarkers that not reached statistical significance in univariate analysis were excluded from further evaluation. Multivariate analysis showed that expression patterns of the biomarkers CA2, EPHA4, and FHL1 provided information on patient survival in addition to NIH risk score. CDKN2A and DPP4 on the other hand, showed strong association with NIH risk score. Since primary tumor location is a prognosticator in GIST (i.e. gastric GIST are less aggressive than tumors with other locations), we investigated the confounding effects between NIH risk score, tumor location and expression patterns for the individual biomarkers. Four biomarkers provided additional information to patient survival in the multivariate analysis: CA2, DPP4, EPHA4, and FHL1. Expression patterns of the immunohistochemical biomarkers in relation to NIH risk score and survival are visualized in a heatmap (Figure 9).

To optimize the prediction of survival in GIST patients, we constructed decision-tree models combining NIH risk score and expression of immunohistochemical biomarkers. NIH risk was divided in a high-risk group and into a group of lower risks (very low-, low-, and intermediate-risk). NIH risk score was found to be the single most effective prognosticator in the model. In Figure 10, we present a decision-tree model sorted by NIH risk score, in which the addition of CA2 and EPHA4 expression patterns were shown to predict patient survival more accurately than the score alone.

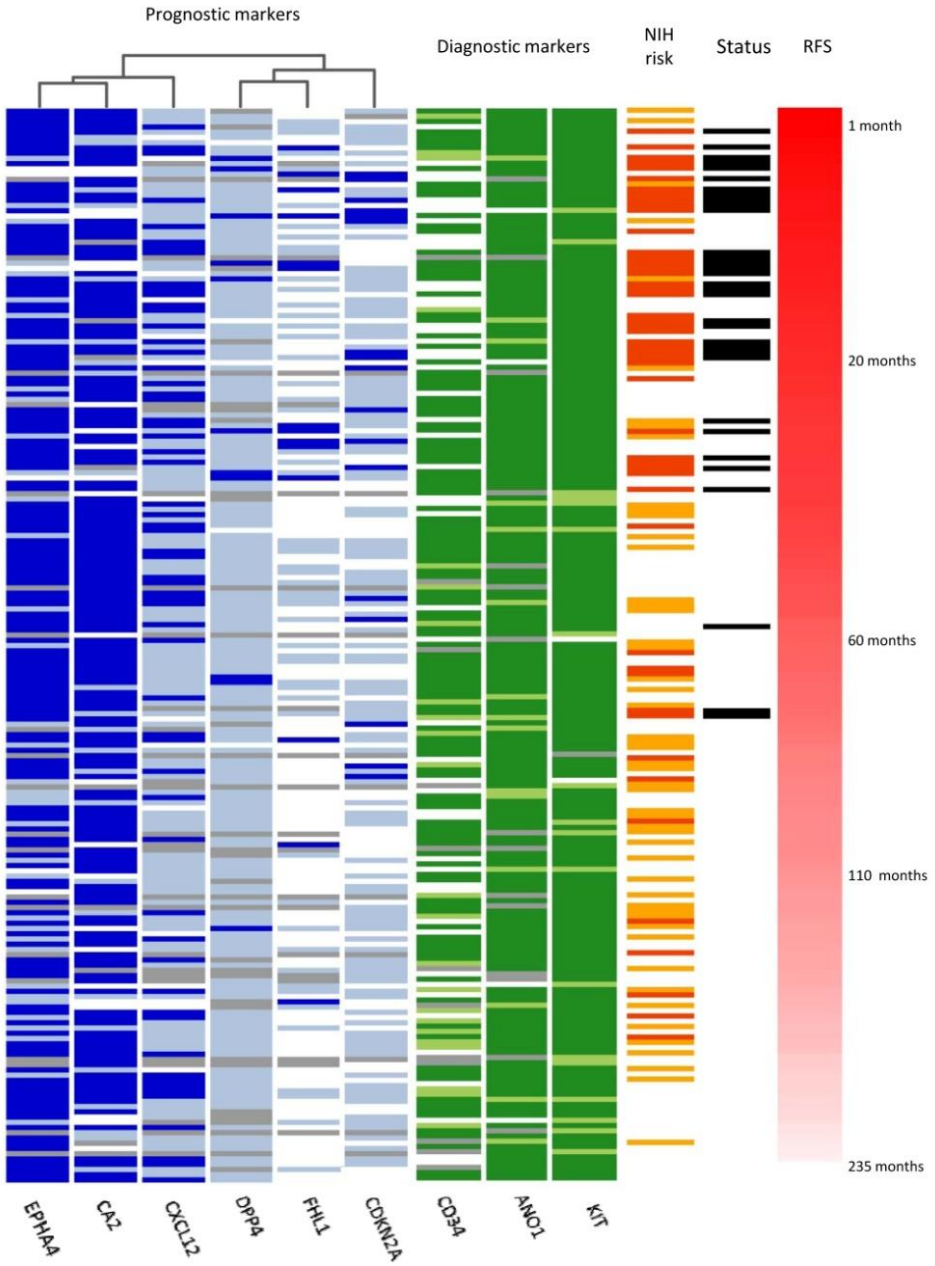

Figure 9. Heatmap representing expression patterns of biomarkers in 204 GISTs, in relation to NIH risk score and patients survival. Protein expressions of biomarkers with prognostic value are presented in blue: EPHA4, CA2, CXCL12, DPP4, FHL1, and CDKN2A. Expressions of diagnostic biomarkers are presented in green: KIT, ANO1, and CD34. Dark blue/green: >90% tumor cells labeled, light blue/green: 10-90% tumor cells labeled, white: no labeling, grey: no data. NIH risk score is presented as red: high risk, orange: intermediate risk, white: low or very low risk. Data is arranged according to increasing recurrence-free survival (RFS) (1 month at the top, 235 months in the bottom), and unsupervised hierarchical clustering. Patients that were dead of disease (DOD) at follow-up are indicated with a black line.

