ANALYSES OF AKT AND PDGF RECEPTOR EXPRESSION AND ACTIVATION IN HUMAN TUMORS

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Stockholm 2009
ABSTRACT

These studies aimed at developing methodology for detection of proteins involved in cancer and the evaluation of their prognostic significance in human tumors. Signal transduction profiling of tumors are becoming increasingly interesting based on the accumulating evidence of its prognostic and drug response-predictive value. Realization of the full potential of this information requires novel assay formats. We have explored the possibility to adapt the microfluidic Gyros platform for determination of Akt phosphorylation and the in situ PLA to investigate phosphorylation of PDGFR. The Gyros analyses clearly demonstrated that the drug-induced reduction in phosphorylation of Akt could be detected in the Gyrolab bioaffy device. A correlation between high pAkt/Akt expression and high Gleason score was seen when prostate tumors were analyzed. These preliminary findings encourage to further evaluation of the Gyrolab bioaffy device as a novel platform for analyses of tumor signal transduction.

By in situ PLA we detected phosphorylated PDGFRβ in cells stably overexpressing the human PDGFRβ but not in cells transfected with the PDGF α-receptor and in immortalized human fibroblasts endogenously expressing the PDGFRβ. We also demonstrated detection of tyrosine phosphorylated PDGFRβ in tissue sections from fresh-frozen human scar tissue undergoing wound healing.

PDGFR signaling has been shown in brain malignancies like glioma and medulloblastoma. We determined PDGF receptor expression in human choroid plexus tumors by immunohistochemistry and found that the majority expressed PDGFR. PDGFRβ was more frequently expressed. Gene amplification was also more frequent for the PDGFRB. Next we also looked at grade I choroid plexus papillomas and grade II atypical choroid plexus papillomas and the activation status of both PDGF α and β receptors by in situ PLA in formalin fixed paraffin embedded tumor tissue. Both the α- and β-receptor was less expressed in the carcinomas compared to the two papillomas. In contrast the activated β-receptor was found more frequently in the carcinomas whereas the activated α-receptor did not differ between the tumor types.

In glioblastoma samples from patients of a randomized trial that compared hydroxyurea monotherapy with combination of hydroxyurea and imatinib, PDGFRα was expressed in 32% of the tumors. It was associated with male sex, young age at presentation, and loss of PTEN expression. PDGFRα expression status was independently associated with short survival in the entire series and associated with poor survival in the subset of patients treated with hydroxyurea monotherapy but not among those treated with the combination.

PDGF receptor expression is common in the tumor stroma of common solid tumors. We found that PDGF α- and β-receptors were independently expressed, at variable frequencies, in the tumor stroma of all tested tumor types. In breast cancer, high stromal PDGFRβ expression was significantly associated with high histopathological grade, ER negativity and high HER2 expression. High stromal PDGFRβ expression was correlated with significantly shorter recurrence-free and breast cancer specific survival. The prognostic significance of stromal PDGF β-receptor expression was particularly prominent in tumors from pre-menopausal women. These findings highlight the prognostic significance of stromal markers, and should be considered in ongoing clinical development of PDGF receptor inhibitors.

In summary, we have developed generic methods for analyses of tumor relevant proteins; Gyrolab bioaffy and in situ PLA. Furthermore, analyses of human tumors have revealed clinically relevant previously unrecognized associations between PDGFR and survival in GBM and breast cancer.
LIST OF PUBLICATIONS

I. **Janna Paulsson**, Josefin Bolík, Lars Egevad, Fredrik Petersson, Mats Inganäs and Arne Östman
   Gyrolab Bioaffy, a novel method for signal transduction profiling of tumor tissue
   *Manuscript*

   *In Situ* Detection of Phosphorylated Platelet-derived Growth Factor Receptor β Using a Generalized Proximity Ligation Method
   *Mol Cell Proteomics*. 2007, 6(9):1500-9

III. Nina N Nupponen*, **Janna Paulsson***, Astrid Jeibmann, Brigitte Wrede, Minna Tanner, Johannes EA Wolff, Werner Paulus, Arne Östman and Martin Hasselblatt
   Platelet-derived growth factor receptor expression and amplification in choroid plexus carcinomas

   Functional role of platelet derived growth factor receptor signaling in choroid plexus tumors
   *Under 2nd revision in Am J Pathol*

V. **Janna Paulsson***, Martin Hasselblatt*, Maja Bradic, Marjut Puputti, Barbara Reismeier, Monica Nistér, Nina Nupponen, Werner Paulus, Gregor Dressemann, Heikki Joensuu and Arne Östman
   Predictive role of PDGF receptors in patients with recurrent glioblastoma treated with hydroxyurea monotherapy or with hydroxyurea and imatinib
   *Manuscript*

   Prognostic significance of stromal PDGF β-receptor expression in human breast cancer
   *In press in Am J Pathol, 2009*

* # Authors contributed equally
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<tr>
<td>aCPP</td>
<td>Atypical choroid plexus papilloma</td>
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<td>AML</td>
<td>Acute myelogenous leukemia</td>
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<tr>
<td>Ang</td>
<td>Angiopoietin</td>
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<td>ALL</td>
<td>Acute lymphoblastic leukemia</td>
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<td>AP</td>
<td>Accelerated phase</td>
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<td>ASM</td>
<td>Aggressive systemic mastocytosis</td>
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<td>ATP</td>
<td>Adenosine triphosphate</td>
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<td>CAF</td>
<td>Cancer associated fibroblast</td>
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<td>CISH</td>
<td>Chromogenic <em>in situ</em> hybridization</td>
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<td>CML</td>
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<td>Chronic phase</td>
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<td>CPC</td>
<td>Choroid plexus carcinoma</td>
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<td>CPP</td>
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<td>DFSP</td>
<td>Dermatofibrosarcoma protuberans</td>
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<td>ECM</td>
<td>Extra cellular matrix</td>
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<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<td>Eph</td>
<td>Erythropoietin-producing human hepatocellular carcinoma</td>
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<tr>
<td>Ephrin</td>
<td>Eph family receptor interacting protein</td>
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<td>ERK</td>
<td>Extracellular regulated kinase</td>
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<td>EWS</td>
<td>Ewing sarcoma oncogene</td>
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<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
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<td>FISH</td>
<td>Fluorescence <em>in situ</em> hybridization</td>
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<td>FLT</td>
<td>Fetal liver tyrosine kinase</td>
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<td>GBM</td>
<td>Glioblastoma multiforme</td>
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<td>GDP</td>
<td>Guanosine diphosphate</td>
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<td>GIST</td>
<td>Gastrointestinal stromal tumor</td>
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<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
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<td>GTP</td>
<td>Guanosine triphosphate</td>
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<td>HES/CEL</td>
<td>Hypereosinophilic syndrome/Chronic eosinophilic leukemia</td>
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<td>HGF</td>
<td>Hepatocyte growth factor</td>
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<td>IGF</td>
<td>Insulin like growth factor</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>IFP</td>
<td>Interstitial fluid pressure</td>
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<td>LBC</td>
<td>Lymphoid blast crisis</td>
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<td>Mitogen activated protein kinase</td>
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<td>MBC</td>
<td>Myeloblast crisis</td>
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<tr>
<td>MDS/MPD</td>
<td>Myelodysplastic/myeloproliferative diseases</td>
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<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
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<td>NSCLC</td>
<td>Non small cell lung cancer</td>
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<td>OS</td>
<td>Overall survival</td>
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<td>PAK</td>
<td>p21-activated kinase</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
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<td>PDGFR</td>
<td>Platelet derived growth factor receptor</td>
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<td>PDK</td>
<td>Phosphoinositide dependent kinase</td>
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<td>PFS</td>
<td>Progression free survival</td>
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<td>Ph</td>
<td>Philadelphia chromosome</td>
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<td>PH</td>
<td>Pleckstrin homology</td>
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<td>PI3K</td>
<td>Phosphoinositide-3-kinase</td>
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<td>PLA</td>
<td>Proximity ligation assay</td>
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<td>PtdIns</td>
<td>Phosphatidylinositols</td>
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<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
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<td>RTK</td>
<td>Receptor tyrosine kinase</td>
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<td>SCCHN</td>
<td>Squamos cell carcinoma of the head and neck</td>
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<td>SH2</td>
<td>Src homology 2</td>
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<td>SOS</td>
<td>Son of sevenless</td>
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<td>S6K1</td>
<td>Ribosomal S6 kinase</td>
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<td>TGF</td>
<td>Transforming growth factor</td>
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<td>TIE</td>
<td>Tyrosine kinase with Ig and EGF homology domains</td>
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<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
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<td>WHO</td>
<td>World health organization</td>
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1 INTRODUCTION
Cancer is not one disease but rather a general name for many different diseases all due to genetic aberrations in numerous genes leading to uncontrolled cell growth. Tumorigenesis (tumor formation) is a slow process arising from changes in one cell that evolve in multiple stages to premalignant lesions and eventually a malignant tumor (Vogelstein and Kinzler, 1993). It has lately become evident in different tumor types that the progenitor cell has features of stem cells and might therefore be viewed as a cancer stem cell (Reya et al., 2001). A normal cell is regulated by many different mechanisms. Cancer cells need to acquire different properties to be able to overcome these regulatory systems. This is done by obtaining a set of common properties which have been divided into six groups; self-sufficiency in growth signals, insensitivity to anti-growth signals, limitless replicative potential, evading apoptosis, sustained angiogenesis and tissue invasion and metastasis (Hanahan and Weinberg, 2000).

