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Effect of Genetic Kinome Alterations on the Response of Cancer Cells to SUTENT Therapy

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ERKLÄRUNG

Ich erkläre an Eides statt, dass ich die der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München vorgelegte Dissertationsarbeit mit dem Titel:

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Torsten Winkler

For Sylvia and Lara

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2. Summary

Genetic alterations are widely known to influence the etiopathology of tumor development as well as the therapeutic outcome of cancer treatment. The anti-cancer drug SUTENT® (sunitinib malate, SU12248), an orally available multi-targeted small molecule inhibitor, has been approved for the treatment of gastrointestinal stromal tumors (GIST) and metastatic renal cell carcinoma (mRCC). So far, only a few genetic alterations in patients with GIST are known to impair the effect of the drug. Since sunitinib is on the verge of being approved for the therapy of various cancer types, the general role of genetic alterations that affect its treatment efficacy is currently of great interest.

This study initially measured the sensitivities of 122 cancer cell lines to sunitinib treatment by determining inhibition of proliferation and migration as well as induction of apoptosis. The resulting sensitivity profiles were then statistically correlated with the distribution of genetic alterations in sunitinib targets. This analysis showed that one allele of the single nucleotide polymorphisms ABL1$^{991S/L}$, RON$^{523R/Q}$, TYK2$^{362V/F}$, TYK2$^{684I/S}$ and RON$^{1335R/G}$ always occurred significantly more often in sensitive or insensitive cell lines. Two of these five candidates, RON$^{1335R/G}$ and TYK2$^{684I/S}$, also showed allele-dependent differences in their sunitinib-induced inhibition of phosphorylation. In particular, the inhibition of phosphorylation of RON decreased in the presence of 1335G. This allele-dependent effect was not detected in the biological response to sunitinib. In contrast, sunitinib only inhibited the phosphorylation of TYK2 if 684S was present. TYK2^{684I} was not inhibited and did not bind to sunitinib, i.e., the amino acid exchange generated a new sunitinib target with high affinity. Moreover, TYK2^{684S} correlated with increased induction of apoptosis during sunitinib treatment. Investigations on the drug's mode of action revealed that the inhibition of TYK2^{684S} subsequently led to the absence of downstream activation of STAT3. Thus, TYK2 represents a new target for directed cancer therapy. Finally, as serine 684 of TYK2 enhances sunitinib sensitivity, it should be considered a marker to improve efficacy of sunitinib therapy.

3. Zusammenfassung

Genetische Veränderungen sind dafür bekannt sowohl die Entwicklung, den Krankheitsverlauf als auch den Therapieerfolg von Krebs beeinflussen zu können. Das Krebsmedikament SUTENT® (Sunitinib Malat, SU12248), ein Hemmer von zahlreichen Proteinen, ist derzeit für die Behandlung von gastrointestinalen Stromatumoren (GIST) sowie von metastasierten Nierenzellkarzinomen (mRCC) zugelassen. Bis heute sind nur wenige genetische Veränderungen in Patienten mit GIST bekannt, welche die Wirksamkeit von Sunitinib beeinträchtigen. Da SUTENT vor der Zulassung zur Behandlung von verschiedensten Tumorarten ist, ist die Aufklärung von genetischen Veränderungen, welche die Therapie mit Sunitinib beeinflussen, von größtem Interesse.

Zu Beginn dieser Studie wurde der Einfluss von Sunitinib auf 122 Krebszelllinien in Bezug auf die Hemmung von Proliferation und Migration sowie das Auslösen von Apoptose bestimmt. Die erhaltenen Sensitivitätsprofile wurden anschließend mit der Verteilung genetischer Veränderungen in Zielproteinen von Sunitinib statistisch korreliert. Dabei zeigte sich, dass die beiden Allele der SNPs ABL1$^{991S/L}$, RON$^{523R/Q}$, TYK2$^{362V/F}$, TYK2$^{684I/S}$ und RON$^{1335R/G}$ in sensitive bzw. insensitiven Zelllinien signifikant unterschiedlich vorkamen. Dieser Unterschied erstreckte sich bei RON$^{1335R/G}$ und TYK2$^{684I/S}$ auch auf die Hemmung der Phosphorylierung durch Sunitinib. Dabei wurde RON^{1335R} wesentlich mehr gehemmt als RON^{1335G}. Ein Einfluss dieses Unterschieds auf die biologische Antwort konnte jedoch nicht gezeigt werden. Im Gegensatz dazu wurde die Phosphorylierung von TYK2 nur in Gegenwart des Serinallels an Position 684 gehemmt. TYK2^{684I} wurde weder gehemmt, noch zeigte es Bindung an Sunitinib. Dies bedeutet, dass durch den Aminosäureaustausch ein neues, hochaffines Zielmolekül entsteht. Darüber hinaus korrelierte bei funktionalen Experimenten bei Behandlung mit Sunitinib TYK2^{684S} mit einer gesteigerten Apoptoserate. Nachforschungen über den Wirkmechanismus offenbarten, dass die Hemmung von TYK2^{684S} nachfolgend zum Ausbleiben der Aktivierung von STAT3 führt.

Zusammenfassend konnte TYK2 als neues Zielmolekül für gerichtete Krebstherapie identifiziert werden. Darüber hinaus konnte in dieser Studie das Serinallel an Position 684 in TYK2 für eine gesteigerte Sensitivität gegenüber Sunitinib verantwortlich gemacht werden. Daher sollte TYK2^{684S} als Marker für die Verbesserung der Therapie mit Sunitinib in Betracht gezogen werden.

4. Abbreviations

ABL	c-abl oncogene 1, receptor tyrosine kinase
ACK	activated Cdc42-associated kinase
ATCC	The American Type Culture Collection
AURKA/B	Aurora kinase A/B
AXL	AXL receptor tyrosine kinase
Bp	Base pair
CadhD	cadherin-like domain
CAM	Calmodulin
CCK	colon carcinoma kinase
cDNA	complementary DNA
COSMIC	Catalogue of somatic mutations in cancer database
CSF1R	Colony stimulating factor 1 receptor
CSK	c-src tyrosine kinase
DDR	discoidin domain receptor
DiscD	discoidin-like domain
DKFZ	Deutsches Krebsforschungszentrum
DMSO	Dimethylsulfoxide
DSMZ	German Collection of Microorganisms and Cell Cultures
ECACC	European Collection of Cell Cultures
EGF	epidermal growth factor
EGFD	epidermal growth factor-like domain
EGFR	epidermal growth factor receptor
EphR	ephrin receptor
FACS	Fluorescence-activated cell sorting
FAK	focal adhesion kinase
FCS	Fetal calf serum
FDA	US Food and Drug administration
FES	feline sarcoma oncogene
FGFR	fibroblast growth factor receptor

FLT3	fms-related tyrosine kinase
FNIII	fibronectin type III-like domain
FRK	fyn-related kinase
GIST	gastrointestinal stromal tumor
HER2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
HGFR	hepatocyte growth factor receptor
IC	Inhibitor concentration
IgD	immunoglobulin-like domain
IL-6	interleukin 6
IL6R	interleukin 6 receptor
INF	Interferon
InsR	insulin receptor
JAK	Janus kinase
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
KrinD	kringle-like domain
LD	Lethal dose
LMR	Lemur
LRD	leucine-rich domain
LTK	leukocyte tyrosine kinase
MET	met proto-oncogene (hepatocyte growth factor receptor)
MoKCa	Mutations of Kinases in Cancer database
mRCC	metastatic renal cell carcinoma
MSP	macrophage-stimulating protein
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MuSK	muscle-specific kinase
NEK9	NIMA (never in mitosis gene a)- related kinase 9
NET	neuroendocrine tumor
NGFR	nerve growth factor receptor
NSCLC	non-small-cell lung cancer
OSM	Oncostatin M
OSMR	Oncostatin M receptor
PDGF	platelet derived growth factor
PDRFR	platelet derived growth factor receptor, receptor tyrosine kinase

PI	Propidium Iodide
PTB domain	Protein tyrosine-binding domain
PYK2	Prolin-rich tyrosine kinase 2
Rb	retinoblastoma protein
RET	ret proto-oncogene
RNA	ribonucleic acid
RNAi	RNA interference
RON	Récepteur d'origine Nantes, receptor tyrosine kinase
ROR	receptor orphan
ROS1	c-ros oncogene 1, receptor tyrosine kinase
RTK	Receptor tyrosine kinase
RYK	receptor related to tyrosine kinases
SCF	stem cell factor
SEM	standard error of the mean
SH2 domain	Scr homology-2 domain
SNP	Single nucleotide polymorphism
SRC	Schmidt-Ruppin A-2 viral oncogene homolog
STAT3	signal transducer and activator of transcription 3
SYK	spleen tyrosine kinase.
TBK1	TANK-binding kinase 1
TGFα	tumor growth factor α
TIE	tyrosine kinase receptor in endothelial cells
TK	tyrosine kinase
TYK2	Tyrosine Kinase 2
TyKiVa	Tyrosine Kinome Variants database
TYRO3	TYRO3 protein tyrosine kinase
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

5. Introduction

5.1 Cancer – A disease with many faces

The term cancer does not refer to a single disease, but rather to various medical conditions, all of which are characterized by uncontrolled growth and spread of degenerated cells. Depending on the tissue and cell type from which the abnormal cells originate, more than 100 different tumor variants are possible, each having a different etiopathology as well as mortality rate (figure 1).

Estimated New Cases*

	Males			Females	
Prostate	217,730	28%		Breast	207,090	28%
Lung & bronchus	116,750	15%		Lung & bronchus	105,770	14%
Colon & rectum	72,090	9%		Colon & rectum	70,480	10%
Urinary bladder	52,760	7%		Uterine corpus	43,470	6%
Melanoma of the skin	38,870	5%		Thyroid	33,930	5%
Non-Hodgkin lymphoma	35,380	4%		Non-Hodgkin lymphoma	30,160	4%
Kidney & renal pelvis	35,370	4%		Melanoma of the skin	29,260	4%
Oral cavity & pharynx	25,420	3%		Kidney & renal pelvis	22,870	3%
Leukemia	24,690	3%		Ovary	21,880	3%
Pancreas	21,370	3%		Pancreas	21,770	3%
All Sites	**789,620**	**100%**		**All Sites**	**739,940**	**100%**

Estimated Deaths

	Males			Females	
Lung & bronchus	86,220	29%		Lung & bronchus	71,080	26%
Prostate	32,050	11%		Breast	39,840	15%
Colon & rectum	26,580	9%		Colon & rectum	24,790	9%
Pancreas	18,770	6%		Pancreas	18,030	7%
Liver & intrahepatic bile duct	12,720	4%		Ovary	13,850	5%
Leukemia	12,660	4%		Non-Hodgkin lymphoma	9,500	4%
Esophagus	11,650	4%		Leukemia	9,180	3%
Non-Hodgkin lymphoma	10,710	4%		Uterine Corpus	7,950	3%
Urinary bladder	10,410	3%		Liver & intrahepatic bile duct	6,190	2%
Kidney & renal pelvis	8,210	3%		Brain & other nervous system	5,720	2%
All Sites	**299,200**	**100%**		**All Sites**	**270,290**	**100%**

Figure 1: Estimated new cancer incidences and deaths in the U.S. for 2010 (figure taken from (Jemal *et al.*, 2010))

Every year about 13 million new cases of cancer occur worldwide, and about 32% result in the patient's death within 5 years. This makes cancer the second-leading cause of human deaths (Jemal *et al.*, 2010).

Cancer can be induced under different internal and external circumstances. Internal factors such as inherited mutations (e.g., BRCA mutations (Hall *et al.*, 1992; Casey *et al.*, 1993; Kent *et al.*, 1995)), hormones (e.g., estrogen (Gray *et al.*, 1977; Ziel, 1982)) or immune conditions (Blair and Cook, 2008; Loose and Van de Wiele, 2009) can support the development of cancer. External factors like tobacco, alcohol (Shabad, 1971; Pelucchi *et al.*, 2006), infections (e.g., human papillomavirus, HPV (Persaud, 1989)), chemicals, or radiation can lead to cancerogenesis. Although the immune system recognizes and eliminates damaged cells (Appelbaum, 1992; Disis and Lyerly, 2005), occasionally incipient cancer cells manage to evade or pass this detection and begin to develop a tumor. After a while cancer cells will leave the initial tumor mass and spread to other organs, forming distant metastases. These metastases will be the cause in 90% of all human cancer deaths (Sporn, 1996).

5.1.1 Characterization of cancer

5.1.1.1 Benign and malignant tumors

Neoplasms can be roughly grouped as benign and malignant tumors. Benign neoplasms, e.g., warts, are never invasive and they generally do not cause the patient's death. Nevertheless they can be harmful and cause severe problems by secreting hormones or compressing blood vessels and organs. Furthermore, some benign tumors like colon polyps can change into invasive malignant forms. A very early stage in this process is the so-called "carcinoma *in situ*" (CIS). In this state the tumor is already malignant but not yet invasive and therefore still located at one spot (=*in situ*) (Valenzuela and Julian, 2007). If, for example, a ductal carcinoma *in situ* (DCIS), the early stage in the development of breast cancer, is diagnosed, there is a good chance of cure (rate of death from breast carcinoma after breast-conserving surgery of DCIS (after 10 years): 0.8%), because of the possibility of surgical removal (Warren *et al.*, 2005). If undetected, however, the CIS will develop into an invasive malignant tumor that not only invades the surrounding area but can also form distant metastases all over the body, ultimately killing the patient (Sporn, 1996; Wiechmann and Kuerer, 2008).

5.1.1.2 Terminology of different tumor types

Tumors are also classified more precisely according to the cell type from which the tumor originates. The following terms are used: adenoma (derived from gland cells), chondroma (derived from cartilage-forming cells), or lipoma (derived from fat cells) for benign tumors. Malignant forms are termed carcinoma (originally referring to transformed epithelial cells); they account for about 80% of all malignant tumors, sarcoma (derived from cells with mesodermal origin: bone, muscle, cartilage), leukemia (derived from blood cells), lymphoma (derived from lymphatic cells), myeloma (derived from plasma B-cells), or glioma (derived from glial cells). Moreover, the tissue or organ where the tumor is located is added to its designation, resulting in expressions like hepatocarcinoma (liver cancer) or mammary ductal carcinoma (breast cancer; figure 2).

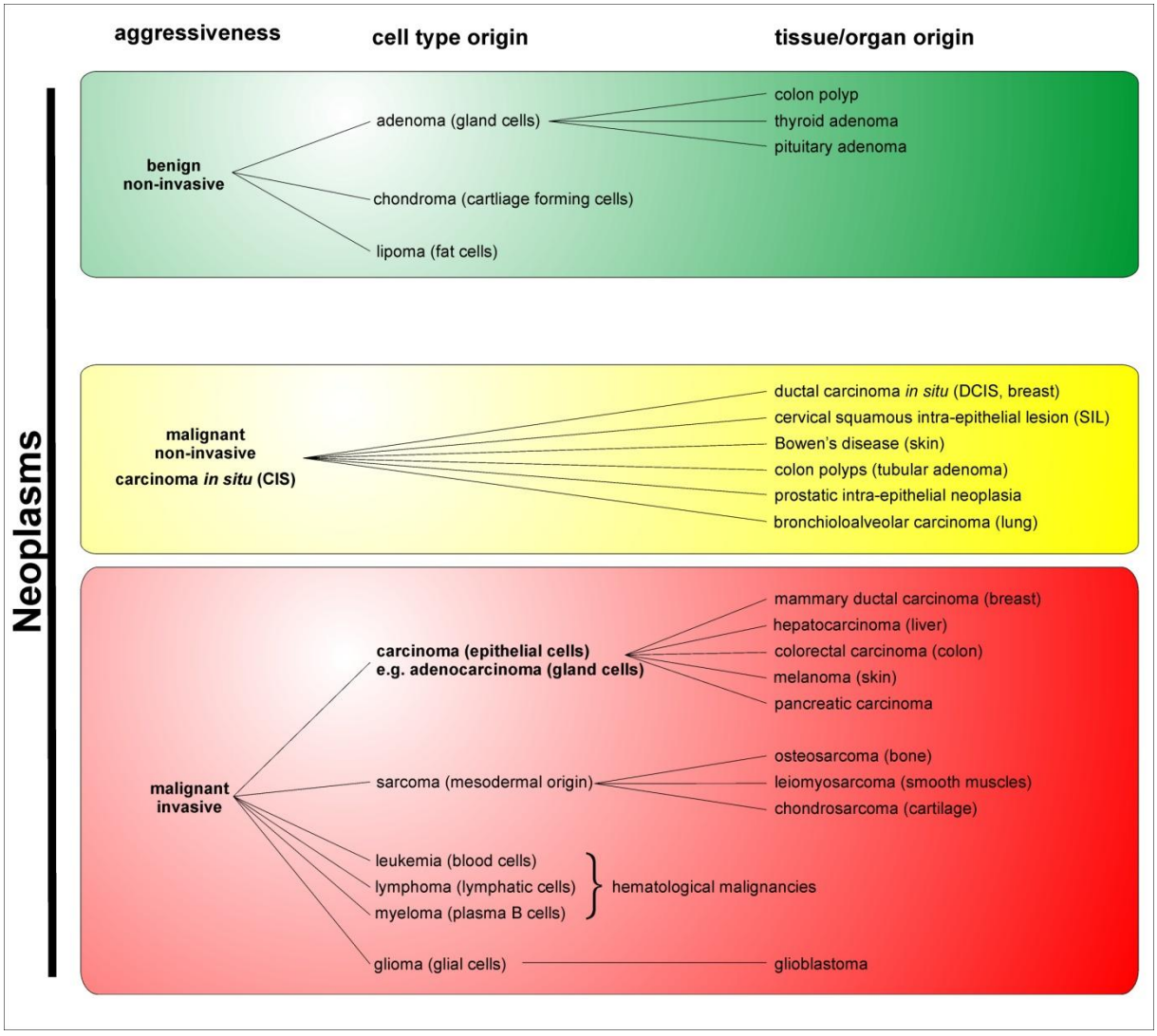

Figure 2: Examples of the terminology of neoplastic tissue. Green box: benign tumors. Yellow box: carcinoma *in situ*. Red box: malignant, invasive tumors.

5.1.2 Development of malignant tumors at the molecular level

Growth, division, proliferation, migration, adhesion, and death of cells are highly regulated processes (Sonnenschein and Soto, 1989; Mori *et al.*, 1999). Control is necessary to ensure coordinated tissue and organ homeostasis. In order to be transformed into a cancer cell, a normal cell has to overcome these controls. Hanahan and Weinberg suggested that virtually all cancer types must acquire the same six hallmark capabilities: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, tissue invasion, and metastasis (Hanahan and Weinberg, 2000). All these prerequisite features may be acquired at different times. Moreover, some genetic alterations may confer several capabilities simultaneously, decreasing the number of mutational steps required for transformation into a cancer cell.

5.1.2.1 Self-sufficiency in growth signals

Normal cells require a stimulus, for example growth factors (GFs), to begin proliferating. Cancer cells can acquire the ability to overcome this obstacle by different strategies. First, some cancer types are able to produce GFs by themselves. For example, PDGF (platelet-derived growth factor) and TGFα (tumor growth factor) are produced by certain glioblastomas or sarcomas, respectively (Goustin *et al.*, 1986). Second, some undergo independent growth by overexpressing surface receptors. This causes the cell to proliferate, although the GF concentration remains normal. For example, EGFR/erbB (epidermal growth factor receptor) or HER2/neu can be overexpressed in mammary carcinoma and several other tumor types (Libermann *et al.*, 1985; Libermann *et al.*, 1985; Slamon *et al.*, 1987). Third, ligand-independent tumor growth can be achieved by structural alteration of a receptor (Goustin *et al.*, 1986; Di Fiore *et al.*, 1987) or of an effector protein located downstream. Thus, the Ras-Raf-MAPK pathway is affected very often by genetic alterations. In about 25% of all human tumors and about 50% of colon carcinomas Ras undergoes a mutation, which leads to permanent activation of mitosis in the absence of a stimulus and thus uncontrolled proliferation (Medema and Bos, 1993; Kinzler and Vogelstein, 1996).

5.1.2.2 Insensitivity to anti-growth signals

The maintenance of cellular quiescence (cell cycle status G_0) is essential for normal tissue homeostasis. Therefore, soluble and/or surface-embedded growth inhibitors, on neighboring cells, e.g., TGFβ, actively block uncontrolled mitosis (Moses *et al.*, 1990). To form a tumor an incipient cancer cell has to evade these anti-proliferative signals by overcoming the G_1-checkpoint. The key protein here is generally Rb (retinoblastoma protein). Hypophosphorylated Rb blocks proliferation by inhibiting the E2F transcription factor, which controls the progression from the G_1 to the S phase. Dysfunction of Rb itself or of proteins located upstream (e.g., SMAD4 or TGFβ receptor) can disrupt this protective E2F suppression, which initiates the S phase and has as a consequence proliferation (Fynan and Reiss, 1993; Markowitz *et al.*, 1995; Schutte *et al.*, 1996; Zuo *et al.*, 1996; Chin *et al.*, 1998)

5.1.2.3 Evasion of apoptosis

Apoptosis, programmed cell death, is triggered by a variety of external or internal signals. While apoptosis is essential for the development and maintenance of organs, it is also important for the elimination of degenerated cells and thus for preventing cancer development. Once the program begins, cellular membranes are disrupted, nuclear and cytoplasmic skeletons are broken down, chromosomes are degraded, and the nucleus is fragmented, resulting in the controlled, coordinated, and intended death of the cell (Wyllie *et al.*, 1980).

There are two major pathways that lead to the induction of apoptosis: the extrinsic and the intrinsic pathways. In the extrinsic pathway external signals are conveyed by means of ligands such as FasL or TNFα (tumor necrosis factor alpha; (Ashkenazi and Dixit, 1999). These ligands bind to their respective receptor (FasR and TNF-R1/2) and thus activate the caspases, which finally lead to cell death (Thornberry and Lazebnik, 1998). The extrinsic pathway is also used, for example, by cytotoxic T-cells of the immune system to induce apoptosis of incipient cancer cells.

In contrast, the intrinsic pathway does not require any external stimulus. Instead the cell permanently monitors its internal condition and induces apoptosis in response to cellular stress such as heat, radiation, nutrient deprivation, viral infection, hypoxia,

increased intracellular calcium concentration, mitochondrial dysfunction, or DNA damage (Levine *et al.*, 1995; Lowy, 2003; Goyeneche *et al.*, 2006; Hess *et al.*, 2007). One of the most important internal inducers of apoptosis is p53, a protein that can "sense" DNA damage and consequently induce apoptosis (Symonds *et al.*, 1994).

Incipient cancer cells have to avoid the induction of apoptosis. They become resistant to programmed cell death in many different ways. The two most prominent ones involve the tumor suppressor p53 and the survival signal pathway PI3-kinase-AKT. Both the loss of function by p53 as well as the gain of function by AKT can impair the cell's ability to overcome apoptosis and promote the development of an unstable genome, which is typical of cancer (Harris, 1996; Levine, 1997; Cantley and Neel, 1999).

5.1.2.4 Limitless replicative potential

The replicative potential of normal cells is limited to about 60-70 doublings due to the length restriction of telomeres (Counter *et al.*, 1992; Hayflick, 1997). Telomeres are composed of thousands of short base pair (bp) repeats at the end of every chromosome. About 50-100 bp are lost during each cell cycle. Once the whole telomere disappears, the cell stops dividing and enters apoptosis (Counter *et al.*, 1992). To overcome this process, cancer cells have to express telomerase, an enzyme that constantly adds new bp repeats to the chromosomal ends, thus allowing unlimited replication (Shay and Bacchetti, 1997; Bryan and Cech, 1999).

5.1.2.5 Sustained angiogenesis

Every eukaryotic cell functions and survives thanks to nutrients and oxygen supplied by the vascular system. The vascularisation of the body is sufficient to reach every single cell. A growing tumor mass requires the formation of new blood vessels. Therefore, the cancer cells have to develop the ability to induce angiogenesis (Bouck *et al.*, 1996; Hanahan and Folkman, 1996). Without such an additional supply, tumors would only reach ca. 1-2 mm^3 in size (Folkman, 1972). Thus, the incipient tumor has to produce angiogenesis-inducing ligands like VEGF (vascular endothelial growth factor), which binds to VEGFR (vascular endothelial growth factor receptor) of endothelial cells (e.g.,

blood vessels). This subsequently leads to angiogenesis, by means of which the newly formed cancer cells are supplied (Goustin *et al.*, 1986; Veikkola and Alitalo, 1999)

5.1.2.6 Tissue invasion and metastasis

If a tumor only grew at its site of origin, cancer would not be a real threat, because it could be removed by surgery. The initial tumor does not kill the patient in 90% of all cases; the metastases formed elsewhere in the body do (Sporn, 1996). Although metastasis remains one of the most poorly understood processes in cancer biology, many cellular signaling pathways are known to facilitate invasion and metastasis (Chiang and Massague, 2008). As most of these pathways are highly dependent on site and tissue, generalization is difficult. It is, however, assumed that three changes must occur in tumor cells before they are able to invade and metastasize. First, the incipient cancer cell has to lose cell-cell contact. Normal epithelial cells are characterized by strong cell-cell adhesion, which is mediated by E-cadherin. It acts like a hook-loop fastener between the cells. Once the function of E-cadherin is lost, the cancer cells become detached from the surrounding cells (Johnson, 1991; Christofori and Semb, 1999; van Roy and Berx, 2008). During this endothelial-to-mesenchymal transition (EMT) a loss of function of Annexin A1 and subsequently an activation of TYK2/STAT3 and ERK1/2 signaling seem to be essential (Maschler *et al.*, 2010). Cells are also attached to proteins of the extracellular matrix (ECM). Therefore, another critical step in metastasis involves the release of cancer cells from the ECM. Extracellular proteases, which degrade proteins of the ECM, play a central role in this process and are therefore upregulated in many types of tumor (Coussens and Werb, 1996; Chambers and Matrisian, 1997). It is not sufficient for the cancer cell to leave the original site to form metastases. A shift in the spectrum of integrin expression has to occur before the tumor cell can remain in a new tissue microenvironment (Ishizaki *et al.*, 1995; Varner and Cheresh, 1996; Giancotti and Ruoslahti, 1999).

In brief, protein kinases are the central hub of all these processes. They are necessary to transform a normal cell into a cancer cell and to maintain tumor progression. For this reason, protein kinases have become the main focus in many investigations of cancer therapies.

5.2 Protein kinases – Key players in cancer development, progression, and therapy

Protein kinases comprise a family of more than 520 enzymes that catalyze the transfer of gamma phosphate groups from ATP to hydroxyl groups of serine, threonine, or tyrosine residues in substrate proteins (Edelman *et al.*, 1987; Yarden and Ullrich, 1988; Ullrich and Schlessinger, 1990; Fantl *et al.*, 1993; Manning *et al.*, 2002). Thanks to reversible phosphorylation, protein kinases, especially tyrosine kinases (TKs), are essential regulators of almost all life processes such as cell cycle control, progression, metabolism, proliferation, apoptosis, and differentiation (Ullrich and Schlessinger, 1990).

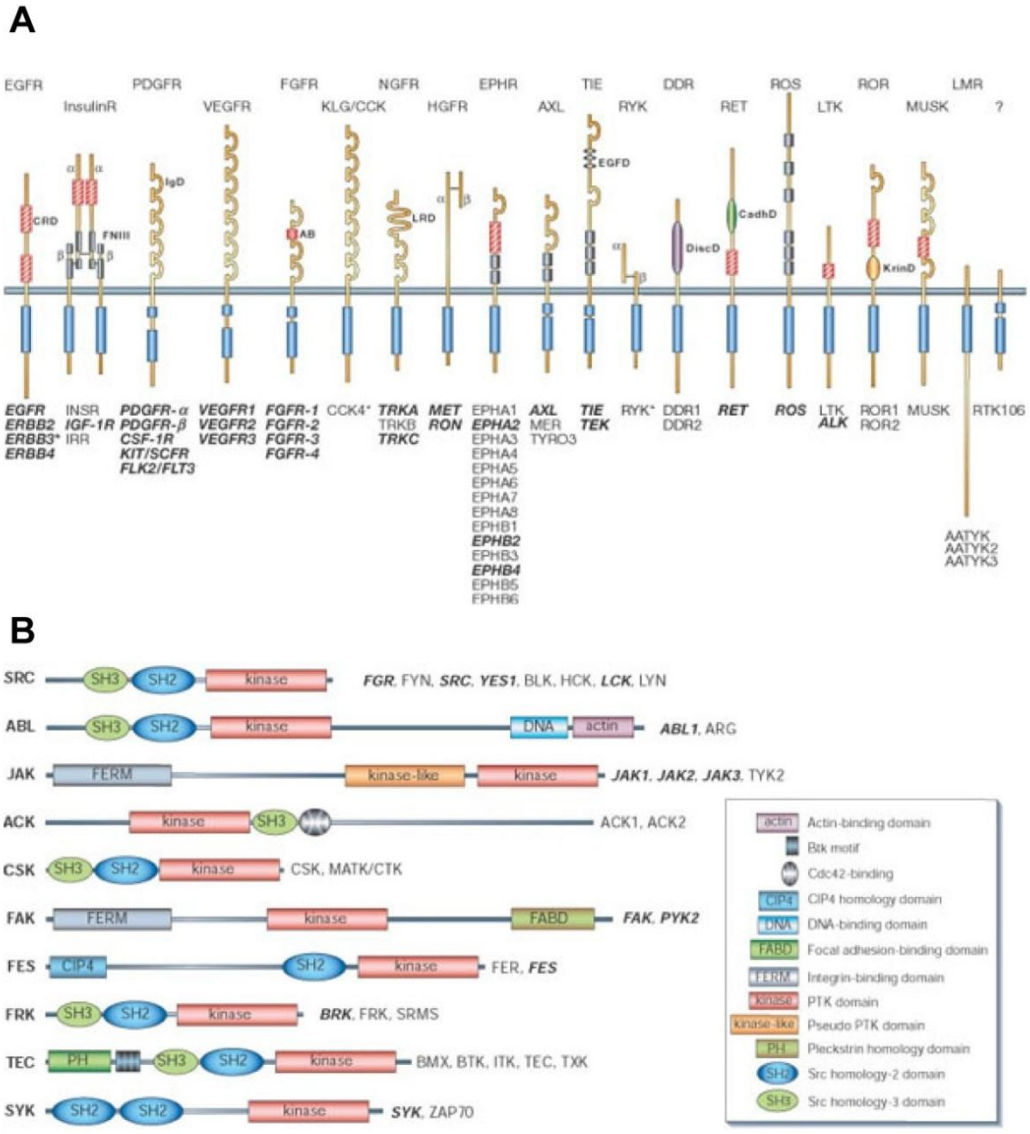

Figure 3: Structure, function, activation and signaling of tyrosine kinases. (A) Receptor tyrosine kinases. The prototypic receptor for each family is mentioned above the receptor; known family members are listed below. (B) Cytoplasmic tyrosine kinases. The prototypic receptor for each family is mentioned on the left of each kinase; known family members are listed on the right. (figure after (Blume-Jensen and Hunter, 2001))

Protein kinases can be assigned to two major categories: receptor tyrosine kinases (RTKs, figure 3/A) and cytoplasmic tyrosine kinases (CTKs, figure 3/B). RTK activation is achieved by ligand-induced receptor oligomerization, which results in the autophosphorylation of tyrosine residues of receptor subunits (Heldin, 1995). This leads to both the activation of the catalytic activity as well as the phosphorylation of additional tyrosine residues, thus mediating the binding of target proteins and subsequently causing downstream signaling.

5.2.1 Involvement of protein kinases in cancer

Tyrosine kinases play the central role in almost every cellular process. Their deregulation has a deep impact on signal transduction pathways and is integral to cancer development. Some of the thus affected pathways include the RAS/RAF/MAPK pathway (Sebolt-Leopold *et al.*, 2007; Lawrence *et al.*, 2008), the WNT pathway (Coombs *et al.*, 2008; Lai *et al.*, 2009; McDonald and Silver, 2009), the Notch and hedgehog pathway (Wang *et al.*, 2008; Li and Harris, 2009; Medina *et al.*, 2009), the PI3-kinase/AKT pathway (Kok *et al.*, 2009; Vogt *et al.*, 2009), and the JAK/STAT pathway (Boudny and Kovarik, 2002; Spano *et al.*, 2006; Nefedova and Gabrilovich, 2007; Li, 2008). Due to their central importance, genes of kinases are very often proto-oncogenes. which when mutated or overexpressed become oncogenes that support the transformation from a normal cell to a tumor cell.

Historically, v-Src (Rous sarcoma virus) was the first oncogene to be discovered. It induces tumor development in chickens (Rous, 1911; Stehelin, 1976). A link to tyrosine kinases was established when v-Src was discovered to be a tyrosine kinase (Collett and Erikson, 1978; Hunter and Sefton, 1980). Axel Ullrich's seminal finding that human EGFR (epidermal growth factor receptor) has a high homology with the v-erbB oncogene raised the question of whether abnormalities in the expression or structure of human endogenous genes could cause human cancer (Ullrich *et al.*, 1984). This idea gained support from the Nobel laureates Michael Bishop and Harold Varmus who found that cancer-inducing genes like v-Src or v-erbB are mutated host genes that had been recombined into the viral genome (Varmus and Bishop, 1986). On the basis of this knowledge, a cDNA-based screen for genetic aberrations was performed with EGFR and

HER2 (human EGFR-related gene). It revealed that HER2 was amplified up to 100-fold in about 30% of patients with invasive breast tumors. Furthermore, HER2 amplification and overexpression strongly correlated with reduced survival and shorter time until relapse (Slamon *et al.*, 1987).