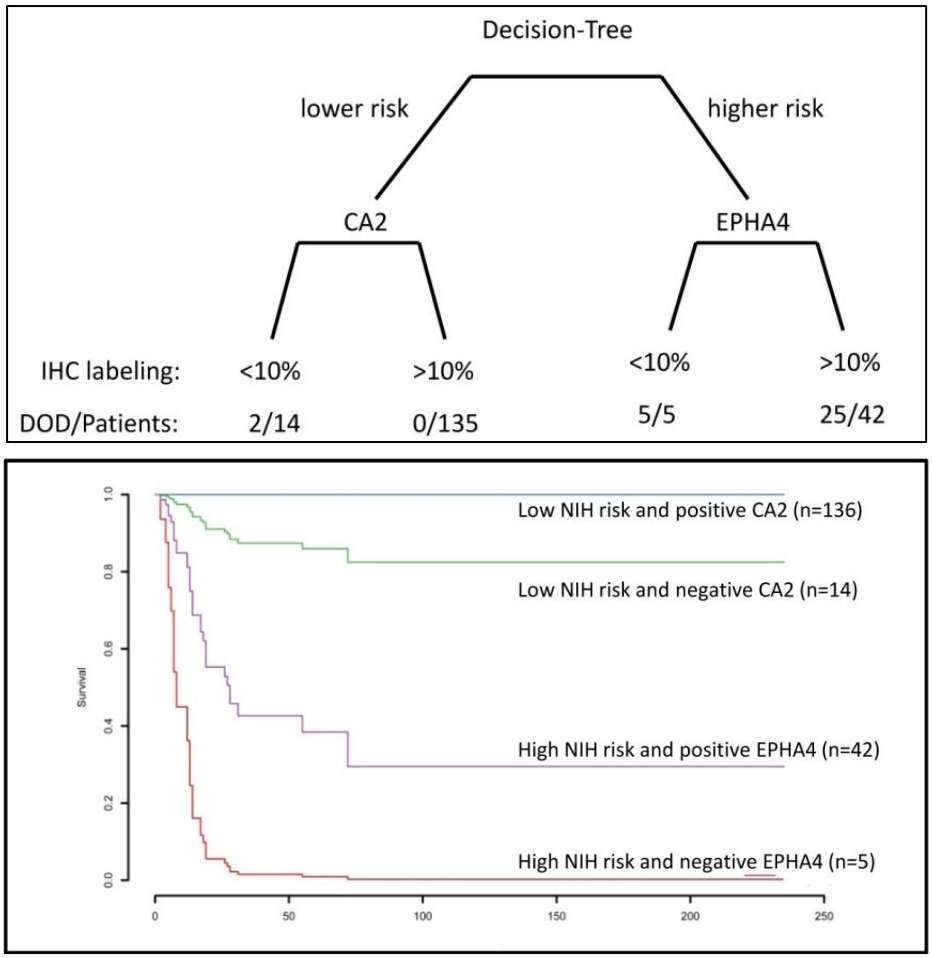

Figure 10. Decision-tree model improves prognostication of GIST tumors. *Top)* In the lower risk group of tumors, negative CA2 staining identified patients with shorter survival. Only two patients in the lower risk group died of GIST disease (DOD), both of which had CA2 negative tumors. In the high-risk group of tumors, negative EPHA4 staining identified patients with short survival. All patients in the high-risk group with negative EPHA4 died of GIST disease (DOD). *Bottom)* Cox survival curves of recurrence-free survival for the GIST patients in the groups created by the decision-tree model.

This is the first study attempting to compare a series of immunohistochemical biomarkers as prognosticators of GIST patient survival. In order to facilitate the comparison of biomarkers we used a single cohort of well-characterized tumors arranged in a TMA and optimized antibodies and dilutions to obtain maximal discrimination between tumor samples. Furthermore, the same scoring system was used for all biomarkers. However, the choices of antibodies, antibody dilutions, and scoring system will affect the observed expression levels and consequently influence the correlations between biomarker expression patterns and clinicopathological parameters. Under these experimental conditions, we could confirm the usefulness of CDKN2A and DPP4 as prognostic markers in imatinib-naïve GIST, and add CA2, CXCL12, EPHA4, and FHL1 as novel biomarkers of GIST patient survival.

GISTs regularly express SSTR1 and SSTR2 (Paper III)

GISTs have been proposed to have a NE phenotype due to their expression of peptide hormones (Ekeblad *et al.*, 2006), synaptic vesicle proteins (Reubi *et al.*, 2004; Bümming *et al.*, 2007), and peptide receptors (Reubi *et al.*, 2004; Palmieri *et al.*, 2007). Expression of SSTR in GIST has been reported, but data on SSTR subtypes and expression levels are limited (Reubi *et al.*, 2004; Palmieri *et al.*, 2007). In order to further characterize the expression of SSTRs in GIST, we performed expression profiling of SSTR subtypes 1–5 on biopsies from 34 GISTs. qPCR analysis demonstrated expression of SSTR subtype 1 & 2 in the majority of tumors, while SSTR3–5 were only expressed at low levels. Median mRNA expression levels of SSTR1 & 2 were similar, but SSTR1 showed a more variable expression pattern with high expression levels in individual tumors. The same tumors were also analyzed by immunohistochemical staining for SSTR1–5 receptor proteins. SSTR1 & 2 showed positive staining in all GISTs and SSTR3–5 in a subset of tumors. Altogether, qPCR or immunohistochemistry thus detected expression of SSTR1 & 2 in all analyzed tumors and SSTR3–5 in a subset of GISTs. The divergent results in individual tumors between qPCR and immunohistochemical analyses most likely reflect intra-tumoral heterogeneity or differences in sensitivity between the methods. From these data we conclude that GISTs express multiple SSTR subtypes. However, GISTs mainly express SSTR1 & 2, as opposed to gastrointestinal carcinoids which mainly express SSTR2 & 5 (Nilsson *et al.*, 1998), suggesting differences in receptor profiles that need to be considered when designing SSTR-mediated radiotherapy. We identified individual patients in our series that might have been candidates for SSTR-mediated therapy, including tumors of both high-risk and low-risk. Patients with high SSTR expression, who had developed primary or secondary resistance to imatinib, could have been suitable for SSTR-mediated therapy.

GIST cells bind and internalize ^{177}Lu–octreotate in vitro (Paper III)

Binding and internalization of a radiolabeled somatostatin analog ^{177}Lu-octreotate in GIST tumor cells were studied in primary cell cultures from one gastric GIST and one small intestinal GIST. GIST cells in primary culture expressed SSTR1 & 2, as determined by immunocytochemistry and confocal laser microscopy. Cultured cells demonstrated specific binding and uptake of ^{177}Lu, as shown in Figure 11. The amount of internalized ^{177}Lu increased continuously during the incubation period, whereas surface-bound ^{177}Lu increased marginally, indicating receptor saturation. The relative amount of internalized *vs.* surface-bound ^{177}Lu was 8-fold at 48 hours. In control experiments, both binding and internalization of ^{177}Lu were effectively reduced by addition of unlabeled octreotide to the culture media. The two primary cell cultures established from GIST patients bound and internalized ^{177}Lu to the same degree, suggesting that these patients could have been candidates for SSTR-mediated radiotherapy.

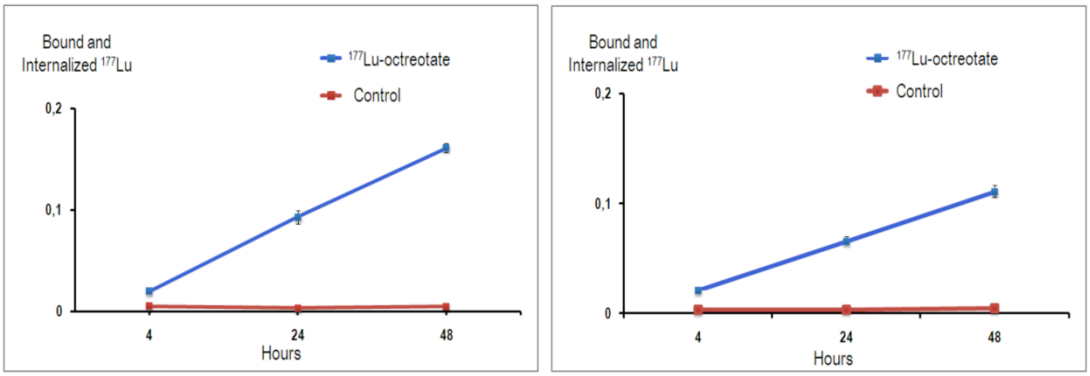

Figure 11. Binding and internalization of ^{177}Lu-octreotate in primary cell culture of GIST. Experiments of a gastric GIST (*left*) and a small intestinal GIST (*right*) showed significant binding and internalization of ^{177}Lu–octreotate, as compared to control experiments. Error bars are smaller than symbols.

GISTs can be visualized by scintigraphy using ^{111}In–octreotide (Paper III)

To assess the binding of radiolabeled somatostatin analogs and the possibility of visualizing SSTR-expressing tumors by scintigraphy, GIST patients were injected with the radiolabeled somatostatin analog ^{111}In-octreotide preoperatively. Positive tumor imaging was obtained in three out of six GIST patients, indicating that radiolabeled somatostatin analogs localize to GIST tumors in significant amounts (Figure 12). Thus, octreotide scintigraphy via SSTR may be a possible diagnostic tool for GIST patients, which necessitates a future prospective study. To assess the amount of internalized ^{111}In in GIST tumors, the ^{111}In activity concentration (%IA/g tissue) was measured in excised tumor biopsies and blood samples from five patients. Measurements confirmed specific uptake of ^{111}In, but the tumor-to-blood activity concentration ratio (T/B) varied between the five patients (range 8 to 96). T/B values in GIST were similar to those obtained in medullary thyroid carcinoma (Forssell-Aronsson *et al.*, 2000 and 2004), but lower than those obtained in midgut carcinoids (Hashemi *et al.*, 2003; Forssell-Aronsson *et al.*, 2004). Relatively high values were observed in individual GIST tumors, indicating high expression of SSTR, internalization of radionuclide, and rapid clearance of ^{111}In-octreotide. The need for new treatment strategies in GIST is evident for those patients that develop primary or secondary resistance to imatinib and sunitinib. Our experimental results indicate that SSTR-targeted radiotherapy may be a future therapeutic option for certain patients.