Two different types of genes are involved in tumor initiation, either tumor suppressor genes are lost or oncogenes are activated. The number of genetic alterations a cell needs to become transformed was originally thought to be between three and six (Vogelstein and Kinzler, 1993). However when tumors have been screened for mutations during the last couple of years the number seems to be much higher than first appreciated (Sjoblom et al., 2006).

During the last couple of years epigenetic changes has also been widely accepted to be involved in tumorigenesis.

A tumor does not only consist of the malignant cells but also different cell types building up the tumor stroma, these includes cells that build up the tumor vasculature (endothelial and pericytes), immune cells (macrophages and NK cells) and tumor fibroblasts.

Malignancies can be divided according to origin of cell type in the main types; carcinoma, sarcoma, leukemia, and lymphoma. A tumor can be either benign or malignant. Benign tumors are non-aggressive and do not invade the nearby tissue but rather localizes to one site. Malignant tumors on the other hand are tumors that readily invade to nearby tissues and commonly metastasize to distant organs.

Studies of epidemiology have found that certain cancers can be linked to genetic predisposition and risk factors such as tobacco, alcohol, food stuff, obesity, chemical or physical carcinogens and virus infections.

The incidence of cancer in Sweden is about 50000 (in 2007) and it is worldwide one of the leading causes of death according to the World Health Organization (WHO). It is the most common cause of death among people between 15 and 75 in Sweden and the second after cardiovascular disease among the total population (Cancerfonden, 2009).

The most common cancer forms in Sweden are breast and prostate cancer with 7088 and 8870 cases, respectively, diagnosed yearly (2007) (Cancerfonden, 2009). The relative survival for patients with breast and colorectal cancer has increased during the last 30 years due to screening and improved treatment. Cervical and stomach cancers have been the best success stories in the cancer treatment field with a decreased incidence due to early detection and changed diet (Cancerfonden, 2009).
1.1 TYROSINE KINASES

Phosphorylation is an important event in regulating protein function. The enzymes responsible for tyrosine phosphorylation and dephosphorylation are the tyrosine kinases and protein tyrosine phosphatases, respectively. Tyrosine phosphorylation is often involved in mitogenic cell signaling. There are 90 tyrosine kinases in the human genome divided into two groups, the cytosolic and the receptor tyrosine kinases (RTKs) (Manning et al., 2002). The RTKs are composed of an extracellular ligand binding domain, a transmembrane domain and an intracellular kinase domain (see Figure 1). RTKs are, in most cases (e.g. PDGFR, VEGFR and FGFR), activated by homo-dimeric ligand binding which leads to receptor dimerization and autophosphorylation of specific tyrosine residues in the kinase domain (Schlessinger, 2000). The epidermal growth factor receptor (EGFR) family on the other hand form hetero-oligomers following ligand binding (King et al., 1988). Activation of the kinase domain leads to up-regulation of the intrinsic tyrosine activity, which in turn recruits intracellular proteins with a SH2 (Src homology 2) domain leading to cellular responses such as migration, proliferation and cell survival. In turn the cytosolic tyrosine kinases commonly act as members of intracellular signaling activated by RTKs or other cell surface proteins (see Figure 2 and 3).

![Figure 1](image1.png)

Figure 1. Structures of the RTK family. Figure adapted from (Blume-Jensen and Hunter, 2001).

1.1.1 Signaling via tyrosine kinase receptors

Ligand binding to RTKs leads to activation of the kinase domain by phosphorylation and recruitment of proteins with a SH2 domain and further downstream signaling via for example the phosphoinositide-3-kinase (PI3K)-Akt or Ras-Mitogen activated protein (MAP) kinase pathways.

PI3K-Akt signaling

PI3K is composed of one regulatory and one catalytic subunit, p85 and p110 respectively (reviewed in (Luo et al., 2003)). PI3K binds to the phospho-tyrosine residues in the intracellular domain of tyrosine kinase receptors via its SH2 domains in the p85 subunit. It can also be activated via adaptor proteins bound via their SH2 domains to phosphorylated RTKs or by direct
activation of the p110 subunit by Ras. Activated PI3K in turn phosphorylates the membrane bound PtdIns(4)P and PtdIns(4,5)P$_2$ to PtdIns(3,4)P$_2$ and PtdIns(3,4,5)P$_3$. PtdIns(3,4,5)P$_3$ functions as membrane docking for proteins with a pleckstrin homology (PH) domain like phosphoinositide dependent kinase (PDK) 1 and Akt (also called protein kinase B, PKB). Akt is a serine/threonine kinase and there are three forms of Akt 1, 2 and 3, Akt1 being the dominant form. PDK1 phosphorylates Akt on Thr308 leading to trans-autophosphorylation of the Ser473 which gives fully active Akt. In turn, Akt then phosphorylates many different proteins leading to cellular processes like proliferation and survival. An example is the inhibitory phosphorylation by Akt of the pro-apoptotic protein BAD. BAD can then no longer form a complex with the other pro-apoptotic proteins Bcl-2 and Bcl-xl leading to inhibition of apoptosis. Another Akt substrate is GSK3, a kinase which regulates break down of Myc and cyclin D1. These proteins are involved in the cell cycle switch from G1 to S-phase. When GSK3 is phosphorylated by Akt it is inactive and can no longer degrade cyclin D1 and Myc resulting in cell cycle progression. Akt can also regulate cell growth by inactivating the protein tuberin which is an inhibitor of the G protein Rheb (a Ras-like protein). When tuberin is inactive it can not inhibit Rheb which in turn then can activate mTOR (mammalian target of rapamycin). mTOR phosphorylates and activates the ribosomal S6 kinase (S6K1) which activates ribosomal S6 leading to protein synthesis and cell growth (reviewed in (Luo et al., 2003)). (see Figure 2)

The phosphatase PTEN is closely regulating the PI3K signaling by dephosphorylating PtdIns(3,4,5)P$_3$ back to PtdIns(4,5)P$_2$.