Transcription, localization, activity, and degradation of tyrosine kinases are highly controlled processes in healthy cells. To become oncogenic, the cells have to circumvent this regulation. Four major events contribute to the oncogenic transformation of tyrosine kinases. First, there is a retroviral transduction of an oncogene with homology to a human tyrosine kinase, for example Src or Myc (Cooper *et al.*, 1980; Robinson and Vande Woude, 1982; Symonds *et al.*, 1989; Fu *et al.*, 2005). Second, an oncogenic fusion protein is created via genomic rearrangements like chromosomal translocations. A well-studied example is the fusion protein BCR-ABL. It emerges from the translocation of a part of chromosome 9 to chromosome 22 (t(9;22)(q34;q11), the so-called Philadelphia chromosome), which results in the permanently active fusion protein BCR-ABL that has oncogenic potential. Especially in leukemia, BCR-ABL is frequently present (Chan *et al.*, 1987; Groffen and Heisterkamp, 1987; Kurzrock *et al.*, 1987; Witte, 1988; Heisterkamp *et al.*, 1990). A third possibility is gene overexpression and / or amplification, as in HER2, which is overexpressed up to 100-fold in 30% of patients with invasive breast tumors (Slamon *et al.*, 1987). Finally, a gain-of-function mutation in proto-oncogenes can lead to oncogenic transformation of tyrosine kinases. Thus, PI3KCA is frequently mutated in various cancer types (Bachman *et al.*, 2004; Samuels and Velculescu, 2004; Samuels *et al.*, 2004; Samuels *et al.*, 2005; Ameur *et al.*, 2009). All four oncogenic events result in enhanced or constitutive activity of the kinase and subsequently lead to an increase of downstream signaling (table 1). Thus, the selective inhibition of oncogenic tyrosine kinase activity seems to be an obvious therapeutic strategy (Baselga, 2006; Cruzalegui, 2010).

Table 1: Examples of tyrosine kinases implicated in human cancer. (Zhang *et al.*, 2009)

	Kinase	Tumor/Cancer Types	Oncogenic Alteration
RTK	EGFR	Breast, lung, glioma	Extracellular domain deletions & point mutations(L858R, G719S, & deletions)
	HER-2/ErbB2	Breast, ovarian, colon, lung, gastric	Overexpression
	IGF-IR	Colorectal, pancreatic, breast, ovarian, MM	Overexpression
	PDGFR-α	Glioma, glioblastoma,ovarian, HES	Overexpression & translocation
	PDGFR-β	CMML, glioma, DFSP	Translocation Tel-, COL1A1
	c-Kit	GIST, seminoma,mastocytosis	Point mutations: D816V
	Flt-4, Flt3	AML	Internal tandem duplication
	FGFR1	CML, Stem cell myeloproliferative disorder	Translocation BCR-, FOP-, ZNF198-, CEP110-
	FGFR3	Multiple myeloma	Translocations & point mutations (S249C)
	FGFR4	Breast, ovary	Overexpression
	c-Met	Glioblastoma, Colorectal, Hepatocellular carcinoma, renal carcinoma, HNSCC metastases	Overexpression, translocation Tpr-, & point mutations(Y1253D)
	RON	Colon, hepatocellular carcinoma	Overexpression
	c-Ret	Thyroid carcinoma, MEN2A, MEN2B, FTMC familial & sporadic	Translocations & point mutations
	ALK	Anaplastic large cell lymphoma, lung, neuroblastoma	Translocations NPM-, EML4; point mutations
CTK	c-SRC	Lung, colon, breast & prostate	Overexpression, C-terminal truncation
	c-YES	Lung, colon, breast & prostate	Overexpression
	Abl	CML	Translocation Bcr-
	JAK-2	CML, T-ALL, solid	Translocation Tel-, point mutation V617F
S/T Kinase	Akt	Multiple	Overexpression
	ATM	Ataxia telangiectasia	Point mutations
	Aurora A & B	Multiple	Overexpression
	CDKs	Multiple	Overexpression
	mTOR	Multiple	Overexpression
	PKCi	Non-small cell lung, ovarian	Overexpression
	PLKs	Multiple	Overexpression
	b-Raf	Colon, thyroid, melanoma	Point mutation (V599M)
	S6K	Multiple	Overexpression
	STK11/LKB1	Peutz-Jeghers syndrome	Point mutations
LK	PI3K	Prostate, colorectal, breast	Overexpression, point mutations (H1047R)
	SK1	Breast, prostate	Overexpression

RTK: receptor tyrosine kinase, CTK: cytoplasmic tyrosine kinase, S/T Kinase: serine/threonine kinase, LK: lipid kinase. MM: Multiple Myeloma; HES: Hypereosinophilic Syndrome; CMML: Chronic Myelomonocytic Leukemias; DFSP: Dermatofibrosarcoma Protuberans; AML:Acute Myelogenous Leukemia; GIST: Gastrointestinal Stromal tumor; HNSCC: Head and neck squamous cell carcinoma; MEN2A: Multiple Endocrine Neoplasia type 2A Syndrome; MEN2B: Multiple Endocrine Neoplasia type 2B Syndrome; FTMC: Familial Medullary Thyroid Carcinoma; CML: Chronic Myelogenous Leukemia; T-ALL: T cell Acute Lymphoblastic Leukemia; (Zhang *et al.*, 2009)

5.2.2 Protein kinases as targets for cancer therapy

Chemotherapeutic agents (e.g., mechlorethamine (Bis(2-chloroethyl)methylamine), a nitrogen mustard) have been used clinically in the treatment of cancer since 1942. These highly toxic drugs act by interfering with the cell metabolism (for example, by alkylating DNA) and thus kill dividing cells. Due to the fact that not only cancer cells but also some healthy cells permanently divide (e.g., bone marrow, cells of the mucous membrane of the oral cavity, or cells of the hair root) adverse side effects are quite frequent. New strategies that better distinguish between cancer and normal cells are needed. Since deregulated protein kinases are key players in tumor formation, proliferation, invasion, angiogenesis, metastasis, apoptosis, and survival (Blume-Jensen and Hunter, 2001), the development of selective kinase inhibitors has been considered imperative (Baselga, 2006; Cruzalegui, 2010). The first targeted anti-cancer drug was trastuzumab (Herceptin®), a therapeutic monoclonal antibody against HER2 for the treatment of HER2-positive invasive breast cancer (Hudziak *et al.*, 1989; Fendly *et al.*, 1990; Baselga *et al.*, 1998; Robertson, 1998). Since then, several other kinase inhibitors have been tested in clinical trials or have even been clinically approved by the Food and Drug Administration (FDA) of the United States.

5.2.2.1 Classification of targeted protein kinase inhibitors

Targeted kinase inhibitors can be grouped into two classes: therapeutic antibodies (suffix: -omab (murine antibody); -imab (primate antibody); -ximab (chimeric antibody); -zumab (humanized antibody); -umab (human antibody)) and small molecule inhibitors (suffix: -ib). Examples of targeted inhibitors are given in figure 4.

Both classes of inhibitors are able to inhibit target activity that blocks cancer-relevant processes (Levitzki and Gazit, 1995), albeit by different modes of action. As therapeutic antibodies do not enter the cell, they work mainly via steric blocking of molecule-molecule interaction. Thus, they target only extracellular antigens such as receptor tyrosine kinases or ligands. However, all kinds of proteins can be blocked; it is not limited to kinases. In contrast, small molecule inhibitors enter the cell and compete with the ATP binding site of the catalytic domain; this blocks kinase activity and subsequently downstream signaling.

Kinase inhibitor	Tumor entity/tumor stadium	SMI target
Imatinib*	Ph^+ CML (since 2001), GIST (since Dec 2008) dermatofibrosarcoma protuberans (since Oct 2006), Ph^+ ALL (since Oct 2006)	TKs (BCR-ABL) c-KIT, PDGFR
Nilotinib* (AMN107)	Imatinib resistant CML (since Oct 2007), ALL, GIST	BCR-ABL
Sunitinib*	Advanced RCC (since Jan 2006), Imatinib resistant GIST (since Jan 2006), advanced hepatocellular cancer, pancreatic neuroendocrine tumors, NSCLC	TK, VEGFR1 + 2; PDGFRα + β, c-KIT, FLT3, RET, CSF1R
Erlotinib*	Advanced/metastatic NSCLC after failure of at least one chemotherapy regimen (since Nov. 2004) + gemcitabine in advanced/metastatic pancreatic cancer (since Nov 2005)	EGFR
Pazopanib*	Advanced RCC (since Oct 2009)	PDGFR, VEGFR, c-KIT
Sorafenib*	Metastatic RCC (since Dec 2005), advanced hepatocellular carcinoma (since Nov 2007)	Multikinase inhibitor, PDGFR, VEGFR, c-KIT and Ser/Thr pecific kinases, e. g. Raf1
Gefitinib*	NSCLC with EGFR mutations (since May 2003)	TK, EGFR
Saracatinib (AZD0530)	Indication by metastatic cancer with increased bone resorption	Src
Danuserbib (PHA-739358)	Solid tumors refactory to standard therapy	Aurora kinase
Dasatinib*	Imatinib resistant CML (since June 2006)	TKs (BCR-ABL) Src, Lyn, Btk
CP-868,596	Advanced solid tumors	PDGFR
ABT-869	Solid tumors refractory to standard therapy	Multiple receptor TKs
Vandetanib (ZD6474)	NSCLC, medullary thyroid cancer	EGFR, VEGFR, RET
Telatinib	Advanced metastatic solid tumors	VEGFR2 + 3, PDGFR-β, c-KIT
Axitinib	Advanced NSCLC	VEGFR1, 2, 3
Bevacizumab (mAb)*	Metastatic CRC (since Feb 2004), RCC, NSCLC, HER2 negative breast cancer, glioblastoma multiforme	VEGFR
Trastuzumab (mAb)*	$HER2^+$ breast cancer (since Sept 1998) Endometrial cancer	HER2
Masitinib	Advanced and metastatic solid tumors	c-KIT
UCN-01 (NSC 638850)	Advanced solid cancers	Chk1
Neratinib (HKI-272)	Advanced solid tumors, Breast cancer, NSCLC	EGFR, HER2
BIBW-2992	NSCLC tumors with activating mutations in EGFR kinase domain.	EGFR, HER2
CI-1033	Advanced metastatic breast cancer	ErbB
Lapatinib*	+ capecitabine, advanced/metastatic breast cancer $HER2^+$ (since Mar 2007)	EGFR, HER2
Cedirnanib	Advanced NSCLC tumors	VEGFR1, 2, 3
Motesanib	Advanced or metastatic medullary thyroid cancer	VEGFR1, 2, 3, c-KIT, PDGFR

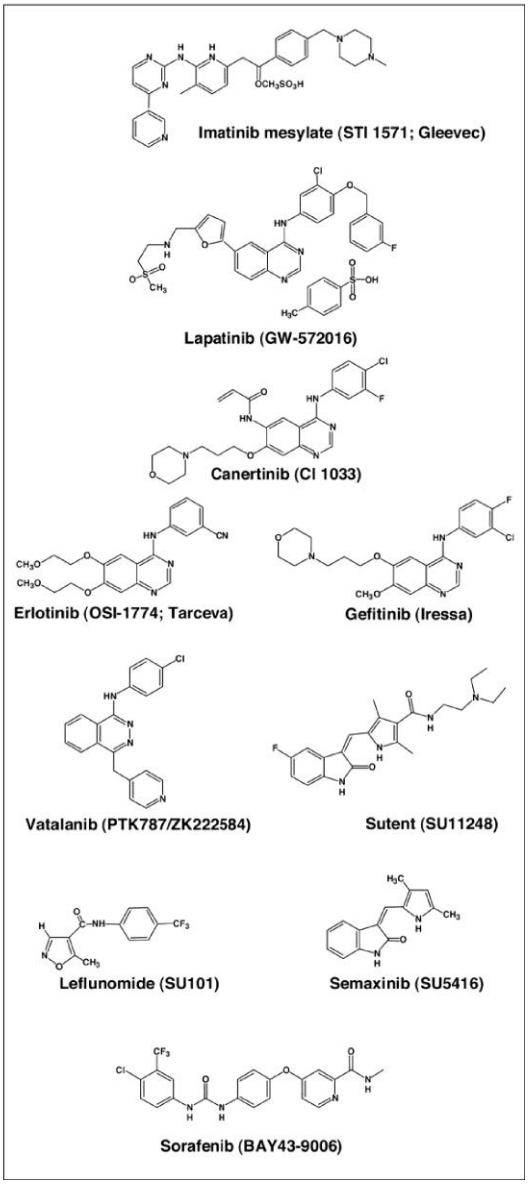

Figure 4: Examples of kinase inhibitors. Left: Kinase inhibitors with their target proteins and tumor indication. (*) signifies that this drug has already been approved by the Food and Drug Administration (FDA) of the United States. Right: Structures of several small molecule inhibitors. Abbreviations: mAb: monoclonal antibody, ALL: acute lymphocytic leukemia, CML: chronic myeloid leukemia, CRC: colorectal cancer, GIST: gastrointestinal stromal tumor, NSCLC: non-small cell lung cancer, Ph+: Philadelphia-chromosome positive, RCC: renal cell carcinoma, TKs: tyrosine kinases, Btk: Bruton's tyrosine kinase, CSF1R: colony stimulating factor 1 receptor, EGFR: epidermal growth factor receptor, FLT: fms-like tyrosine kinase receptor, mTOR: mammalian target of rapamycin, PDGFR: platelet derived growth factor receptor, PI3K: phosphatidylinositol 3-kinase, PKC: protein kinase C, VEGFR: vascular endothelial growth factor receptor, CNS: central nervous system, GI: gastrointestinal. (table after (Giamas *et al.*, 2010). Image taken from (Arora and Scholar, 2005))

5.2.2.2 A closer look at small molecule kinase inhibitors

Protein kinases catalyze the transfer of the gamma-phosphate of ATP to a serine, threonine, or tyrosine residue of substrate proteins. This transfer activates the target protein and subsequently leads to downstream signaling (Ullrich and Schlessinger, 1990). The required ATP-binding to the kinases occurs at a homologue region: the ATP-binding pocket. Additionally, all protein kinases share an activation loop; it is important for the regulation of kinase activity (Johnson *et al.*, 1998; Manning *et al.*, 2002). The enzyme can switch between an active and an inactive state, depending on the ATP-binding status, by changing the conformation of the binding pocket and the active loop (Johnson *et al.*, 1998; Traxler and Furet, 1999).

Most small molecule inhibitors compete with ATP for binding to the ATP-binding site. Inhibitors can bind either to the active (type 1 inhibitor) or the inactive (type 2 inhibitor) conformation of a protein kinase (Zhang *et al.*, 2009). Most inhibitors are type 1 inhibitors. They typically consist of a heterocyclic core scaffold with the purine binding site and several side chains, which attach to the hydrophobic pockets of the ATP binding site (figure 5). Examples of type 1 inhibitors are sunitinib (SUTENT®), a potent inhibitor of VEGFR, PDGFR, cKIT, and several others, or gefitinib (Iressa®) and erlotinib (Tarceva®), both inhibitors of EGFR (Sun *et al.*, 1999; Laird *et al.*, 2000; Mendel *et al.*, 2000; Wakeling *et al.*, 2002; Bulgaru *et al.*, 2003; Perez-Soler, 2004; Cohen *et al.*, 2005; Comis, 2005; Wakelee and Schiller, 2005; Cabebe and Wakelee, 2006; Faivre *et al.*, 2007; Roskoski, 2007; Shibuya, 2009; Eichholz *et al.*, 2010). Type 2 inhibitors recognize the inactive conformation of protein kinases. In the inactive state, an additional hydrophobic binding site right next to the ATP-binding site is accessible, which serves as anchorage for type 2 inhibitors. Examples of this type are imatinib (Gleevec®), an inhibitor of the BCR-ABL fusion protein, PDGFRα/β and cKIT, and the Raf inhibitor sorafinib (Joensuu *et al.*, 2001; Druker, 2002; Druker, 2002; Kantarjian *et al.*, 2003; Wan *et al.*, 2004).

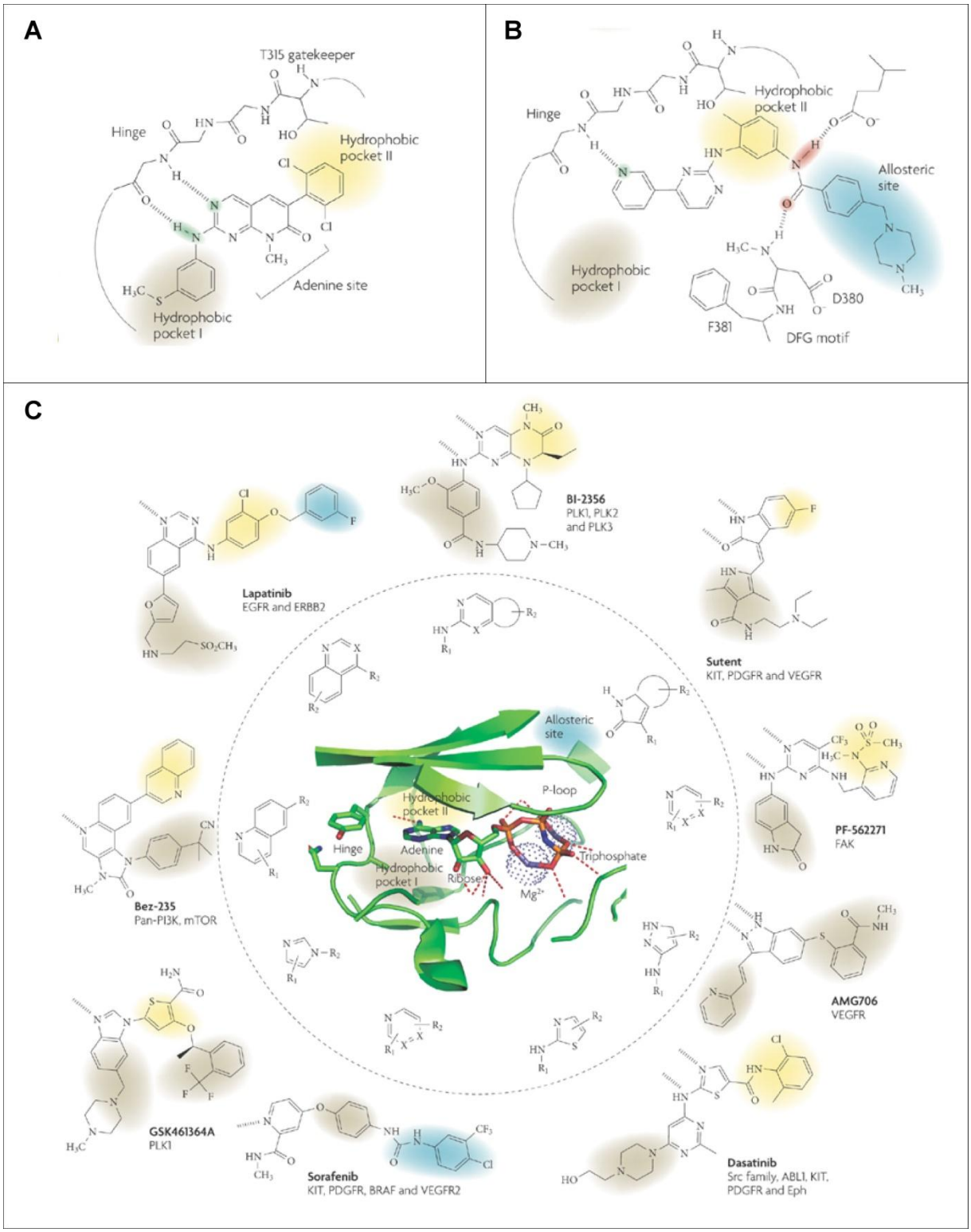

Figure 5: Binding of ATP-competitive small molecule inhibitors. (A) Example of the binding of a type 1 inhibitor. ABL1 in complex with the type 1 ATP-competitive inhibitor PD166326. (B) Example of the binding of a type 2 inhibitor. ABL1 in complex with the type 2 inhibitor imatinib. The allosteric pocket exposed in the DFG-out conformation is indicated by the blue shaded area. (C) Diversity of kinase inhibitors. The ATP binding site of AKT1 in complex with ATP . The middle ring shows commonly used heterocyclic core scaffolds (X = C, N). The outer ring shows examples of structurally diverse type 1 inhibitors and their reported kinase targets. Colors of parts of the inhibitors indicate their binding to the hydrophobic pocket I (gray) or II (yellow) or the allosteric binding site (blue) EGFR, epidermal growth factor receptor; Eph, ephrin receptor tyrosine kinases; FAK, focal adhesion kinase; PDGFR, platelet-derived growth factor; PLK, Polo-like kinase; VEGFR, vascular endothelial growth factor receptor. (figure after (Zhang *et al.*, 2009))

5.3 The small molecule inhibitor sunitinib malate (SU12248, SUTENT®)

Sunitinib malate (SUTENT®, SU12248 (named after Joseph Schlessinger and Axel Ullrich, founder of the biotech company SUGEN, which was later acquired by Pharmacia and subsequently Pfizer), (5-[5-fluoro-2-oxo-1,2-dihydroindol-(3Z)-ylidenemethyl]-2,4-dimethyl-1H-pyrrole-3-carboxylic acid [2-diethylaminoethyl]amide)) is an orally available, multi-targeted ATP-competitive small molecule inhibitor. It was simultaneously approved for the treatment of imatinib-intolerant or resistant gastrointestinal stromal tumors (GIST) and metastatic renal cell carcinoma (mRCC) by the U.S. Food and Drug Administration (FDA) in 2006 (Demetri *et al.*, 2006; Motzer *et al.*, 2006; Motzer *et al.*, 2006; Goodman *et al.*, 2007; Motzer *et al.*, 2007; Motzer *et al.*, 2007). In 2007 the European Union also approved sunitinib malate for the treatment of both cancer types.

5.3.1 Evolution of sunitinib malate – from single-targeting to multi-targeting

For a long time it was believed that as few targets as possible should be inhibited in order to avoid severe side effects. Thus, the long search for the "magic bullet" protein, which if inhibited would cure cancer, continued for many years. To halt angiogenesis and consequently tumor growth beyond a size of 1-2 mm^3, the initial target during the development of sunitinib was the vascular endothelial growth factor receptor (VEGFR) (Folkman, 1972; Gimbrone *et al.*, 1972; Folkman, 1990; Millauer *et al.*, 1994). SU5416, a potent inhibitor of VEGFR1/2/3, was the prototype (Fong *et al.*, 1999; Shaheen *et al.*, 1999). For better clinical efficacy, the drug was chemically modified to yield SU6668. The chemical modifications not only resulted in better properties but also in a broader spectrum of target inhibition, which included VEGFR1/2/3, platelet-derived growth factor receptor (PDGFR), and fibroblast growth factor receptor (FGFR) (Sun *et al.*, 1999). Although both drugs showed antitumor activity *in vivo* (Laird *et al.*, 2000; Mendel *et al.*, 2000), they failed clinical trials because of their inadequate, toxic, and pharmacokinetic properties. Further investigation finally resulted in SU12248 (sunitinib, figure 6). Again, the additional chemical modifications increased the pharmacological properties but also decreased target specificity. Although the complete spectrum of inhibited proteins was unknown, targets included PDGFRα/β, ret proto-oncogene (RET), Fms-like tyrosine kinase-3 receptor (FLT3), VEGFR1/2/3, colony-stimulating factor 1 receptor (CSF1R),

and stem cell factor receptor (cKIT) (Abrams *et al.*, 2003; Mendel *et al.*, 2003; Murray *et al.*, 2003; O'Farrell *et al.*, 2003; Sun *et al.*, 2003; Faivre *et al.*, 2007).

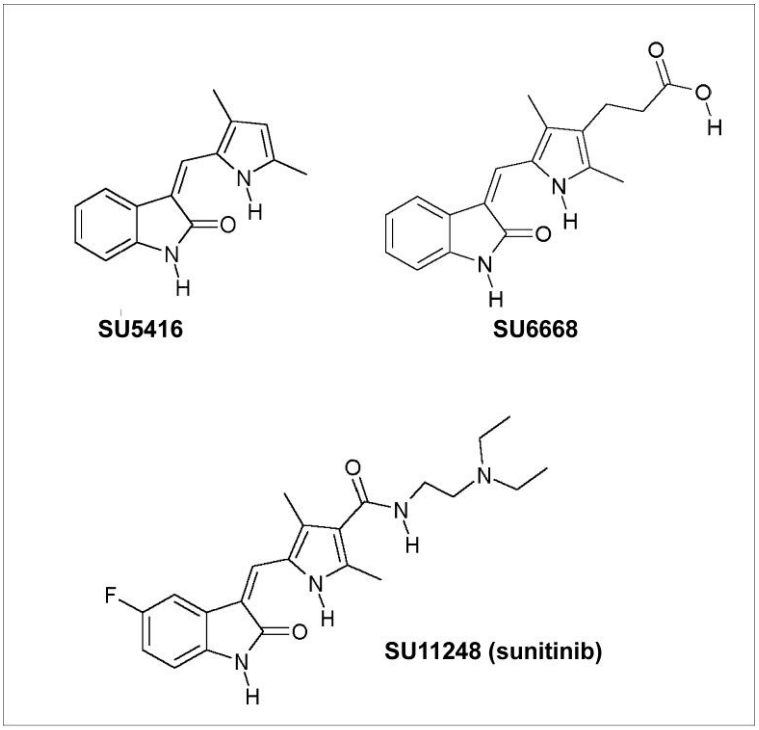

Figure 6: Evolution of SU12248. (5- [5-fluoro-2-oxo-1,2- dihydroindol-(3Z)-ylidenemethyl]-2, 4-dimethyl-1H-pyrrole-3-carboxylic acid [2-diethylaminoethyl]amide). (structures after (Schlessinger, 2005))

Sunitinib showed time-dependent and dose-dependent antitumor activity in mice with human tumor xenografts of breast, lung, renal, and liver carcinoma, as well as melanoma (Abrams *et al.*, 2003; Abrams *et al.*, 2003; Mendel *et al.*, 2003; Murray *et al.*, 2003; Morimoto *et al.*, 2004; Yee *et al.*, 2004; Huynh *et al.*, 2009). Thus, the FDA approved sunitinib malate (SUTENT) for the treatment of metastatic renal cell carcinoma (mRCC) and gastrointestinal stromal tumors (GIST) in 2006 (Goodman *et al.*, 2007). In addition SU12248 showed anticancer activity in various other tumor types such as non-small-cell lung cancer (NSCLC), thyroid cancer, melanoma, hepatocellular carcinoma, head and neck squamous cell carcinoma, metastatic thymic carcinomas, neuroendocrine tumors (NET), as well as colon cancer (Abrams *et al.*, 2003; Mendel *et al.*, 2003; Murray *et al.*, 2003; O'Farrell *et al.*, 2003; Faivre *et al.*, 2006; Motzer *et al.*, 2007; Strobel *et al.*).

Surprisingly, the initial fear of inhibiting several kinases at once has changed into an awareness of its advantage. Multi-targeted kinase inhibitors like sunitinib cause only

mild side effects like fatigue, hand-foot syndrome, diarrhea, or skin discoloration (Kollmannsberger *et al.*, 2007). Such agents also decrease the risk of developing resistances due to mutations or the bypassing of signals. Moreover, as the number of drugs a patient is required to take is reduced, the risk of drug interactions and toxicity is also decreased (Faivre *et al.*, 2007).

5.3.2 The spectrum of target proteins inhibited by sunitinib malate

Initially, sunitinib was known to inhibit only a few protein kinases (PDGFRα/β, RET, FLT3, VEGFR1/2/3, CSF1R, and cKIT (Abrams *et al.*, 2003; Mendel *et al.*, 2003; Murray *et al.*, 2003; O'Farrell *et al.*, 2003; Sun *et al.*, 2003; Faivre *et al.*, 2007)). Just recently, our group (PhD thesis, Michaela Bairlein, 2010, TU Munich) successfully elucidated the whole spectrum of all inhibited proteins. More than 200 cellular proteins were found to bind to sunitinib *in vitro*. Of these about 50-60 are considered mediators of the drug response due to their high-affinity binding to sunitinib (figure 7).

The link to cancer has been well established for several of these targets including non-metastatic cells 4 (NME4), c-ros oncogene 1 (ROS1), BMP2 inducible kinase (BMP2K), aurora kinase (AURKA/B), never in mitosis gene a-related kinase 9 (NEK9), and TANK-binding kinase (TBK1) (Kang *et al.*; Chen *et al.*, 1991; Liu *et al.*, 1996; Pomerantz and Baltimore, 1999; Belham *et al.*, 2003; Waite and Eng, 2003; Horvath *et al.*, 2004; Tan and Lee, 2004; Furukawa *et al.*, 2006; Kracmarova *et al.*, 2008; Veerakumarasivam *et al.*, 2008; Jun *et al.*, 2009). However, it is less understood how tyrosine kinase 2 (TYK2), another high-affinity sunitinib target, is involved in tumor development and progression.

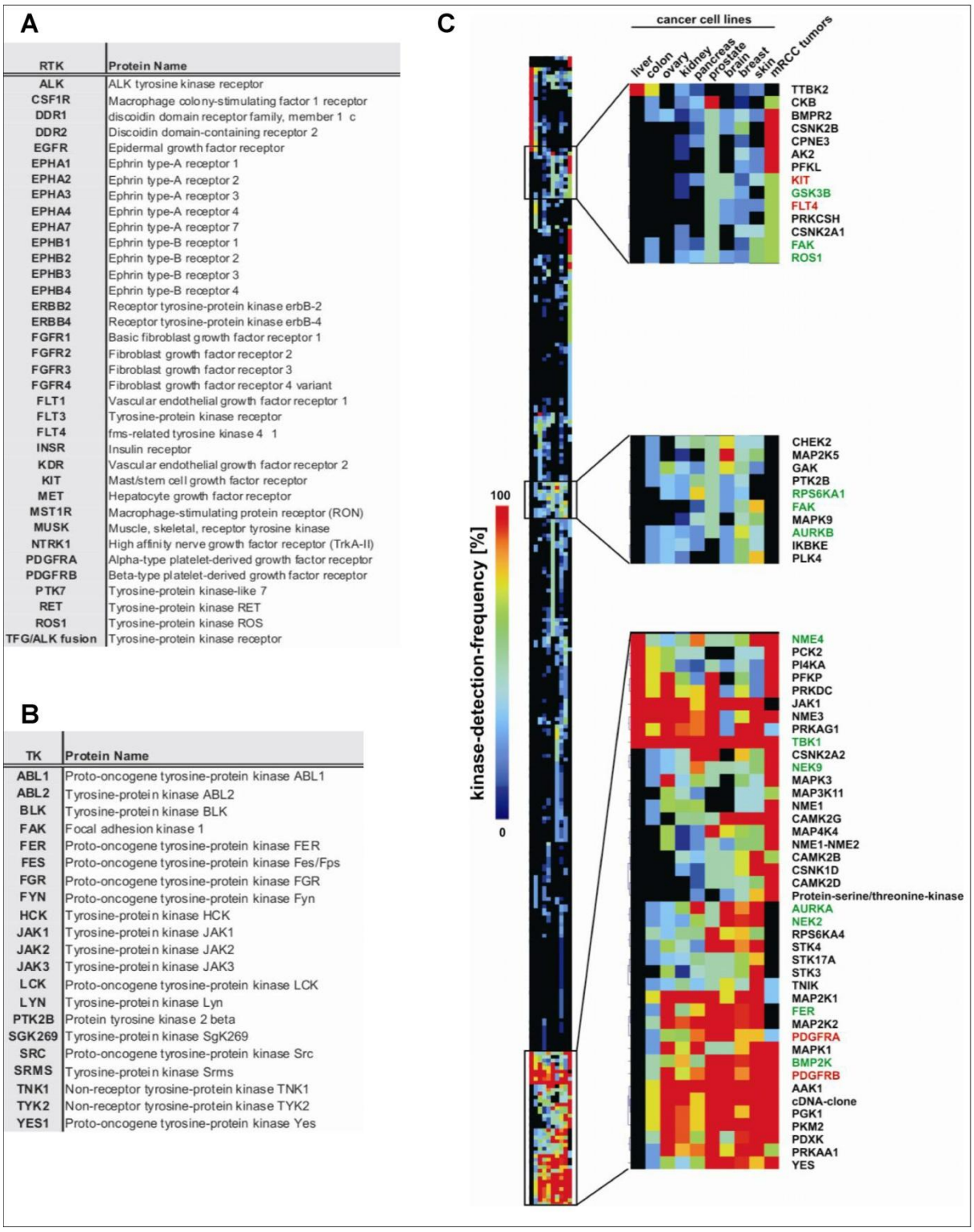

Figure 7: Tyrosine kinase targets of sunitinib malate thought to mediate drug response. (A) Receptor tyrosine kinases targeted by sunitinib malate. (B) Cytoplasmic tyrosine kinases targeted by sunitinib malate. Targets are sorted alphabetically. (C) Target interaction map for sunitinib in cancer cell lines and mRCC tumors. Selectivity profiles of sunitinib are sorted by tissue, shown in percentile kinase detection per tissue, and analyzed by a two-dimensional hierarchical cluster algorithm Euclidean distance-based. Three clusters of frequently detected kinase targets based on identification coverage per tissue or occurrence among different cancer types are magnified. Those sunitinib targets already described are marked in red; interesting new hits are highlighted in green. (Taken from PhD thesis, Michaela Bairlein, TU Munich, 2010)

5.4 Genetic alterations in cancer

Genetic alterations can be cancerogenic and influence the etiopathology and aggressiveness of the tumor as well as patient response to cancer therapy (Sasaki, 1982; Hynes, 1993; Fukasawa, 2005). A distinction is made between mutations, insertions, deletions, amplifications, and single nucleotide polymorphisms (SNPs). In contrast to all other genetic alterations, the different alleles of SNPs occur naturally with a certain distribution among the population and thus never cause cancer. However, alleles of SNPs can strongly influence progression and / or treatment of the disease (Bange *et al.*, 2002; Jezequel *et al.*, 2004; Thussbas *et al.*, 2006; Naidu *et al.*, 2009). Extensive investigations are now in progress on the central role of genetic alterations in the development, progression, and cure of cancer. This work is supported by databases like TyKiVa (tyrosine kinome variants) (Ruhe *et al.*, 2007), which lists all genetic alterations of 254 established cancer cell lines and thus provides extensive system information for the design and interpretation of cell line-based cancer research.