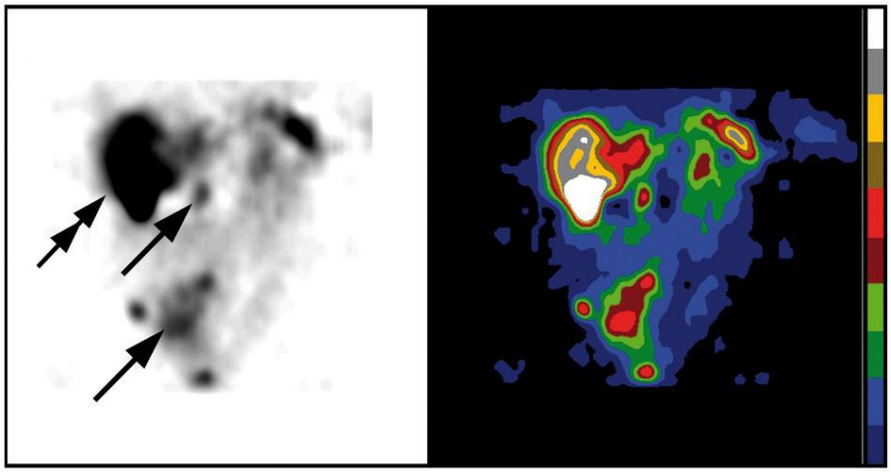

Figure 12. Octreotide scintigraphy of a GIST patient injected with ^{111}In-octreotide, demonstrating an unresectable primary small intestinal GIST, and multiple abdominal (arrows) and liver (double arrow) recurrences.

Our present data on SSTR1 & 2 expression indicate, heterogeneity between GIST lesions in the same patient. Good tumor visualization was obtained by scintigraphy in two patients despite low SSTR mRNA expression in the analyzed biopsies. Such tumor heterogeneity, with marked differences in ^{111}In binding has also been observed in patients with NETs, e.g. carcinoids and endocrine pancreatic tumors (Forssell-Aronsson *et al.*, 2004).

The somatostatin analogs used in this study (i.e. octreotide and octreotate) are preferentially designed to bind to SSTR2 & 5. NET cells mainly express SSTR2 and have been demonstrated to bind and internalize radiolabeled octreotide *in vitro* (Andersson *et al.*, 1996). A majority of NETs are also positive by ^{111}In-octreotide scintigraphy (Teunissen *et al.*, 2011). In contrast, GISTs showed a relatively lower expression of SSTR2, which may explain a lower frequency of scintigraphically positive tumors. The high uptake of ^{111}In and ^{177}Lu observed in GIST most likely represents internalization together with octreotide/octreotate after binding to SSTR2. To optimize the diagnostic imaging in GIST, the somatostatin analog should instead be tailored for binding to SSTR1, or to all SSTR subtypes (pan analogs).

SUMMARY AND CONCLUSIONS

The main observations and conclusions of these investigations can be summarized as follows.

- GISTs with different mutations in *KIT* and *PDGFRA* showed distinct gene expression profiles. PROM1 (CD133) is highly expressed in GIST with *KIT* exon 11 mutations, gastric location, and poor prognosis. PROM1 is a prognostic biomarker and may serve as a therapeutic target in GIST.

- Several immunohistochemical biomarkers predict survival in GIST patients. Some of these correlate with NIH risk score (e.g. CDKN2A, DPP4), while others (e.g. CA2, EPHA4, and FHL1) provided additional prognostic information to this score. A decision-tree model combining NIH risk score and the expression of CA2 and EPHA4 improves the prediction of patient survival.

- Several GISTs express SSTR1 & 2. Primary tumor cell cultures bind and internalize ^{177}Lu when incubated with radiolabeled somatostatin analog ^{177}Lu-octreotate. Diagnostic imaging by ^{111}In-octreotide visualizes GIST in individual patients. Tumor-to-blood activity ratios in excised tumor biopsies indicated ^{111}In uptake ratios, adequate for radiotherapy, which may be a novel treatment strategy in case of resistance to TKI.

FUTURE PERSPECTIVES

Identification of cancer stem cells (CSCs) in GIST

CSCs are promising targets for curative cancer therapy. CSCs may be targeted by interfering with self renewal pathways such as Wnt/β-catenin, sonic hedgehog, and Notch. Alternatively, CSCs can be targeted by epigenetic manipulations (e.g. microRNA modifications and HDAC inhibition) or by induced cell differentiation (e.g. by BMP or retinoic acid stimulation) (Alison *et al.*, 2011). Targeted therapy in GIST requires identification of CSCs and characterization of their signaling pathways. Our gene expression profiling study (Paper I) has identified genes associated with *KIT* mutational status, including *PROM1* (*CD133*), which is often regarded as a marker for CSCs in several tumor types, including sarcomas (Suvà *et al.*, 2009; Tirino *et al.*, 2011). However, recent studies by Bozzi *et al.* (2011) and Chen *et al.* (2011) have suggested that PROM1 is a lineage marker for GIST, as supported by studies in GIST cell lines (Chi *et al.*, 2010). ETV1 was identified as a lineage survival factor for ICC and GIST. A molecular signature was associated with *ETV1* activation, including high expression of *PROM1, DUSP6*, *TIMP3, CTSL1,* and *PTPRE* (Chi *et al.*, 2010). In our profiling study on tumor biopsies from GIST, we did not find any correlation between *ETV1* and *PROM1* expression, suggesting that *PROM1* may be regulated by alternative mechanisms. Characterization of CSC and ICC phenotype in GIST is therefore essential for the development of CSC targeted therapy.

Identification of predictive biomarkers for therapy in GIST

Biomarkers in oncology may be used to assist in diagnosis, prognostication, or prediction of response to therapy. In GIST, several diagnostic biomarkers have been proposed (e.g. KIT, ANO1, PKCΘ, CA2, and CD34), which have greatly facilitated the diagnosis of GIST. In this thesis, we have evaluated a number of prognostic biomarkers using a cohort of GIST patients treated with surgery only. We were able to identify several prognostic biomarkers and study their relationship to survival and clinicopathological variables. These biomarkers need to be validated prospectively. Biomarkers for therapeutic response are rare, but *KIT* and *PDGFRA* mutations have been correlated to TKI resistance. Interestingly, one of the prognostic biomarkers identified in this study, i.e. PROM1, was recently shown to correlate to imatinib response on isolated GIST cells (i.e. imatinib-sensitive GIST cell lines expressed PROM1, while imatinib-resistant cell lines did not) (Chen *et al.*, 2011). Further studies evaluating prognostic biomarkers in relation to the response to TKI inhibition should be performed.

Optimizing peptide receptor mediated radiotherapy (PRRT) in GIST

Resistance to TKIs develops in the majority of GIST patients and many patients lack therapeutic alternatives despite a good performance status. The results presented in this thesis suggest that SSTR-mediated therapy may become a treatment option in GIST, in selected patients. The optimal setting for PPRT may be as adjuvant treatment after intentionally curative surgery, in a situation where residual small, or microscopic (R1), tumors may be present. Optimizing peptide receptor-mediated radiotherapy in GIST will require implementation of innovative approaches, including the use of new targeting molecules and novel delivery systems. Optimization of radiolabeled somatostatin analogs including affinity studies, as well as studies on biodistribution and dosimetry, will be needed before clinical trials of PRRT via SSTR can be initiated in GIST patients. An alternative approach would be to develop PRRT using other peptide receptors that are highly expressed in GIST (e.g. bombesin 2, CCK2, VPAC2). Radiolabeled bombesin analogs are available (Faintuch *et al.*, 2008) and should be evaluated in GIST.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to everyone who has contributed to this thesis in one way or another. My warmest thanks to all of you!