![Figure 2. PI3K-Akt signaling. Figure adapted from (Blume-Jensen and Hunter, 2001).](image)

**Ras- MAP kinase signaling**

The membrane bound G-protein Ras gets activated by SOS (son of sevenless) which exchanges GDP to GTP (reviewed in (Hilger et al., 2002)). Ras activation is dependent on SOS binding via Grb2 (containing a SH2-domain) to activated tyrosine kinase receptors to get it to the cell membrane where Ras is bound. Ras in turn then binds to Raf thereby recruiting Raf to the membrane where it gets activated by different kinases, for example Src and PAK. Activated Raf can then phosphorylate MEK1/2 (also called MAPK kinases) which in turn phosphorylates extracellular signal regulated kinases, ERK1/2 (also called MAPKs). ERK1/2 translocates to the
nucleus where it phosphorylates a number of proteins leading to cellular responses like proliferation and differentiation (reviewed in (Hilger et al., 2002)). (see Figure 3)

Figure 3. Ras-MAP kinase signaling. Figure adapted from (Hilger et al., 2002).

Mutational activation of PI3K-Akt and Ras-MAP kinase signaling in cancer
Many tumors have lost the tumor suppressor PTEN and thereby also the regulation or have activating mutations in the oncogene PI3K leading to uncontrolled signaling. Glioblastomas frequently have PTEN mutations, but also prostate, breast and renal cancers harbor PTEN mutations (Li et al., 1997; Wang et al., 1998; Wang et al., 1997). PI3K mutations are found in ovarian, breast and colorectal cancers (Ikenoue et al., 2005; Levine et al., 2005).

The oncogenes Ras and Raf are also often mutated in tumors, leading to constitutive signaling. Examples of tumors with Ras mutations are pancreatic, colorectal, lung and ovarian (reviewed in (Hilger et al., 2002)). Raf on the other hand is frequently mutated in melanomas and lung cancer (Brose et al., 2002; Madhunapantula and Robertson, 2008).

1.1.2 Tyrosine kinases and cancer

Tyrosine kinase mutations driving the growth of tumor cells
Tyrosine kinases can drive tumor growth through different genetic alterations like chromosomal translocations, gene amplifications, point mutations or interstitial deletions (Blume-Jensen and Hunter, 2001).

In chronic myeloid leukemia (CML) and a fraction of acute lymphoblastic leukemias (ALL) the tumor growth is dependent on a chromosomal translocation between chromosome 9 and 22, giving rise to the “Philadelphia chromosome” (Ph) (Rowley, 1973). This translocation leads to the fusion between Bcr and the tyrosine kinase Abi (Bcr-Abi) displaying a constitutive activation of Abi. Also in chronic myelomonocytic leukemia the tumor cells are driven by a translocation, in this case chromosome 5 and 12 giving rise to a constitutively active platelet derived growth factor receptor β (Tel-PDGFRβ fusion protein) (Golub et al., 1994). In other forms of myeloproliferative neoplasms with eosinophilia the fibroblast growth factor receptor 1 (FGFR1) is constitutively active due to chromosomal translocations forming fusion genes with FGFR1 and for example BCR (Cross and Reiter, 2008). Chromosomal rearrangements between the RTK RET and intracellular activating genes leading to fusion proteins that dimerizes independently of ligand binding have been found in many thyroid carcinomas (Kodama et al., 2005).
Amplification of tyrosine kinases is another type of genetic alteration leading to overproduction of a structurally normal protein in tumors. In many breast, ovarian, gastric, colon and non-small cell lung cancers (NSCLC) the HER2 is amplified (Blume-Jensen and Hunter, 2001). Over-expression of HER2 (ErbB2) is found in about 25% of all breast cancers and is often expressed in the more aggressive tumors (Piccart-Gebhart et al., 2005). Amplification of the EGFR is common in many solid tumor types leading to over-expression of EGFR in tumors like head and neck, breast, colon, gastric, glioblastoma (GBM), lung, liver and prostate (Salomon et al., 1995). In tumors from 12 of 25 (48%) GBM patients EGFR amplification was found (Mellinghoff et al., 2005). Another study of primary and secondary glioblastomas found that EGFR amplifications were only seen in the primary glioblastomas, but then to a degree of 70% (Joensuu et al., 2005).

Over-expression of the MET receptor is found in many solid tumors such as gastric and lung carcinomas and is usually correlated with bad prognosis. Mutations have been found in metastases from squamos-cell carcinoma of the head and neck (SCCHN) and in aggressive forms of hepatocellular carcinomas (Gentile et al., 2008).

Examples of tumors harboring activating point mutations in tyrosine kinases are gastrointestinal stromal tumors (GIST), NSCLC and GBM. GISTs are the most common mesenchymal tumors of the gastro-intestinal tract expressing constitutively active mutations in either c-Kit (85%) or PDGF receptor α (Heinrich et al., 2003a; Heinrich et al., 2003b). In many cases of NSCLC the EGFR has been found to be mutated (Kobayashi et al., 2005; Kosaka et al., 2004; Paez et al., 2004).

Another tyrosine kinase receptor, FLT3 (fetal liver tyrosine kinase) is frequently expressed in acute myelogenous leukemia (AML) and is mutated in many cases by internal tandem duplications resulting in a constitutively active kinase (Small, 2008).

**Tyrosine kinases involved in recruitment of tumor stroma**

Solid tumors consist not only of mutationally transformed malignant cells but also of normal supporting cells like fibroblasts and immune cells that build up the tumor stroma and contribute to the tumor angiogenesis. Tyrosine kinases are involved in stimulation of these different cell types. In experimental tumors where PDGF production has been genetically manipulated the importance of PDGF signaling in the recruitment of tumor stroma have been shown (Anderberg et al., 2009; Forsberg et al., 1993; Skobe and Fusenig, 1998).

**Tyrosine kinases involved in angiogenesis**

Angiogenesis is the formation of new blood vessels by sprouting of existing vessels and is a crucial event in solid tumors growing beyond 2mm³. During angiogenesis the endothelial cells needs to get stimulated to proliferate and migrate. The vascular endothelial growth factor (VEGF) family is involved in both normal and tumor angiogenesis. VEGF receptor 2 (VEGFR2) expressed on normal and tumor endothelial cells is responsible for tumor blood vessel production and VEGFR3 is involved in tumorigenesis by formation of tumor lymphatics (Jussila and Alitalo, 2002; Shibuya and Claesson-Welsh, 2006). VEGF, produced by fibroblasts and tumor cells, recruit VEGFR2 expressing endothelial cells (Kalluri and Zeisberg, 2006).