5.4.1 Genetic alterations – friend and foe of cancer therapy

The potential resistance-mediating influence of mutations on cancer therapy has been known for decades (Schimmer *et al.*, 1984; el Rouby *et al.*, 1993). In recent years genetic alterations were also identified which increased sensitivities. One of the best studied examples is the influence of genetic alterations on the efficacy of gefinitib (Iressa®), a selective small molecule inhibitor of EGFR (Hirata *et al.*, 2004; Settleman, 2004). Gefitinib has been approved for the treatment of patients with advanced non-small cell lung cancer (NSCLC) who failed to respond to conventional chemotherapy (Cohen *et al.*, 2003; Cohen *et al.*, 2004). Although gefitinib inhibits not only EGFR but also several other kinases such as ROCK or IRAK1, mutations in EGFR are sufficient to mediate an altered drug response (Brehmer *et al.*, 2005). Interestingly, all mutations affecting the efficacy of gefitinib are located in exons 18-21, part of the tyrosine kinase domain of EGFR. While mutations such as L858R or G719S impair increased gefitinib response (Tracy *et al.*, 2004; Chen *et al.*, 2006; Jackman *et al.*, 2006; Sasaki *et al.*, 2006), T790M causes resistance to gefitinib treatment (figure 8) (Bell *et al.*, 2005; Regales *et al.*, 2007; Vikis *et al.*, 2007; Yun *et al.*, 2008). In view of their strong impact on EGFR inhibition, these genetic alterations have become genetic markers that allow estimation of the

success of gefitinib therapy (Kobayashi *et al.*, 2005; Shih *et al.*, 2005; Taja-Chayeb *et al.*, 2005; Han *et al.*, 2006; Okamoto *et al.*, 2006; Wu *et al.*, 2008).

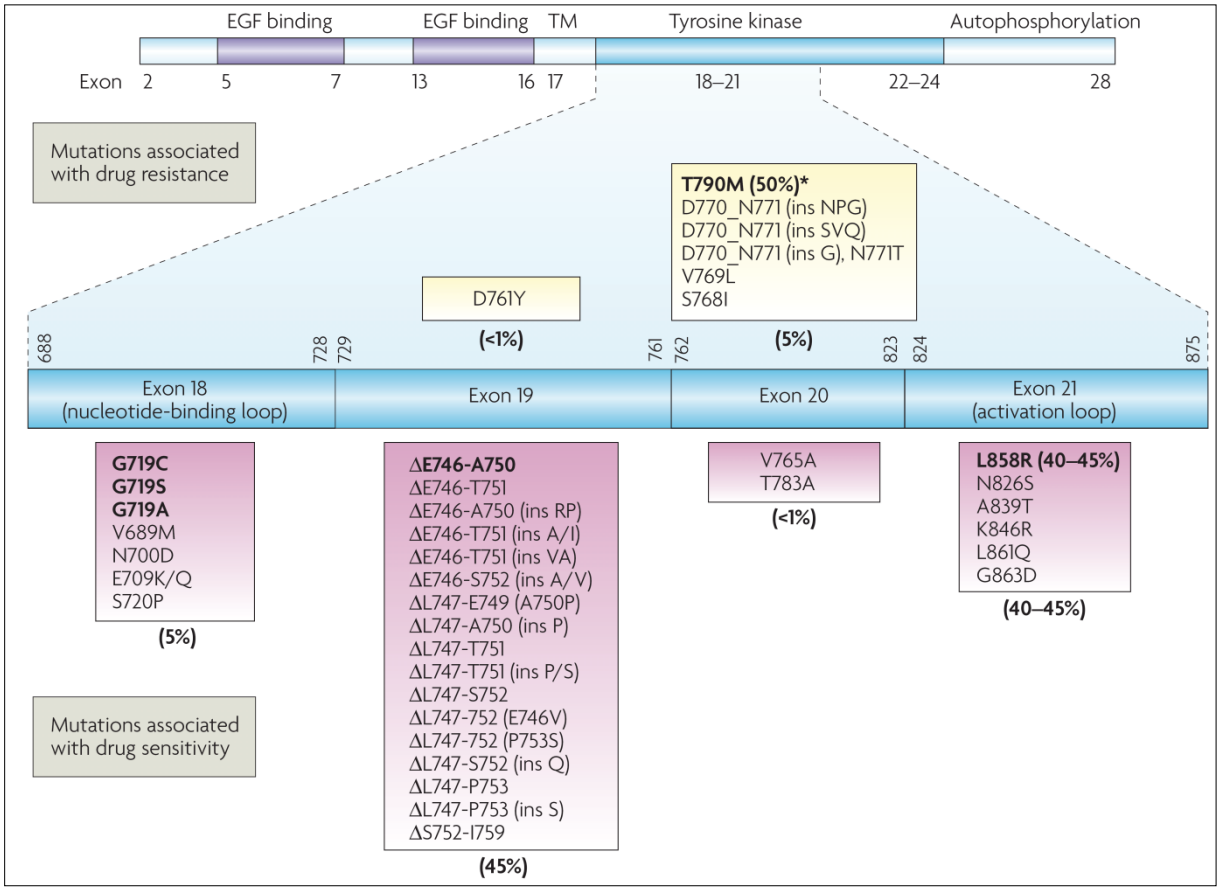

Figure 8: Genetic alterations in EGFR that influence the response to gefitinib therapy in NSCLC. A diagram of the epidermal growth factor receptor (EGFR) showing the distribution of exons in the extracellular domain (EGF binding), transmembrane domain (TM), and intracellular domain. Exons 18–21 in the tyrosine kinase region, where the relevant mutations are located, is enlarged. A detailed list of EGFR mutations that are associated with sensitivity (magenta boxes) or resistance (yellow boxes) to gefitinib is given. The most important genetic alterations are in bold. (*) indicates the clinically most relevant mutation associated with resistance, T790M. (figure taken from (Sharma *et al.*, 2007))

5.4.2 Genetic alterations in tumors treated with sunitinib malate

Several years ago it was predicted that genetic alteration would play a role in the efficacy of sunitinib. For example, FLT3 is frequently mutated in acute myeloid leukemia (AML) (Naoe and Kiyoi, 2004; Ozeki *et al.*, 2004), and RET can be altered in thyroid carcinoma or in multiple endocrine neoplasia (Eng *et al.*, 1995; Chang *et al.*, 1999; Rey *et al.*, 2001). Likewise the activation of cKIT and PDGFR mutations in GIST is known to influence therapy response (Heinrich *et al.*, 2003; Duensing *et al.*, 2004; Duensing *et al.*, 2004). Nowadays, several mutations with impact on sunitinib therapy in GIST have been

documented: the cKIT mutations D816H/V, D820G, N822K, Y823D, A829P, and the PDGFRα mutation D842V result in resistance to sunitinib treatment. In contrast, the mutations V645A and T670I in cKIT and V561D in PDGFRα mediate an enhanced response (figure 9) (Heinrich *et al.*, 2008).

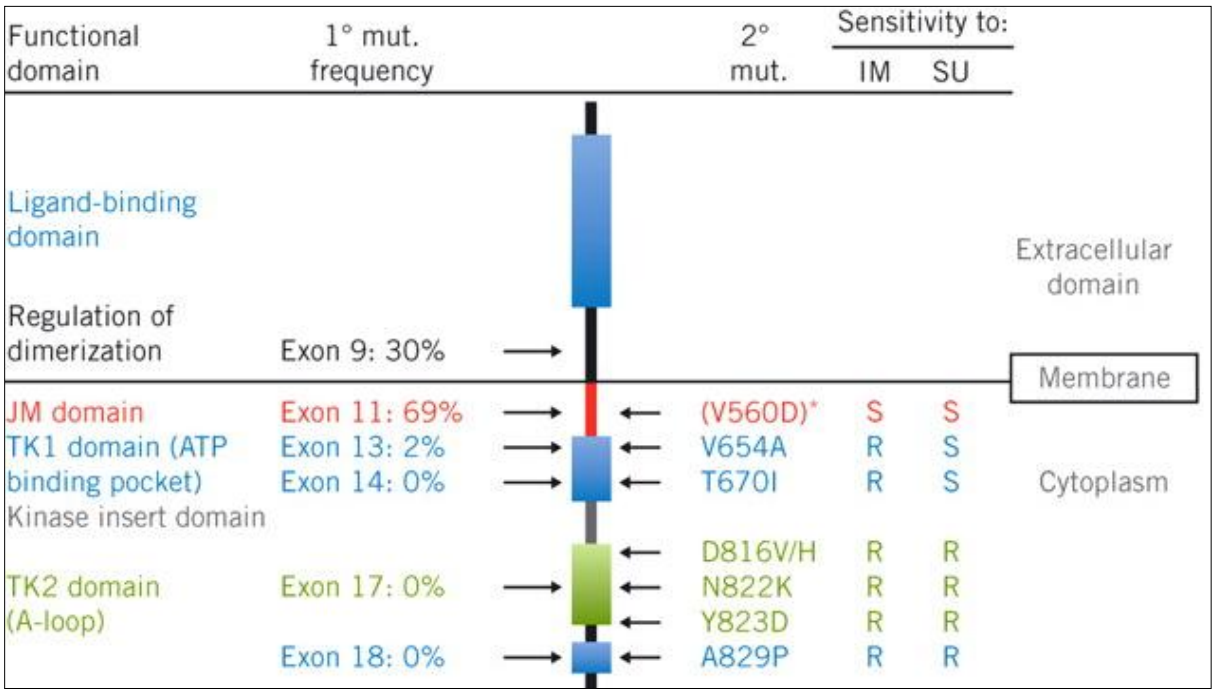

Figure 9: cKIT mutations that alter the efficacy of sunitinib. Primary (1°) and secondary (2°) mutations (mut.). Frequencies of primary KIT genotypes, specific secondary KIT mutations, and resistance (R) or sensitivity (S) to imatinib (IM) or sunitinib (SU) (figure taken from (Gajiwala *et al.*, 2009))

As GIST is mainly driven by PDGFR and/or cKIT, the knowledge of mutations in these two receptor tyrosine kinases might suffice as a prognostic indicator for the efficacy of GIST treatment (Heinrich *et al.*, 2003; Lasota *et al.*, 2008; Lasota and Miettinen, 2008; Gajiwala *et al.*, 2009; Nishida *et al.*, 2009). However, since sunitinib exhibits anti-tumor activity in many different tumor types, the global analysis of genetic alterations that influence the efficacy of sunitinib malate in various cancer types is of great interest.

6. Aims of this PhD thesis

Cancer is still the second leading cause of human deaths worldwide. Major efforts are under way to find new drug targets, to develop new anti-cancer drugs, to find new indications for already approved drugs and to improve already existing therapies. For the latter task, mutations, single nucleotide polymorphisms (SNPs) and other genetic alterations are in the focus of investigation.

This study focused on the small-molecule inhibitor sunitinib malate, which has been approved for the treatment of gastrointestinal stromal tumors (GIST) and metastatic renal cell carcinoma (mRCC). It is currently undergoing clinical trials for use in the treatment of an expanded range of various tumor types. The aim of this PhD thesis was to elucidate the genetic alterations that influence the response to sunitinib treatment and in the process improve the ability to predict the success of sunitinib-based therapy.

7. Materials and Methods

7.1 Materials

7.1.1 Laboratory chemicals, biochemicals and inhibitors

Acrylamide	Serva, Heidelberg
Aprotinin	Sigma, Taufkirchen
APS (Ammonium peroxodisulfate)	Bio-Rad, München
Bisacrylamide	Roth, Karlsruhe
Bromphenol blue	Sigma, Taufkirchen
BSA (Bovine serum albumin)	Sigma, Taufkirchen
Coomassie G250	Serva, Heidelberg
Crystal Violet	Sigma, Taufkirchen
Gefitinib (IRESSA)	LC Laboratories, USA
HEPES (N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid))	Serva, Heidelberg
L-Glutamine (GibCo)	Invitrogen, Eggenstein
Matrigel	BD Biosciences, Heidelberg
MTT	Sigma, Taufkirchen
PMSF (Phenylmethanesulfonyl fluoride)	Sigma, Taufkirchen
Ponceau S	Sigma, Taufkirchen
Propidium iodide	Roche, Mannheim
SDS (Sodium dodecyl sulfate)	Roth, Karlsruhe
Sodium azide	Serva, Heidelberg
Sodium fluoride	Sigma, Taufkirchen
Sodium orthovanadate	Sigma, Taufkirchen
SU11248 (SUTENT, sunitinib)	ACC Corporation, USA
TEMED (N,N,N',N'-Tetramethylethylenediamine)	Serva, Heidelberg
Triton X-100	Serva, Heidelberg

All other chemicals were purchased in analytical grade from Merck (Darmstadt).

7.1.2 Enzymes

DNAse I, RNAse free	Roche, Mannheim
LA Taq-DNA Polymerase	Takara, Japan
Trypsin (GibCo)	Invitrogen, Eggenstein
Reverse Transcriptase (AMV)	Roche, Mannheim

7.1.3 Kits and other materials

Cell culture materials	Greiner, Solingen
Cellulose nitrate 0.45 µm	Schleicher & Schüll, Dassel
Caspase 3/7 Glo Assay	Promega, USA
ECL Kit	PerkinElmer/NEN, Köln
Hyperfilm MP	Amersham, Freiburg
Micro BCA Protein Assay Kit	Pierce, Sankt Augustin
Parafilm	Dynatech, Denkendorf
Protein A-Sepharose	Amersham, Freiburg
Protein G-Sepharose	Amersham, Freiburg
QIAGEN Plasmid Maxi Kit	Qiagen, Hilden
QIAGEN Plasmid Mini Kit	Qiagen, Hilden
QIAGEN RNeasy Mini Kit	Qiagen, Hilden
QIAquick PCR Purification Kit (50)	Qiagen, Hilden
Sterile filter 0.22 µm, cellulose acetate	Nalge Company, USA
Sterile filter 0.45 µm, cellulose acetate	Nalge Company, USA
Transwells, 0.8 µM pore-size	BD Biosciences, Heidelberg
Whatman 3MM	Whatman, Rotenburg/Fulda

7.1.4 Growth factors and ligands

EGF (human)	Peprotech, USA
PDGF-BB (human)	Peprotech, USA
PDGF-AA (human)	Peprotech, USA
SCF (human)	Peprotech, USA
MSP (human)	Peprotech, USA

IL-6 (human)	Peprotech, USA
OSM (human)	Peprotech, USA

7.2 Cell culture media

GibcoTM media and additives were obtained from Invitrogen (Eggenstein). Media were supplemented to the requirements of each cell line. Freeze medium contained 95% heat-inactivated FCS and 5% DMSO.

7.3 Stock solutions and commonly used buffers

BBS (2x)	50 mM BES 280 mM NaCl 1.5 mM Na2HPO4 pH 6.96
Collecting gel	0,5 M Tris/HCl pH6.8 buffer (4x) 0,4 % SDS HBS (2x) 46 mM HEPES, pH 7.5 274 mM NaCl 1.5 mM Na2HPO4 pH 7.0
HNTG	20.0 mM HEPES, pH 7.5 150 mM NaCl 0.1% TritonX-100 10.0% Glycerol 10.0 mM Na4P2O7
DNA loading buffer (6x)	0.05% Bromphenol blue 0.05% Xylencyanol 30.0% Glycerol

	100.0 mM EDTA pH 8.0
Laemmli buffer (3x)	100 mM Tris/HCl pH 6.8 3.0% SDS 45.0% Glycerol 0.01% Bromphenol blue 7.5% ß-Mercaptoethanol
NET	50.0 mM Tris/HCl pH 7.4 5.0 mM EDTA 0.05% Triton X-100 150.0 mM NaCl
PBS	137.0 mM NaCl 27.0 mM KCl 80.9 mM Na2HPO4 1.5 mM KH2PO4 pH 7.4
SD-Transblot	50.0 mM Tris/HCl pH 7.5 40.0 mM Glycine 0.004% SDS
Separating gel buffer (4x)	0,5 M Tris/HCl pH 8.8 0,4% SDS
“Strip” buffer	62.5 mM Tris/HCl pH 6.8 2.0% SDS 100.0 mM β-Mercaptoethanol
TAE	40.0 mM Tris/Acetate pH 8.0 1.0 mM EDTA TE10/0.1 10.0 mM Tris/HCl pH 8.0

Tris-Glycine-SDS	25.0 mM Tris/HCl pH 7.5 200.0 mM Glycine 0.1% SDS

7.4 Cell lines

Table 2: Cell lines with tissue origins

Nr.	Name	Origin	ATCC/DSMZ number	Obtained from
Nr.	**Brain**			
1	1321N1	astrocytoma		ECACC
2	CCF-STTG1	astrocytoma		ECACC
3	IMR-32	neuroblastoma		DSMZ
4	SF-767	glioblastoma		Tissue Bank of the Brain Tumor Research Center, UCSF, CA, USA
5	SH-SY-5Y	neuroblastoma	CRL-2266	ATCC
6	SK-N-SH	neuroblastoma	HTB-11	ATCC
7	U-188-MG	glioblastoma	HTB-15	ATCC
8	U-373	glioblastoma	HTB-17	ATCC
9	U-1240	glioblastoma		(Nister *et al.*, 1988)
10	U-1242	glioblastoma		(Nister *et al.*, 1988)
	Breast			
11	BT-20	breast carcinoma	HTB-19	ATCC
12	BT-474	breast ductual carcinoma	HTB-20	ATCC
13	BT-549	breast ductual carcinoma	HTB-122	ATCC
14	DAL	breast carcinoma		Pier Giorgio Natali Regina Elena Cancer Institute Rome, Italy
15	Hs-578T	breast ductual carcinoma	HTB-126	ECACC
16	MCF-7	breast carcinoma	HTB-22	ATCC
17	MDA-MB-231	breast carcinoma	HTB-26	ATCC
18	MDA-MB-361	breast carcinoma	HTB-27	ATCC
19	MDA-MB-415	breast carcinoma	HTB-128	ATCC
20	MDA-MB-435S	breast ductual carcinoma	HTB-129	ATCC
21	MDA-MB-436	breast carcinoma	HTB-130	ATCC
22	MDA-MB-468	breast carcinoma	HTB-132	ATCC
23	SK-Br-3	breast carcinoma	HTB-30	ATCC
24	T-47D	breast ductual carcinoma	HTB-133	ATCC
25	ZR-75-1	breast ductual carcinoma	CRL-1500	ATCC
26	ZR-75-30	breast ductual carcinoma	CRL-1504	ATCC

	Cervix and Vulva			
27	HeLa S3	cervix carcinoma	CCl-2.2	ATCC
	Colon			
28	CaCo2	colon carcinoma	HTB-37	ATCC
29	DLD-1	colon carcinoma	CCL-221	ATCC
30	LoVo	colon carcinoma	CCL-229	ATCC
31	SW-480	colon carcinoma	CCL-228	ATCC
32	SW-620	colon carcinoma	CCL-227	ATCC
33	SW-837	rectum carcinoma	CCL-235	ATCC
34	WiDr	colon carcinoma	CCL-218	ATCC
	Head and Neck			
35	UM-SCC-10A	pharynx squamous cell carcinoma		(Vlock *et al.*, 1989)
36	UM-SCC-10B	pharynx squamous cell carcinoma		(Vlock *et al.*, 1989)
37	UM-SCC-17A	larynx squamous cell carcinoma		(Vlock *et al.*, 1989)
38	UM-SCC-17B	larynx squamous cell carcinoma		(Vlock *et al.*, 1989)
39	UM-SCC-22A	pharynx squamous cell carcinoma		(Vlock *et al.*, 1989)
40	UM-SCC-22B	pharynx squamous cell carcinoma		(Vlock *et al.*, 1989)
	Kidney and Adrenal Gland			
41	769-p	kidney carcinoma	CRL-1923	ATCC
42	786-0	kidney carcinoma	CRL-1933	ATCC
43	A-498	kidney carcinoma	HTB-44	ATCC
44	CaKi-1	kidney clear cell carcinoma	HTB-46	ATCC
45	CaKi-2	kidney clear cell carcinoma	HTB-47	ATCC
46	G-401	Wilms tumor	CRL-1441	Ambion
	Liver			
47	HepG-2	hepatocellular carcinoma	HB-8065	ATCC
	Lung			
48	A-427	lung carcinoma	ACC-234	DSMZ
49	A-549	lung carcinoma	CCL-185	DKFZ
50	Calu-1	lung epidermoid carcinoma	HRB-54	ATCC
51	Calu-3	lung adenocarcinoma	HTB-55	ATCC
52	Calu-6	anaplastic carcinoma, probably lung	HTB-56	ATCC
53	NCI-H292	lung mucoepidermoid carcinoma	CRL-1848	ATCC
54	NCI-H441	lung papillary adenocarcinoma	HTB-174	ATCC
55	NCI-H460	lung large cell carcinoma	HTB-177	ATCC
56	NCI-H520	lung squamous cell carcinoma	HTB-182	ATCC
57	NCI-H596	lung adenosquamous carcinoma	HTB-178	ATCC
58	NCI-H661	lung large cell carcinoma	HTB-183	ATCC
59	SK-LU-1	lung adenocarcinoma	HTB-57	ATCC

60	SK-Mes-1	lung squamous cell carcinoma	HTB-58	ATCC
	Ovary			
61	CaOv-3	ovary papillary adenocarcinoma	HTB-75	ATCC
62	OAW-42	ovary carcinoma		DKFZ
63	OVCAR-3	ovary adenocarcinoma	HTB-161	ATCC
64	PA-1	ovary adenocarcinoma	CRL-1572	ATCC
65	Sk-OV-3	ovary adenocarcinoma	HTB-77	ATCC
	Pancreas			
66	818-7	pancreas adenocarcinoma		(Schmiegel *et al.*, 1993)
67	AsPc-1	pancreas adenocarcinoma	CRL-1682	ATCC
68	BxPc3	pancreas adenocarcinoma	CRL-1687	ATCC
69	Capan-1	pancreas adenocarcinoma	HTB-79	DKFZ
70	Capan-2	pancreas adenocarcinoma	HTB-80	DKFZ
71	CF-PAC-1	pancreas adenocarcinoma	CRL-1918	ATCC
72	Colo-357	pancreas carcinoma		(Kalthoff *et al.*, 1991)
73	DANG-G	pancreas carcinoma	ACC-249	DSMZ
74	Hs766T	pancreas carcinoma	HTB-134	ATCC
75	Mia-PaCa-2	pancreas carcinoma	CRL-1420	ATCC
76	PANC-1	pancreas epitheloid carcinoma	CRL-1420	ATCC
77	PANC-TU-1	pancreas carcinoma		(Kalthoff *et al.*, 1991)
78	PaTu-8988T	pancreas carcinoma	ACC-162	DSMZ
79	PT-45P1	pancreas carcinoma		(Kalthoff *et al.*, 1991)
80	SW-850	pancreas carcinoma		(Kalthoff *et al.*, 1991)
	Prostate			
81	BM-1604	prostate adenocarcinoma	ACC-298	DSMZ
82	DU-145	prostate carcinoma	HTB-81	ATCC
83	PC-3	prostate adenocarcinoma	CRL-1435	ATCC
84	PPC-1	prostate adenocarcinoma		(Chen, 1993)
85	TSU-PR-1	prostate adenocarcinoma		Dr. Isaacs, John Hopkins Oncology Center, Baltimore, USA
	Skin			
86	A-375	malignant melanoma	CRL-1619	ATCC
87	BOW-G	melanosarcoma		DKFZ
88	C-32	malignant melanoma	CRL-1585	ATCC
89	C-8161	malignant melanoma		(Welch *et al.*, 1991)
90	Colo-16	skin squamous cell carcinoma		(Moore *et al.*, 1975)
91	Colo-829	malignant melanoma	CRL-1974	ATCC
92	F-01	melanoblastoma		DKFZ
93	G-361	malignant melanoma	CRL-1424	ATCC
94	Hs-294T	malignant melanoma	HTB-140	ATCC
95	HAT-144	malignant melanoma	HTB-63	ATCC
96	KA-II	malignant melanoma		(Soruri *et al.*, 1998)
97	Malme 3M	malignant melanoma	HTB-64	ATCC

98	MeWo	malignant melanoma	HTB-65	ATCC
99	MM-DU	malignant melanoma		Koerner et al., 2008
100	MM-LEH	malignant melanoma		Koerner et al., 2008
101	MM-LO	malignant melanoma		Koerner et al., 2008
102	MM-SU	malignant melanoma		Koerner et al., 2008
103	RPMI-7951	malignant melanoma	HTB-66	ATCC
104	SBCL-2	malignant melanoma		The Wistar Institute, Philadelphia, PA, USA
105	SK-Mel-24	malignant melanoma	HTB-71	ATCC
106	SK-Mel-28	malignant melanoma	HTB-72	ATCC
107	WM-35	malignant melanoma	CRL-2807	ATCC
108	WM-115	primary melanoma	CRL-1675	ATCC
109	WM-239A	primary melanoma		The Wistar Institute, Philadelphia, PA, USA
110	WM-266-4	primary melanoma	CRL-1676	ATCC
111	WM-1617	primary melanoma	CRL-2809	ATCC
112	WM-793	malignant melanoma	CRL-2806	ATCC
113	WM-852	malignant melanoma		The Wistar Institute, Philadelphia, PA, USA
114	WM-902B	primary melanoma		The Wistar Institute, Philadelphia, PA, USA
115	WM-983A	primary melanoma		The Wistar Institute, Philadelphia, PA, USA
116	WM-983B	primary melanoma		The Wistar Institute, Philadelphia, PA, USA
117	WM-1341D	primary melanoma		The Wistar Institute, Philadelphia, PA, USA
	Testes			
118	NT-2	teratocarcinoma	CRL-1973	ATCC
119	TERA-2	embryonal carcinoma	HTB-106	ATCC
	Thyroid			
120	FTC-133	thyroid carcinoma		ECACC
121	FTC-238	thyroid carcinoma		ECACC
122	TT	thyroid carcinoma		ECACC

7.5 Antibodies

7.5.1 Primary antibodies

The following antibodies were used for immunoprecipitation or as primary antibodies in immunoblot or immunofluorescence analysis.

Table 3: Primary antibodies

Antibody	Specification	Origin	Order number
cABL	24-11	Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA	sc-23
EGFR		Cell Signaling Technology, Danvers, MA, USA	2232
FLT3	8F2	Cell Signaling Technology, Danvers, MA, USA	3462
HER2		Millipore Corporate, Billercia, MA, USA	06-562
JAK1	HR-785	Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA	sc-277
JAK2	HR-758	Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA	sc-278
P-EGFR	53A5	Cell Signaling Technology, Danvers, MA, USA	4407
P-HER2	Tyr1221/1222	Cell Signaling Technology, Danvers, MA, USA	2249
P-JAK1	Tyr1022/1023	Millipore Corporate, Billercia, MA, USA	07-849
P-JAK2	Tyr1007/1008	Abcam plc, UK	ab32101
P-STAT3	Tyr705	Cell Signaling Technology, Danvers, MA, USA	9131
P-TYK2	Tyr1054/1055	Cell Signaling Technology, Danvers, MA, USA	9321
P-Tyr	4G10	Millipore Corporate, Billercia, MA, USA	05-321
PYK2	N-19	Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA	sc-1514
RON	C-20	Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA	sc-322
RON	RB-1356	Abgent, Inc., San Diego, CA,USA	AP7674c
STAT3		Cell Signaling Technology, Danvers, MA, USA	9132
TNK	C44F9	Cell Signaling Technology, Danvers, MA, USA	4570
TYK2		Cell Signaling Technology, Danvers, MA, USA	9312
TYK2	EP1127Y	Abcam plc, UK	ab52645

7.5.2 Secondary antibodies

For immunoblot analysis corresponding secondary antibodies conjugated with horseradish peroxidase (HRP) were utilized.

Table 4: Secondary Antibodies

Antibody	Origin
Goat anti-mouse-HRP	Sigma, Taufkirchen
Goat anti-rabbit-HRP	BioRad, München
Rabbit anti-goat-HRP	BioRad, München

7.6 Oligonucleotides

7.6.1 siRNA oligonucleotides

Table 5: siRNA oligonucleotides

Name	Sequence sense	Sequence antisense	Company
RON	validated		Ambion, Carlsbad, USA
TYK2	validated		Ambion, Carlsbad, USA
Gl2	5' - CGUACGCGGAAUACUUCGAtt - 3'	5' - UCGAAGUAUUCCGCGUACGtt - 3'	Dharmacon, Lafayette, CO, USA

7.6.2 Primers

Table 6: Primers for RT-PCR

Name	Sequence	Company
TYK2-Forward	5' - ACG GCC TGT ACC TCA TTC AC - 3'	Metabion Martinsried, Germany
TYK2-Reverse	5' - CTG TCT CGT AGA AGG CCA GG - 3'	Metabion Martinsried, Germany
RON-Forward	5' - GTC GCG GGC CAA ACC AAA GTC AG - 3'	Metabion Martinsried, Germany
RON-Reverse	5' - CCA GGC CCA GAA TCG AAT CCA ATG - 3'	Metabion Martinsried, Germany
GAPDH-Forward	5' - ACC ACA GTC CAT GCC ATC AC - 3'	Metabion Martinsried, Germany
GAPDH-Reverse	5' - TCC ACC ACC CTG TTG CTG TA - 3'	Metabion Martinsried, Germany

7.7 Methods

7.7.1 Cellular Assays

7.7.1.1 MTT Assay

In a 96-well flat-bottomed plate, 1000- 2000 cells/100 µl cell suspension was seeded. After 24h, cells were exposed to different concentrations of compound. Each treatment was tested in triplicate wells. At the end of exposure (24h, 48h and 72h), 20 µl of MTT (5 mg/ml in PBS) [3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide; thiazolyl blue, SIGMA, St. Louis, MO] was added to each well, and the plates were incubated at 37 ºC for 4h. Then 50 µl triplex solution (10% SDS, 5% isobutanol, 0.012 M HCl) was added and the plates were incubated at 37ºC overnight in a cell incubator. The optical density (OD) was measured using a multiwell spectrophotometer at a wavelength of 570 nm. The inhibitory rate of cell proliferation was calculated by the following formula: Inhibition Rate (%) = [1-(ODtreated- ODtreated (day0)/ODcontrol-

ODcontrol (day0))]×100%. The IC50-value (i.e. the drug concentration that reduced the absorbance observed in untreated cells by 50%) was calculated by using Hill threeparameter log fit or the sigmoidal dose-response curve fitting algorithm in SIGMA Plot 10 on log-transformed data.

7.7.1.2 Flow Cytometry

Transfected or compound- treated cells were trypsinized after 72h of siRNA transfection or drug application and collected by centrifugation. For fixation, cells were washed once with PBS, resuspended in 1ml cold 70% ethanol and stored overnight at 4°C. Cells were then collected by centrifugation, washed once with PBS and incubated with 0.01% Triton, 0.1% sodium citrate, 0.02mM propidium iodide (Sigma) in the dark for 2h at 4°C. Cells were analyzed by flow cytometry (FACS Calibur, BD Bioscience). Using the CellQuestPro software, each of the three peaks (representing cells in G1, S, and G2/M phases, respectively) obtained in the flow cytometry profile of fluorescence plotted against cell number was gated and quantified.

7.7.1.3 Caspase 3/7- Assay

Caspase 3/7 activity of siRNA transfected or compound- treated cells was measured using the Caspase 3/7 Glo- Assay from Promega according to manufacturer`s instruction.

7.7.1.4 Wound Assay

Cancer cells were seeded in 6-well cell culture plates and grown to confluence under serum conditions (10 % FCS (w/v)) for two to three days. Confluent monolayers were scratched with a pipette tip and maintained under standard conditions. Plates were washed once with fresh medium to remove non-adherent cells. Migrating cells were monitored by photomicroscopy.

7.7.1.5 Transwell Migration Assay

The lower chamber of a transwell plate (8- µm pore size polycarbonate membrane; Corning Costar Corp., Cambridge, MA) was filled with 600 µl normal cell culture media (10% (w/v) FCS) and 15 x 103 to 30 x 103 cells were resuspended in 200 µl starvation media (0% (w/v) FCS) and seeded in the upper chamber containing either increasing compound concentrations or DMSO as vehicle control. After 16h the cells were

methanol- fixed and stained with crystal violet. After taking images stained cells were dissolved in 5% AcCOOH and the optical density (OD) was measured at 590 nm in an ELISA Reader. The transwell migration was expressed as a percentile “migration index” (number of migrating cells treated with compound divided by the number of migrating cells from the control multiplied by one hundred). The SEM was calculated from the migration indices of independent performed experiments. The statistical significance of the data was analyzed using the Student` s t test unpaired.