I would particularly like to thank:

My supervisor, Professor ***Ola Nilsson,*** for giving me the opportunity and inspiration to complete this thesis in your group. Your expertise and enthusiasm for science have been invaluable. Also, thank you for hours of interesting discussions by the microscope ('etta, nolla, tvåa, bom'). I greatly appreciate you for always being so supportive and encouraging in topics concerning both work and life.

My extended group and co-authors. My co-supervisor Associate professor ***Bengt Nilsson***, for sharing your clinical expertise and assistance. Professor ***Håkan Ahlman*** and Professor ***Eva Forssell-Aronsson***, for interesting discussions and bringing new energy to this project. ***Johanna Dalmo***, for waiting for me in the culvert.

My closest group of colleagues, including ***Ellinor, Gülay, Yvonne, Pauline, Linda, Malin, Anki, Siw,*** and ***Anders,*** for your experienced lab skills, and for contributions and engagement in my research. I especially value your friendship, and all lunches talking about cooking and house renovations. I also thank ***Ann*** and ***Lilian***, for helping me in the isotope-lab and for always being so friendly.

Erik Kristiansson, my amazing statistical expert and co-author, for your never-ending positive energy and for always taking time for my questions. I appreciate our inspiring lunches and discussions regarding everything from statistical methods to ice-hockey. I will always value our friendship.

Members of my first group. Professor ***Lars-Gunnar Kindblom*** for introducing me to the field of cancer research during my initial year. ***Johanna***, for letting me choose green color at the test tube racks. ***Sibylle***, ***Anna-Carin,*** and ***Carina*** for 'daring' to handle the TMA block. Thank you for always being so nice!

All other *friends* and *co-workers* at Lundberg Laboratory for Cancer Research and Sahlgrenska Cancer Center for always making it a pleasure to go to work. Special thanks to my dear friends ***Marta***, ***Annika***, and ***Stina*** for laughs and valuable discussions, and for sharing all the greatest things in life at the same time. ***Fredrik*** (den onde) and ***Fredrik*** (den gode) for always making me feel special. ***Emman***, ***Gittan***, ***Mattias***, ***Christer***, ***Pernilla, Ywonne***, ***Barbro, Karin***, ***Tajana***, ***Anna***, ***Birgitta***, and ***Camilla*** for nice coffe breaks, great 'Kräftskivor', and many laughs.

Ulric for brilliant assistance with color and print, and ***Ivan*** for helping me with my computer (especially when the text is very small).

My wonderful ***friends*** outside of work, you make my life rich. I hope you know how much you mean to me! ***Tove***, for your wise reflections of the essence of life. ***Annica***, ***Sandra***, and ***Susanna*** for everything that we share and discussions about everything but science. All friends in Göteborg - ***Anisi-Eriksson***, ***Asting***, ***Claesson***, ***Ehnar***, ***Högberg***, ***Larsson***, ***Persson***, ***Vänerhav,*** and ***Wickström-Andersson*** for making Göteborg the best place on earth. Thank you for dinners, play dates, and ski-trips! And all my other wonderful friends who give me positive energy, no one forgotten!

All lovely members of my crazy family in Linköping - ***cousins***, ***aunts,*** and ***uncles***, for your never-ending love and energy. You mean the world to me!

My closest family, for your endless support through the years.
My wonderful ***Mamma*** & ***Pappa***, I love you so much for always believing in me and always caring for our best. Ni är världsklass! My parents in law, ***Håkan*** & ***Gunnel*** for your warmth and support with our children, and ***Gunilla*** & ***Lennart*** for care and encouragements. ***Johanna*** & ***Edvard*** for your love and generosity, and for making us laugh. ***Katarina***, ***Lars***, ***Erika*** and ***My*** for the joy you give me and my family when we meet.

Finally, all my love to ***Torbjörn***, ***Elmer***, and ***Melvin***. You are the best family I could ever wish for! Thank you for showing me the true meaning of life. Jag älskar er!

This work was supported by grants from: *the Swedish Cancer Society, the Swedish Research Council, Sahlgrenska Academy (the government ALF agreement), the Assar Gabrielsson Research Foundation, the Johan Jansson Research Foundation, the Sahlgrenska University Hospital Foundations, the Royal Society of Arts and Sciences in Gothenburg, and BioCARE - a National Strategic Research Program at University of Gothenburg.*

REFERENCES

Agaram NP, Wong GC, Guo T, Maki RG, Singer S, Dematteo RP, Besmer P, Antonescu CR. 2008. Novel V600E BRAF mutations in imatinib-naive and imatinib-resistant gastrointestinal stromal tumors. Genes Chromosomes Cancer 47(10):853-9.

Al-Hajj M, Clarke MF. 2004. Self-renewal and solid tumor stem cells. Oncogene 23(43):7274-82.

Alison MR, Lim SM, Nicholson LJ. 2011. Cancer stem cells: problems for therapy? J Pathol 223(2):147-61.

Ambros V. 2004. The functions of animal microRNAs. Nature 431(7006):350-5.

Andersson J, Bümming P, Meis-Kindblom JM, Sihto H, Nupponen N, Joensuu H, Oden A, Gustavsson B, Kindblom LG, Nilsson B. 2006. Gastrointestinal stromal tumors with KIT exon 11 deletions are associated with poor prognosis. Gastroenterology 130(6):1573-81.

Andersson P, Forssell-Aronsson E, Johanson V, Wängberg B, Nilsson O, Fjälling M, Ahlman H. 1996. Internalization of indium-111 into human neuroendocrine tumor cells after incubation with indium-111-DTPA-D-Phe1-octreotide. J Nucl Med 37(12):2002-6.

Andrae J, Gallini R, Betsholtz C. 2008. Role of platelet-derived growth factors in physiology and medicine. Genes Dev 22(10):1276-312.

Antonescu CR, Sommer G, Sarran L, Tschernyavsky SJ, Riedel E, Woodruff JM, Robson M, Maki R, Brennan MF, Ladanyi M and others. 2003. Association of KIT exon 9 mutations with nongastric primary site and aggressive behavior: KIT mutation analysis and clinical correlates of 120 gastrointestinal stromal tumors. Clin Cancer Res 9(9):3329-37.

Bardsley MR, Horvath VJ, Asuzu DT, Lorincz A, Redelman D, Hayashi Y, Popko LN, Young DL, Lomberk GA, Urrutia RA and others. 2010. Kitlow stem cells cause resistance to Kit/platelet-derived growth factor alpha inhibitors in murine gastrointestinal stromal tumors. Gastroenterology 139(3):942-52.

Baselga J, Norton L, Albanell J, Kim YM, Mendelsohn J. 1998. Recombinant humanized anti-HER2 antibody (Herceptin) enhances the antitumor activity of paclitaxel and doxorubicin against HER2/neu overexpressing human breast cancer xenografts. Cancer Res 58(13):2825-31.

Bauer S, Duensing A, Demetri GD, Fletcher JA. 2007. KIT oncogenic signaling mechanisms in imatinib-resistant gastrointestinal stromal tumor: PI3-kinase/AKT is a crucial survival pathway. Oncogene 26(54):7560-8.

Bauer S, Yu LK, Demetri GD, Fletcher JA. 2006. Heat shock protein 90 inhibition in imatinib-resistant gastrointestinal stromal tumor. Cancer Res 66(18):9153-61.

Benesch M, Wardelmann E, Ferrari A, Brennan B, Verschuur A. 2009. Gastrointestinal stromal tumors (GIST) in children and adolescents: A comprehensive review of the current literature. Pediatr Blood Cancer 53(7):1171-9.

Biomarker Definitions Working Group. 2001. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin Pharmacol Ther 69(3):89-95.

Bishop JM. 1991. Molecular themes in oncogenesis. Cell 64(2):235-48.

Blay P, Astudillo A, Buesa JM, Campo E, Abad M, Garcia-Garcia J, Miquel R, Marco V, Sierra M, Losa R and others. 2004. Protein kinase C theta is highly expressed in gastrointestinal stromal tumors but not in other mesenchymal neoplasias. Clin Cancer Res 10(12 Pt 1):4089-95.

Bonci D. 2010. MicroRNA-21 as therapeutic target in cancer and cardiovascular disease. Recent Pat Cardiovasc Drug Discov 5(3):156-61.