Another tyrosine kinase family involved in angiogenesis is the TIE (Tyrosine kinase with Ig and EGF homology domains) receptors 1 and 2, both expressed by endothelial cells of the vascular
and lymphatic system (Augustin et al., 2009). There are four known ligands that bind to TIE2, the angiopoietins (Ang) with Ang1 and Ang2 as the most studied. Ang1 has an agonistic role and is important for vessel maturation and stability by promoting endothelial-pericyte interactions whereas Ang2 works antagonistically and is important for vascular permeability (Armulik et al., 2005). In tumor angiogenesis Ang-TIE2 signaling works together with the VEGF-VEGFR system. Both ligands (Ang1 and 2) and TIE2 receptors have been found expressed in tumors (Augustin et al., 2009).

PDGF receptors have been shown to be important for vessel stabilization and maturation by recruitment of pericytes, cells surrounding the endothelial cells, to capillary walls (Betsholtz, 2004).

The Eph (erythropoietin-producing human hepatocellular carcinoma) receptors are a relatively new class of RTKs which interact with ligands (ephrins, Eph family receptor interacting proteins) that are cell surface bound leading to cell adhesion, extra cellular matrix (ECM) attachment and migration. There are 14 known receptors and 8 ligands and their signaling have mostly been studied in the nervous system but they are also important for angiogenesis (Cheng et al., 2002). EphA2 receptor and its ligand ephrinA1 have been found expressed in many human tumors (Cheng et al., 2002).

1.1.3 Tyrosine kinase inhibitors

Up to date there are eleven approved tyrosine kinase inhibitors, including both antibodies and low molecular weight inhibitors (Baselga, 2006).

Tyrosine kinase antibodies

The first FDA (United States Food and Drug Administration) approved tyrosine kinase antibody was trastuzumab (Herceptin), for the use against HER2 positive metastatic breast cancer (table1) (Cobleigh et al., 1999; Slamon et al., 2001). The three other approved antibodies are cetuximab (Erbilux), bevacizumab (Avastin) and panitumumab (Vectibix). Cetuximab is approved for the treatment of colorectal cancer and advanced or metastatic SCCHN, blocking the EGFR (Bonner et al., 2006; Cunningham et al., 2004; Saltz et al., 2004). Bevacizumab block the VEGFR pathway by binding to VEGF-A and thereby inhibiting binding of the ligand to any VEGFRs, it was first approved as treatment for metastatic colorectal cancer in combination with chemotherapy in 2004 (table 1) (Hurwitz et al., 2004). Now it is also approved for HER2 negative breast cancer in combination with paclitaxel, for NSCLC in combination with chemotherapy and in combination with chemotherapy for colorectal cancer (Cohen et al., 2007; Johnson et al., 2004; Miller et al., 2007; Sandler et al., 2006). Panitumumab (Vectibix) is the second approved EGFR antibody and is now in use for treatment of EGFR positive metastatic colorectal cancer (table 1) (Van Cutsem et al., 2007).

Low molecular weight inhibitors

The first low molecular weight tyrosine kinase inhibitor to be approved was imatinib (Glivec/Gleevec). It was first clinically used to block the Abl kinase in CML since Bcr-Abl was understood to be the driving force of tumor growth (Druker et al., 2001a; Druker et al., 2001b). Imatinib inhibits, apart from Abl, also c-kit and the two PDGFRs (α and β). It has later also been approved for the treatment of GIST where the target molecule is c-kit (table1) (Demetri et al., 2002). In October 2006 imatinib was also approved for dermatofibrosarcoma protuberans.
(DFSP), meylodysplastic/myeloproliferative diseases (MDS/MPD) with PDGFR gene rearrangements, hypereosinophilic syndrome/chronic eosinophilic leukemia (HES/CEL), aggressive systemic mastocytosis (ASM) and Ph-positive refractory ALL (http://www.fda.gov/cder/Offices/OODP/whatsnew.htm).

Erlotinib (Tarceva) is an inhibitor blocking the EGFR and is used for the treatment of NSCLC resistant to chemotherapy and is also approved for use in combination with gemcitabine for first-line treatment of advanced pancreatic cancer (table1) (Moore et al., 2007; Shepherd et al., 2005). Gefitinib (Iressa) is another EGFR small molecule inhibitor which is approved in some Asian countries for NSCLC (Fukuoka et al., 2003; Kris et al., 2003; Takano et al., 2008).

Another type of kinase inhibitors in this group are the “multi-kinase” inhibitors sorafenib (Nexavar) and sunitinib (Sutent), both approved for advanced kidney cancer giving anti-angiogenic therapy through blocking of both VEGFR and PDGFR (Kane et al., 2006; Motzer et al., 2006a; Motzer et al., 2006b). Sorafenib has now also been granted approval for hepatocellular carcinoma (Kane et al., 2009; Llovet et al., 2008). Sunitinib is also approved for imatinib resistant GIST where the major target molecule is c-kit (table 1) (Demetri et al., 2006). Dasatinib (Sprycel) and nilotinib (Tasigna) are both second generation inhibitors developed when resistance to imatinib in CML was seen and the mechanisms were identified (table 1) (Shah et al., 2004; Weisberg et al., 2005). They are both approved for Ph-positive imatinib resistant CML and dasatinib is also approved for treatment of Ph-positive imatinib resistant ALL (Hazarika et al., 2008; Kantarjian et al., 2007; Talpaz et al., 2006). Lapatinib (Tykerb) is also a second generation inhibitor targeting HER2 and is approved for treatment of trastuzumab resistant HER2 positive breast cancer in combination with chemotherapy (table 1) (Geyer et al., 2006).
<table>
<thead>
<tr>
<th>Substance</th>
<th>Antibody or Low molecular weight molecule</th>
<th>Target protein (target cell)</th>
<th>Type of cancer approved for</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trastuzumab</td>
<td>Antibody</td>
<td>HER2 (epithelial)</td>
<td>HER2 positive metastatic breast cancer</td>
</tr>
<tr>
<td>(Herceptin)</td>
<td></td>
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<tr>
<td>Imatinib</td>
<td>Low molecular weight molecule</td>
<td>Bcr-Abl (leukemia)</td>
<td>CML refractory Ph+ ALL</td>
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<tr>
<td>(Glivec/</td>
<td></td>
<td>c-kit (epithelial)</td>
<td>Adjuvant for c-KIT positive GIST</td>
</tr>
<tr>
<td>Gleevec)</td>
<td></td>
<td>PDGFR (mesenchymal and</td>
<td>DFSP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>leukemia)</td>
<td></td>
</tr>
<tr>
<td>Erlotinib</td>
<td>Low molecular weight molecule</td>
<td>EGFR (epithelial)</td>
<td>Chemotherapy resistant lung cancer</td>
</tr>
<tr>
<td>(Tarceva)</td>
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<tr>
<td>Cetuximab</td>
<td>Antibody</td>
<td>EGFR (epithelial)</td>
<td>EGFR positive metastatic colorectal cancer</td>
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<tr>
<td>(Erbitux)</td>
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<td>Squamos cell carcinoma of the head and neck (with RT)</td>
</tr>
<tr>
<td>Bevacizumab</td>
<td>Antibody</td>
<td>VEGF (endothelial)</td>
<td>Metastatic colorectal cancer</td>
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<td>(Avastin)</td>
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<tr>
<td>Sorafenib</td>
<td>Low molecular weight molecule</td>
<td>VEGFR (endothelial)</td>
<td>Advanced renal cell carcinoma</td>
</tr>
<tr>
<td>(Nexavar)</td>
<td></td>
<td>PDGFR (pericytes) c-rat (?) (epithelial)</td>
<td></td>
</tr>
<tr>
<td>Sunitinib</td>
<td>Low molecular weight molecule</td>
<td>VEGFR (endothelial)</td>
<td>Advanced renal cell carcinoma</td>
</tr>
<tr>
<td>(Sutent)</td>
<td></td>
<td>PDGFR (pericytes) c-kit (epithelial)</td>
<td></td>
</tr>
<tr>
<td>Dasatinib</td>
<td>Low molecular weight molecule</td>
<td>Bcr-Abl (leukemia)</td>
<td>Imatinib resistant Ph+ ALL</td>
</tr>
<tr>
<td>(Sprycel)</td>
<td></td>
<td>PDGFRβ (leukemia)</td>
<td></td>
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<td></td>
<td></td>
<td>Src family kinases c-kit</td>
<td></td>
</tr>
<tr>
<td>Panitumumab</td>
<td>Antibody</td>
<td>EGFR (epithelial)</td>
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<td>(Vectibix)</td>
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<tr>
<td>Lapatinib</td>
<td>Low molecular weight molecule</td>
<td>EGFR (epithelial)</td>
<td>HER2 positive trastuzumab resistant</td>
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<tr>
<td>(Tykerb)</td>
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<td>HER2 (epithelial)</td>
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<tr>
<td>Nilotinib</td>
<td>Low molecular weight molecule</td>
<td>Bcr-Abl (leukemia)</td>
<td>Imatinib resistant Ph+ CML</td>
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</tbody>
</table>
1.2 CLINICAL RESULTS OF TYROSINE KINASE INHIBITORS