7.7.1.6 Matrigel outgrowth assay

Determination of the morphology of cells grown on matrigel was carried out in a matrigel-outgrowth assay. Briefly, in a 96-well flat-bottomed plate, 5000-10000 cells/100 µl cell suspension was seeded on the surface of pre-coated matrigel. Colony outgrowth was visualized with a Zeiss Axiovert S100 microscope (Carl Zeiss UK, Welwyn Garden City, UK).

7.7.2 Molecular methods

7.7.2.1 RNA interference

Cancer cells were cultured in DMEM, MEM or RPMI medium supplemented with 10% fetal bovine serum (FBS). 24 h prior to RNAi transfection 15.000 cells/ml were seeded into 6-well cell culture plates. At 30% confluency cells were transfected with 30 pmol of validated or pre-designed siRNA from Ambion RNAiMax (Invitrogen) according to the manufacturer`s instruction. Gl2 siRNA was taken as control. 5 d after transfection cells were used for cell cycle analysis by flow cytometry, MTT-assay and western blotting. The knock-down efficiency was monitored by RT-PCR and Western Blotting.

7.7.2.2 RNA extraction, cDNA synthesis, PCR

Total RNA extraction was performed using the RNeasy Protect Mini-Kit (Qiagen) according to the manufacturer's instruction. The resulting pellet was dissolved in nuclease-free water. RNA concentrations were measured using a spectrophotometer (260 nm/280 nm). After heating at 65°C for 5 min to denature RNA and to inactivate RNases, 3 µg total RNA was subjected to reverse transcription using 25 U AMV Reverse Transcriptase, 125 pmol Oligo(dT)n- primer, 200 µm dNTPs (each) and 5x RT buffer

containing 7.5 mM Mg2+ in a total volume of 20 µl at 42°C for 2 h. The reaction was terminated by heating at 65°C for 10 min.

For each PCR, 5 µl cDNA (diluted 1:10 in nuclease-free water), 5 µl RedTaq PCR Master Mix, 125 pmol forward and reverse primer and nuclease-free water were added to a final volume of 20 µl. Amplification was performed with an Eppendorf Cycler. The thermal cycle used was 3 min at 94°C, 25 cycles of 1min denaturation at 94°C, 1 min annealing at 60°C, 1 min elongation at 72°C and a final elongation step for 10 min at 72°C. Detection of the PCR-products was done on a 1% agarose-gel. Analysis and quantification was performed with the AIDA Image Reader.

7.7.2.3 Retroviral gene transfer in cell lines

The pLXSN (Clontech, Palo Alto, CA) constructs have been described before (Gschwind, Hart et al. 2003). All protein constructs included a C-terminal HA tag, detectable with an anti-HA monoclonal antibody (Babco, Richmond, CA). The amphotropic packaging cell line Phoenix was transfected with pLXSN retroviral expression plasmids by the calcium phosphate/ chloroquine method as described previously (Kinsella and Nolan 1996). 24 h after transfection the viral supernatant was collected and used to infect subconfluent kidney and bladder cancer cells (5x 10^4 cells/6-well plate). Retroviral supernatant was then replaced with fresh medium. 2d following infection, target protein expression was monitored by western blot. Polyclonal ACHN kidney cancer and TccSup bladder cancer cell lines stably expressing target proteins were generated by growing retrovirally infected cells in medium containing G418 (1 g/ml) for 2 weeks.

7.7.2.4 Western Blotting

Cells were lysed in RIPA- buffer and equal amounts of protein were resolved by SDS-PAGE. Proteins were transferred to PVDF (Perkin Elmer Polyscreen) membranes, blocked for 1 h in TBS containing 0.1% Tween-20 (TBST) + 4% nonfat dry milk and incubated overnight at 4°C with primary antibody in TBST + 3% BSA. Membranes were washed three times with TBST and incubated with horseradish peroxidase-conjugated antimouse, antirabbit or antigoat secondary antibody in TBST + 4% nonfat dry milk for 1h at room temperature. Membranes were washed three times with TBST and visualized by ECL (Western Lightning, Perkin Elmer) on X-ray films.

7.7.2.5 Cellular Kinase Assay

Cancer cells were seeded at a density of 150.000 cells/well in 6-well flat-bottom cell culture dishes. 24 h prior to SU11248 treatment, cells were starved for 24 h in medium containing 0% FCS. Drug incubation was performed for 2 h, followed by pervanadate stimulation for 5 min at 37°C. Cells were lysed and subjected to immunoprecipitation.

7.7.3 Databases and Statistics

7.7.3.1 Databases

Following data bases were used: "NCBI SNP database": http://www.ncbi.nlm.nih.gov/projects/SNP/. "COSMIC" (Forbes *et al.*, 2010); "TyKiVa" (Ruhe *et al.*, 2007); "MoKCa" (Richardson *et al.*, 2009); "IARCP53" (Petitjean *et al.*, 2007).

7.7.3.2 Statistics

To correlate the SUTENT-sensitivities of the cancer cell lines with their inherited genetic alteration the median of the sensitivities of the whole population was calculated. After this, every genetic alteration in the respective cell line got the value of the difference from the median. The alignment of the sums of these values for every single genetic alteration results in a ranking of the probability that the respective genetic alteration has an influence on the SUTENT-response. All *P* values were calculated using the Student's *t* test and values ≤ 0.05 were considered statistically significant.

8. Results

Genetic alterations are known to potentially influence the efficacy of different types of anti-cancer drugs (el Rouby *et al.*, 1993; Thussbas *et al.*, 2006; Yun *et al.*, 2008). Thus, their influence on small molecule inhibitors such as the EGFR-inhibitor gefitinib or the multi-targeted kinase inhibitor sunitinib is in the focus of investigation. Various mutations and deletions in EGFR altering the response to gefitinib therapy are already described in patients with NSCLC (Lynch *et al.*, 2004; Han *et al.*, 2005; Han *et al.*, 2005; Sharma *et al.*, 2007) and several mutations that influence the efficacy of sunitinib in patients with GIST are known (Heinrich *et al.*, 2008; Gajiwala *et al.*, 2009). Beyond GIST, sunitinib shows anti-cancer activity in various cancer types (Abrams *et al.*, 2003; O'Farrell *et al.*, 2003; Motzer *et al.*, 2007; Huynh *et al.*, 2009; Strobel *et al.*, 2010; Yang *et al.*, 2010). To elucidate genetic alterations that influence patient response to sunitinib treatment it was first determined whether an already known sensitizing mutation could in general also mediate sensitivity to cancer of other tissue origins.

8.1 Proof of principle: Influence of mutations in EGFR on the response to gefitinib treatment

As cell lines harboring published mutations that alter sunitinib response were not available, the EGFR-inhibitor gefitinib was chosen. Gefitinib has been approved for monotherapy of patients with locally advanced or metastatic non-small cell lung cancer (NSCLC) in whom both platinum-based and docetaxel chemotherapies failed (Cohen *et al.*, 2004). Many mutations that alter the response to gefitinib treatment have been published (Tracy *et al.*, 2004; Kobayashi *et al.*, 2005; Shih *et al.*, 2005; Jackman *et al.*, 2006; Okamoto *et al.*, 2006; Sasaki *et al.*, 2006; Sharma *et al.*, 2007; Zhu *et al.*, 2008). To investigate the influence of one of these mutations on the efficacy of gefitinib in cancers other than lung cancer, mutation databases that include cancer cell line information were screened. The available databases (TyKiVa (Tyrosine Kinome Variants) (Ruhe *et al.*, 2007), COSMIC (Catalogue of somatic mutations in cancer) (Forbes *et al.*, 2010), MoKCa (Mutations of kinases in cancer) (Richardson *et al.*, 2009) and IARCP53 (Petitjean *et al.*, 2007)) list more than 4500 genetic alterations occurring in cancer cell lines. Sixteen of these were located in the EGF receptor, 13 in cell lines originating from

tissue other than lung tissue. Only one cancer cell line (SW-48, colon) was found to have an already published sensitizing mutation EGFR^{G719S} (TyKiVa, figure 10). It was therefore selected for further investigation (Ruhe *et al.*, 2007). Additionally, the mutation EGFR^{P753S} (figure 10), which is located at the end of the known sensitizing deletion Del_L747-P753, but has not been reported to alter the response to gefitinib treatment so far, was included in this study.

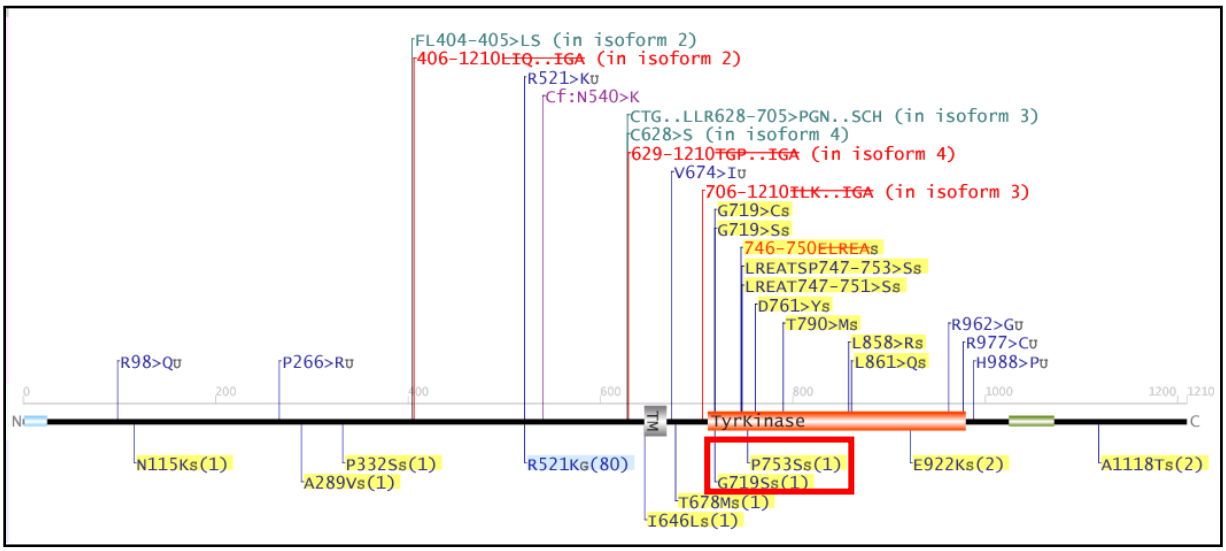

Figure 10: Genetic alterations of EGFR listed in the database TyKiVa. A simplified diagram of the EGF receptor showing the distribution of the transmembrane domain (TM) and tyrosine kinase domain (TyrKinase). Genetic alterations listed above the receptor have already been published and are not necessarily in cancer cell lines. Genetic alterations listed below show newly identified somatic mutations (yellow boxes) or germ line polymorphisms (blue box). Numbers in parentheses indicate the number of cancer cell lines harboring the respective genetic alteration. The red box indicates selected mutations. (image taken from "TyKiVa" (Ruhe *et al.*, 2007))

8.1.1 Allele-dependent influence of $EGFR^{G719S}$ and $EGFR^{P753S}$ on the gefitinib-induced inhibition of proliferation

Gefitinib (Iressa®) is known to inhibit mainly EGFR and several other kinases such as BRK, RICK, and GAK (Brehmer *et al.*, 2005). A number of genetic alterations in EGFR of patients with NSCLC are known to influence the efficacy of the drug. Some of these, i.e., the somatic mutations $EGFR^{G719S}$, $EGFR^{L858R}$, or $EGFR^{del:L747\text{-}P753}$ improve the response to gefitinib, whereas $EGFR^{T790M}$ impairs it (Sharma *et al.*, 2007). To determine whether the sensitizing mutation $EGFR^{G719S}$ might also mediate increased sensitivity to gefitinib in tissue other than lung tissue, data already published were reproduced with NSCLC cell lines. The lung cancer cell lines H-1666 ($EGFR^{858L/790T}$), PC3 ($EGFR^{L858R}$, sensitizing mutation), and H-1975 ($EGFR^{T790M}$, resistance mutation) were chosen to cover the whole spectrum of possible sensitivities. After 72 h of treatment with gefitinib, the anti-proliferative effect of the drug was measured. As expected, the EGFR genotype correlated with the gefitinib sensitivity of the respective cell line (figure 11/A).

Nanomolar concentrations of gefitinib ($IC_{50} \cong 21$ nM) already inhibited the proliferation of PC3 ($EGFR^{L858R}$, sensitizing mutation), whereas H-1975 ($EGFR^{T790M}$, resistance mutation) caused almost no impairment in growth ($IC_{50} > 10$ µM). The sensitivity of the cell line H-1666 ($EGFR^{858L/790T}$) fell between these two extremes ($IC_{50} = 1.65$ µM). On the basis of this proof of principle the sensitivities of the cell line SW-48 (colon, $EGFR^{G719S}$) was compared with that of SW-1417 (colon, $EGFR^{719G}$; figure 11/B).

So far, this mutation was only known to mediate increased sensitivity to gefitinib in patients with NSCLC. Interestingly, the sensitizing mutation $EGFR^{G719S}$ also correlated in colon cancer cell lines with an increased response to gefitinib treatment. While SW-48, which harbors the sensitizing serine allele at position 719, had an IC_{50} value of 0.15 µM, SW-1417 had an IC_{50} value of 7.5 µM: this change of sensitivity is approximately 50-fold (figure 11/B). Thus, the mutation $EGFR^{G719S}$ also correlates with cancer cell lines from tissue other than lung tissue with an altered sensitivity to gefitinib.

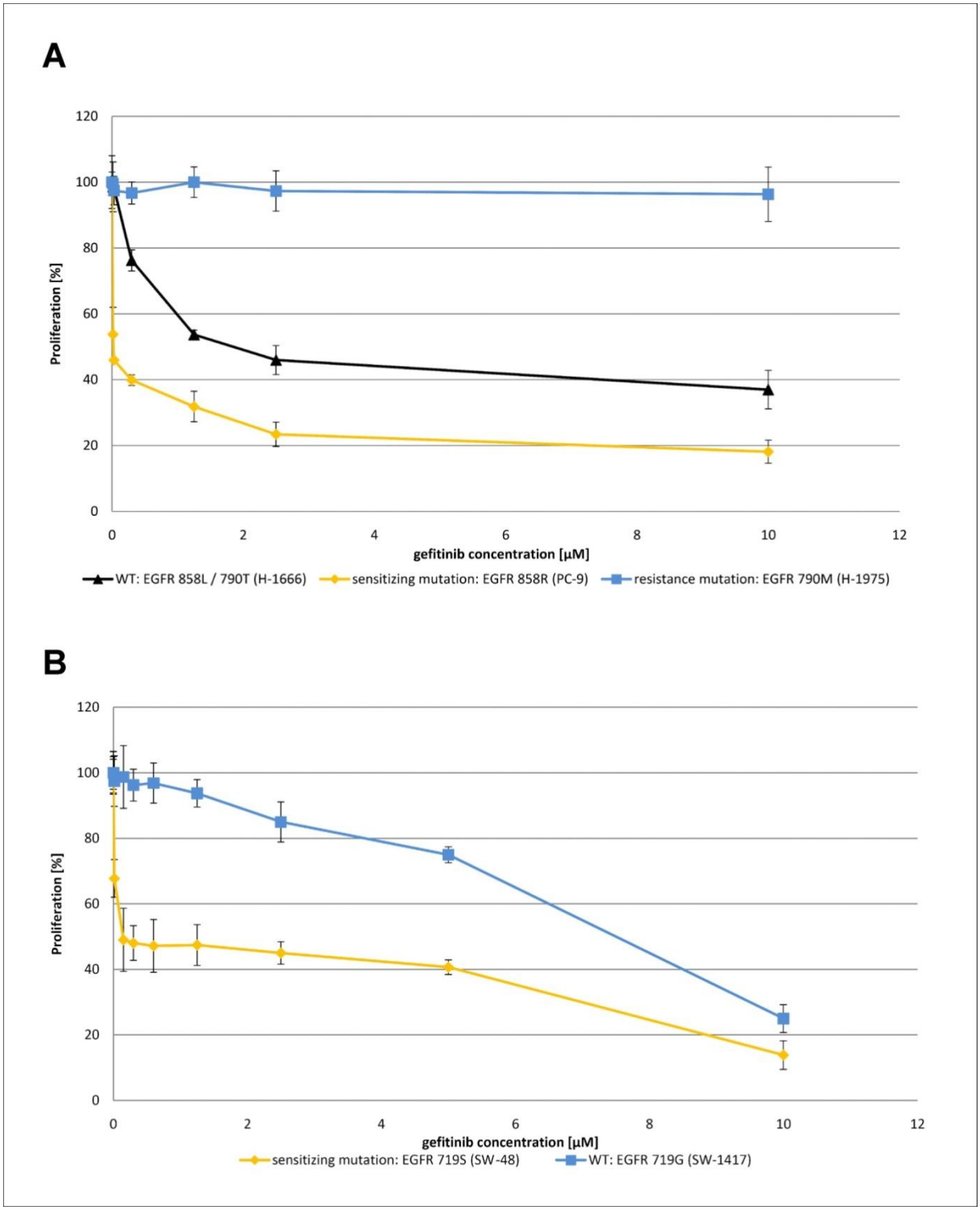

Figure 11: Influence of genetic alterations in EGFR on the response to gefitinib treatment. Cancer cell lines were treated with various concentrations of gefitinib (0.0015 – 10 µM) for 72 h followed by the quantification of viable cells using MTT. (A) Reproduction of already published data of mutations in EGFR that alter the sensitivity to gefitinib in NSCLC cell lines. $EGFR^{858L/790T}$: H-1666; $EGFR^{L858R}$ (sensitizing mutation): PC3; $EGFR^{T790M}$ (resistance mutation): H-1975. (B) Influence of $EGFR^{G719S}$ in a colon cancer cell line on the response to gefitinib. $EGFR^{719G}$: SW-1417; $EGFR^{G719S}$ (sensitizing mutation): SW-48.

In addition, the influence of the different alleles of $EGFR^{P753S}$ was compared: (SK-Mel-28 (skin, $EGFR^{P753S}$) vs. RPMI-7951 (skin, $EGFR^{753P}$; figure 12). The somatic mutation $EGFR^{P753S}$ has not been connected with a change of sensitivity so far, but it is located at the end of an already known sensitizing deletion ($EGFR^{del:L747-P753}$). Thus, this mutation was selected to ascertain its influence on the inhibitory effect of gefitinib on proliferation. The sensitivities of both cell lines showed high micro-molar IC_{50} values (SK-Mel-28 ($EGFR^{P753S}$): 5.6 µM; RPMI-7951 ($EGFR^{735P}$): 6.8 µM). This indicated that the mutation $EGFR^{P753S}$ has no effect on the response to gefitinib treatment.

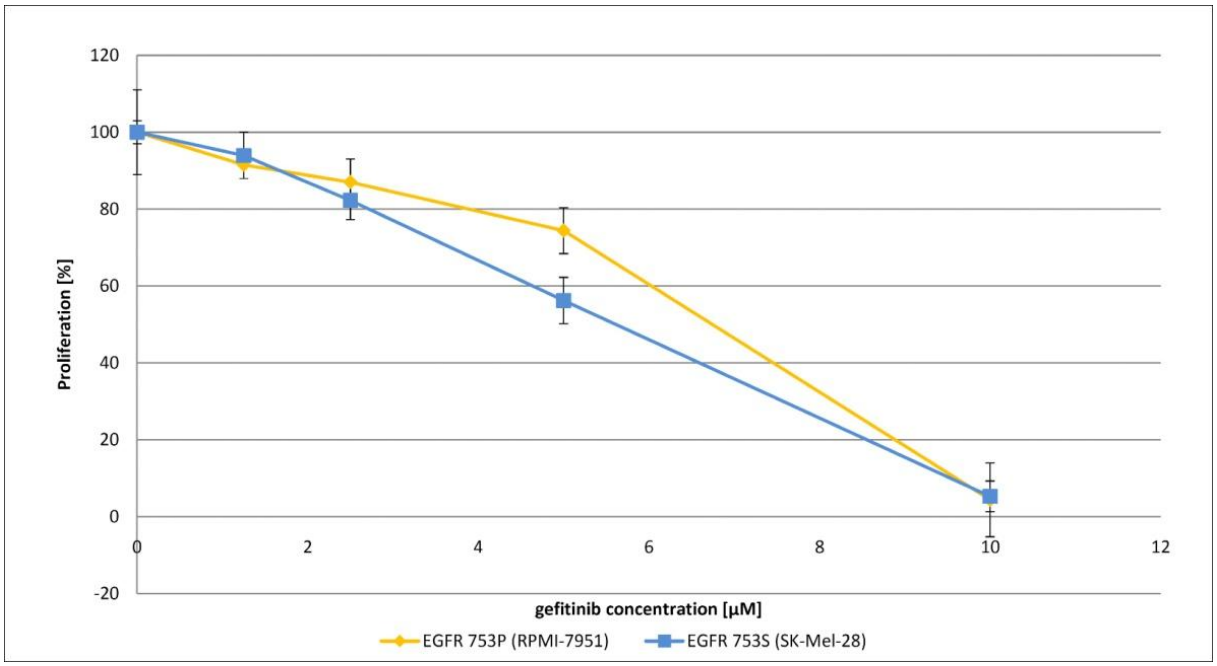

Figure 12: Influence of $EGFR^{P753S}$ on the response to gefitinib. $EGFR^{753P}$: RPMI-7951 (skin); $EGFR^{P753S}$ (mutation with unknown function, located at the end of the published sensitizing mutation $EGFR^{deletion:L747-P753}$): SK-Mel-28 (skin).

8.1.2 Influence of EGFR^{G719S} on the efficacy of gefitinib at the molecular level

The crucial aspect in small molecule inhibitor efficacies is their ability to inhibit target phosphorylation (Ullrich and Schlessinger, 1990; Levitzki and Gazit, 1995). The main target of gefitinib is EGFR (Brehmer *et al.*, 2005). A change in its phosphorylation status suffices to alter the cellular response in NSCLC (Lynch *et al.*, 2004; Zhu *et al.*, 2008). So far, the observed mutation-dependent alteration of the inhibition of proliferation in the colon cancer cell line SW-48 was only a correlation. To verify that the improved response was connected to the serine allele at position 719 in the EGF receptor, the mutation-dependent efficacy of gefitinib to inhibit the phosphorylation of EGFR was determined (figure 13). Therefore, the two colon cancer cell lines, each harboring one of the two alleles, were analyzed (SW-48: EGFR^{719S}; SW-1417: EGFR^{719G}). After being starved for 24 h, the cells were treated with various concentrations of gefitinib (0.0015 – 10 µM) or DMSO control for 1 h followed by EGF stimulation for 5 min.

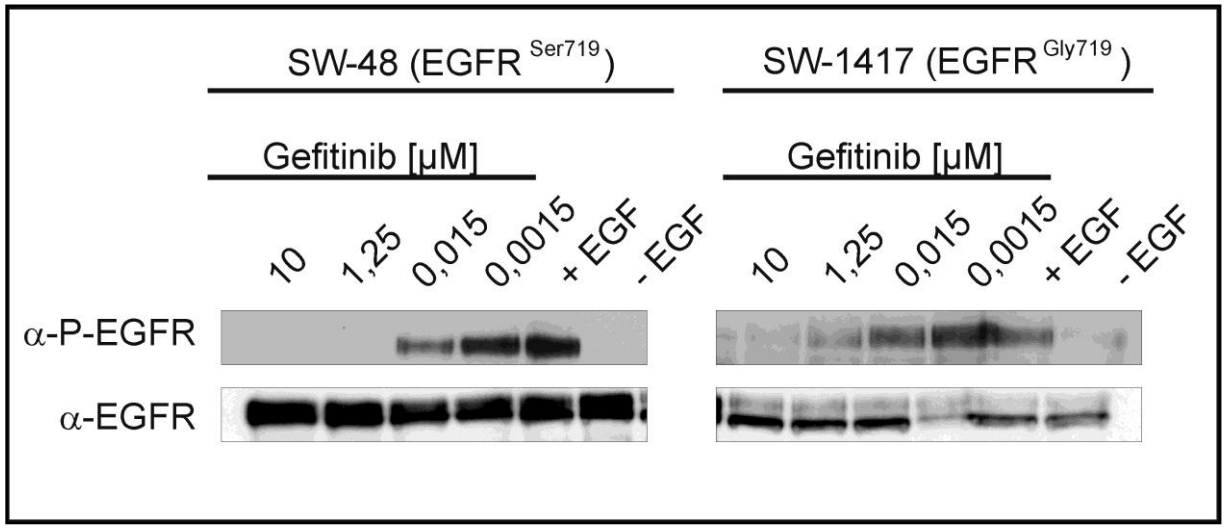

Figure 13: Influence of EGFR^{G719S} on the gefitinib-induced inhibition of EGFR phosphorylation. Cancer cell lines harboring one of the two alleles (SW-48: EGFR^{G719S}; SW-1417: EGFR^{719G}) were starved for 24 h. After 1 h treatment with various concentrations of gefitinib (0.0015 – 10 µM) or DMSO control cells were first stimulated with EGF (10 ng/ml; 5 min) and then cell lyses and Western blot analysis were performed.

A total of 15 nM gefitinib was sufficient to significantly reduce EGFR phosphorylation in the cell line SW-48, which harbors the sensitizing mutation EGFR^{G719S} (figure 13, left). In contrast, EGFR phosphorylation of the control cell line SW-1417 (EGFR^{719G}) remained present up to a concentration of 1.25 µM gefitinib (figure 13, right), i.e., an increase of approximately 600-fold. This observation matched the results of the mutation-

dependent alteration of the inhibitory effect on proliferation and served as the proof of principle for the sunitinib project. In brief, the serine allele at position 719 in the EGF receptor also mediates an increased response to gefitinib treatment in a colon cancer cell line at the cellular and the molecular levels. To estimate the clinical relevance of these findings the frequency of the sensitizing serine allele in colon cancer was checked among the world population. Thus, the database COSMIC (Catalogue Of Somatic Mutations In Cancer; (Forbes *et al.*, 2010)), which lists 88727 somatic mutations in 366477 tumor samples, was used. It revealed that the serine allele at position 719 in EGFR is only present in 0.09% of all colon cancer samples. Compared with 25.4% in lung cancer, the clinical relevance of the mutation G719S in EGFR for the treatment of colon cancer with gefitinib is negligible. However, the finding that a mutation can influence sensitivity across cancer types of different tissue origins, served as proof of principle and thus as the basis for the work with sunitinib malate.

8.2 Influence of genetic alterations on the response to sunitinib treatment

Sunitinib has been approved for the treatment of GIST and mRCC (Goodman *et al.*, 2007). It has been known for several years that sunitinib inhibits PDGFRα/β, cKIT, RET, FLT3, CSF1R, and VEGFR1/2/3 (Abrams *et al.*, 2003; Mendel *et al.*, 2003; Murray *et al.*, 2003; O'Farrell *et al.*, 2003; Faivre *et al.*, 2006; Faivre *et al.*, 2007). Especially PDGFR and cKIT play a very important role in the development and progression of GIST (Lasota and Miettinen, 2008). Thus, the sensitivity-influencing mutations in these two receptor tyrosine kinases are sufficient to be of prognostic value for the treatment of GIST (Heinrich *et al.*, 2008; Gajiwala *et al.*, 2009). The whole spectrum of more than 200 proteins that bind to sunitinib was only recently elucidated (PhD thesis, Michaela Bairlein, 2010, TU Munich). Fifty to sixty of these targets are considered those most responsible for sunitinib response. Currently ongoing clinical studies are being conducted on different types of tumors such as liver cancer, small cell lung cancer (SCLC), and acute myeloid leukemia (AML) (O'Farrell *et al.*, 2003; Fiedler *et al.*, 2005; Polyzos, 2008; Zhu and Raymond, 2009; Zhu *et al.*, 2009). It is thus important to elucidate the sensitizing or desensitizing genetic alterations in all sunitinib targets, in order to improve the prognosis of sunitinib-based therapy.

8.2.1 Selection of genetic alterations

First, all genetic alterations listed in the database TyKiVa (Tyrosine Kinome Variants; (Ruhe *et al.*, 2007)) were assigned to sunitinib targets. Only those genetic alterations that occurred with a certain frequency (≥ 5% ≅ 14 cell lines) in TyKiVa were considered (table 7).

Table 7: Genetic alterations in sunitinib targets with a frequency ≥ 5% in 254 tested cancer cell lines of TyKiVa. Numbers of genetic alterations indicate the position affected in the respective amino acid sequence. Abbreviations of amino acids before the number refer to the variant of the majority of all tested cell lines. Abbreviations behind the number show the amino acid variant present in the number of cell lines mentioned in the right column. (Ruhe *et al.*, 2007)

Target	Genetic alteration	Frequency (% of all cell lines)
ABL1	S991L	14 (5%)
EPHA1	V900M	20 (7%)
EPHA2	R876H	16 (6%)
EPHA3	R914H	15 (5%)
EPHA3	W924R	74 (26%)
EPHA10	G749E	17 (6%)
FAK	Insertion ETDD..QTRP415E	13 (5%)
FAK	Insertion L926PWRL	52 (18%)
FES	Deletion SWAE..ELTK72-	17 (6%)
FGFR2	Deletion HSGI..PKQQ199-	54 (19%)
FGFR4	L136P	37 (13%)
FGFR4	G388R	58 (20%)
FLT3	M227T	36 (13%)
FRK	G122R	74 (26%)
MER	V870I	14 (5%)
PDGFRα	S478P	13 (5%)
RET	G691S	33 (12%)
RON	R523Q	57 (20%)
RON	1335R/G	128 (45%)
RON	Insertion R813RQ	115 (41%)
RON	Deletion RPVP..PRPT627RSQC	38 (13%)
ROS	T145P	13 (5%)
ROS	S1109L	21 (7%)
ROS	D2213N	20 (7%)
ROS	K2228Q	22 (8%)
ROS	S2229C	22 (8%)
ROS	Insertion C76CNDTYATVCE	41 (14%)
TNK1	M598V	23 (8%)
TNK1	Insertion EMEL..LARP597EVRSH	64 (23%)
TNK1	Deletion DR472-	15 (5%)
TYK2	V362F	64 (23%)
TYK2	684I/S	26 (9%)
TYK2	P1104A	13 (5%)
TYRO3	I346N	85 (30%)
VEGFR2	V297I	16 (6%)
VEGFR2	Q472H	24 (8%)
VEGFR3	Q890H	20 (7%)

This list shows 37 genetic alterations in 21 sunitinib targets. To determine which genetic alteration is significantly overrepresented in sunitinib-sensitive or -resistant cancer cell lines, it was necessary to measure the sunitinib sensitivity spectrum.

8.2.2 Analysis of the sunitinib sensitivity spectrum

Sunitinib has anti-proliferative, anti-migratory, and pro-apoptotic effects on cancer cells (Mendel *et al.*, 2003; Motzer *et al.*, 2006; Motzer *et al.*, 2007; Huynh *et al.*, 2009). To identify which genetic alteration is statistically overrepresented in sunitinib-sensitive or -resistant cancer cell lines, 122 cell lines were screened for all three biological effects.

8.2.2.1 Sunitinib-dependent inhibition of proliferation

Sunitinib is known to have a strong anti-proliferative effect on cancer cells (Mendel *et al.*, 2003; Huynh *et al.*, 2009). To create the basis for a correlation of sunitinib sensitivities with the presence of alleles of genetic alterations, all tested cell lines were treated with various concentrations of sunitinib (0.6 – 20 µM) for 72 h. Then the Hill three-parameter log fit or the sigmoidal dose-response curve fitting algorithm was applied to calculate IC_{50} values (examples of typical regressions for sensitive, low-sensitive, and resistant cell lines: figure 14/A. All cancer cell line sensitivities are displayed in figure 14/B). Interestingly, the majority of all cancer cell lines (81.8%) responded to the treatment with sunitinib within 72 h. Only 18.2% had no inhibitory effect on proliferation.

A strong tissue dependency was also detected (figure 14/C). On average, cancer cell lines originating in kidney, brain, prostate, ovary, pancreas, or lung tissue showed a higher sensitivity for the inhibition of proliferation than those from breast, skin colon, thyroid, or head/neck tissue. While the proliferation of kidney cancer cell lines was already inhibited at an IC_{50} value of about 2.7 µM, head/neck cancer cell lines kept growing on average up to sunitinib concentrations of 16.1 µM, which can be considered resistant (figure 14/C). The IC_{50} values are listed in table 8.