Bozzi F, Conca E, Manenti G, Negri T, Brich S, Gronchi A, Pierotti MA, Tamborini E, Pilotti S. 2011. High CD133 expression levels in gastrointestinal stromal tumors. Cytometry B Clin Cytom 80(4):238-47.

Braconi C, Bracci R, Bearzi I, Bianchi F, Sabato S, Mandolesi A, Belvederesi L, Cascinu S, Valeri N, Cellerino R. 2008. Insulin-like growth factor (IGF) 1 and 2 help to predict disease outcome in GIST patients. Ann Oncol 19(7):1293-8.

Brooks JD. 2012. Translational genomics: The challenge of developing cancer biomarkers. Genome Res 22(2):183-7.

Buchdunger E, Cioffi CL, Law N, Stover D, Ohno-Jones S, Druker BJ, Lydon NB. 2000. Abl protein-tyrosine kinase inhibitor STI571 inhibits in vitro signal transduction mediated by c-kit and platelet-derived growth factor receptors. J Pharmacol Exp Ther 295(1):139-45.

Bümming P, Nilsson O, Ahlman H, Welbencer A, Andersson MK, Sjölund K, Nilsson B. 2007. Gastrointestinal stromal tumors regularly express synaptic vesicle proteins: evidence of a neuroendocrine phenotype. Endocr Relat Cancer 14(3):853-63.

Bümming P, Andersson J, Meis-Kindblom JM, Klingenstierna H, Engstrom K, Stierner U, Wängberg B, Jansson S, Ahlman H, Kindblom LG and others. 2003. Neoadjuvant, adjuvant and palliative treatment of gastrointestinal stromal tumours (GIST) with imatinib: a centre-based study of 17 patients. Br J Cancer 89(3):460-4.

Bümming P, Nilsson B, Sörensen J, Nilsson O, Ahlman H. 2006. Use of 2-tracer PET to diagnose gastrointestinal stromal tumour and pheochromocytoma in patients with Carney triad and neurofibromatosis type 1. Scand J Gastroenterol 41(5):626-30.

Charafe-Jauffret E, Ginestier C, Iovino F, Wicinski J, Cervera N, Finetti P, Hur MH, Diebel ME, Monville F, Dutcher J and others. 2009. Breast cancer cell lines contain functional cancer stem cells with metastatic capacity and a distinct molecular signature. Cancer Res 69(4):1302-13.

Chen J, Guo T, Zhang L, Qin LX, Singer S, Maki RG, Taguchi T, Dematteo R, Besmer P, Antonescu CR. 2011. CD133 and CD44 are universally overexpressed in GIST and do not represent cancer stem cell markers. Genes Chromosomes Cancer 51(2):186-95.

Chi P, Chen Y, Zhang L, Guo X, Wongvipat J, Shamu T, Fletcher JA, Dewell S, Maki RG, Zheng D and others. 2010. ETV1 is a lineage survival factor that cooperates with KIT in gastrointestinal stromal tumours. Nature 467(7317):849-53.

Chow LQ, Eckhardt SG. 2007. Sunitinib: from rational design to clinical efficacy. J Clin Oncol 25(7):884-96.

Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ. 2005. Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res 65(23):10946-51.

Corless CL, Barnett CM, Heinrich MC. 2011. Gastrointestinal stromal tumours: origin and molecular oncology. Nat Rev Cancer 11(12):865-78.

Corless CL, Heinrich MC. 2008. Molecular pathobiology of gastrointestinal stromal sarcomas. Annu Rev Pathol 3:557-86.

Corless CL, McGreevey L, Haley A, Town A, Heinrich MC. 2002. KIT mutations are common in incidental gastrointestinal stromal tumors one centimeter or less in size. Am J Pathol 160(5):1567-72.

Croce CM. 2008. Oncogenes and cancer. N Engl J Med 358(5):502-11.

de Matteis A. 1992. Tissue markers in the diagnosis and prognosis of prostatic carcinoma. Eur Urol 21 Suppl 1:66-70.

Dematteo RP, Gold JS, Saran L, Gonen M, Liau KH, Maki RG, Singer S, Besmer P, Brennan MF, Antonescu CR. 2008. Tumor mitotic rate, size, and location independently predict recurrence after resection of primary gastrointestinal stromal tumor (GIST). Cancer 112(3):608-15.

DeMatteo RP, Lewis JJ, Leung D, Mudan SS, Woodruff JM, Brennan MF. 2000. Two hundred gastrointestinal stromal tumors: recurrence patterns and prognostic factors for survival. Ann Surg 231(1):51-8.

Demetri GD, Benjamin RS, Blanke CD, Blay JY, Casali P, Choi H, Corless CL, Debiec-Rychter M, DeMatteo RP, Ettinger DS and others. 2007. NCCN Task Force report: management of patients with gastrointestinal stromal tumor (GIST)--update of the NCCN clinical practice guidelines. J Natl Compr Canc Netw 5 Suppl 2:S1-29.

Duensing A, Joseph NE, Medeiros F, Smith F, Hornick JL, Heinrich MC, Corless CL, Demetri GD, Fletcher CD, Fletcher JA. 2004. Protein Kinase C theta (PKCtheta) expression and constitutive activation in gastrointestinal stromal tumors (GISTs). Cancer Res 64(15):5127-31.

Ekeblad S, Nilsson B, Lejonklou MH, Johansson T, Stålberg P, Nilsson O, Ahlman H, Skogseid B. 2006. Gastrointestinal stromal tumors express the orexigen ghrelin. Endocr Relat Cancer 13(3):963-70.

Espinosa I, Lee CH, Kim MK, Rouse BT, Subramanian S, Montgomery K, Varma S, Corless CL, Heinrich MC, Smith KS and others. 2008. A novel monoclonal antibody against DOG1 is a sensitive and specific marker for gastrointestinal stromal tumors. Am J Surg Pathol 32(2):210-8.

Faintuch BL, Teodoro R, Duatti A, Muramoto E, Faintuch S, Smith CJ. 2008. Radiolabeled bombesin analogs for prostate cancer diagnosis: preclinical studies. Nucl Med Biol 35(4):401-11.

Faivre S, Demetri G, Sargent W, Raymond E. 2007. Molecular basis for sunitinib efficacy and future clinical development. Nat Rev Drug Discov 6(9):734-45.

Faussone-Pellegrini MS. 1992. Histogenesis, structure and relationships of interstitial cells of Cajal (ICC): from morphology to functional interpretation. Eur J Morphol 30(2):137-48.

Feldman SA, Eiden LE. 2003. The chromogranins: their roles in secretion from neuroendocrine cells and as markers for neuroendocrine neoplasia. Endocr Pathol 14(1):3-23.

Fleischman RA. 1993. From white spots to stem cells: the role of the Kit receptor in mammalian development. Trends Genet 9(8):285-90.

Fletcher CD, Berman JJ, Corless C, Gorstein F, Lasota J, Longley BJ, Miettinen M, O'Leary TJ, Remotti H, Rubin BP and others. 2002. Diagnosis of gastrointestinal stromal tumors: A consensus approach. Hum Pathol 33(5):459-65.

Floris G, Debiec-Rychter M, Wozniak A, Stefan C, Normant E, Faa G, Machiels K, Vanleeuw U, Sciot R, Schoffski P. 2011. The heat shock protein 90 inhibitor IPI-504 induces KIT degradation, tumor shrinkage, and cell proliferation arrest in xenograft models of gastrointestinal stromal tumors. Mol Cancer Ther 10(10):1897-908.

Forssell-Aronsson E, Bernhardt P, Nilsson O, Tisell LE, Wängberg B, Ahlman H. 2004. Biodistribution data from 100 patients i.v. injected with 111In-DTPA-D-Phe1-octreotide. Acta Oncol 43(5):436-42.

Forssell-Aronsson EB, Nilsson O, Bejegard SA, Kölby L, Bernhardt P, Mölne J, Hashemi SH, Wängberg B, Tisell LE, Ahlman H. 2000. 111In-DTPA-D-Phe1-octreotide binding and somatostatin receptor subtypes in thyroid tumors. J Nucl Med 41(4):636-42.