1.2.1 Antibodies

Trastuzumab
In one of the phase III studies which led to trastuzumab approval for HER2 overexpressing metastatic breast cancer trastuzumab alone showed 4% complete responders, about 15% partial responders and 29% stable disease (Cobleigh et al., 1999). Median duration of response was 9.1 months and median time to treatment failure was 11 months compared to 5.4 months for earlier chemotherapy treatment in patients who were categorized as partial or complete responders (Cobleigh et al., 1999). In the other study trastuzumab was used in combination with chemotherapy as first line treatment. The study showed that patients receiving the combination had a longer median time to progression (7.4 months vs. 4.6 months for chemotherapy alone) and longer median survival, 25.1 months compared to 20.3 months for chemotherapy alone (Slamon et al., 2001). Trastuzumab is also used as adjuvant in early stage breast cancer, where it results in significantly better disease free survival (Piccart-Gebhart et al., 2005; Smith et al., 2007).

Cetuximab
Cetuximab as single agent showed 9% partial response and 37% stable disease in colorectal cancer patients who had relapsed on chemotherapy (Saltz et al., 2004). In a study comparing cetuximab alone or in combination with irinotecan in metastatic colorectal cancer patients who had progressed on irinotecan, response rates were higher (22.9% vs. 10.8%), progression-free survival (PFS) was better (4.1 months vs. 1.5 months), and median survival was longer (8.6 months vs. 6.9 months) in the combination group compared to in the cetuximab alone group (Cunningham et al., 2004).

A randomized study comparing best supportive care with cetuximab in colorectal cancer showed a benefit of the treatment in patients who had failed the chemotherapy pretreatment (Jonker et al., 2007). As first-line treatment of colorectal cancer patients cetuximab addition to chemotherapy (FOLFIRI) gave no difference in overall survival (OS) although the combination reduced the risk of progression (Van Cutsem et al., 2009a).

In SCCHN patients, cetuximab in combination with radiation therapy increased the median OS by almost double, 49 months compared to 29.3 months for radiation therapy alone (Bonner et al., 2006). When instead cetuximab was used in combination with platinum-fluorouracil (cis- or carboplatin) as first-line therapy in patients with metastatic or recurrent SCCHN the median OS was about three months longer (10.1 vs. 7.4 months for chemotherapy alone) (Vermorken et al., 2008).

Panitumumab
Panitumumab has been shown to increase the PFS compared to best supportive care (mean PFS 13.8 weeks vs. 8.5 weeks) in patients with colorectal cancer progressing on chemotherapy although no difference in OS was noted (Van Cutsem et al., 2007; Van Cutsem et al., 2008).

Bevacizumab
Many clinical trials with bevacizumab in different combinations with chemotherapy regimens are ongoing. In a study to compare bevacizumab in combination with chemotherapy (irinotecan, fluorouracil and leucovorin) for patients with metastatic colorectal cancer the response rate was
higher in the combination group (44.8% vs. 34.8%) (Hurwitz et al., 2004). Also median duration of response (10.4 months vs. 7.1 months), median duration of PFS (10.6 months vs. 6.2 months) and the OS (20.3 months vs. 15.6 months) were longer in the combination group (Hurwitz et al., 2004).

Patients with advanced NSCLC who were treated with carboplatin and paclitaxel in combination with bevacizumab had higher response rates (35% vs. 15% for carboplatin and paclitaxel) and longer median survival (12.3 months vs. 10.3 months) compared to chemotherapy alone (Sandler et al., 2006).

In metastatic renal cell carcinoma bevacizumab monotherapy (high-dose) gave significant delay in time to progression (64% no tumor progression vs. 20% in the placebo group) but no difference in OS (Yang et al., 2003). In combination with interferon α-2a, bevacizumab increased the PFS by almost double, 10.2 months versus 5.4 months in placebo+interferon α-2a in patients with metastatic renal cell carcinoma (Escudier et al., 2007b).

Patients with metastatic breast cancer treated with paclitaxel in combination with bevacizumab had a significantly longer PFS (11.8 months vs. 5.9 months) than when treated with paclitaxel alone but no significant difference in OS was seen (Miller et al., 2007).

1.2.2 Low molecular weight inhibitors

Imatinib
Almost all patients with CML showed a complete response after imatinib treatment (300mg or more) with 53 of 54 patients having a complete hematological response already after about one month (Druker et al., 2001b). CML patients in blast crisis had lower response (55% responders) and 70% of ALL patients were responders (Druker et al., 2001a). After a 60 months follow up of CML patients on imatinib, 97% who had a complete cytogenetic response twelve months after beginning of treatment still had not progressed and estimated OS was 87% (Druker et al., 2006). The same was seen in a different study after a five-year follow up with an 83.2% estimated OS (de Lavallade et al., 2008).