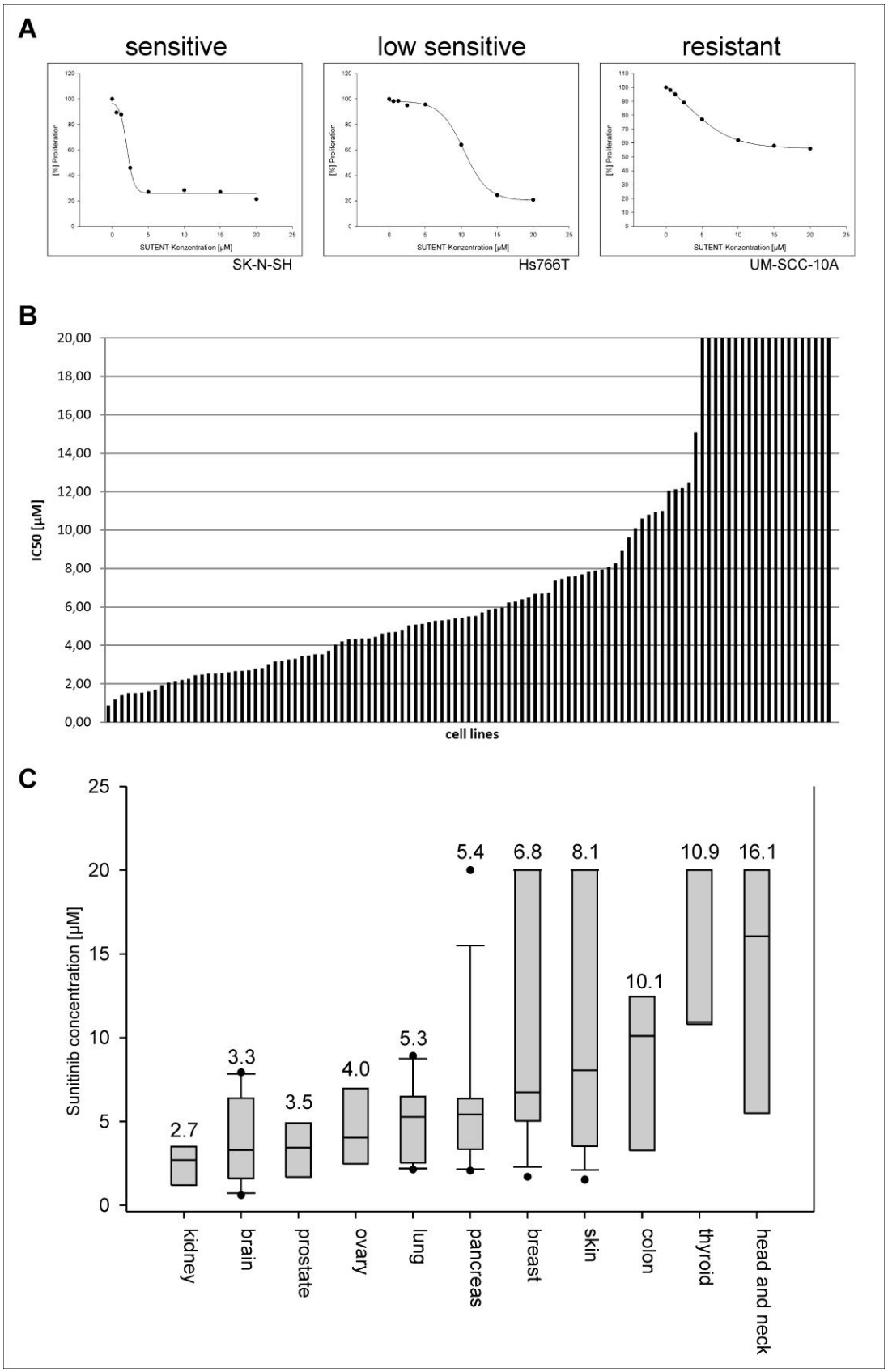

Figure 14: Analysis of sunitinib-dependent inhibition of proliferation of various cancer cell lines. Cancer cell lines were treated with various concentrations of sunitinib (0.6 – 20 µM) for 72 h. IC_{50} values were calculated using the Hill three-parameter log fit or sigmoidal dose-response curve fitting algorithm. (A) Examples for sensitive, low-sensitive, and resistant curve fits. (B) Sensitivities of various cancer cell lines for the inhibition of proliferation after sunitinib treatment. (C) Tissue dependency of cancer cell line sensitivities. IC_{50} values of cancer cell lines with the same tissue origin were grouped according to their median. Numbers above the box plots indicate the median IC_{50} value of the respective cell line tissue.

Table 8: IC_{50} values for the sunitinib-dependent inhibition of proliferation

cell line	IC50	cell line	IC50	cell line	IC50
1321N1	6.27	Hs578T	2.67	SK-Mes-1	2.45
769-P	4.20	Hs766T	11.00	SK-N-SH	1.19
786-O	2.82	HT144	12.07	SK-OV3	9.61
818-4	5.97	KAII	1.52	SW-480	12.45
A375P	4.43	LOVO	4.68	SW-620	10.60
A-427	4.62	MDA-MB-231	3.53	SW-850	2.06
AsPc-1	2.25	MDA-MB-361	7.70	T47D	>20
BM1604	4.37	MDA-MB-415	6.75	T98G	4.67
BOW-G	>20	MDA-MB-435S	1.70	TERA-2	>20
BT-20	5.51	MDA-MB-436	>20	TSU-PR-1	5.09
BT474	>20	MDA-MB-453	5.04	TT	>20
BT-549	7.61	MDA-MB-468	6.70	U118	2.21
BxPc3	5.42	MEWO3	>20	U1240	7.47
C8161	7.37	MIAPaCa-2	2.80	U1242	2.48
CaCO2	>20	MM-Leh	7.83	U-373	3.30
CaKi-1	0.87	MM-LO	3.72	UM-SCC-10A	>20
CaKi-2	1.52	MM-SU	>20	UM-SCC-10B	12.13
Calu-1	5.30	NCI-H292	5.33	UM-SCC-17A	>20
Calu-3	5.12	NCI-H441	8.07	UM-SCC-17B	4.36
CaOV-3	4.03	NCI-H460	2.14	UM-SCC-22A	5.87
Capan-1	5.43	NCI-H520	6.48	UM-SCC-22B	>20
Capan-2	5.72	NCI-H596	5.28	WIDR	3.27
CCSTTG1	7.93	NCI-H661	2.53	WM-115	5.93
CF-Pac1	7.57	NT2	2.60	WM-1341D	>20
Colo16	8.27	OAW42	3.02	WM-1617	5.20
Colo-357	4.80	OVCAR3	4.33	WM-239A	15.07
Colo829	3.44	PA-1	1.92	WM-266-4	3.20
DAL	>20	PANC-1	3.53	WM-793	>20
DAN-G	>20	PANC-TU1	5.53	WM-852	>20
DLD-1	10.10	PaTu8988T	4.32	WM-902B	>20
DU-145	1.40	PPC1	2.53	WM-983B	>20
FTC133	10.80	RPMI	2.67	ZR-75-1	12.20
FTC238	10.93	RT-4	2.56		
G361	3.47	SF-126	1.60		
G401	2.70	SF-767	0.60		
HCT-15	3.17	SH-SY-5Y	6.40		
Hela-S3	7.90	SK-BR-3	6.22		
HepG2	6.68	SK-LU-1	8.92		
Hs294T	1.53	SK-Mel-24	>20		

8.2.2.2 Sunitinib-dependent induction of apoptosis

Sunitinib not only inhibits proliferation of cancer cell lines, but it also induces apoptosis (Mendel *et al.*, 2003; Huynh *et al.*, 2009). Thus, the sensitivity spectrum for the induction of apoptosis was measured. The workflow was similar to the analysis of the inhibitory effect on proliferation. In detail, all tested cell lines were treated with various concentrations of sunitinib (1.25 – 20 µM) for 72 h, then the Hill three-parameter log fit or the sigmoidal dose-response curve fitting algorithm was used to calculate LD_{50} values (figure 15/A; table 9).

As in the analysis of the inhibitory effect of sunitinib on proliferation, most of the cancer cell lines (79.6%) induced apoptosis in a low micro-molar range after sunitinib treatment. Like the inhibition of proliferation, the induction of apoptosis was also tissue dependent (figure 15/B). Cell lines derived from kidney and brain were the most sensitive. In contrast, the proliferative, highly sensitive prostate tissue (IC_{50} = 3.5 µM) had a median LD_{50} value of 20 µM, which equals a 5.7-fold change, and proved completely insensitive to the induction of apoptosis. Compared with the fold-changes of all the other cell line groups, the values disperse around a mean value of 2.4 ± 0.96-fold change. Cancer cell lines from lung and breast tissue also showed a similar, but not as pronounced shift. Interestingly, cell lines derived from colon had a similar median of inhibition of proliferation (median: 10.1 µM) compared with the induction of apoptosis (median: 9.6 µM).

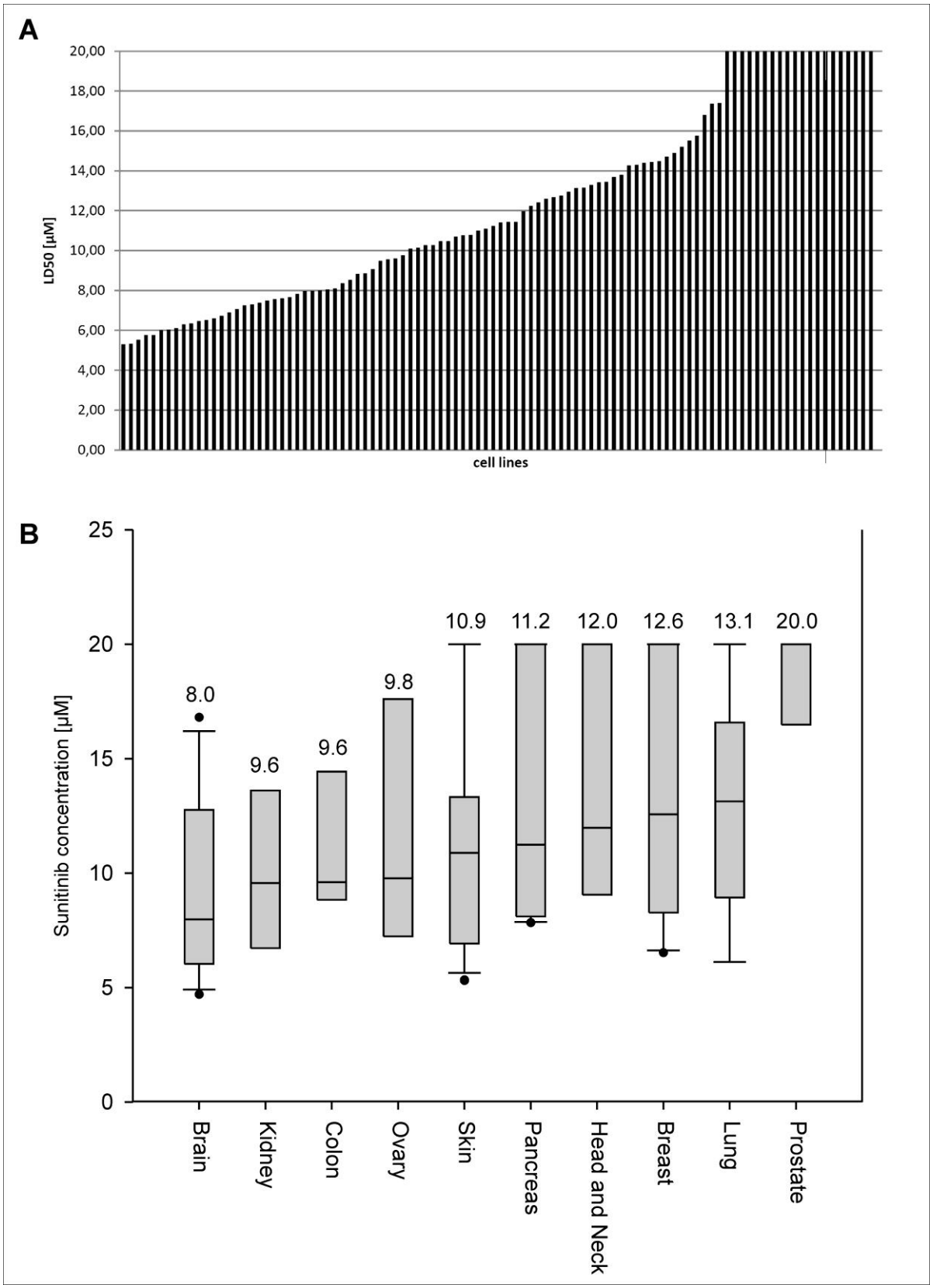

Figure 15: Analysis of sunitinib-dependent induction of apoptosis of various cancer cell lines. Cancer cell lines were treated with various concentrations of sunitinib (1.25 – 20 µM) for 72 h. LD_{50} values were calculated using Hill three-parameter log fit or the sigmoidal dose-response curve fitting algorithm. (A) Sensitivities of various cancer cell lines for the induction of apoptosis after sunitinib treatment. Sensitivities are ranked from sensitive to resistant. (B) Tissue dependency of cancer cell line sensitivities. LD_{50} values of cancer cell lines with the same tissue origin were grouped and ranked according to their median. Numbers above the box plots indicate the median LD_{50} value of the respective cell line tissue group.

Table 9: LD_{50} values for the sunitinib-dependent induction of apoptosis

cell line	LD50	cell line	LD50	cell line	LD50
1321N1	6.04	Hs294T	7.60	SF-767	7.38
769-P	14.72	Hs578T	6.52	SH-SY-5Y	4.70
786-O	10.27	Hs766T	11.43	SK-BR-3	10.47
818-4	7.83	IMR32	13.79	SK-LU-1	9.49
A375P	5.77	KAII	5.33	SK-N-SH	5.77
A-549	17.40	LOVO	9.60	SK-OV3	15.20
A590	6.11	MCF-7	14.40	SW-850	>20
BM1604	>20	MDA-MB-231	6.73	T98G	7.30
BOW-G	10.15	MDA-MB-361	13.69	TERA-2	6.60
BT-20	>20	MDA-MB-415	9.07	TSU-PR-1	12.95
BT474	14.49	MDA-MB-435S	8.53	U118	7.98
BT-549	11.43	MDA-MB-436	>20	U1240	10.28
BxPc3	8.10	MDA-MB-468	7.49	U1242	16.80
C-32	>20	MEWO3	7.67	U-373	10.70
C8161	13.16	MIAPaCa-2	11.09	UM-SCC-10A	>20
CaCO2	14.43	MM-DU	>20	UM-SCC-10B	11.98
CaKi-1	>20	MM-Leh	8.06	UM-SCC-17A	8.00
CaKi-2	6.01	MM-LO	7.07	UM-SCC-17B	>20
Calu-3	8.37	MM-SU	5.31	UM-SCC-22A	10.10
Calu-6	13.13	NCI-H460	12.41	WIDR	8.83
CaOV-3	9.77	NCI-H520	>20	WM-115	12.60
Capan-1	10.79	NCI-H596	14.26	WM-1341D	6.30
CCSTTG1	12.76	NCI-H661	15.76	WM-1617	6.35
CF-Pac1	7.97	NT2	5.53	WM-239A	10.77
Colo16	11.00	OAW42	>20	WM-35	12.23
Colo829	6.47	OVCAR3	7.57	WM-793	>20
DAL	>20	PA-1	6.90	WM-852	13.45
DAN-G	15.52	PANC-1	>20	WM-902B	>20
DU-145	>20	PaTu8988T	11.23	WM-983A	13.43
F-01	9.57	PC-3	>20	WM-983B	13.28
FCT133	14.30	PPC1	>20	ZR-75-1	>20
FTC238	10.47	PT-5Y-T5	>20		
G401	8.86	RPMI	11.42		
Hela-S3	14.90	RT-4	17.37		
HepG2	7.25	SBC-12	12.68		

8.2.2.3 Sunitinib-dependent inhibition of migration

Besides inhibiting proliferation and inducing apoptosis, sunitinib also inhibits the migration of cancer cells (Liamina *et al.*, 2009; Zhang *et al.*, 2009). To subsequently correlate the sensitivities of various cancer cell lines with the predominant presence of genetic alterations in migratory sensitive or insensitive cell lines, their migratory sensitivity profile was explored.

For this purpose, a trans-well migration assay was performed. Cells were treated with various concentrations of sunitinib (0.15 – 5 µM) and allowed to migrate against an FCS gradient from 0% to 10% for 24 h. To quantify the migrated cells, all were stained, either counted or photo-spectrometrically measured. Then the Hill three-parameter log fit or the sigmoidal dose-response curve fitting algorithm was used to calculate IC_{50} values (examples for typical migration pictures for sensitive (cell line RPMI-7951, tissue: skin), low-sensitive (cell line: U-373, tissue: brain), and resistant (cell line: DU-145, tissue: prostate) cell lines: figure 16/A; IC_{50} values: table 10). A list of all cancer cell line sensitivities is shown in figure 16/B. Unfortunately, not all cell lines do migrate. Thus, the analyzed spectrum consisted of only 32 cancer cell lines for further investigation.

Table 10: IC_{50} values for the sunitinib-dependent inhibition of migration

cell line	IC50	cell line	IC50	cell line	IC50	cell line	IC50
1321N1	2.5	CaOV-3	1.5	MM-Leh	4.6	T98G	0.45
A-498	>5	Colo829	>5	PPC1	4.0	TERA-2	0.6
A590	4.1	DU-145	>5	RPMI7951	0.2	TSU-PR-1	>5
BT-20	4.1	FCT133	2.7	SBC-12	1.8	U1240	0.2
BT-549	2.0	FTC238	3.4	SF-126	0.3	U1242	0.3
CaKi-1	3.0	Hs294T	3.0	SF-767	0.3	U-373	2.5
CaKi-2	2.1	HT-29	2.3	SK-N-SH	0.2	WM-239A	1.0
Calu-6	4.0	MDA-MB-231	1.0	SK-OV3	0.7	WM-983A	1.8

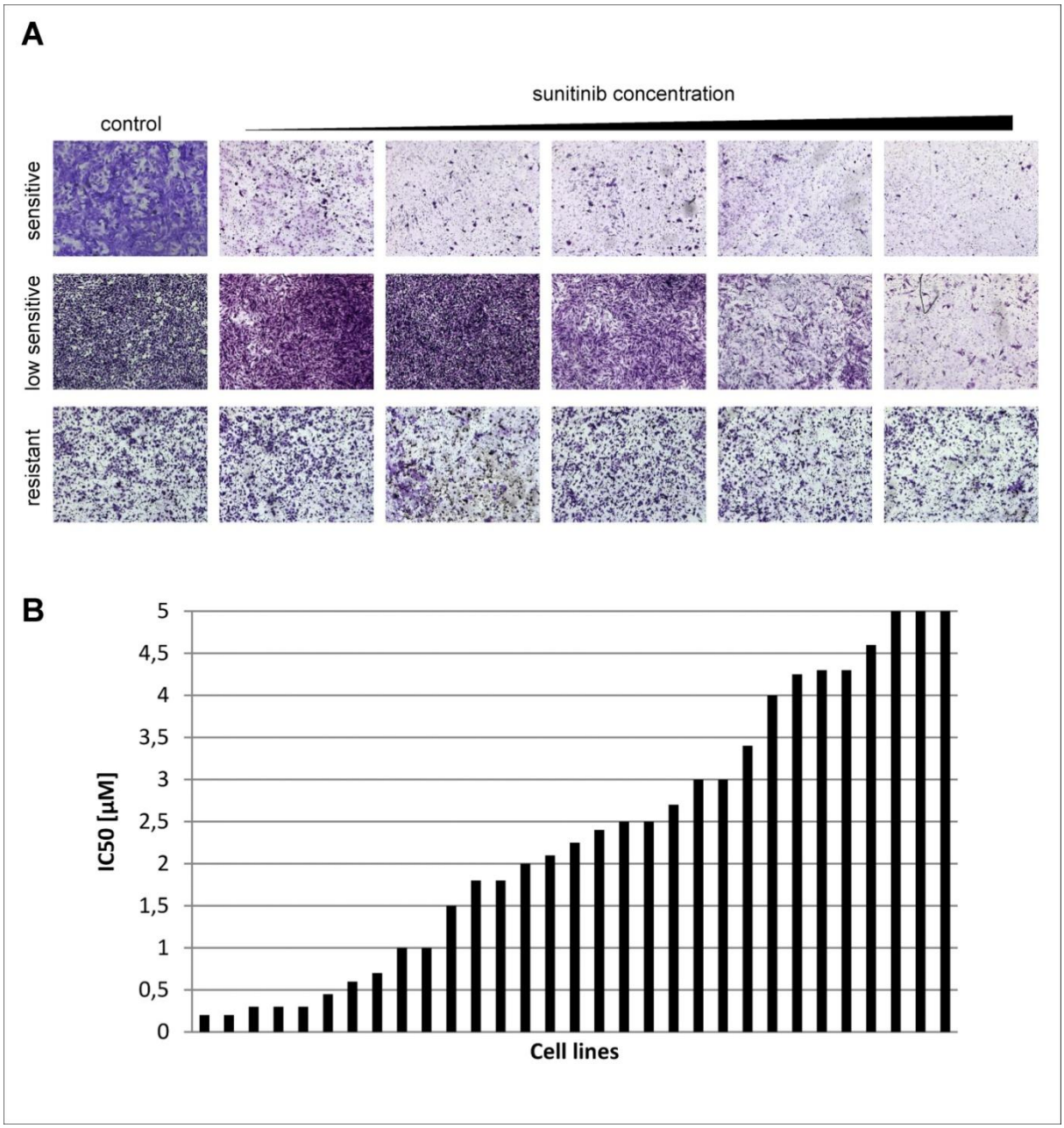

Figure 16: Analysis of sunitinib-dependent inhibition of migration of various cancer cell lines. In a trans-well migration assay cancer cell lines were treated with various concentrations of sunitinib (0.15 – 5 µM) and allowed to migrate against an FCS gradient for 24 h. IC_{50} values were calculated using the Hill three-parameter log fit or the sigmoidal dose-response curve fitting algorithm. (A) Examples for sensitive (RPMI-7951), low-sensitive (U-373), and resistant (DU-145) cell lines. (B) Sensitivities of various cancer cell lines for the inhibition of migration after treatment with sunitinib. Sensitivities are ranked from sensitive to resistant.

8.2.2.4 Comparison of frequencies of genetic alterations: analyzed cell line population vs. cell lines listed in TyKiVa

For significant results, the distribution of genetic alterations among the analyzed cell line population has to equal the distribution listed in TyKiVa. Therefore, the distribution of all selected genetic alterations was compared among both cell line populations (figure 17).

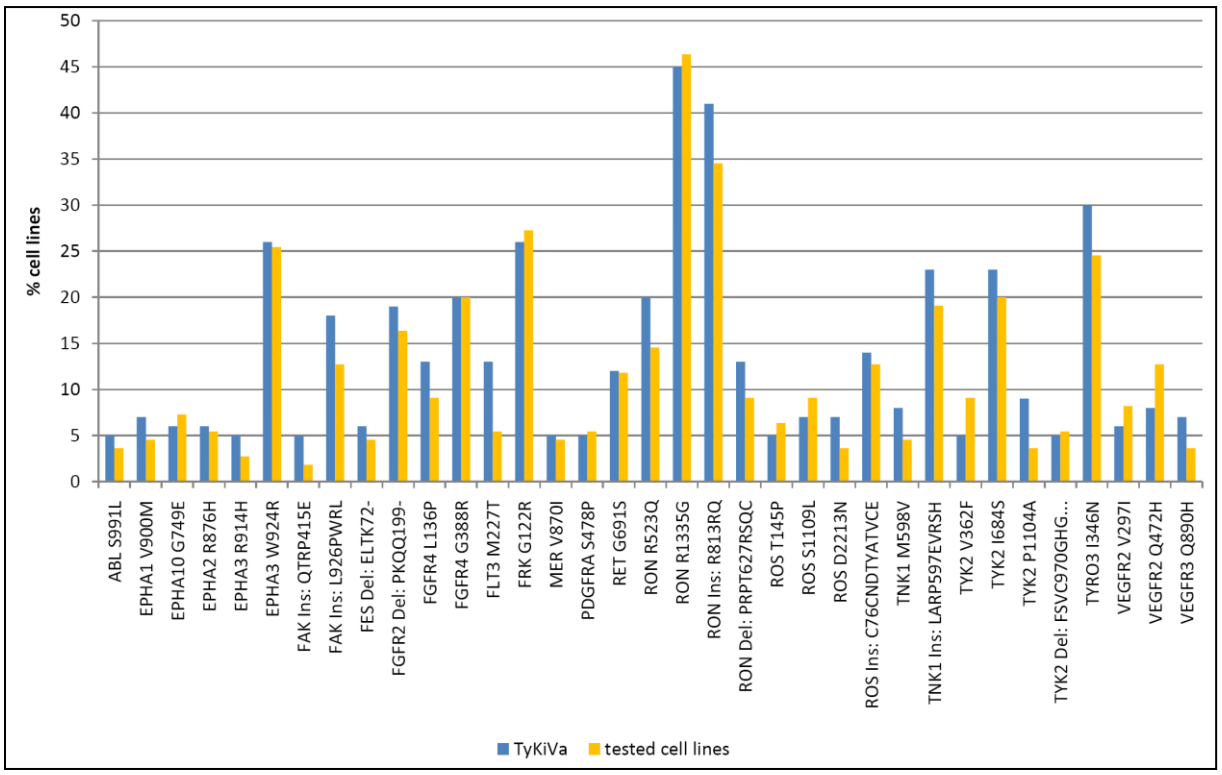

Figure 17: Comparison of the frequency of genetic alterations among the analyzed cell line population and cell lines listed in TyKiVa. Blue bars: Frequency of genetic alteration in 254 cancer cell lines listed in TyKiVa. Orange bars: Frequency of genetic alterations in 122 analyzed cancer cell lines.

The distribution of genetic alterations among the analyzed cell line populations was found to represent the distribution of genetic alterations in TyKiVa quite well (a mean deviation of 2.74±1.63%). Thus, the measured sensitivity spectra as regards the inhibition of proliferation, the induction of apoptosis, as well as the inhibition of migration could be used for statistical analysis.

8.2.3 Statistical analysis of the distribution of alleles

The database TyKiVa includes 155 polymorphisms and 234 somatic mutations in 254 established cancer cell lines (Ruhe *et al.*, 2007). Thirty-seven of these genetic alterations in 21 sunitinib targets occur with a frequency higher than 5% and thus were selected for further investigation of their influence on sunitinib response. In order to check, which genetic alteration is significantly overrepresented in sunitinib-sensitive or -resistant cancer cell lines, the distribution of each allele among the cell line population was statistically analyzed. The sensitivities, obtained from the anti-proliferative, anti-migratory, and pro-apoptotic response to sunitinib treatment, of the two cell line groups harboring one allele were compared using Student's t-test (figure 18).

The analysis of sensitivities obtained from the sunitinib-induced inhibition of proliferation revealed four alleles that were significantly overrepresented in sunitinib-sensitive cell lines compared with the other alleles and thus they might mediate increased sunitinib sensitivity. These alleles were $ABL1^{911L}$ of the SNP $ABL1^{911S/L}$ ($p=0.037$), RON^{523Q} of the SNP $RON^{523R/Q}$ ($p=0.024$), $TYK2^{362F}$ of the SNP $TYK2^{362V/F}$ ($p=0.013$), and $TYK2^{684S}$ of the SNP $TYK2^{684I/S}$ ($p=0.012$; figure 18/A). The statistical analysis of the sensitivity profile obtained from the sunitinib-induced apoptosis showed that the allele RON^{1335G} of the SNP $RON^{1335R/G}$ was over-represented in sunitinib-resistant cancer cell lines ($p=0.0036$), whereas the allele $TYK2^{684S}$ of the SNP $TYK2^{684I/S}$ ($p=0.0022$) was overrepresented in sunitinib-sensitive cell lines (figure 18/B). Furthermore, a statistical analysis of sensitivity data obtained from the anti-migratory effect of sunitinib was also performed. Due to the limited number of tested cell lines, only genetic alterations with a high frequency ($\geq$ 15%) could be considered. This revealed that the two alleles $TYK2^{362F}$ ($p=0.038$) and $TYK2^{684S}$ ($p=0.018$), which had also been identified by the analysis of the proliferation data, were significantly overrepresented in sunitinib-sensitive cell lines (figure 18/C).

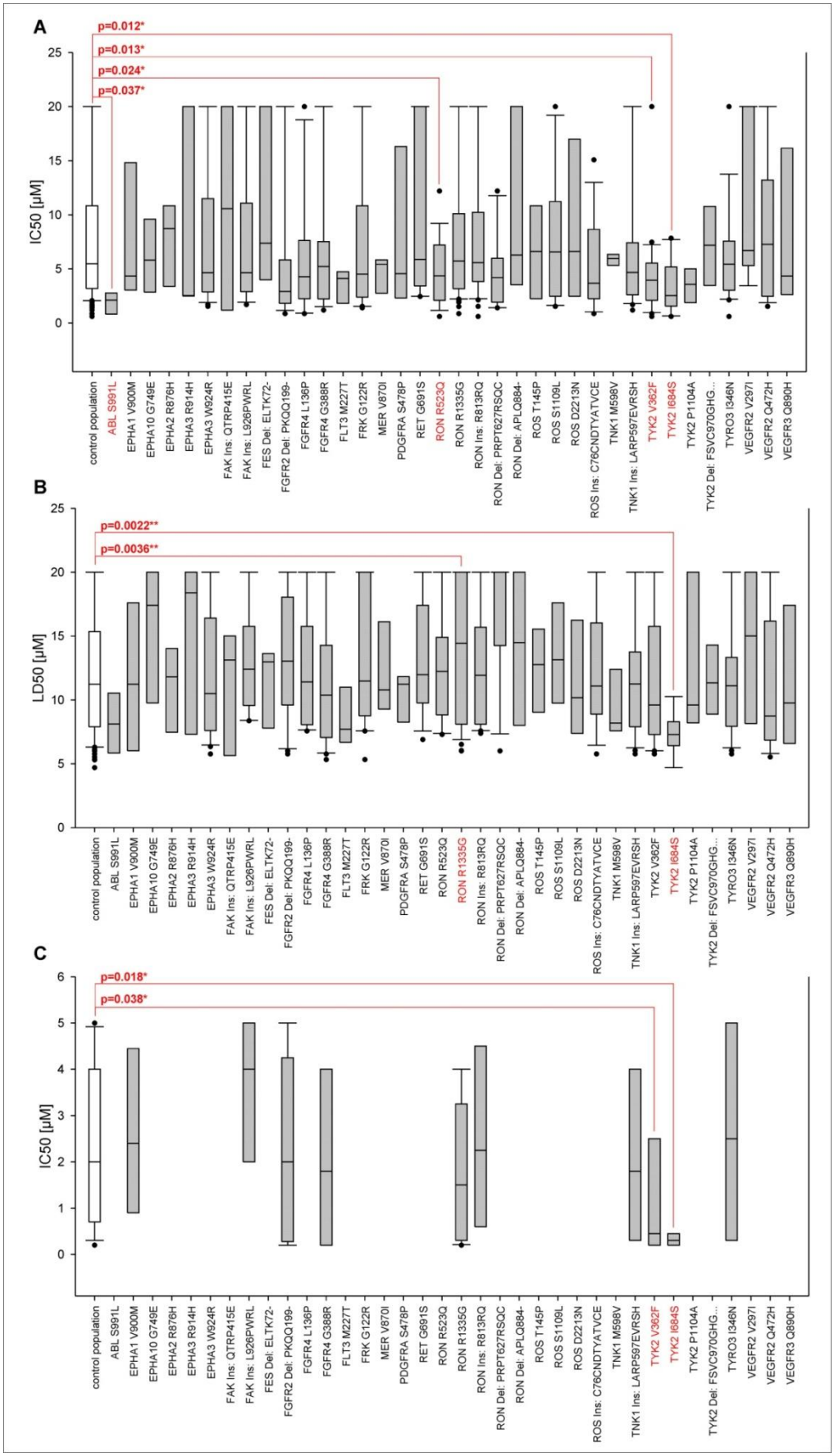

Figure 18: Statistical analysis of the distribution of alleles. Groups, which consist of cell lines harboring one allele, were statistically compared using Student's t-test. The calculation was done with IC_{50}/LD_{50} values obtained from the measurement of sunitinib-induced (A) inhibition of proliferation, (B) induction of apoptosis, and (C) inhibition of migration.

In brief, four alleles that were significantly overrepresented in sensitive cancer cell lines (ABL1^{991L}, RON^{523Q}, TYK2^{362F}, TYK2^{684S}) and one allele with increased occurrence in resistant cell lines (RON^{1335G}) were identified. They then became candidates for further investigation. Interestingly, TYK2^{362F} was identified by the analysis of two different biological responses (inhibition of proliferation and migration). Moreover, TYK2^{684S} appeared in all three statistical analyses as highly overrepresented in sunitinib-sensitive cell lines and thus could be responsible for the increased response to sunitinib treatment (figure 19).

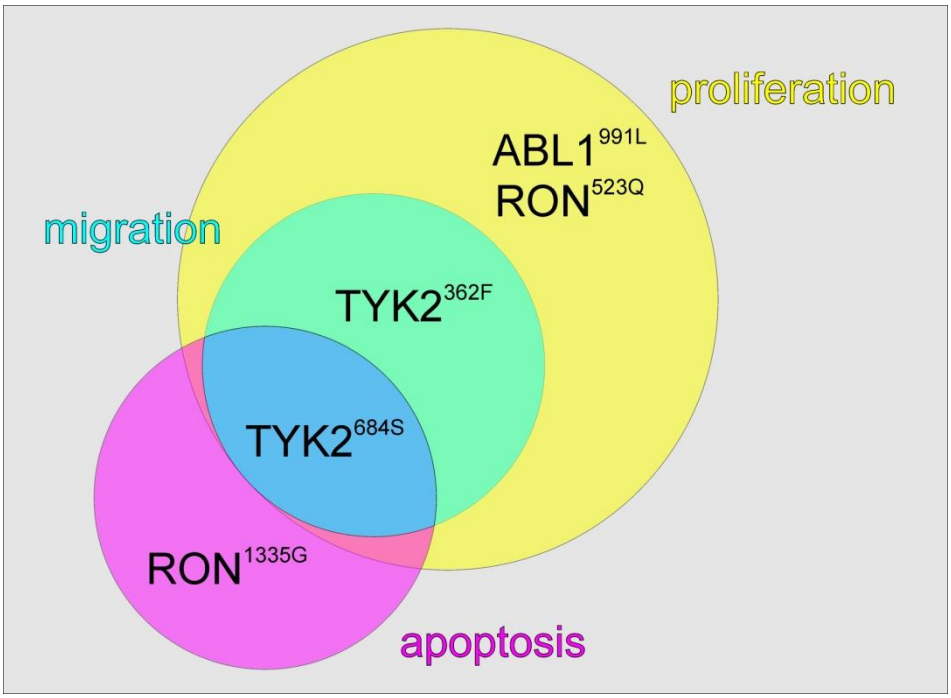

Figure 19: Frequency of identification of the five statistically overrepresented alleles. Candidates identified by statistical analysis of data obtained from the effect of sunitinib on migration (cyan circle), proliferation (yellow circle), and apoptosis (magenta circle) are shown. Overlapping areas indicate the frequency of identification. TYK2^{684S} , for example, was identified in all three statistical correlations.