Friedberg EC. 2003. DNA damage and repair. Nature 421(6921):436-40.

Gasi D, van der Korput HA, Douben HC, de Klein A, de Ridder CM, van Weerden WM, Trapman J. 2011. Overexpression of full-length ETV1 transcripts in clinical prostate cancer due to gene translocation. PLoS One 6(1):e16332.

Goh BK, Chow PK, Yap WM, Kesavan SM, Song IC, Paul PG, Ooi BS, Chung YF, Wong WK. 2008. Which is the optimal risk stratification system for surgically treated localized primary GIST? Comparison of three contemporary prognostic criteria in 171 tumors and a proposal for a modified Armed Forces Institute of Pathology risk criteria. Ann Surg Oncol 15(8):2153-63.

Gold JS, Gonen M, Gutierrez A, Broto JM, Garcia-del-Muro X, Smyrk TC, Maki RG, Singer S, Brennan MF, Antonescu CR and others. 2009. Development and validation of a prognostic nomogram for recurrence-free survival after complete surgical resection of localised primary gastrointestinal stromal tumour: a retrospective analysis. Lancet Oncol 10(11):1045-52.

Gunawan B, von Heydebreck A, Sander B, Schulten HJ, Haller F, Langer C, Armbrust T, Bollmann M, Gasparov S, Kovac D and others. 2007. An oncogenetic tree model in gastrointestinal stromal tumours (GISTs) identifies different pathways of cytogenetic evolution with prognostic implications. J Pathol 211(4):463-70.

Guzman ML, Swiderski CF, Howard DS, Grimes BA, Rossi RM, Szilvassy SJ, Jordan CT. 2002. Preferential induction of apoptosis for primary human leukemic stem cells. Proc Natl Acad Sci U S A 99(25):16220-5.

Hahn WC, Weinberg RA. 2002. Modelling the molecular circuitry of cancer. Nat Rev Cancer 2(5):331-41.

Haller F, Agaimy A, Cameron S, Beyer M, Gunawan B, Happel N, Langer C, Ramadori G, von Heydebreck A, Fuzesi L. 2010. Expression of p16INK4A in gastrointestinal stromal tumours (GISTs): two different forms exist that independently correlate with poor prognosis. Histopathology 56(3):305-18.

Hanahan D, Weinberg RA. 2011. Hallmarks of cancer: the next generation. Cell 144(5):646-74.

Hashemi SH, Benjegard SA, Ahlman H, Wängberg B, Forssell-Aronsson E, Billig H, Nilsson O. 2003. 111In-labelled octreotide binding by the somatostatin receptor subtype 2 in neuroendocrine tumours. Br J Surg 90(5):549-54.

Heinrich MC, Corless CL, Duensing A, McGreevey L, Chen CJ, Joseph N, Singer S, Griffith DJ, Haley A, Town A and others. 2003a. PDGFRA activating mutations in gastrointestinal stromal tumors. Science 299(5607):708-10.

Heinrich MC, Corless CL, Demetri GD, Blanke CD, von Mehren M, Joensuu H, McGreevey LS, Chen CJ, Van den Abbeele AD, Druker BJ and others. 2003b. Kinase mutations and imatinib response in patients with metastatic gastrointestinal stromal tumor. J Clin Oncol 21(23):4342-9.

Herman JG, Baylin SB. 2003. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 349(21):2042-54.

Hirota S, Isozaki K, Moriyama Y, Hashimoto K, Nishida T, Ishiguro S, Kawano K, Hanada M, Kurata A, Takeda M and others. 1998. Gain-of-function mutations of c-kit in human gastrointestinal stromal tumors. Science 279(5350):577-80.

Hornick JL, Fletcher CD. 2002. Immunohistochemical staining for KIT (CD117) in soft tissue sarcomas is very limited in distribution. Am J Clin Pathol 117(2):188-93.

Hsu KH, Tsai HW, Lin PW, Hsu YS, Shan YS, Lu PJ. 2010. Osteopontin expression is an independent adverse prognostic factor in resectable gastrointestinal stromal tumor and its interaction with CD44 promotes tumor proliferation. Ann Surg Oncol 17(11):3043-52.

Huang HY, Li CF, Huang WW, Hu TH, Lin CN, Uen YH, Hsiung CY, Lu D. 2007. A modification of NIH consensus criteria to better distinguish the highly lethal subset of primary localized gastrointestinal stromal tumors: a subdivision of the original high-risk group on the basis of outcome. Surgery 141(6):748-56.

Im YH, Kim HT, Lee C, Poulin D, Welford S, Sorensen PH, Denny CT, Kim SJ. 2000. EWS-FLI1, EWS-ERG, and EWS-ETV1 oncoproteins of Ewing tumor family all suppress transcription of transforming growth factor beta type II receptor gene. Cancer Res 60(6):1536-40.

Jakobsen AM, Andersson P, Saglik G, Andersson E, Kölby L, Erickson JD, Forssell-Aronsson E, Wängberg B, Ahlman H, Nilsson O. 2001. Differential expression of vesicular monoamine transporter (VMAT) 1 and 2 in gastrointestinal endocrine tumours. J Pathol 195(4):463-72.

Jane-Valbuena J, Widlund HR, Perner S, Johnson LA, Dibner AC, Lin WM, Baker AC, Nazarian RM, Vijayendran KG, Sellers WR and others. 2010. An oncogenic role for ETV1 in melanoma. Cancer Res 70(5):2075-84.

Janeway KA, Kim SY, Lodish M, Nose V, Rustin P, Gaal J, Dahia PL, Liegl B, Ball ER, Raygada M and others. 2011. Defects in succinate dehydrogenase in gastrointestinal stromal tumors lacking KIT and PDGFRA mutations. Proc Natl Acad Sci U S A 108(1):314-8.

Joensuu H, Roberts PJ, Sarlomo-Rikala M, Andersson LC, Tervahartiala P, Tuveson D, Silberman S, Capdeville R, Dimitrijevic S, Druker B and others. 2001. Effect of the tyrosine kinase inhibitor STI571 in a patient with a metastatic gastrointestinal stromal tumor. N Engl J Med 344(14):1052-6.

Joensuu H, Vehtari A, Riihimaki J, Nishida T, Steigen SE, Brabec P, Plank L, Nilsson B, Cirilli C, Braconi C and others. 2011. Risk of recurrence of gastrointestinal stromal tumour after surgery: an analysis of pooled population-based cohorts. Lancet Oncol.

Johnstone RW. 2002. Histone-deacetylase inhibitors: novel drugs for the treatment of cancer. Nat Rev Drug Discov 1(4):287-99.

Jones PA, Baylin SB. 2002. The fundamental role of epigenetic events in cancer. Nat Rev Genet 3(6):415-28.

Kang HJ, Nam SW, Kim H, Rhee H, Kim NG, Hyung WJ, Noh SH, Kim JH, Yun CO, Liu ET. 2005. Correlation of KIT and platelet-derived growth factor receptor alpha mutations with gene activation and expression profiles in gastrointestinal stromal tumors. Oncogene 24(6):1066-74.

Kastan MB. 2008. DNA damage responses: mechanisms and roles in human disease: 2007 G.H.A. Clowes Memorial Award Lecture. Mol Cancer Res 6(4):517-24.

Kim EJ, Zalupski MM. 2011. Systemic therapy for advanced gastrointestinal stromal tumors: beyond imatinib. J Surg Oncol 104(8):901-6.

Kindblom LG, Remotti HE, Aldenborg F, Meis-Kindblom JM. 1998. Gastrointestinal pacemaker cell tumor (GIPACT): gastrointestinal stromal tumors show phenotypic characteristics of the interstitial cells of Cajal. Am J Pathol 152(5):1259-69.

Kinzler KW, Vogelstein B. 1997. Cancer-susceptibility genes. Gatekeepers and caretakers. Nature 386(6627):761, 763.

Klein G. 1987. The approaching era of the tumor suppressor genes. Science 238(4833):1539-45.

Klein G, Klein E. 1985. Evolution of tumours and the impact of molecular oncology. Nature 315(6016):190-5.