In imatinib treated GIST patients 53.7% had partial response and 27.9% had stable disease (Demetri et al., 2002). In a study of imatinib as adjuvant in patients with GIST the estimated 1-year recurrence-free survival was 98% in the imatinib group compared to 83% in the placebo group (Demitteo et al., 2009).

Erlotinib
NSCLC patients, previously treated, were given either erlotinib or placebo. Response rate in the erlotinib group was 8.9% compared to less than 1% in the placebo group, PFS was similar in both groups but OS was longer in the erlotinib group (6.7 months vs. 4.7 months for placebo) (Shepherd et al., 2005). Patients with advanced pancreatic carcinoma treated with erlotinib in combination with gemcitabine had a significantly longer median OS (6.24 months vs. 5.91 months) and 1-year survival (23% vs. 17%) compared to gemcitabine alone (Moore et al., 2007).
**Sunitinib**
In GIST patients who previously progressed on imatinib, sunitinib was shown to be effective with a median time to progression of 27.3 weeks compared to 6.4 weeks in the placebo group (Demetri et al., 2006).

Patients with metastatic renal cell carcinoma progressing on standard therapy had a 40% partial response after sunitinib and another 27% had stable disease (Motzer et al., 2006a). To confirm this study a second study with similar patients was conducted giving similar results with 34% partial responders (Motzer et al., 2006b).

**Sorafenib**
In the study which granted sorafenib approval for hepatocellular carcinoma the median OS was 10.7 months compared to 7.9 in the placebo treated group (Llovet et al., 2008). Patients with advanced renal cell carcinoma, resistant to standard therapy, treated with sorafenib also showed about three months benefit in PFS (5.5 months vs. 2.8 months in the placebo group) (Escudier et al., 2007a).

**Lapatinib**
Lapatinib is used in combination with the chemotherapy capecitabine in breast cancer patients who have relapsed on trastuzumab with about two months delayed time to progression when compared with capecitabine alone (6.2 vs. 4.3 months) (Cameron et al., 2008). In patients who developed brain metastases after trastuzumab treatment lapatinib had not much effect on its own but in combination with capecitabine 20% had an objective response (Lin et al., 2009).

**Dasatinib and Nilotinib**
The second generation Bcr-Abl inhibitor dasatinib is very effective in imatinib resistant CML patients in chronic phase with complete hematologic response in 92% of patients (Talpaz et al., 2006). Patients with accelerated phase CML, CML with blast crisis or Ph-positive ALL had lower response with 70% receiving a major hematologic response (Talpaz et al., 2006). In imatinib resistant CML patients with either myeloid or lymphoid blast crisis about 35% had major hematologic responses after dasatinib treatment (Cortes et al., 2008).

Nilotinib have great effects in Ph positive CML patients resistant to imatinib for all but one known resistant mutant (T315I), with complete response in 48% and partial response in 16% (Kantarjian et al., 2007). In patients with accelerated phase CML hematologic response was seen in 47% and major cytogenetic response in 29% (le Coutre et al., 2008).

### 1.2.3 Sensitivity and resistance
During the last few years the mechanisms behind response to tyrosine kinase inhibitors, or lack of response, have been evaluated.

**Amplified or mutated drug target**
Treatment with EGFR inhibitors in NSCLC have shown that response is associated with tumors harboring EGFR mutations (Han et al., 2005; Mitsudomi et al., 2005; Paez et al., 2004).
In leukemias and myeloproliferative disorders like CML, HES/CEL and ALL patients with tumors that have mutations in either Bcr-Abl or PDGF receptors respond to imatinib (Apperley et al., 2002; Cools et al., 2003; Druker et al., 2006).

Patients with metastatic GIST harboring Kit mutations in exon 11 had a higher partial response to imatinib compared to patients with no mutation or exon 9 mutations (83.5% and 47.8% respectively) (Heinrich et al., 2003a).

Sunitinib which is a small molecule targeting both the PDGFR and c-Kit is effective against several imatinib resistant GISTs but response is dependent on specific tumor Kit mutations (Heinrich et al., 2008). In contrast to imatinib response, tumors with exon 11 mutations had a lower response to sunitinib (34% compared to 58% for exon 9 mutations and 56% for wt KIT and PDGFRA) and thereby worse progression free and overall survival (Heinrich et al., 2008).

Alterations downstream of the drug target

Breast tumors expressing HER2 which initially do not respond to trastuzumab or become resistant to treatment have been shown to have PI3K activating mutations or loss of PTEN (Berns et al., 2007; Nagata et al., 2004).

In the case of response to cetuximab in patients with colorectal cancer it was shown that mutations in K-Ras were a characteristic of nonresponders (Di Fiore et al., 2007; Khambata-Ford et al., 2007; Lievre et al., 2006). Patients treated with the combination of cetuximab and chemotherapy (FOLFIRI) and had tumors harboring wt Ras had a higher response rate compared to patients whose tumors had mutated Ras (59.3% vs. 36.2%) (Van Cutsem et al., 2009a). PTEN loss also confers resistance to cetuximab in metastatic colorectal patients, none of the patients with tumors harboring PTEN mutations responded to therapy (Frattini et al., 2007).

Also, patients with metastatic colorectal cancer, treated with either cetuximab or panitumumab show no response if the tumor contains mutations in the PI3K gene (Sartore-Bianchi et al., 2009). PI3K mutations also correlate with worse clinical outcome with shorter PFS (Sartore-Bianchi et al., 2009).

Response to panitumumab has also been shown to be dependent on wt K-Ras. In colorectal cancer patients treated with panitumumab or best supportive care response rate was 17% in patients with wt K-Ras tumors and 0% in patients with mutated K-Ras tumors (Amado et al., 2008). In the wt K-Ras group the PFS was 12.3 weeks vs. 7.3 for best supportive care (Amado et al., 2008).

In additional studies of NSCLC to understand the response to the EGFR inhibitors, erlotinib and gefitinib, it was seen that even though EGFR mutations was present, if the tumor also harbored K-Ras mutations the response was lower (Eberhard et al., 2005; Han et al., 2006).

Secondary mutations

Resistance to imatinib has developed after initial response to the drug in CML, HES/CEL and GIST through secondary activating mutations in Bcr-Abl, PDGFRa and c-Kit, respectively (Cools et al., 2003; Daub et al., 2004; Gorre et al., 2001). These secondary mutations are often present in the kinase domain making it impossible for the inhibitor to bind, although the tyrosine
kinase can still bind ATP (Daub et al., 2004). The most commonly secondary mutation found responsible for imatinib resistance in CML is Abl T315I (Gorre et al., 2001). Analogous mutations are found in the other imatinib targets, PDGFRα (T674I) and Kit (T670I) (Daub et al., 2004). Other tumor types with alterations in different tyrosine kinase receptors have developed similar resistant mutants, an example being EGFR mutations (T790M) resistant for gefitinib or erlotinib in lung cancer patients (Blencke et al., 2004; Kobayashi et al., 2005; Pao et al., 2005).

Another resistance mechanism found in EGFR inhibitor treated NSCLC is amplification of the MET oncogene, which was found in 22% of tumors that had developed resistance (Engelman et al., 2007).

The resistance led to development of second generation inhibitors, targeting these drug resistant mutants. Unfortunately also after treatment with the new generation drugs tumors have acquired resistance. Secondary mutations non-sensitive to sunitinib have been found in Kit in GIST patients (Gajiwala et al., 2009).