8.2.4 Verification of candidate selection

The identification of five candidates that potentially influence sunitinib response (ABL1^{991L}, RON^{523Q}, TYK2^{362F}, TYK2^{684S}, RON^{1335G}) was based on the manual selection of alleles with a certain frequency among sunitinib targets and thus on a multi-criteria approach. To exclude the possibility that important findings were neglected, the selection of candidates was verified using a less stringent, single-criterion approach. Therefore, more than 4500 genetic alterations of all available cancer cell line databases (TyKiVa (Tyrosine Kinome Variants) (Ruhe *et al.*, 2007), COSMIC (Catalogue of somatic mutations in cancer) (Forbes *et al.*, 2010), MoKCa (Mutations of kinases in cancer) (Richardson *et al.*, 2009), and IARCP53 (Petitjean *et al.*, 2007) were included. Furthermore, sunitinib targets and non-targets were not differentiated, in case a genetic alteration would enable inhibition of a sunitinib non-target. To narrow down the 4500 genetic alterations, sunitinib sensitivities were correlated with the increased occurrence of alleles in sensitive or insensitive cancer cell lines (figure 20/A: proliferation; figure 20/B: apoptosis). Therefore, IC_{50}/LD_{50} values obtained by measuring sunitinib-dependent inhibition of proliferation as well as induction of apoptosis were ranked (figure 20, y-axis). Afterwards, the respective alleles were assigned to each cell line (figure 20, horizontal to each cell line: black squares indicate the presence of an insertion, mutation, deletion, or the rarer allele of a SNP). To visualize accumulations of genetic alterations in sensitive or resistant cancer cell lines, each genetically altered event was examined with respect to its presence in a sensitive or insensitive cell line. Therefore, the value of the difference from the median of each cell line population (figure 20, red lines; median (IC_{50} proliferation) = 5.42 µM; median (LD_{50} apoptosis) = 11.3 µM) was assigned to each genetically altered event. Thus, genetic alterations in cell lines more sensitive than the median of all cell lines were assigned a negative value; genetic alterations more insensitive than the median were assigned a positive value. The numerical value mirrored the difference to the median. The sum of these values for each genetic alteration over all cell lines resulted in a measurand that quantified the probability that a genetic alteration would occur in a sensitive (negative measurand) or resistant (positive measurand) cell line, respectively. The subsequent ranking of these values led to the visualization of genetic alterations that mainly occurred in sensitive (figure 20, left end of x-axis) or insensitive (figure 20, right end of x-axis) cell lines.

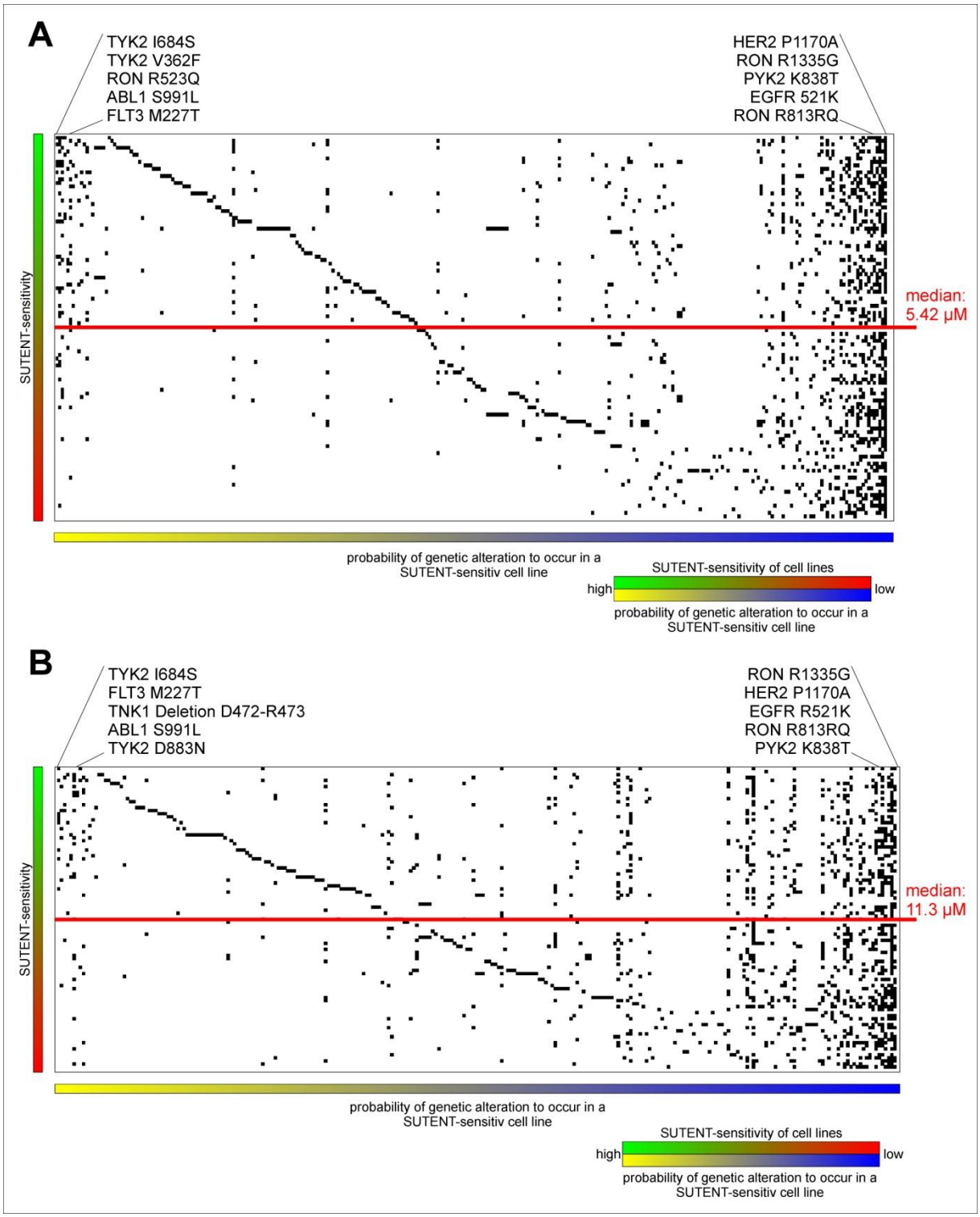

Figure 20: Correlation of sunitinib sensitivities with the presence of genetic alterations. (A) Correlation of genetic alterations with sunitinib sensitivities for the inhibition of proliferation. (B) Correlation of genetic alterations with sunitinib sensitivities for the induction of apoptosis. The y-axis represents cell line sensitivities in increasing order. Horizontal black squares of every cell line indicate the presence of a genetic alterations. The median of the sensitivities of the whole cell line population is indicated by a red line. The x-axis represents a ranking of the probability that the genetic alteration influences the sunitinib response. The further left a genetic alteration ranks, the more likely it is that this genetic alteration occurs only in sunitinib-sensitive cell lines and the more likely it is that this genetic alteration causes the sensitivity. The further right it ranks, the more likely it is that the genetic alteration mediates resistance. The TOP-5 hits on both sides are listed above each correlation.

Interestingly, although this approach considered only the frequency of occurrence of an allele in sensitive or resistant cell lines and did not discriminate between sunitinib targets and non-targets, all identified genetic alterations, with the exception of EGFR and HER2, were located in sunitinib targets. Furthermore, the TOP-5 hits of both clusters were generally identical. These genetic alterations were $TYK2^{I684S}$, $TYK2^{V362F}$, RON^{R523Q}, $ABL1^{S991L}$, $FLT3^{M227T}$, $TYK2^{D883N}$, $TNK1^{Del:\ D472\text{-}R473}$, RON^{R1335G}, $HER2^{P1170A}$, $EGFR^{R521K}$, $RON^{Ins:R813RQ}$ and $PYK2^{K838T}$. Especially the two alleles $TYK2^{684S}$ and RON^{1335G} appeared in both correlations as top hits. Even more interesting was the fact that all five candidates identified by the multi-criteria selection ($ABL1^{991L}$, RON^{523Q}, $TYK2^{362F}$, $TYK2^{684S}$, RON^{1335G}) were among the less stringent, single-criterion selection (figure 21). This not only validated the findings of the multi-criteria selected targets but underlined the potential importance of these genetic alterations for the response to sunitinib treatment. Seven additional genetic alterations were identified in addition to these five genetic alterations ($FLT3^{M227T}$, $TYK2^{D883N}$, $TNK1^{Del:\ D472\text{-}R473}$, $HER2^{P1170A}$, $EGFR^{R521K}$, $RON^{Ins:\ R813RQ}$ and $PYK2^{K838T}$).

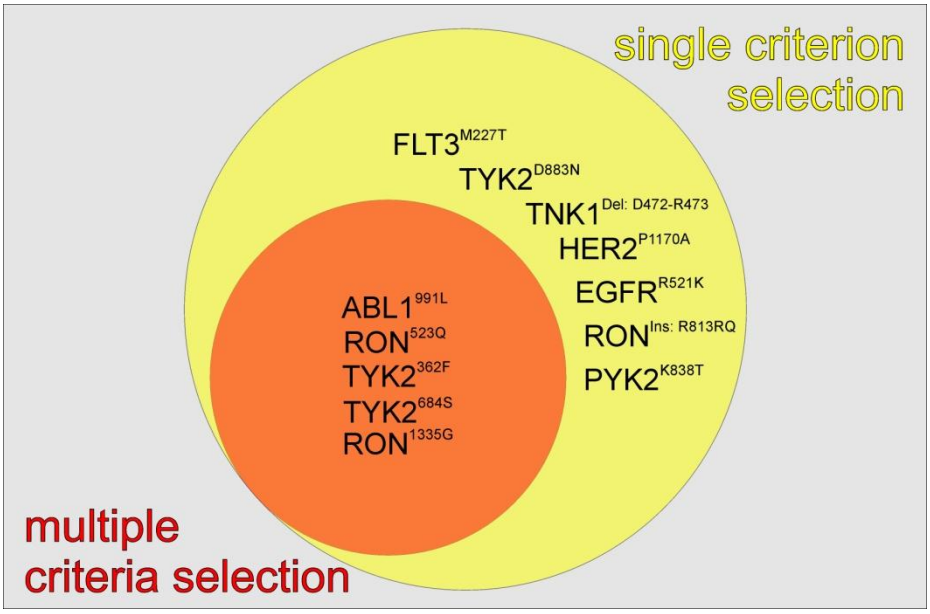

Figure 21: Comparison of single-criterion selected candidates with multiple-criteria selected candidates. Genetic alterations listed in the red circle indicate hits identified by the multiple-criteria approach. The yellow circle represents genetic alterations identified via a single-criterion approach and includes all hits of the multiple- criteria selection.

None of these additionally identified genetic alterations was statistically overrepresented in sunitinib-sensitive or -insensitive cancer cell lines (figure 22; A: proliferation; B: apoptosis). However, to ensure that all potentially influential genetic alterations were considered, all 12 genetic alterations were selected for further investigation (table 11).

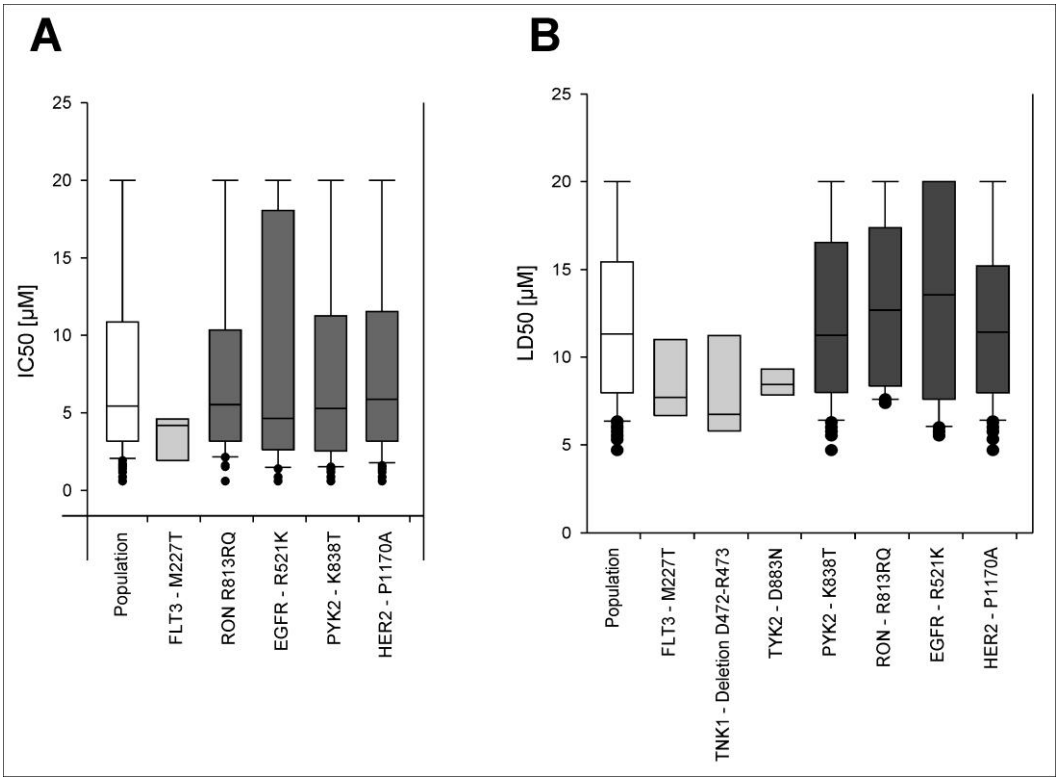

Figure 22: Statistical analysis of the distribution of additionally identified alleles. Always two groups, which consist of cell lines harboring one allele, were formed. IC50/LD50 values of these groups were statistically compared using Student's t-test. The calculation was done with IC50/LD50 values obtained from the measurement of sunitinib-induced (A) inhibition of proliferation and (B) induction of apoptosis. The distribution of all tested cell lines is indicated by a white box. Genetic alteration identified as potentially sensitivity- increasing are indicated by light gray boxes, potentially sensitivity-decreasing, by dark gray boxes.

Table 11: Summary of candidates selected for further investigation. Known sunitinib targets are in italics. Genetic alterations located in the tyrosine kinase domain are indicated in bold.

potentially sunitinib-sensitivity increasing	potentially sunitinib-sensitivity decreasing
TYK2 I684S	***RON R1335G***
TYK2 V362F	HER2 P1170A
RON R523Q	EGFR R521K
ABL1 S991L	*RON Ins: R813RQ*
FLT3 M227T	*PYK2 K838T*
TYK2 D883N	
TNK1 Del: D472-R473	

8.2.5 Influence of identified candidates on the response to sunitinib treatment

Genetic alterations are widely known to potentially influence the efficacy of small molecule inhibitors (Tracy *et al.*, 2004; Jackman *et al.*, 2006; Sasaki *et al.*, 2006; Sharma *et al.*, 2007; Yun *et al.*, 2008). In recent years several genetic alterations that influence the sensitivity to sunitinib treatment have been identified in patients with GIST (Heinrich *et al.*, 2008). As all sunitinib targets were known, the central aim of this study was to identify genetic alterations that influence the sensitivity to sunitinib. Thus, the 12 identified candidates underwent further analysis with respect to their influence on sunitinib efficacy at the molecular as well as the cellular levels.

8.2.5.1 Correlation of potentially influential alleles with sunitinib response depending on endogenous target expression level

Target expression levels frequently correlate with drug sensitivities. Thus, mRNA levels are routinely profiled to estimate several drug efficacies (Los *et al.*, 2002; Gunther *et al.*, 2003; Gunther *et al.*, 2005). In this context, the amount of VEGF and soluble KIT (sKIT) in the blood serum correlates with sunitinib efficacy (DePrimo and Bello, 2007; Deprimo *et al.*, 2009; Kontovinis *et al.*, 2009). In order to determine if the most frequent genetic alterations influence sunitinib sensitivity due to their change of target expression, the endogenous expression levels were measured at the mRNA level (table 12).

Table 12: Analysis of endogenous target expression at the mRNA level. X = normalized (to GAPDH) target expression < 0.2; XX = normalized target expression < 1.0. XXX = normalized target expression ≥ 1.0

tissue	cell line	cKIT	FLT3	PDGFRα	PDGFRβ	RET	RON	TYK2	EGFR	ABL
breast	MDA-MB-435S			X	X	X	X	XXX	X	XX
	MDA-MB-231		XX		X	X	XX	XXX	X	XX
	MDA-MB-436		XX	X		X	XX	XXX		X
	MDA-MB-468		X		X	X	XXX	XXX	X	XXX
	Hs 578T				XXX	X		XXX	XXX	X
	MDA-MB-453		X			X	XX	XXX	XXX	X
	BT-474		X				XX	XX	XX	XX
	BT-549						XX	XXX		X
	BT-20						XX	XXX		XX
	T-47D			X		X	XXX	XX	XXX	X
prostate	DU-145		XX				XX	XXX	XX	XXX
	PPC-1					XX	XX	XX	XXX	XXX
	PC-3	X			X	X	XX	XXX	XXX	XXX
HGBM	SF-126	XX			X	X	X	XXX	XXX	XX
	SF-767	X					XX	XXX		XXX
	U-373	X	XX		XX	X	X	XXX	X	XX
	U-1242				X			XX	XX	X
	U-1240			X	X	X	X	XX	XXX	XX
	U-118			X	XXX		X	XXX	XX	XXX
	A-704 (kidney)							XXX	XX	XXX
ovary	TT (thyroid)		XX		X	X	X	XXX	XX	XXX
	CaOV3		XX		X		XX	XXX	XX	XX
	PA-1	X	X		X			XXX		XX
colon	HCT-15		X		X	X	XXX	XXX	XX	XXX
	DLD-1	XX	X	X	XX	X	XXX	XXX	X	XXX
	SW-480				XX		XXX	XXX	XX	XXX
	SW-620				XX	X	XXX	XXX	X	XXX
lung	A-549	X		X	X	X	XXX	XX	X	XXX
	NCI-H661		X		XX	X	X	XX	XX	XXX
	NCI-H441		X			X	XXX	XXX	XX	XXX
	NCI-H292		X	X	XX	X	XXX	X		XXX
	NCI-H596			X			XX	XXX	XX	XXX
	Sk-Mes-1	X	X	XX	XX	X	XXX	XXX	XXX	XXX
	A-427		XX	XXX		X	XXX	XX		XXX
	Calu-1		X		XXX		XXX	XXX	XX	XXX
pancreas	BxPc-3						XXX	XXX	X	X
	PaTu-8902		X			X	XXX	XXX	XX	X
	Colo-357		XX	X		X	XXX	XXX	X	XX
	SW-850		XX				XXX	XXX	XX	X
	Hs 766T		X			X	XXX	XX		X
	PANC-1		X			X	XXX	XXX		XX

	AsPc-1	XX	X	XX		X	XXX	XXX	XXX	X
	Capan-1	XX	X	X	X	X	XXX	XX	XXX	XX
	A-590	X						XXX	X	X
skin	A-375-P	X		XXX	X	X		XXX		X
	Sk-Mel-24	X		XXX	XX			XX		XX
	RPMI-7951				XXX	X	X	XXX	X	X
	WM-902-B	X				X		XX		XXX
	WM-266-4					X	X	XXX	X	XX
	WM-239-A					X		XX		X
	WM-115		X	X	XX	X	X	XXX		XXX
	C-8161		X		X	X	XX	XXX		XXX
	Mamle-3M		X	X	X	X	XX	XXX	X	XX
	Hs 294T		X		XX	X	X	XX	X	X

Although no genetic alterations were identified in PDGFR and cKIT, they were included in this study since they are very potent sunitinib targets and thus should serve to elucidate the general influence of target expression level on sunitinib sensitivity. Subsequently, PDGFR and cKIT expression levels were correlated with sunitinib efficacy with respect to both the induction of apoptosis (figure 23/A) and the inhibition of proliferation; they served as a positive control (figure 23/B). In contrast, EGFR is not inhibited by sunitinib. Consequently, its expression level did not influence sunitinib sensitivity of cancer cell lines as regards the inhibition of proliferation (figure 23/B) or the induction of apoptosis (figure 23/A); it served as a negative control.

To elucidate the influence of genetic alterations on this correlation, the impact of each sunitinib target on sunitinib sensitivity had to be determined first (induction of apoptosis: figure 23/A; inhibition of proliferation: figure 23/B).

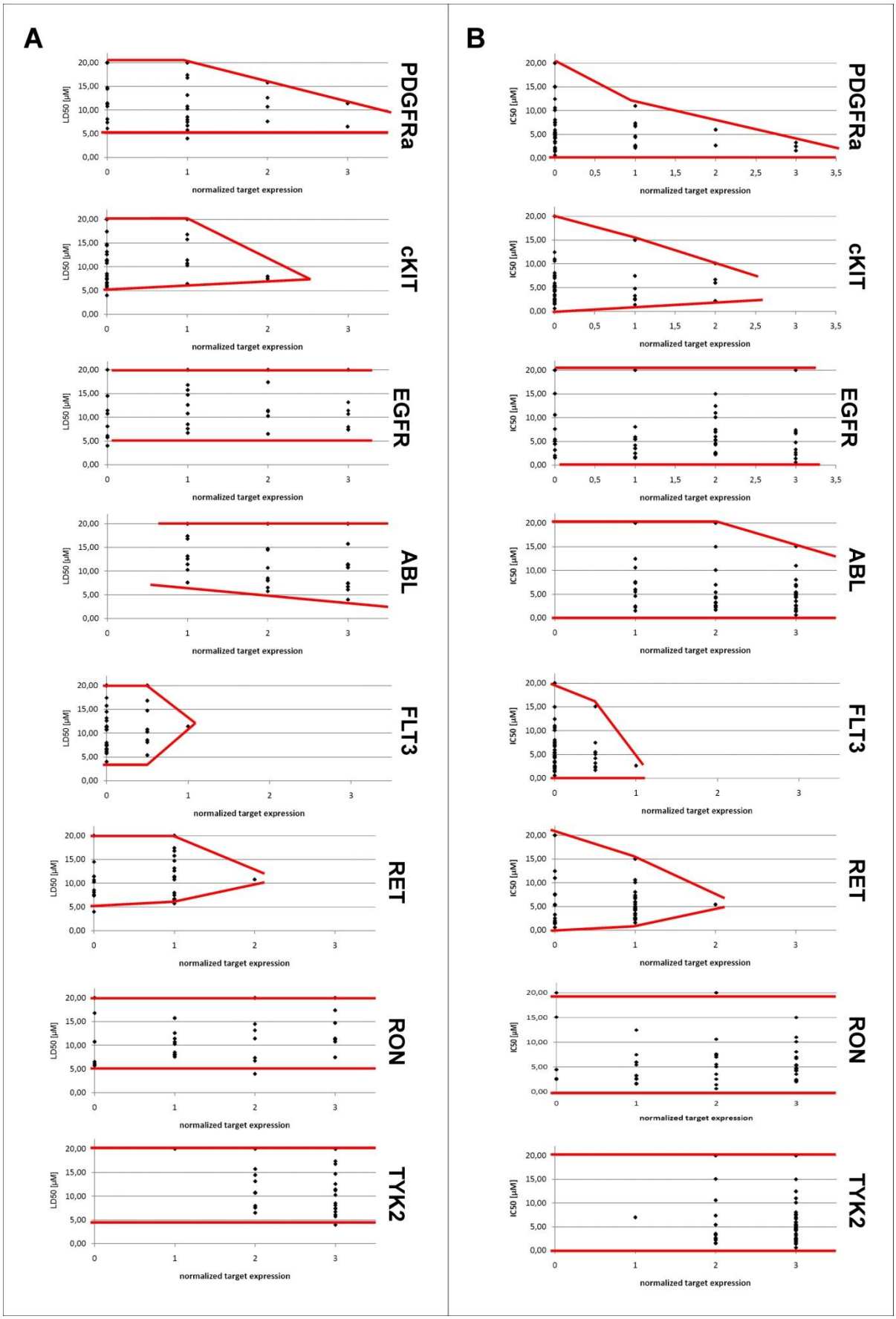

Figure 23: Correlation of endogenous target expression with sensitivity to sunitinib. Every dot represents one cell line with the respective normalized (against GAPDH) target expression (x-axis) and sunitinib sensitivity (LD_{50}/IC_{50} values; y-axis). Red lines mark the limits of sensitivities, measured at a certain target expression level. (A) induction of apoptosis (B) inhibition of proliferation

This revealed that the endogenous expression levels of FLT3, RET, and ABL correlated with sunitinib sensitivity, whereas the expression levels of RON and TYK2 showed no influence. It must be emphasized that this gave no information about the quality of the sunitinib target, only information as to which sunitinib target influences the response to sunitinib treatment in an expression level-dependent manner. Therefore the question was addressed as to whether genetic alterations influence this dependency on endogenous target expression level. Expression levels were assigned to two groups of cell lines harboring one allele (induction of apoptosis: figure 24/A; inhibition of proliferation: figure 24/B). With the exception of $TYK2^{684S}$, all alleles were equally distributed over the given, target expression level- dependent area. Cell lines harboring the serine allele at position 684 in TYK2 grouped at the highly sensitive area of the plot. A closer look at this grouping showed that it was allele-dependent, but unfortunately not connected with the TYK2 expression level. Thus, all genetic alterations did not influence the dependency of sunitinib sensitivity on the sunitinib target expression level.

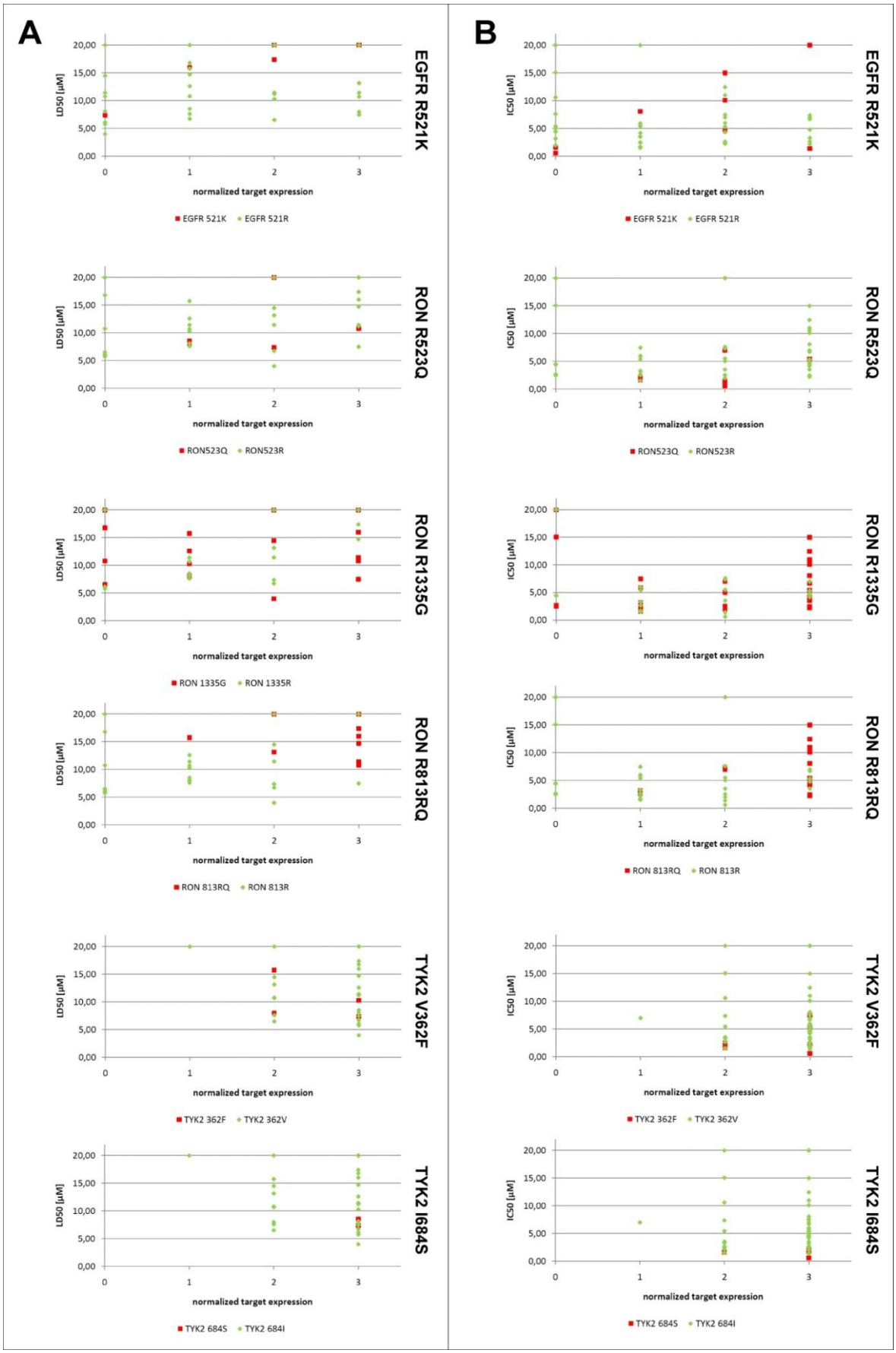

Figure 24: Influence of genetic alterations on the endogenous target expression level dependent on sensitivity to sunitinib. Every dot represents one cell line with the respective normalized (against GAPDH) target expression (x-axis) and sunitinib sensitivity (y-axis). Green and red dots indicate cell lines harboring one allele. (A) induction of apoptosis. (B) inhibition of proliferation.

Apart from the correlation with sunitinib sensitivities, the mRNA level of RON^{R813RQ} correlated with the insertion (figure 25). Interestingly, the expression of RON was significantly higher (p = 0.0007) in cells harboring the insertion RON^{813RQ}. Since this observation was sunitinib independent, no further investigations were done.

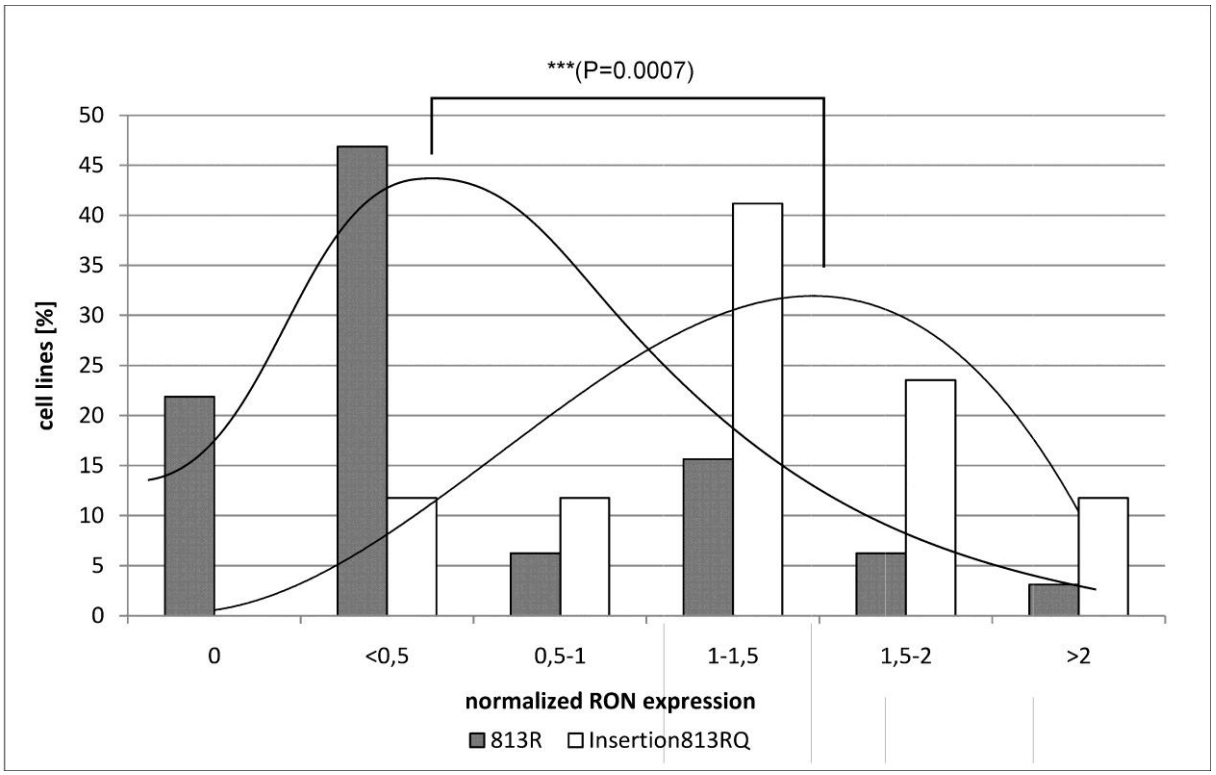

Figure 25: Endogenous expression level of RON depending on the insertion RON^{R813RQ}. mRNA expression levels were determined and normalized to GAPDH expression. Cell lines were grouped according to their RON expression level (0; <0.5; 0.5-1; 1-1.5; 1.5-2; >2). Bars indicate the percentage of cell lines within one group. Lines indicate the RON^{813} genotype-dependent distribution of RON expression. Insertion-dependent differences in expression levels were calculated using Student's t-test.