Knudson AG, Jr. 1971. Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci U S A 68(4):820-3.

Kubota D, Orita H, Yoshida A, Gotoh M, Kanda T, Tsuda H, Hasegawa T, Katai H, Shimada Y, Kaneko K and others. 2011. Pfetin as a prognostic biomarker for gastrointestinal stromal tumor: validation study in multiple clinical facilities. Jpn J Clin Oncol 41(10):1194-202.

Kwekkeboom DJ, Kam BL, van Essen M, Teunissen JJ, van Eijck CH, Valkema R, de Jong M, de Herder WW, Krenning EP. 2010. Somatostatin-receptor-based imaging

and therapy of gastroenteropancreatic neuroendocrine tumors. Endocr Relat Cancer 17(1):R53-73.

Lasota J, Corless CL, Heinrich MC, Debiec-Rychter M, Sciot R, Wardelmann E, Merkelbach-Bruse S, Schildhaus HU, Steigen SE, Stachura J and others. 2008. Clinicopathologic profile of gastrointestinal stromal tumors (GISTs) with primary KIT exon 13 or exon 17 mutations: a multicenter study on 54 cases. Mod Pathol 21(4):476-84.

Lasota J, Stachura J, Miettinen M. 2006. GISTs with PDGFRA exon 14 mutations represent subset of clinically favorable gastric tumors with epithelioid morphology. Lab Invest 86(1):94-100.

Lennartsson J, Jelacic T, Linnekin D, Shivakrupa R. 2005. Normal and oncogenic forms of the receptor tyrosine kinase kit. Stem Cells 23(1):16-43.

Liegl B, Hornick JL, Antonescu CR, Corless CL, Fletcher CD. 2008. Rhabdomyosarcomatous Differentiation in Gastrointestinal Stromal Tumors After Tyrosine Kinase Inhibitor Therapy: A Novel Form of Tumor Progression. Am J Surg Pathol.

Marks P, Rifkind RA, Richon VM, Breslow R, Miller T, Kelly WK. 2001. Histone deacetylases and cancer: causes and therapies. Nat Rev Cancer 1(3):194-202.

Marotta LL, Polyak K. 2009. Cancer stem cells: a model in the making. Curr Opin Genet Dev.

Martin J, Poveda A, Llombart-Bosch A, Ramos R, Lopez-Guerrero JA, Garcia del Muro J, Maurel J, Calabuig S, Gutierrez A, Gonzalez de Sande JL and others. 2005. Deletions affecting codons 557-558 of the c-KIT gene indicate a poor prognosis in patients with completely resected gastrointestinal stromal tumors: a study by the Spanish Group for Sarcoma Research (GEIS). J Clin Oncol 23(25):6190-8.

Martinho O, Gouveia A, Silva P, Pimenta A, Reis RM, Lopes JM. 2009. Loss of RKIP expression is associated with poor survival in GISTs. Virchows Arch 455(3):277-84.

Miettinen M, El-Rifai W, L HLS, Lasota J. 2002. Evaluation of malignancy and prognosis of gastrointestinal stromal tumors: a review. Hum Pathol 33(5):478-83.

Miettinen M, Lasota J. 2006. Gastrointestinal stromal tumors: review on morphology, molecular pathology, prognosis, and differential diagnosis. Arch Pathol Lab Med 130(10):1466-78.

Miettinen M, Wang ZF, Lasota J. 2009. DOG1 antibody in the differential diagnosis of gastrointestinal stromal tumors: a study of 1840 cases. Am J Surg Pathol 33(9):1401-8.

Negrini M, Nicoloso MS, Calin GA. 2009. MicroRNAs and cancer--new paradigms in molecular oncology. Curr Opin Cell Biol 21(3):470-9.

Negrini S, Gorgoulis VG, Halazonetis TD. 2008. Genomic instability--an evolving hallmark of cancer. Nat Rev Mol Cell Biol 11(3):220-8.

Nilsson B, Bümming P, Meis-Kindblom JM, Odén A, Dortok A, Gustavsson B, Sablinska K, Kindblom LG. 2005. Gastrointestinal stromal tumors: the incidence, prevalence, clinical course, and prognostication in the preimatinib mesylate era--a population-based study in western Sweden. Cancer 103(4):821-9.

Nilsson B, Nilsson O, Ahlman H. 2009. Treatment of gastrointestinal stromal tumours: imatinib, sunitinib -- and then? Expert Opin Investig Drugs 18(4):457-68.

Nilsson O, Kölby L, Wängberg B, Wigander A, Billig H, William-Olsson L, Fjälling M, Forssell-Aronsson E, Ahlman H. 1998. Comparative studies on the expression of somatostatin receptor subtypes, outcome of octreotide scintigraphy and response to octreotide treatment in patients with carcinoid tumours. Br J Cancer 77(4):632-7.

Nowell PC. 1976. The clonal evolution of tumor cell populations. Science 194(4260):23-8.

Oldenhuis CN, Oosting SF, Gietema JA, de Vries EG. 2008. Prognostic versus predictive value of biomarkers in oncology. Eur J Cancer 44(7):946-53.

Pages F, Galon J, Dieu-Nosjean MC, Tartour E, Sautes-Fridman C, Fridman WH. 2010. Immune infiltration in human tumors: a prognostic factor that should not be ignored. Oncogene 29(8):1093-102.

Palmieri G, Montella L, Aiello C, Barbieri F, Di Vizio D, Schulz S, Beninati S, Budillon A, Caraglia M, Insabato L and others. 2007. Somatostatin analogues, a series of tissue transglutaminase inducers, as a new tool for therapy of mesenchimal tumors of the gastrointestinal tract. Amino Acids 32(3):395-400.

Parkkila S, Lasota J, Fletcher JA, Ou WB, Kivela AJ, Nuorva K, Parkkila AK, Ollikainen J, Sly WS, Waheed A and others. 2009. Carbonic anhydrase II. A novel biomarker for gastrointestinal stromal tumors. Mod Pathol 23(5):743-50.

Patel YC. 1999. Somatostatin and its receptor family. Front Neuroendocrinol 20(3):157-98.

Peltomäki P. 2001. DNA mismatch repair and cancer. Mutat Res 488(1):77-85.

Reubi JC, Korner M, Waser B, Mazzucchelli L, Guillou L. 2004. High expression of peptide receptors as a novel target in gastrointestinal stromal tumours. Eur J Nucl Med Mol Imaging 31(6):803-10.

Reya T, Morrison SJ, Clarke MF, Weissman IL. 2001. Stem cells, cancer, and cancer stem cells. Nature 414(6859):105-11.

Rindi G, Leiter AB, Kopin AS, Bordi C, Solcia E. 2004. The "normal" endocrine cell of the gut: changing concepts and new evidences. Ann N Y Acad Sci 1014:1-12.

Rossi S, Miceli R, Messerini L, Bearzi I, Mazzoleni G, Capella C, Arrigoni G, Sonzogni A, Sidoni A, Toffolatti L and others. 2011. Natural history of imatinib-naive GISTs: a retrospective analysis of 929 cases with long-term follow-up and development of a survival nomogram based on mitotic index and size as continuous variables. Am J Surg Pathol 35(11):1646-56.

Rubin BP. 2010. Bioinformatic mining of gene expression datasets identifies ETV1 as a critical regulator of oncogenesis in gastrointestinal stromal tumors. Cancer Cell 18(5):407-8.

Sabah M, Cummins R, Leader M, Kay E. 2004. Loss of heterozygosity of chromosome 9p and loss of p16INK4A expression are associated with malignant gastrointestinal stromal tumors. Mod Pathol 17(11):1364-71.

Schwab M. 1997. MYCN Amplification in Neuroblastoma: a Paradigm for the Clinical Use of an Oncogene. Pathol Oncol Res 3(1):3-7.

Schöffski P, Reichardt P, Blay JY, Dumez H, Morgan JA, Ray-Coquard I, Hollaender N, Jappe A, Demetri GD. 2010. A phase I-II study of everolimus (RAD001) in combination with imatinib in patients with imatinib-resistant gastrointestinal stromal tumors. Ann Oncol 21(10):1990-8.