### 1.2.4 Treatment of glioblastomas with EGFR and PDGFR antagonists

Two EGFR tyrosine kinase inhibitors have been used in clinical trials for GBMs, gefitinib (Rich et al., 2004) and erlotinib. Erlotinib has been shown to be most effective against GBM in patients with high expression levels of the EGFR and low levels of activated Akt (Haas-Kogan et al., 2005). Coexpression of the deletion mutant form of EGFR (EGFRvIII), commonly found in GBMs, and PTEN was also found to be associated with response to EGFR tyrosine kinase inhibitors like gefitinib and erlotinib (Mellinghoff et al., 2005).

Imatinib is the only PDGFR inhibitor which has been used in clinical trials for the treatment of glioblastomas. As monotherapy it had no effect, but in two independent studies imatinib in combination with hydroxyurea has been shown to have antitumor activity (Dressemann, 2005; Reardon et al., 2005; Wen et al., 2006).

### 1.2.5 Combination therapy

The strategy in the beginning of targeted therapies was to use very specific drugs with known mechanisms in contrast to the cytostatic drugs. Over the last few years it has become apparent that inhibition of one tyrosine kinase might lead to upregulation of another tyrosine kinase that may surmount the inhibition (Stommel et al., 2007). Therefore combination therapies targeting more than one kinase has been proposed as a better way of targeted therapies, either with use of multikinase inhibitors or combinations of drugs. Downstream molecules are also important in understanding drug response, many tumors have activating mutations in PI3K or mutations leading to loss of PTEN which both lead to a highly activated Akt signaling which explains drug resistance in for example gliomas and breast tumors. In such tumors drugs targeting the PI3K pathway, like inhibitors of mTOR, might be more effective.

In patients with metastatic renal cell carcinoma bevacizumab alone was compared to combination with erlotinib but no benefit in response rate (14% responders vs. 13%) or PFS (9.9 months vs. 8.5 months) with combination therapy was seen (Bukowski et al., 2007). When instead sunitinib was combined with bevacizumab in patients with metastatic renal cell carcinoma an objective response rate of 52% was seen, but since 48% had to stop treatment due to severe side-effects this dose regimen will not be further evaluated (Feldman et al.,
In patients with NSCLC bevacizumab in combination with erlotinib or chemotherapy was compared with chemotherapy alone; one year survival was 57.4% for bevacizumab+erlotinib, 53.8% for bevacizumab+chemotherapy and 33.1% for chemotherapy (Herbst et al., 2007). Bevacizumab addition to gemcitabine and erlotinib for patients with metastatic pancreatic cancer did not further improve OS (Van Cutsem et al., 2009b). Addition of panitumumab was shown to give a worse outcome in patients with metastatic colorectal cancer (PFS 10 months vs. 11.4 months) when added to chemotherapy and bevacizumab (Hecht et al., 2009).

The synergistic effects expected when inhibiting more than one target has unfortunately thus not been observed. This might be due to the late stage treatment of metastatic disease but also to lack of proper patient selection.

1.3 PLATELET-DERIVED GROWTH FACTOR (PDGF)

PDGFs are the major growth factors for connective tissue forming cells such as fibroblasts, glial cells, pericytes and vascular smooth muscle cells (Heldin et al., 1998). It was first isolated from platelets but has later been found expressed in many different cell types like for example epithelial cells, fibroblasts, endothelial cells, vascular smooth muscle cells, kidney mesangial cells and macrophages (reviewed in (Heldin and Westermark, 1999). PDGF signaling is important during embryogenesis. Mice knock-outs of the genes for PDGF-A and -B and both α- and β-receptors are all embryonically or perinatally lethal (Bostrom et al., 1996; Leveen et al., 1994; Soriano, 1994, 1997). In the adult PDGF signaling is important during wound healing and the major cell fate of PDGF signaling is cell growth and motility. PDGFs are stored in the extra cellular matrix by being bound via the retention sequence to heparin sulfate, collagens or osteopontin/SPARC (secreted protein acidic and rich in cysteines) (Heldin and Westermark, 1999).

1.3.1 PDGF ligands and receptors

There are five known isoforms of PDGF, the homodimers -AA, -BB, -CC and -DD and the heterodimer -AB (Heldin et al., 2002). They are all disulfide-bonded dimers with a conserved motif of eight cysteines (Heldin et al., 1998). The different isoforms bind to different forms of the PDGF receptors. PDGF-AA and -CC binds to the α-receptor, giving rise to α-α receptor homodimers (Hart et al., 1988; Heldin et al., 1988; Li et al., 2000). PDGF-DD binds to β-receptors giving rise to β-β receptor homodimers (Bergsten et al., 2001) while -AB gives α-α receptor homodimers or α-β receptor heterodimers (Hart et al., 1988; Heldin et al., 1988). PDGF-BB can bind to both receptor types giving rise to all forms of receptor dimers (Hart et al., 1988; Heldin et al., 1988). (see Figure 4)
PDGF receptor α and β are structurally similar, both consisting of five extracellular immunoglobulin (Ig)-like domains responsible for ligand binding and dimerization, a transmembrane domain and an intracellular kinase domain (Heldin et al., 1998). After ligand binding the receptors get activated through dimerization and autophosphorylation of a critical regulatory tyrosine in the kinase domain; Tyr849 and Tyr857 in the α- and β-receptor, respectively (Kazlauskas and Cooper, 1989). This leads to up-regulation of the inherent tyrosine activity, recruitment of docking proteins with SH2 domains binding to the phosphotyrosines, examples are Src, PI3K, SHP-2 and PLCγ which transmit the signal to downstream molecules in the cell (Ostman and Heldin, 2007). The tyrosine kinase Src, bind to the phosphorylated Tyr579 or Tyr581 in the β-receptor and corresponding Tyr572 or Tyr574 in the α-receptor via its SH2 domains. PI3K can bind to two autophosphorylated tyrosine residues in the β-receptor, Tyr740 and Tyr751 leading to conformational changes and activation of the catalytic subunit and downstream activation of Akt. Grb2 binds to phosphorylated (p) Tyr716 and Tyr775 in the β-receptor but only to one residue in the α-receptor, pTyr993 which via SOS leads to activation of Ras and downstream signaling via the MAPK pathway (or PI3K-Akt). PLCγ 1 and 2 both bind both the PDGF receptor subtypes via two different pTyr residues (Tyr1021 and 1009 in the β-receptor and Tyr988 and 1018 in the α-receptor) (Heldin et al., 1998).

PDGFRα are expressed by astrocytes, fibroblasts, and pericytes whereas PDGFRβ are normally expressed on perivascular cells, fibroblasts, smooth muscle cells and macrophages (Heldin and Westermark, 1999; Ostman, 2004).

### 1.3.2 Autocrine PDGF signaling in tumors

PDGF was first described to be able to transform cells following the discovery that the simian sarcoma virus oncogene, v-sis, was derived from the gene encoding PDGB (Doolittle et al., 1983; Waterfield et al., 1983). Cells expressing the PDGF, like fibroblasts, can be transformed by the v-sis gene leading to constitutive PDGF-B production (Deuel et al., 1983).