8.2.5.2 Influence of genetic alterations on the inhibition of phosphorylation

The crucial aspect in small molecule inhibitor efficacies is their ability to inhibit target phosphorylation (Ullrich and Schlessinger, 1990; Levitzki and Gazit, 1995). Twelve potentially influential genetic alterations were identified. Their influence on the inhibitory effect of sunitinib on target phosphorylation was determined. Ten of these genetic alterations were located in sunitinib targets and two in the sunitinib non-targets EGFR and HER2. As was seen in the effect of G719S in EGFR on gefitinib sensitivity in colon cancer cell lines, the genetic background is subordinate as regards the inhibition of respective targets at the molecular level (in contrast to the resulting effect at cellular level). Thus, it was initially sufficient to compare only two cell lines harboring one allele homozygously. To elucidate the allele-dependent difference in sunitinib-induced target inhibition, cell lines were starved over night and then incubated in various concentrations of sunitinib (1.25 – 10 µM) for 2 h. After stimulation with pervanadate the sunitinib-dependent inhibition of target-phosphorylation was analyzed by phospho-tyrosine (4G10) immune precipitation (IP) and target detection (figure 26 (sunitinib targets)) or by phospho-target detection followed by target reblotting (figure 27 (sunitinib non-targets HER2 and EGFR)).

8.2.5.2.1 Influence of genetic alterations on the inhibition of sunitinib targets

Surprisingly, the two hits RON$^{1335R/G}$ and TYK2$^{684I/S}$ also showed an allele-dependent difference in the sunitinib-induced inhibition of the respective target at the molecular level (figure 26, asterisks). In other words, the gylcine allele at position 1335 in RON mediated a decreased inhibition compared with the arginine allele. In contrast, the serine allele at position 684 in TYK2 dramatically increased the inhibition of TYK2 phosphorylation. While TYK2^{684I} was not inhibited at all, the phosphorylation of TYK2^{684S} was already decreased at low sunitinib concentrations. Moreover, phosphorylation of the well-known sunitinib target FLT3 was inhibited at low sunitinib concentrations ($IC_{50} \approx 2$ µM). Furthermore, ABL1, PYK2, and TNK showed good responses to sunitinib treatment ($IC_{50} \approx$ 2-5 µM). Thus, the analysis of the remaining eight genetic alterations in the sunitinib targets exhibited no allele-dependent difference in the sunitinib-induced inhibition of phosphorylation (figure 26).

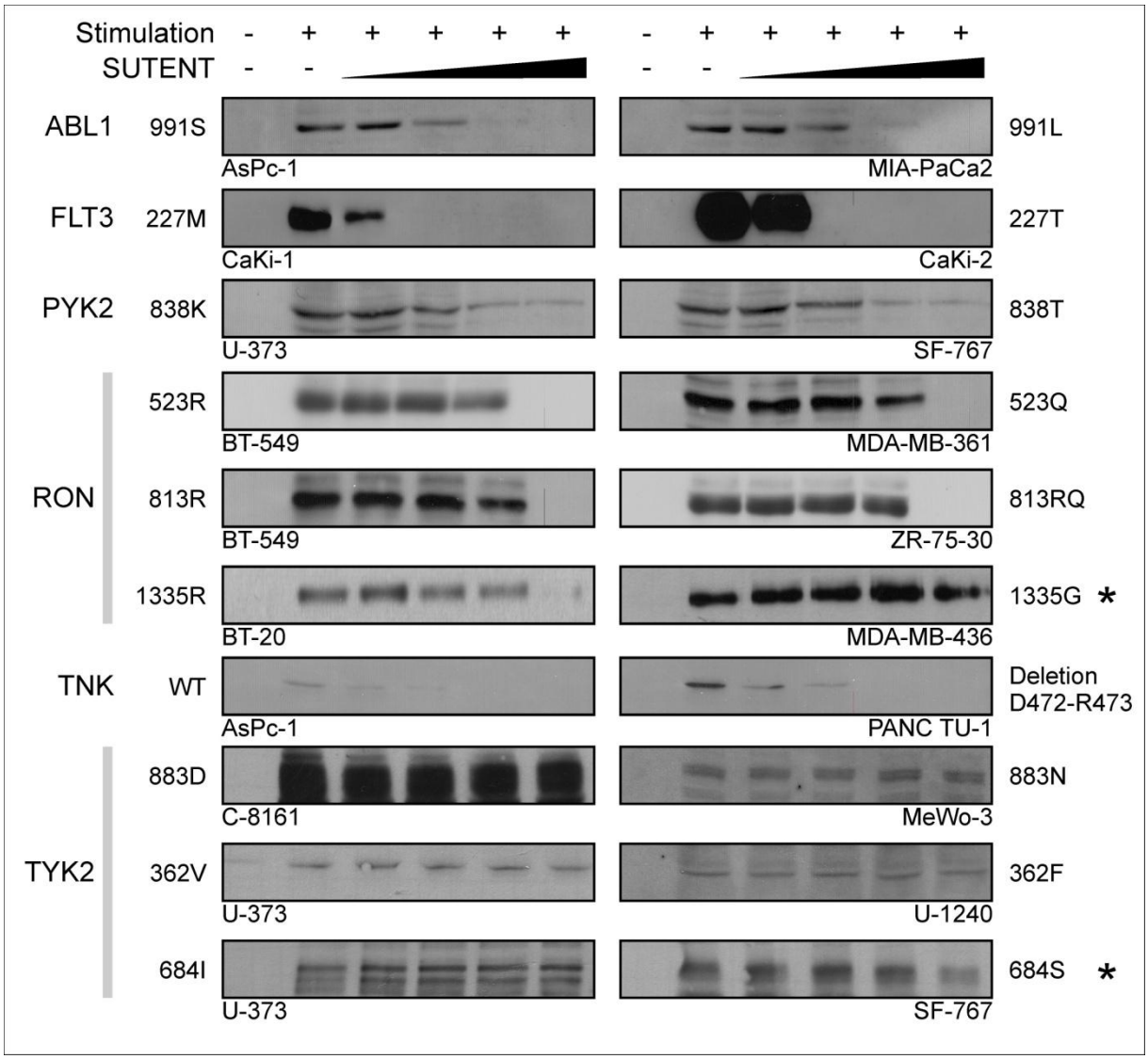

Figure 26: Sunitinib-induced inhibition of target phosphorylation dependent on target genotype. Cell lines harboring one of the two alleles were starved over night. After 2 h treatment with sunitinib the cells were treated with pervanadate (10 nM; 5 min) followed by 4G10-IP and target detection. (*) indicates allele-dependent differences in target inhibition. Cell lines: ABL^{991S}: AsPc1, pancreas; ABL^{991L}: MIA-PaCa2, pancreas; FLT3^{227M}: CaKi-1, kidney; FLT3^{227T}: CaKi-2, kidney; PYK2^{838K}: U-373, brain; PYK2^{838T}: SF-767, brain; RON^{523R}: BT-549, breast; RON^{523Q}: MDA-MB-361, breast; RON^{813R}: BT-549, breast; RON^{813RQ}: ZR-75-30, breast; RON^{1335R}: BT-20, breast; RON^{1335G}: MDA-MB-436, breast; TNK^{WT}: AsPc1; pancreas; TNK$^{Del:D472-R473}$: PANC-TU1, pancreas; TYK2^{883D}: C8161, skin; TYK2^{883N}: MeWo3, skin; TYK2^{362V}: U-373, brain; TYK2^{362F}: U-1240, brain; TYK2^{684I}: U-373, brain; TYK2^{684S}: SF-767, brain.

8.2.5.2.2 Influence of genetic alterations on the inhibition of sunitinib non-targets

In addition to the identified sunitinib targets, the single-criterion approach revealed that two alleles in the sunitinib non-targets $EGFR^{521R/K}$ and $HER2^{1170P/A}$ were overrepresented in sunitinib-resistant cancer cell lines. To determine the influence of these alleles, the sunitinib-dependent inhibition of target-phosphorylation and the target binding to sunitinib-coupled beads were analyzed (figure 27). As expected, phosphorylation of neither HER2 nor EGFR was inhibited by sunitinib (figure 27/A). Furthermore, HER2 did not bind to sunitinib (figure 27/B). Interestingly, in contrast to $EGFR^{521R}$, $EGFR^{521K}$ bound to sunitinib-coupled beads (figure 27/C). Since the SNP is located in the extracellular part of the EGF receptor and did not influence its inhibition of phosphorylation by sunitinib, $EGFR^{521R/K}$ was not further investigated in this study.

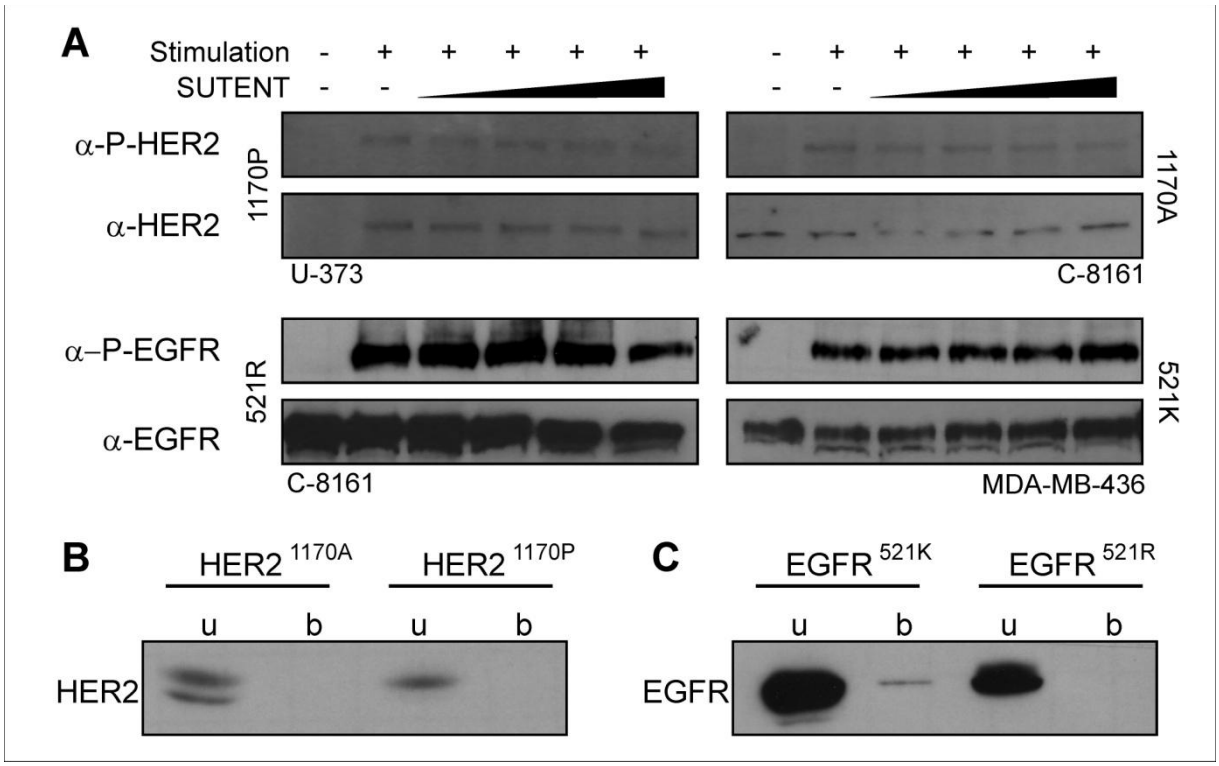

Figure 27: Sunitinib induced inhibition of non-target phosphorylation (EGFR and HER2) depending on target genotype. (A) Cell lines harboring one of the two alleles homozygously were starved over night. After 2 h treatment with sunitinib the cells were treated with pervanadate or EGF (EGF: 10 ng/ml; 5 min; pervanadate: 10 nM; 5 min) followed by phosphorylation-target detection and target reblotting. (B) HER2 binding to sunitinib- coupled beads depending on the SNP $HER2^{1170P/A}$. (C) EGFR binding to sunitinib-coupled beads depending on the SNP $EGFR^{521R/K}$. For binding, cells were lysed and incubated with the SUTENT-coupled beads over night at 4°C. The next day bound proteins were eluted with free inhibitor. Unbound protein (u) was compared with bound protein (b).

8.2.5.2.3 Verification of the allele-dependent difference of RON^{1335G} and TYK2^{684S} on the sunitinib-dependent inhibition of target phosphorylation

Due to the great heterogeneity of cancer, it is always possible that observations in one cancer patient or cancer cell line are only context dependent and cannot be generalized. To prove that the allele-dependent differences in the inhibition of RON$^{1335R/G}$ and TYK2$^{684I/S}$ were not cell line dependent or due to any artifact, the observations were reproduced in additional cell lines. Specific ligands - not pervanadate - were used to stimulate phosphorylation to reduce the risk of artifacts. In the case of RON this meant stimulation with macrophage-stimulating protein (MSP).

Interestingly, the allele-dependent difference in target phosphorylation also showed up in additional cell lines. RON^{1335R} was inhibited at sunitinib concentrations between 5-10 µM (figure 28, right side). In the presence of the gylcine allele at position 1335, RON was not inhibited at all (figure 28, left side). This difference was observed in all three tested cell line pairs of different tissue origin (brain, pancreas, and breast).

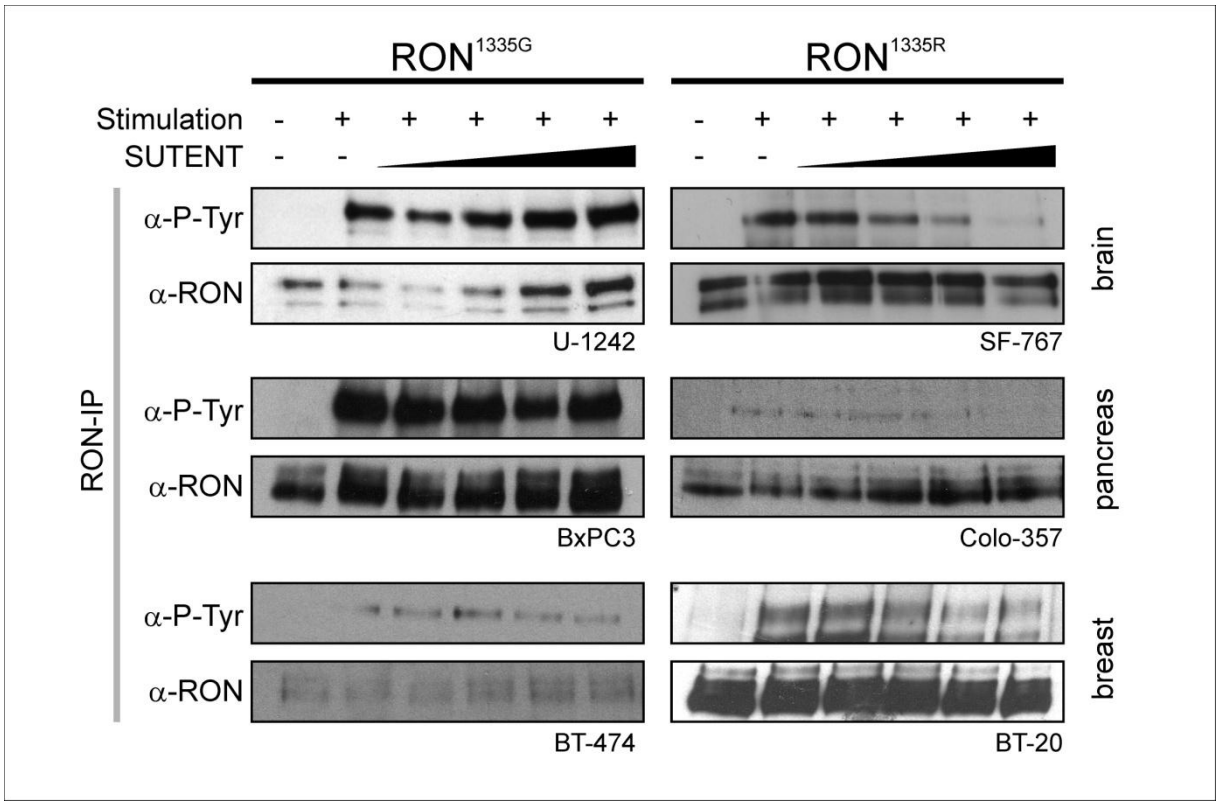

Figure 28: Sunitinib-induced inhibition of RON$^{1335R/G}$ phosphorylation dependent on target genotype. Cell lines harboring one of the two alleles homozygously were starved over night. After 2 h treatment with sunitinib, the cells were treated with MSP (10 ng/ml; 5 min)). After cell lyses a RON-IP was carried out followed by tyrosine phosphorylation detection using 4G10 anti-phospho-tyrosine antibody and RON-re-blot.

Phosphorylation of cytoplasmic TYK2 was stimulated with oncostatin-M (OSM) or interleukin 6 (IL-6) (depending on the expression of IL6R and OSMR). Additionally, a 4G10-IP was not used to detect phosphorylation levels. To allow target reblotting and thus the exclusion of loading errors and subsequently false-positive results, a phospho-TYK2 antibody was used to directly detect phosphorylation level without an IP being relevant (figure 29).
This revealed that TYK2 was only inhibited if the serine allele at position 684 was present; otherwise, not (figure 29/A). This allele-dependent difference of inhibition was found in all eight tested cell lines. Sunitinib was able to bind to TYK2 only in the presence of the serine allele (figure 29/B).

In brief, the two SNPs, $RON^{1335R/G}$ and $TYK2^{684I/S}$, showed an allele-dependent difference in the sunitinib-dependent inhibition of target phosphorylation. Thus, these two genetic alterations were investigated further.

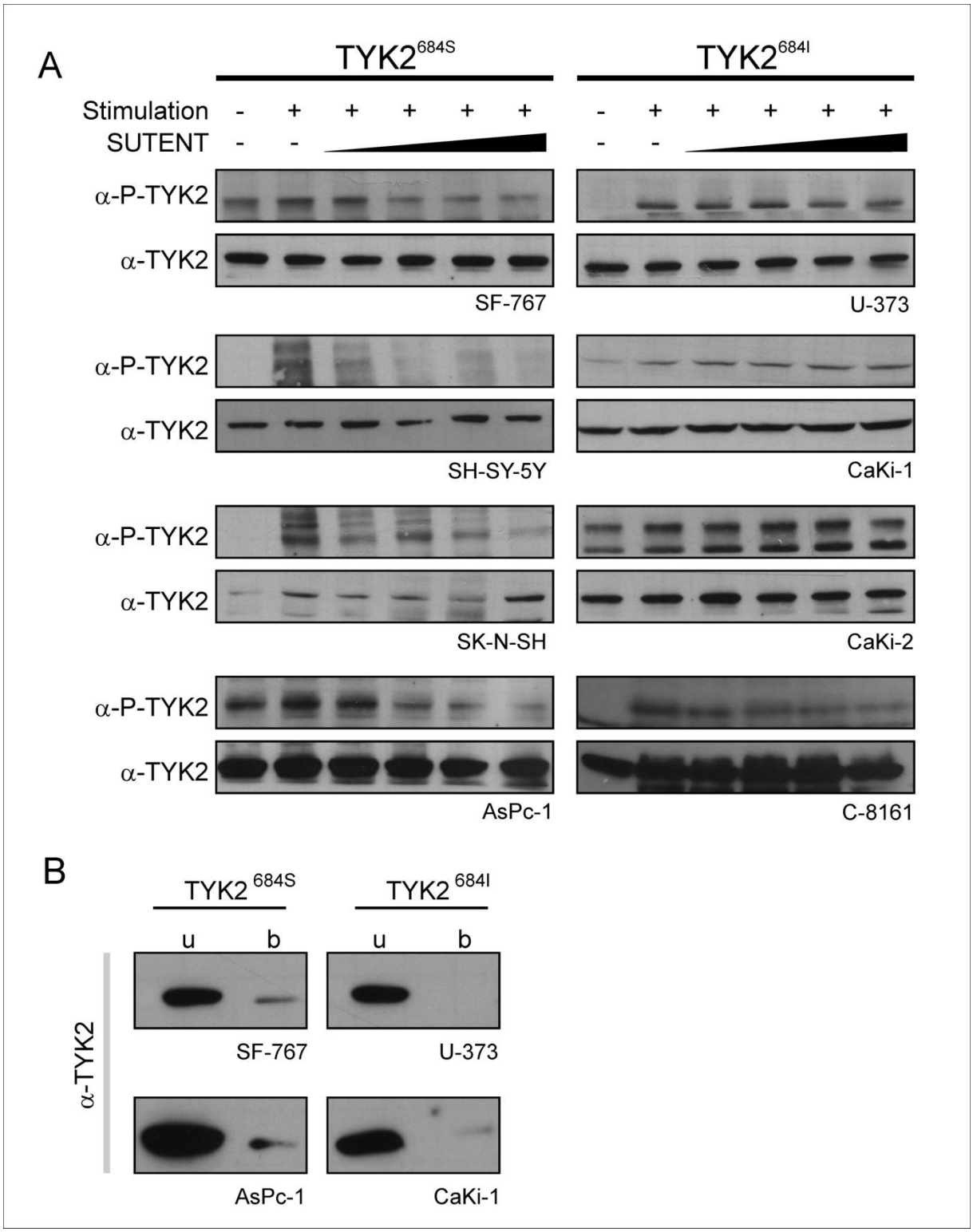

Figure 29: Sunitinib-induced inhibition of TYK2$^{684I/S}$ phosphorylation dependent on target genotype. (A) Cell lines harboring one of the two alleles homozygously were starved over night. After 2 h treatment with sunitinib cells were treated with IL-6 (5 ng/ml; 5 min) or Oncostatin-M (20 ng/ml; 5 min). Phosphorylation levels were analysed using a phospho-TYK2 antibody. (B) 684I/S-dependent binding of TYK2 to sunitinib-coupled beads. For binding, cells were lysed and incubated with SUTENT-coupled beads over night at 4°C. The next day bound proteins were eluted with free inhibitor. Unbound TYK2 (u) was compared with sunitinib-bound protein (b).

8.2.6 General role of TYK2 in cancer

The alleles of the two identified SNPs, $RON^{1335R/G}$ and $TYK2^{684I/S}$, impart different responses to sunitinib-dependent inhibition of target phosphorylation. The impact of RON on cancer was quite clear; its involvement in apoptosis, adhesion, migration, proliferation, invasion, and metastasis has been known for decades (Li *et al.*, 1995; Collesi *et al.*, 1996; Santoro *et al.*, 1996; Wang *et al.*, 1996; Danilkovitch and Leonard, 1999; Danilkovitch *et al.*, 1999). In contrast, the involvement of TYK2 in cancer was not as well understood. TYK2, as a member of the JAK-kinase family, plays a role in issues connected with the immune system such as hyper IgE syndrome, viral and microbial defense, mitochondrial respiration in B-lymphocytes, systemic lupus syndrome, or multiple sclerosis (Potla *et al.*, 2006; Li *et al.*, 2007; Su *et al.*, 2007; Woellner *et al.*, 2007; Nakamura *et al.*, 2008; Ban *et al.*, 2009; Hellquist *et al.*, 2009). Its sole involvement with cancer was limited to reports of influence on prostate carcinoma invasiveness (Ide *et al.*, 2008) and interferon α/β signaling (Barbieri *et al.*, 1994). However, the general role of TYK2 in cancer remained vague.

To estimate the impact of the allele-dependent difference in the inhibition of $TYK2^{684I/S}$ the general influence of TYK2 on cancer had to be explained. Therefore, 20 cancer cell lines of different tissue origins were screened for the influence of a TYK2 knockdown on proliferation, apoptosis, and invasion (figure 30). The knockdown of TYK2 had only a minimal effect on proliferation, i.e., a change of 0 – 0.28 fold (figure 30/A). In contrast, the knockdown of TYK2 caused an up to 2-fold induction of apoptosis (figure 30/B). In this context there was a strong tissue dependency. Cancer cell lines of brain responded most to the knockdown, whereas cell lines from the kidney, prostate, and pancreas tissue showed a moderate response. Some cell lines of these tissues like PPC1 (prostate), PANC-1 (pancreas), or CaKi-2 (kidney) responded very strongly to the TYK2 knockdown, whereas PC3 (prostate), MIAPaCa-2 (pancreas), or CaKi-1 were inert. Breast cancer cell lines hardly responded at all. In contrast, the effect of the TYK2 knockdown on invasion (figure 30/C) was very pronounced. Due to the time window of 48 h, in which the transient target knockdown was still efficient, only five of the tested cell lines showed an invasion phenotype. The invasion of all of these cell lines was surprisingly almost completely blocked by the knockdown of TYK2.

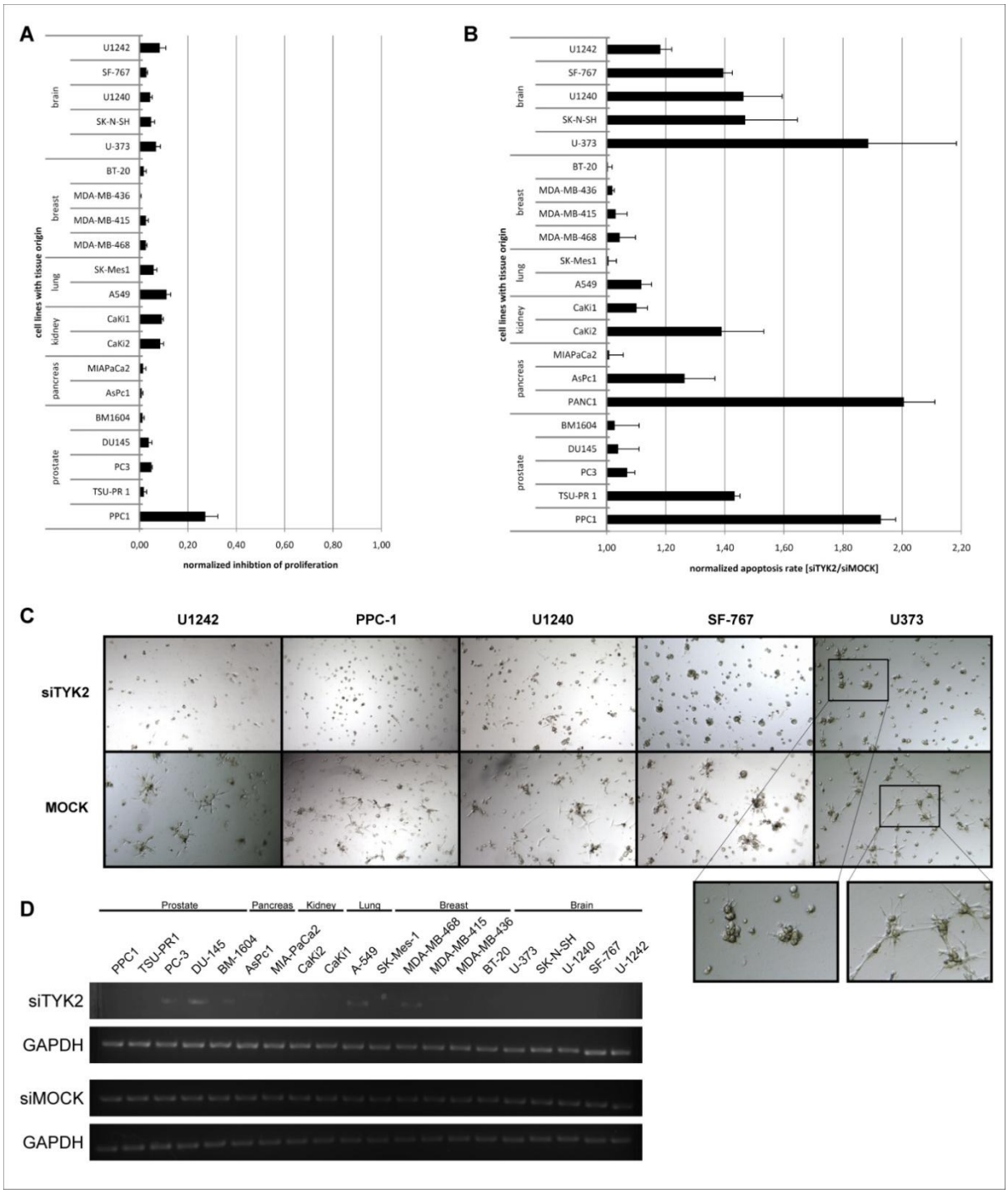

Figure 30: General role of TYK2 in cancer. The knockdown of TYK2 was performed by using siRNA. TYK2-deprived cells were incubated for 48 h and then the respective phenotype was analyzed. Effect of a TYK2 knockdown on (A) proliferation, (B) apoptosis, and (C) invasion (matri gel outgrowth assay). (D) Efficiency of the TYK2 knockdown in all tested cell lines.

This showed the important role of TYK2 in apoptosis and invasion which had not yet been reported. This link between TYK2 and cancer was decisive for continuing the study of the influence of the different alleles 684I/S in TYK2 on sunitinib.

8.2.7 Influence of RON$^{1335R/G}$ and TYK2$^{684I/S}$ at the cellular level

The response to sunitinib treatment does not consist of a single mechanism but involves an interplay of many different pathways. In general, the signals of kinases can be compensated or enhanced by other signals or feedback loops (Dreher and Hanley, 1988; Ullrich and Schlessinger, 1990; Malarkey *et al.*, 1995; Brook *et al.*, 2000; Chabannes *et al.*, 2001; Lin *et al.*, 2009). Consequently knowledge of allele-dependent differences of the specific target inhibition is insufficient to elucidate its role in sunitinib therapy. The influence of the respective genetic alteration must be analyzed at the level of biological response.

8.2.7.1 Influence of RON$^{1335R/G}$ at the cellular level

RON is involved in the induction of apoptosis, mediation of pro-survival signals, invasion, adhesion, migration, proliferation, and metastasis (Li *et al.*, 1995; Collesi *et al.*, 1996; Santoro *et al.*, 1996; Wang *et al.*, 1996; Danilkovitch and Leonard, 1999; Danilkovitch *et al.*, 1999). All these processes play key roles in cancer development and progression.

The questions posed were the following. 1) What does RON contribute to the biological response to sunitinib treatment? 2) Was the allele-dependent difference present not only at the molecular but also at the cellular level? For this purpose, cell lines were selected that harbor one of the two alleles (RON^{1335R}: U-373, brain; RON^{1335G}: U-1242, brain). Small-interfering RNA (siRNA) was used to deprive every cell line of RON. Afterwards the sunitinib-dependent induction of apoptosis (figure 31/A) and inhibition of proliferation (figure 31/B) were determined.