Sharma S, Kelly TK, Jones PA. 2010. Epigenetics in cancer. Carcinogenesis 31(1):27-36.

Sherr CJ. 2004. Principles of tumor suppression. Cell 116(2):235-46.

Singer S, Rubin BP, Lux ML, Chen CJ, Demetri GD, Fletcher CD, Fletcher JA. 2002. Prognostic value of KIT mutation type, mitotic activity, and histologic subtype in gastrointestinal stromal tumors. J Clin Oncol 20(18):3898-905.

Sircar K, Hewlett BR, Huizinga JD, Chorneyko K, Berezin I, Riddell RH. 1999. Interstitial cells of Cajal as precursors of gastrointestinal stromal tumors. Am J Surg Pathol 23(4):377-89.

Slamon DJ. 1987. Proto-oncogenes and human cancers. N Engl J Med 317(15):955-7.

Smith LM, Nesterova A, Ryan MC, Duniho S, Jonas M, Anderson M, Zabinski RF, Sutherland MK, Gerber HP, Van Orden KL and others. 2008. CD133/prominin-1 is a potential therapeutic target for antibody-drug conjugates in hepatocellular and gastric cancers. Br J Cancer 99(1):100-9.

Subramanian S, West RB, Corless CL, Ou W, Rubin BP, Chu KM, Leung SY, Yuen ST, Zhu S, Hernandez-Boussard T and others. 2004. Gastrointestinal stromal tumors (GISTs) with KIT and PDGFRA mutations have distinct gene expression profiles. Oncogene 23(47):7780-90.

Suvá ML, Riggi N, Stehle JC, Baumer K, Tercier S, Joseph JM, Suva D, Clement V, Provero P, Cironi L and others. 2009. Identification of cancer stem cells in Ewing's sarcoma. Cancer Res 69(5):1776-81.

Swärd C, Bernhardt P, Ahlman H, Wängberg B, Forssell-Aronsson E, Larsson M, Svensson J, Rossi-Norrlund R, Kölby L. 2010. [177Lu-DOTA 0-Tyr 3]-octreotate treatment in patients with disseminated gastroenteropancreatic neuroendocrine tumors: the value of measuring absorbed dose to the kidney. World J Surg 34(6):1368-72.

Swärd C, Bernhardt P, Johanson V, Schmitt A, Ahlman H, Stridsberg M, Forssell-Aronsson E, Nilsson O, Kölby L. 2008. Comparison of [177Lu-DOTA0,Tyr3]-octreotate and [177Lu-DOTA0,Tyr3]-octreotide for receptor-mediated radiation therapy of the xenografted human midgut carcinoid tumor GOT1. Cancer Biother Radiopharm 23(1):114-20.

Tabin CJ, Bradley SM, Bargmann CI, Weinberg RA, Papageorge AG, Scolnick EM, Dhar R, Lowy DR, Chang EH. 1982. Mechanism of activation of a human oncogene. Nature 300(5888):143-9.

Tan J, Cang S, Ma Y, Petrillo RL, Liu D. 2010. Novel histone deacetylase inhibitors in clinical trials as anti-cancer agents. J Hematol Oncol 3:5.

Tarn C, Rink L, Merkel E, Flieder D, Pathak H, Koumbi D, Testa JR, Eisenberg B, von Mehren M, Godwin AK. 2008. Insulin-like growth factor 1 receptor is a potential therapeutic target for gastrointestinal stromal tumors. Proc Natl Acad Sci U S A 105(24):8387-92.

Teunissen JJ, Kwekkeboom DJ, Valkema R, Krenning EP. 2011. Nuclear medicine techniques for the imaging and treatment of neuroendocrine tumours. Endocr Relat Cancer 18 Suppl 1:S27-51.

Tirino V, Desiderio V, Paino F, De Rosa A, Papaccio F, Fazioli F, Pirozzi G, Papaccio G. 2011. Human primary bone sarcomas contain CD133+ cancer stem cells displaying high tumorigenicity in vivo. FASEB J 25(6):2022-30.

Tognon CE, Sorensen PH. 2011. Targeting the insulin-like growth factor 1 receptor (IGF1R) signaling pathway for cancer therapy. Expert Opin Ther Targets.

Tomlins SA, Laxman B, Dhanasekaran SM, Helgeson BE, Cao X, Morris DS, Menon A, Jing X, Cao Q, Han B and others. 2007. Distinct classes of chromosomal rearrangements create oncogenic ETS gene fusions in prostate cancer. Nature 448(7153):595-9.

Tryggvason G, Gislason HG, Magnusson MK, Jonasson JG. 2005. Gastrointestinal stromal tumors in Iceland, 1990-2003: the icelandic GIST study, a population-based incidence and pathologic risk stratification study. Int J Cancer 117(2):289-93.

Tzen CY, Wang JH, Huang YJ, Wang MN, Lin PC, Lai GL, Wu CY. 2007. Incidence of gastrointestinal stromal tumor: a retrospective study based on immunohistochemical and mutational analyses. Dig Dis Sci 52(3):792-7.

Van Glabbeke MM, Owzar K, Rankin C, C R. GIST Meta-analysis Group (MetaGIST). Comparison of two doses of imatinib for the treatment of gastrointestinal stromal tumors (GIST): a meta-analysis based on 1640 patients; 2007. Journal of Clinical Oncology.

Wang CH, Chiou SH, Chou CP, Chen YC, Huang YJ, Peng CA. 2011a. Photothermolysis of glioblastoma stem-like cells targeted by carbon nanotubes conjugated with CD133 monoclonal antibody. Nanomedicine 7(1):69-79.

Wang WL, Conley A, Reynoso D, Nolden L, Lazar AJ, George S, Trent JC. 2011b. Mechanisms of resistance to imatinib and sunitinib in gastrointestinal stromal tumor. Cancer Chemother Pharmacol 67 Suppl 1:S15-24.

Wei YC, Li CF, Yu SC, Chou FF, Fang FM, Eng HL, Uen YH, Tian YF, Wu JM, Li SH and others. 2009. Ezrin overexpression in gastrointestinal stromal tumors: an independent adverse prognosticator associated with the non-gastric location. Mod Pathol 22(10):1351-60.

Weinberg RA. 1994. Oncogenes and tumor suppressor genes. CA Cancer J Clin 44(3):160-70.

Weinberg RA. The biology of cancer. Garland Science, Taylor & Francis Group, LLC, New York, NY, USA. 2007.

Vogelstein B, Kinzler KW. 1993. The multistep nature of cancer. Trends Genet 9(4):138-41.

Vogelstein B, Kinzler KW. 2004. Cancer genes and the pathways they control. Nat Med 10(8):789-99.

Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, Visone R, Iorio M, Roldo C, Ferracin M and others. 2006. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A 103(7):2257-61.

Wozniak A, Sciot R, Guillou L, Pauwels P, Wasag B, Stul M, Vermeesch JR, Vandenberghe P, Limon J, Debiec-Rychter M. 2007. Array CGH analysis in primary gastrointestinal stromal tumors: cytogenetic profile correlates with anatomic site and tumor aggressiveness, irrespective of mutational status. Genes Chromosomes Cancer 46(3):261-76.

Yamaguchi U, Nakayama R, Honda K, Ichikawa H, Hasegawa T, Shitashige M, Ono M, Shoji A, Sakuma T, Kuwabara H and others. 2008. Distinct gene expression-defined classes of gastrointestinal stromal tumor. J Clin Oncol 26(25):4100-8.

Yang J, Du X, Lazar AJ, Pollock R, Hunt K, Chen K, Hao X, Trent J, Zhang W. 2008. Genetic aberrations of gastrointestinal stromal tumors. Cancer 113(7):1532-43.

Yokota J. 2000. Tumor progression and metastasis. Carcinogenesis 21(3):497-503.

Younus J, Verma S, Franek J, Coakley N. 2010. Sunitinib malate for gastrointestinal stromal tumour in imatinib mesylate-resistant patients: recommendations and evidence. Curr Oncol 17(4):4-10.