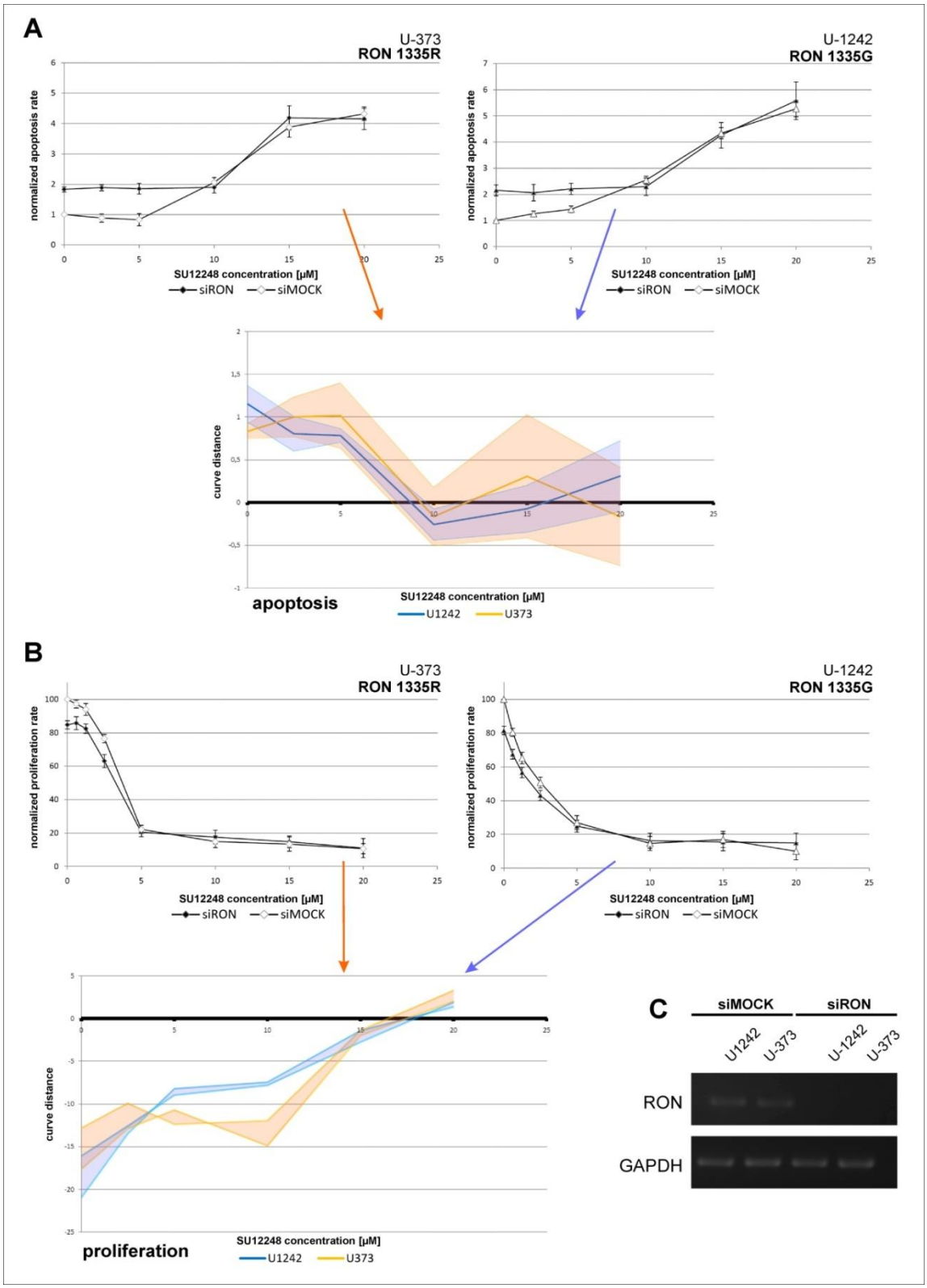

Figure 31: Influence of RON$^{1335R/G}$ on the response to sunitinib treatment. Cell lines harboring one allele homozygously were deprived of RON by using siRNA. Then sunitinib was administered. (A) Influence of RON$^{1335R/G}$ on the induction of apoptosis. Upper diagrams show the fold-change of the induction of apoptosis. The lower diagram displays the curve distances between the upper curves. Colors of the arrows indicate correspond to the respective curve distance graph. Orange and blue lines show the mean distance of the respective curves. Orange and blue areas indicate their S.E.M. (B) Influence of RON$^{1335R/G}$ on the inhibition of proliferation. Upper diagrams show the inhibition of proliferation. The lower diagram displays the curve distances between the upper curves with respect to their S.E.M. (C) Control of knockdown efficiency.

As described in previous publications (Schlabach *et al.*, 2008), the knockdown of RON also induced apoptosis as well as inhibited proliferation in this study (figure 31/A and 31/B, c(sunitinib) = 0 µM). Moreover, with increasing concentrations of sunitinib all curves approximated each other. At concentrations of about 6-8 µM the curves intersected and from then on were aligned. This phenomenon might be due to the fact that sunitinib inhibited RON at the concentration of the intersection point. Such an explanation would also fit the observations made at the molecular level. From this point on it was apparently unimportant for the cell if RON was not able to fulfill its role because it was inhibited or deprived. Thus, the intersection point also represented the concentration of the target inhibition. For RON no allele-dependent difference was detected for induction of apoptosis or for inhibition of proliferation. Thus, it can be assumed that the SNP 1335R/G in the tyrosine kinase domain of RON likely has no influence on the response to sunitinib treatment.

8.2.7.2 Influence of TYK2$^{684I/S}$ at the cellular level

The same experiment was done with TYK2 (figure 32). Since the knockdown of TYK2 had very little effect on proliferation but a more pronounced effect on apoptosis, only the effect on apoptosis was determined. Additionally, two cell lines were used for each genotype to exclude artifacts or cellular context-dependent effects. In contrast to RON, the approximation of the rates of induction of apoptosis with and without TYK2 knockdown depended on the respective allele. The two curves never intersected in the presence of the isoleucin allele, they showed a parallel curve progression (figure 32/A, lower diagrams). In the presence of the serine allele the curves intersected in both cell lines already at a sunitinib concentration of about 6-7 µM (figure 32/A, upper diagrams, red arrows). After the intersection, the curves aligned. From this concentration on it was apparently negligible, whether TYK2 was inactive due to the inhibition by sunitinib or due to the depletion of the whole protein. Consequently, the two curves aligned. This supported observations at the molecular level showing that the allele-dependent difference of TYK2 inhibition was also present at the cellular level. Thus, it can be assumed that the alleles of the SNP 684I/S in the pseudo-tyrosine kinase domain of TYK2 influence the response to sunitinib treatment.

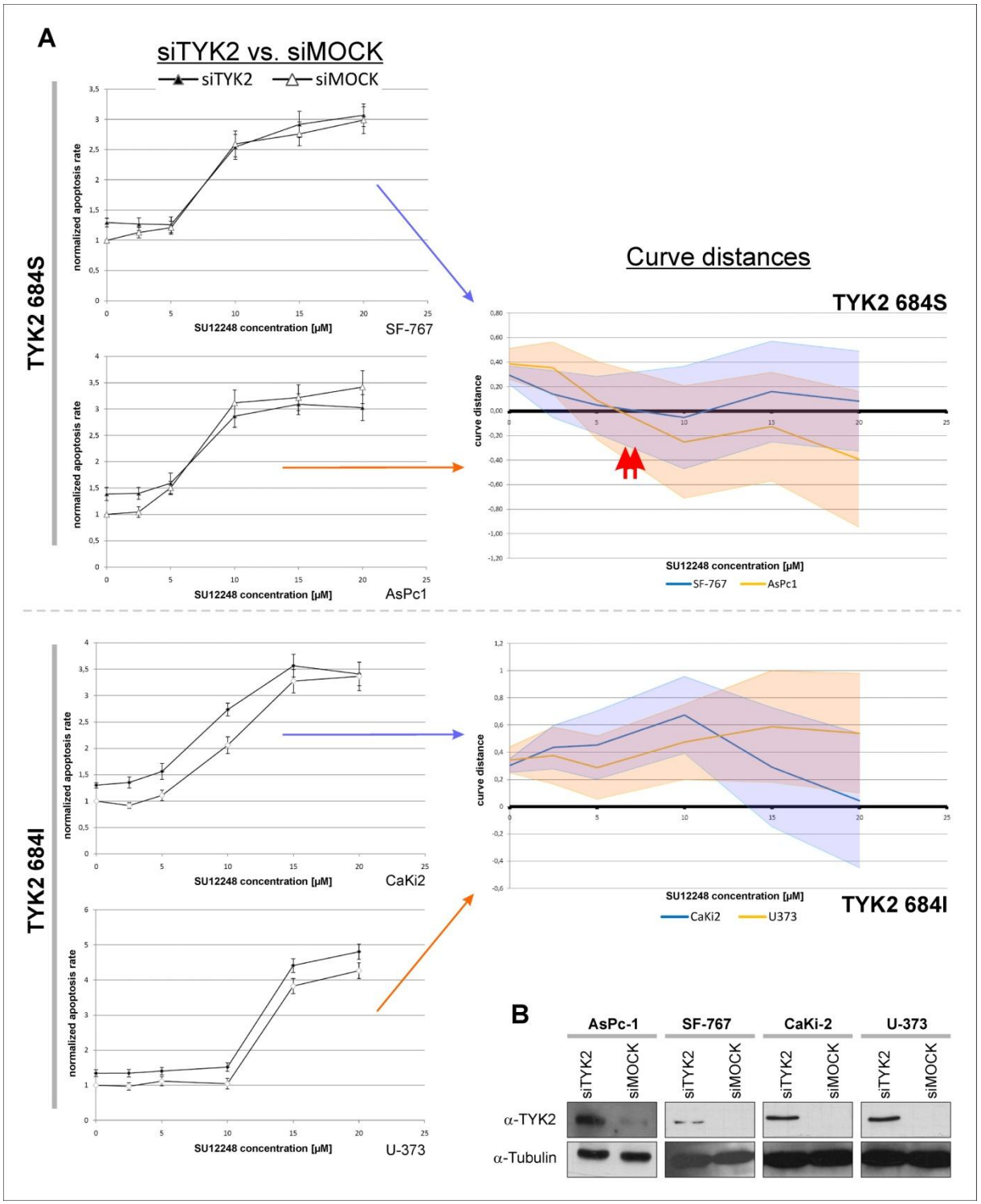

Figure 32: Influence of TYK2$^{684I/S}$ on the response to sunitinib treatment. Cell lines harboring one allele homozygously were deprived of RON by using siRNA followed by sunitinib treatment (A) Influence of TYK2$^{684I/S}$ on the induction of apoptosis. Left diagrams show the fold-change of the induction of apoptosis. Diagrams on the right side show the curve distances. The color of the arrows (blue and orange) indicates which digram became which curve distance graph. In the curve distance graphs orange and blue lines show the mean distance of the respective curves. Orange and blue areas indicate their S.E.M. Red arrows indicate the intersection point (curve distance = 0) (B) Control of knockdown efficiency.

8.2.8 Mode of TYK2 action

TYK2 is a member of the JAK-kinase family (Verma *et al.*, 2003). JAKs are essential for activating STATs (Yu and Jove, 2004). After STAT activation, two STAT molecules dimerize and translocate directly into the nucleus, where they induce transcription of different target genes depending on the activating ligand (Ihle and Kerr, 1995; Briscoe *et al.*, 1996; Ihle, 1996; Darnell, 1997; Ivashkiv and Hu, 2004). Accordingly, STATs fulfill different duties in the cell (figure 33). As a member of the JAK-kinase family, TYK2 is also able to activate several STATs and thus induce different cellular responses (figure 33, right side).

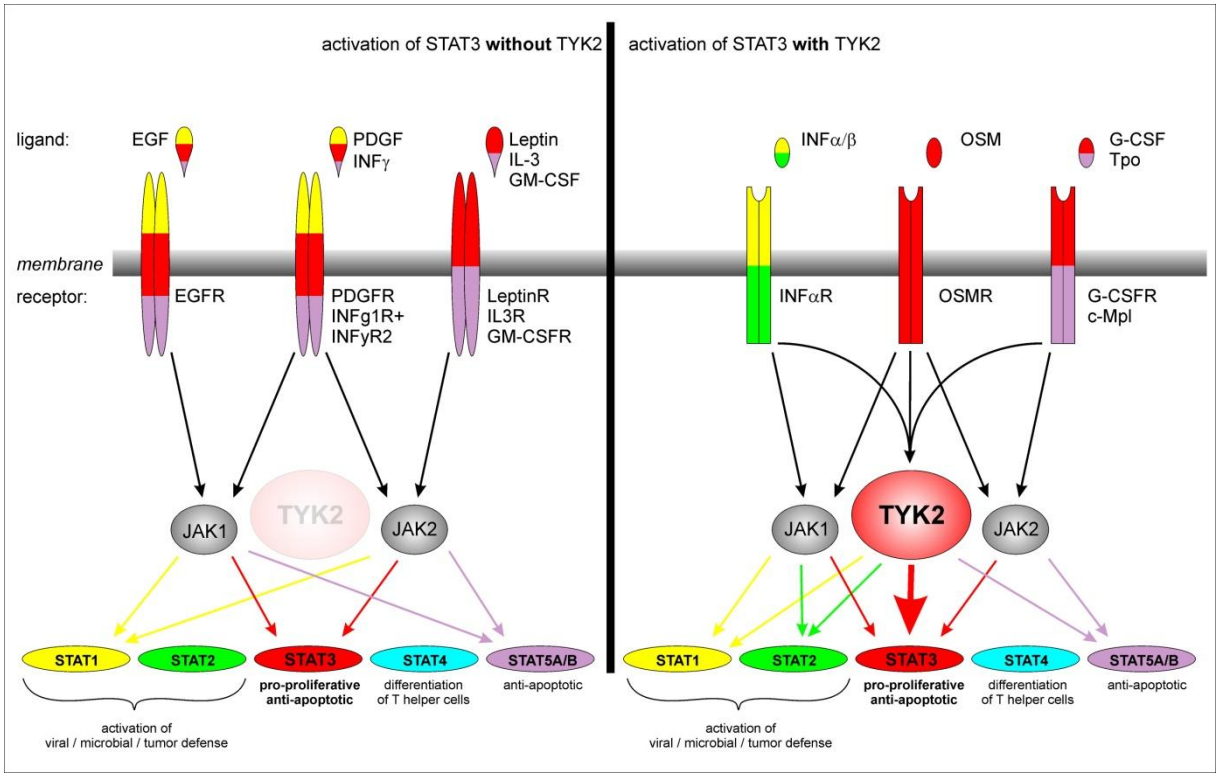

Figure 33: Overview of a selection of possible STAT activations by JAK1, JAK2 and TYK2. On the left are some examples of possibilities for the activation of different STATs without participation of TYK2. On the right are possibilities of STAT activations with participation of TYK2. The colors of ligands and receptors indicate which STATs are activated. (The scheme compiled by extracting and combining information from the following publications (Liao *et al.*; Yu *et al.*; Ihle and Kerr, 1995; Briscoe *et al.*, 1996; Ihle, 1996; Li *et al.*, 1996; Look *et al.*, 1998; Guren *et al.*, 1999; Zhou *et al.*, 2001; Leong *et al.*, 2002; Ruvolo *et al.*, 2003; Bates and Myers, 2004; Chiang *et al.*, 2004; Gavrilescu *et al.*, 2004; Li *et al.*, 2004; Clarkson *et al.*, 2006; Hirai *et al.*, 2006; Ye *et al.*, 2006; Zhou *et al.*, 2006; Basham *et al.*, 2008; Groner *et al.*, 2008; Simeone-Penney *et al.*, 2008; Cascio *et al.*, 2009; Kimura *et al.*, 2009; Liang *et al.*, 2009)

Interferon α/β, for example, activates STAT1 and STAT2 via JAK1 and/or TYK2, which leads to the activation of natural killer cells, the up-regulation of MCH (class I and II)-molecule expression, and subsequently to viral, microbial, and tumor defense (Goldstein and Laszlo, 1988; Verma *et al.*, 2003; Fensterl and Sen, 2009). In such a situation TYK2 serves as tumor suppressor. In contrast, stimulation with oncostatin-M leads via TYK2 and/or JAK1 and/or JAK2 to activation of STAT3, a very important supporter of tumor progression, proliferation, and anti-apoptosis. Under such circumstances TYK2 assumes the role of a tumor promoter, and the JAK/STAT pathway becomes central for cancer development. (Bowman *et al.*, 2000; Battle and Frank, 2002; Verma *et al.*, 2003; Groner *et al.*, 2008). This bidirectional function characterizes not only TYK2 but all JAK-kinase family members. Nevertheless, JAK1 and JAK2 became the focus of many anti-cancer drug investigations (Nefedova and Gabrilovich, 2007; Costantino and Barlocco, 2008; Kim *et al.*, 2008; Zhou *et al.*, 2010). Furthermore, some ligands induce the stimulation of STATs without the participation of TYK2 (figure 33, left side). EGF, for example, activates the EGF receptor, which cross-talks with JAK1, leading to the activation of STAT3 and supporting the pro-proliferative function of EGF.

To understand the underlying mechanism of how TYK2$^{684I/S}$ mediates its allele-dependent influence on the response to sunitinib treatment, the mode of TYK2 action was investigated further. Thus, cell lines harboring either the serine or the isoleucin allele at position 684 (for each genotype two cell lines were selected to exclude cell line-specific phenotypes) were stimulated with oncostatin-M, which primarily activates STAT3 via TYK2 but also via JAK1 and JAK2 (figure 33, right side).

Interestingly, in the presence of the serine allele but not the isoleucin allele at position 684 not only was TYK2 inhibited but also the STAT3 located downstream (figure 34/A). In contrast, the activity of JAK1 and JAK2 was not affected by sunitinib, which transferred the observed effect directly to TYK2. Actually STAT3 is not a sunitinib target and thus not inhibited (PhD thesis, Michaela Bairlein, 2010, TU Munich).

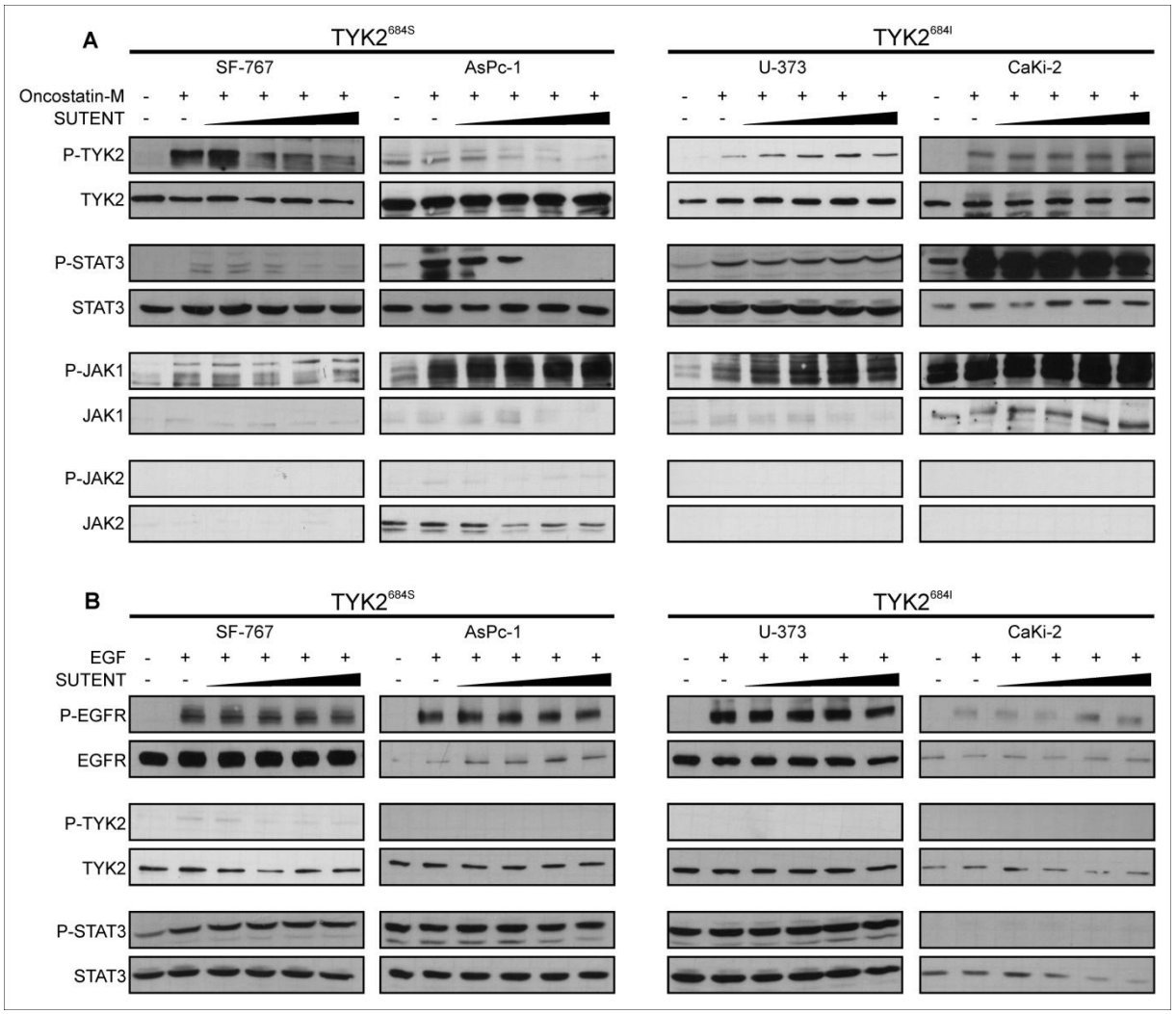

Figure 34: Allele-dependent inhibition of TYK2$^{684I/S}$ leads to subsequent lack of activation of STAT3. Cell lines harboring one of the two alleles homozygously were starved over night. After 1 h treatment with sunitinib cells were treated with the respective ligand to induce target phosphorylation as well as downstream STAT3 activation (A) Stimulation with Oncostatin-M (20 ng/ml; 5 min). (B) Control experiment: Stimulation with EGF (10 ng/ml; 5 min), which activates STAT3 via JAK1 and JAK2 without participation of TYK2. Phosphorylation levels were analyzed by Western blotting.

To exclude the possibility that STAT3 was directly inhibited in these two cell lines by chance, EGFR was stimulated as a control experiment. EGF is known to activate STAT3 only via crosstalk with JAK1 and without the participation of TYK2 (figure 33). Interestingly, under these conditions STAT3 was not inhibited by sunitinib (figure 34/B). Thus, the inhibition of STAT3 observed before was actually an absence of activation due to the direct inhibition of TYK2^{684S}. Thus, it can be assumed that TYK2 most likely mediates its influence on the response to sunitinib treatment by not activating STAT3.

9. Discussion

Paul Ehrlich's idea of "chemical targeting" became a reality with the development of Herceptin, a monoclonal antibody against HER2. (Hudziak *et al.*, 1989; Fendly *et al.*, 1990; Fendly *et al.*, 1990; Baselga *et al.*, 1998). This signaled the beginning of a new era of targeted cancer therapy. In the search for a drug with the potency to inhibit VEGFR and subsequently to prevent angiogenesis, sunitinib malate (SU12248) was developed. This multi-targeted small molecule inhibitor is active against more than 200 proteins. The initial doubts about the danger of inhibiting a multitude of kinases was realized to be an advantage: the inhibition of multiple substrates effectively deals with the variability of tumors and thus lowers the incidence of resistance (Fong *et al.*, 1999; Laird *et al.*, 2000; Mendel *et al.*, 2000; Abrams *et al.*, 2003; Abrams *et al.*, 2003; Mendel *et al.*, 2003; Murray *et al.*, 2003; Schlessinger, 2005; Goodman *et al.*, 2007; Huynh *et al.*, 2009). Genetic alterations can influence cancer therapy (Sasaki, 1982; Hynes, 1993; Fukasawa, 2005). Mutations caused the development of resistances even when only chemotherapeutic regimens were available (Gallie *et al.*, 1991; Wood *et al.*, 1992; Pasman and Schouten, 1993; Makris *et al.*, 1995; Buttitta *et al.*, 1997). Unfortunately, several sensitivity-increasing and -decreasing genetic alterations in the PDGF receptor and cKIT in patients with GIST also developed during treatment with sunitinib (Heinrich *et al.*, 2003; Heinrich *et al.*, 2008; Gajiwala *et al.*, 2009). In addition to GIST, sunitinib has been approved for the treatment of mRCC. Ongoing clinical trials are investigating its relevance for the treatment of other tumor types (O'Farrell *et al.*, 2003; Fiedler *et al.*, 2005; Motzer *et al.*, 2006; Polyzos, 2008; Huynh *et al.*, 2009; Zhang *et al.*, 2009; Zhu and Raymond, 2009). Thus, this was the motivation for examining the general influence of genetic alterations on patient response to sunitinib treatment.

To identify candidates with such potential efficacy, all genetic alterations in sunitinib targets listed in TyKiVa with a frequency ≥5% were selected (37 genetic alterations) and statistically correlated with the overrepresentation of one allele in sunitinib-sensitive or -insensitive cancer cell lines. This analysis yielded five candidates ($ABL1^{991L}$, RON^{523Q}, $TYK2^{362F}$, $TYK2^{684S}$, RON^{1335G}). To ensure that no important result was overlooked, a second approach was taken to identify candidates. This time all available genetic alterations of all available mutation databases of cancer cell lines were used and

correlated with statistical overrepresentation in sunitinib-sensitive or -insensitive cancer cell lines. The five most frequent genetic alterations in sensitive and insensitive cell lines were selected. Interestingly, this correlation covered all previously identified five genetic alterations and thus validated the first finding. To ensure that no important finding was missed, all 12 candidates (TYK2$^{684I/S}$, TYK2$^{362V/F}$, RON$^{523R/Q}$, ABL1$^{991S/L}$, FLT3$^{227M/T}$, TYK2$^{883D/N}$, TNK1$^{Del:\ D472\text{-}R473}$, RON$^{1335R/G}$, HER2$^{1170P/A}$, EGFR$^{521R/K}$, RON$^{Ins:R813RQ}$, PYK2$^{838K/T}$) underwent further investigation. The two non-targets of sunitinib, EGFR and HER2, also appeared among these candidates. It was even more surprising that sunitinib bound to the EGF receptor in the presence of the lysine allele at position 518. Unfortunately, EGFR was still not inhibited by sunitinib. Thus, the binding of sunitinib in the extracellular domain of EGFR might have been unspecific. Although a direct influence of the SNPs EGFR$^{518R/K}$ and HER2$^{1107P/A}$ on sunitinib was not detected, it is still conceivable that the SNPs indirectly modulate response to sunitinib treatment, for example, by altering protein-protein interaction profiles. This would consequently also lead to the appearance of positive hits in the correlation.

The two alleles of RON$^{1335R/G}$ and TYK2$^{684I/S}$ impaired different efficacy of sunitinib on the sunitinib-induced inhibition of target phosphorylation. Since this influence was reproduced in further cell lines for each allele, a cell line-dependent effect can be excluded. An altered target inhibition does not necessarily influence the response to drug treatment at the cellular or even patient level. This is due to the fact that with increasing complexity of the system, more and more influences, either enhancing or silencing, come to bear. Additionally, the influence that the respective target has on the general drug response affects the possible impact of an altered target inhibition at the molecular level. If, for example, a target was not involved in the sunitinib response at all, it would make no difference if the target was inhibited or not. Thus, the allele-dependent difference in sunitinib efficacy at the cellular level was determined. In the case of RON its involvement in cancer had been known for quite a long time (Li *et al.*, 1995; Collesi *et al.*, 1996; Santoro *et al.*, 1996; Wang *et al.*, 1996; Danilkovitch and Leonard, 1999; Danilkovitch *et al.*, 1999). Unfortunately, there was no allele-dependent (RON$^{1335R/G}$) difference in the response to sunitinib treatment in the tested cell lines as regards induction of apoptosis and inhibition of proliferation. Moreover, the attempt to determine an allele-dependent effect on migration failed, because the knockdown of RON was sufficient to almost block migration completely and it could not be

differentiated from additional sunitinib-induced inhibition. However, there is still the possibility that the different alleles mediate altered sunitinib response in cell lines from tissue other than brain tissue or even in patients. This should be addressed in further studies.

Whereas the involvement of RON in cancer was quite clear, TYK2 was more connected with immune response, systemic lupus syndrome, hyper IgE syndrome, mitochondrial respiration in B-lymphocytes, and multiple sclerosis (Seto *et al.*, 2003; Shaw *et al.*, 2003; Ho *et al.*, 2005; Potla *et al.*, 2006; Schischmanoff *et al.*, 2006; Watford and O'Shea, 2006; Li *et al.*, 2007; Su *et al.*, 2007; Woellner *et al.*, 2007; Nakamura *et al.*, 2008; Ban *et al.*, 2009; Hellquist *et al.*, 2009). However, TYK2 also mediates interferon α/β signaling. This would attribute a tumor suppressor function to TYK2 which would be counterproductive to its inhibition. Moreover, TYK2 seemed to also be involved in invasiveness of prostate carcinoma (Goldstein and Laszlo, 1988; Barbieri *et al.*, 1994; Li *et al.*, 1996; Zhou *et al.*, 2001; Ide *et al.*, 2008). Thus, the role of TYK2 in cancer had to be explored.

The JAK/STAT-pathway has been in the center of several anti-cancer drug investigations for a long time, whereas only JAK1 and JAK2 are known to be involved in cancer progression (Behera *et al.*; Yamauchi *et al.*, 2000; Verma *et al.*, 2003; Neilson *et al.*, 2007; Wagner and Rui, 2008; Xiong *et al.*, 2008). So it was very surprising that TYK2 also participates in apoptosis as well as invasiveness. These findings agree with the observations of Ide *et al.*, who repoted that TYK2 was involved in prostate invasiveness (Ide *et al.*, 2008). Furthermore, these results received further support just a few weeks ago, when Maschler *et al.* found that Annexin A1 attenuates endothelial-mesodermal transition (EMT), which is critical for invasiveness and metastasis. Annexin A1 knockdown upregulates TYK2/STAT3 and ERK1/2 signaling. Thus, the authors claim that only TYK2/STAT3 and ERK1/2 are relevant for EMT and consequently invasiveness and metastasis (Maschler *et al.*, 2010). Moreover, a role in interferon-induced apoptosis in brain cancer cell lines was attributed to TYK2 (Dedoni *et al.*, 2010). Further investigations were thus carried out on TYK2 to determine the influence of the SNP 684I/S on the response to sunitinib treatment.

In contrast to $RON^{1335R/G}$, which showed no allele-dependent effect on sunitinib response at the cellular level, $TYK2^{684I/S}$ contributed to the induction of apoptosis after

sunitinib treatment, but only if the serine allele was present. This effect was present in both cancer cell lines (SF-767, brain and AsPc-1, pancreas) that harbored the serine allele. Further experiments revealed that the allele-dependent inhibition of TYK2 via sunitinib leads to no downstream activation of STAT3, which apparently results in the induction of apoptosis as well as the inhibition of invasion. It has been reported that TYK2 influences invasiveness of prostate cancer (Ide *et al.*, 2008) and breast cancer (Maschler *et al.*, 2010). The current study extended our knowledge on cancer types of other tissue origins. Interestingly, all of the analyzed brain cancer cell lines responded to a TYK2 knockdown, indicating first that STAT3 is predominantly activated by TYK2 in this tissue and second that brain tissue is STAT3 dependent. However, this finding concurred with results showing that only TYK2 but not JAK1 or JAK2 overexpression leads to a constitutive activation of STAT3 (Knoops *et al.*, 2008). Other studies have demonstrated that STAT3 is the hub in glioblastoma and its phosphorylation status correlates with the prognostic outcome (Rahaman *et al.*, 2002; Mizoguchi *et al.*, 2006; Brantley *et al.*, 2008; de la Iglesia *et al.*, 2009; Sherry *et al.*, 2009). Furthermore, it is known that sunitinib works via the inhibition of STAT3 in some brain tumors (Yang *et al.*, 2010). Since the stimulation was done with OSM, and it is known that neither JAK1 and JAK2 nor STAT3 itself are inhibited by sunitinib, it is quite likely that the STAT3 inhibition was indirect via an inhibition of TYK2. Moreover, the sole ability of TYK2, in contrast to JAK1 and JAK2, to activate NFκB might contribute to this effect (Yang *et al.*, 2010). Furthermore, it was also shown that TYK2 is significantly overexpressed in prostate carcinoma (fold change: 2.320; p=9.15E-5; figure 35/A) as well as in glioblastoma (fold change: 2.626; p=7.36E-6; figure 35/B)(Magee *et al.*, 2001; Bredel *et al.*, 2005). This supports our findings showing the importance of TYK2 for both cancer types and thus the importance of the SNP TYK2$^{684I/S}$ for treatment with sunitinib.

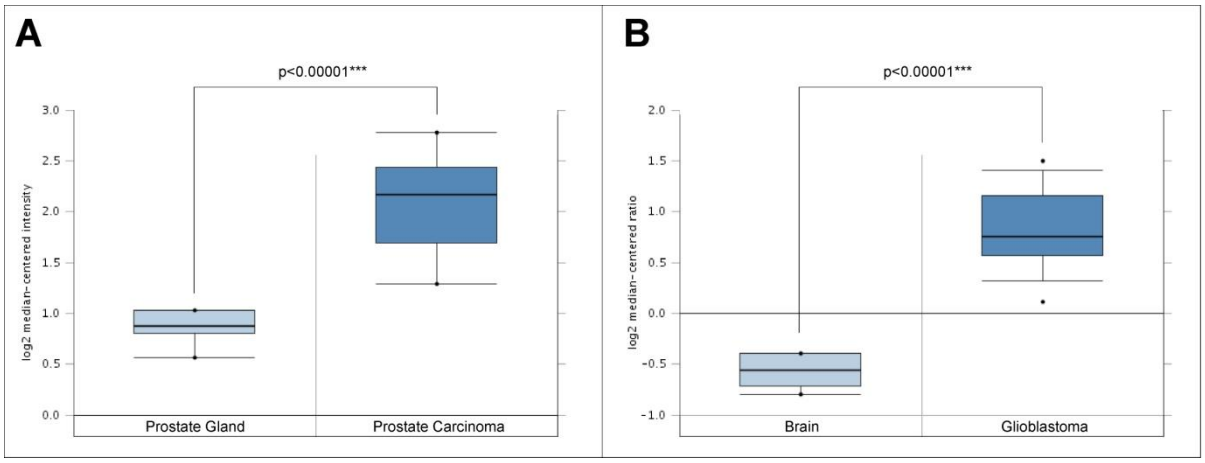

Figure 35: Overexpression of TYK2 in (A) glioblastoma and (B) prostate carcinoma. (Image after (Magee *et al.*, 2001; Bredel *et al.*, 2005))

The improvement of sunitinib-based therapy necessitates knowledge of its underlying mode of action and parameters that influence the response. This study has provided evidence that TYK2$^{684I/S}$ is an additional piece of the puzzle, which once complete will lead to personalized therapy with sunitinib. Moreover, this is the first reported proof that an amino acid exchange switched a sunitinib non-target into a target, which subsequently influenced the cellular response to treatment. Since the serine allele of TYK2$^{684I/S}$ increases sensitivity and occurs in 18% of Caucasians (according to the NCBI SNP database), it should be considered a marker for improving sunitinib therapy. The next step could be a retrospective correlation of the presence of TYK2$^{684I/S}$ in tumor samples with the patient response to sunitinib treatment in terms of overall survival, partial response, or stable disease.

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