**Mutational activated PDGF and PDGFR**

In many myeloproliferative neoplasms and eosinophilia PDGFR are constitutively activated by chromosomal rearrangements leading to different fusion proteins. One example is the hematological disease HES/CEL where a fusion protein of FIP1-LI and PDGFRα has been detected (Cools et al., 2003). The PDGFRα is also constitutively active in a subset of GISTs expressing wild type c-Kit (Heinrich et al., 2003b).
Both chronic myelomonocytic leukemia (CMML) and dermatofibrosarcoma protuberans (DFSP) are examples of autocrine transformation by activating mutations in genes encoding the PDGFRβ and the PDGF-B chain, respectively. CMML has a chromosomal translocation that produces the fusion protein Tel-PDGFRβ, which gives rise to a constitutively active receptor (Golub et al., 1994). DFSP in turn contains the COLIA1-PDGF-B fusion protein (chromosome 17/22) which is processed to mature PDGF-BB giving constitutive PDGF-BB production that leads to PDGFRβ phosphorylation, transformation and tumor growth (Shimizu et al., 1999; Sjoblom et al., 2001).

Other tumors with autocrine PDGF signaling

In glioblastoma multiforme (GBM) it has been shown that both PDGF ligands and PDGFRα are expressed on the tumor cells suggesting autocrine growth signaling (Hermanson et al., 1992). The PDGFRα has been shown to be amplified and overexpressed in a fraction of human malignant gliomas. In a study of glioblastomas using fluorescence in situ hybridization (FISH) the PDGFRα gene was amplified in 29% of the tumors (Joensuu et al., 2005). Also in some ovarian and breast tumors autocrine PDGFR signaling have been suggested since both PDGF and the α-receptor was found expressed on the epithelial tumor cells (Henriksen et al., 1993; Jechlinger et al., 2006).

Soft tissue tumors frequently express PDGFRβ (Franklin et al., 1990). Ewing Sarcomas, a family of tumors characterized by chromosomal translocations leading to fusions between Ewing sarcoma oncogene, EWS, and another ETS transcription factor have been shown to induce PDGF ligands (Lee et al., 1997; Zwerner and May, 2001). In desmoplastic small round-cell tumors (part of the Ewing sarcomas) expressing the fusion protein EWS-WT1, PDGF-A was found (Lee et al., 1997). Another example is PDGF-C, upregulated by EWS/FLI1 in NIH3T3 cells (Zwerner and May, 2001). Blocking of the PDGF-C in Ewing family tumor cell lines was shown to have a growth inhibitory effect (Zwerner and May, 2002). The PDGFRβ has also been found expressed in the tumor cells in an immunohistochemistry (IHC) analysis of Ewing Sarcoma tumor samples. Ewing sarcoma cell lines were also found to be growth inhibited by PDGFR antagonists (Uren et al., 2003). In addition a subset of synovial sarcomas has been shown to have an autocrine PDGF signaling by expressing both PDGF-B and activated PDGFRβ (Tamborini et al., 2004).

1.3.3 PDGF signaling in tumor angiogenesis

Angiogenesis is promoted by endothelial growth factors as the VEGF, produced mostly by the tumor cells but also the stromal fibroblasts. Recruitment of pericytes to the vessel wall is part of the development of normal capillaries and important for vessel stability (Hellstrom et al., 2001). The recruitment to the vessels is mediated by PDGF-B (Abramsson et al., 2003; Guo et al., 2003) and PDGF-induced pericyte recruitment has been shown to increase tumor growth rate (Furuhashi et al., 2004). PDGFRβ have been found to be expressed on pericytes surrounding both normal and tumor vessels (Sundberg et al., 1993).

In an experimental model of pancreatic cancer (Rip1-Tag2) the PDGFRβ expressing perivascular cells was found to be pericyte progenitor cells (expressing a marker for immature perivascular cells, RGS-5) that differentiate to pericytes expressing markers for mature pericytes like NG-2, desmin and αSMA (Song et al., 2005).
Some studies also reveal expression of PDGFRα and PDGFRβ on endothelial cells of vessels in animal models of bone metastases from ovarian, breast and prostate tumors (Apte et al., 2004; Hwang et al., 2003; Lev et al., 2005; Uehara et al., 2003). The same group also found PDGFRα and PDGFRβ expression in both tumor and endothelial cells of experimental pancreatic tumors and their metastasis (Hwang et al., 2003). Validation of these interesting findings needs to be done in human systems.

1.3.4 PDGF involvement in recruitment of tumor fibroblasts and regulation of IFP

PDGFRβ are expressed on fibroblasts and important for recruitment of the tumor stroma (Sundberg et al., 1997). The ligands for PDGF receptors α and β, PDGF-A, -B, and -C are expressed by many tumor epithelial cells, both in human and experimental models (Coltrera et al., 1995; Coombes et al., 1990; Peres et al., 1987; Pietras et al., 2008; Sariban et al., 1988). In different tumor models PDGF have been shown to stimulate the recruitment of tumor stromal cells, expressing PDGF receptors, by either over-expression of PDGF (Anderberg et al., 2009; Skobe and Fusenig, 1998) or transfection of PDGF in to cells with no endogenous PDGF expression (Forsberg et al., 1993).

The interstitial fluid pressure (IFP) is increased in solid tumors and has been shown to be a reason for decreased drug uptake and delivery (Heldin et al., 2004; Jain, 2001). Treatment with PDGF receptor antagonists of tumors with PDGF receptor expression restricted to tumor stroma, leads to increased drug uptake and reduction in IFP (Baranowska-Kortylewicz et al., 2005; Pietras et al., 2001; Pietras et al., 2002; Pietras et al., 2003b). In a NSCLC model imatinib treatment was also shown to reduce the expression of VEGF and level of hypoxia in addition to lowering the IFP (Vlahovic et al., 2006).

1.3.5 PDGF antagonists

A number of inhibitors of PDGF signaling have been described, including tyrosine kinase inhibitors and antibodies against the extra cellular part of the receptors and the ligands (Ostman and Heldin, 2001). Three inhibitors of the PDGFR are now FDA approved drugs; Imatinib/Glivec, SU11248/Sunitinib/Sutent and Sorafenib/Nexavar. All these drugs inhibit more than one tyrosine kinase (see table 1). The only antibody involved in clinical trials is a polyethyl glycol, PEG, -conjugated anti-PDGFRβ Fab-fragment (CDP860) (Jayson et al., 2005). PTK787 is another tyrosine kinase inhibitor with activity against the PDGFRβ, but with about 10-fold lower affinity as for the VEGFR-1 which it was developed against (Bold et al., 2000). Another PDGFR inhibitor is CP-673451 which also blocks VEGF, TIE2 and FGF receptors but at a much lower affinity. It has been shown to block tumor growth in experimental models (Roberts et al., 2005).

1.3.6 Clinical results of PDGFR inhibition

In PDGFRβ dependant CMML effective clinical responses of imatinib have been reported with normal blood count after four weeks treatment and no detectable t(5;12) translocations after about 30 weeks (Apperley et al., 2002). Imatinib have also been shown to reduce tumor volume and thereby making surgical resection possible in DFSP patients, with the t(17;22) translocation, indicating good effects of the drug (McArthur et al., 2005; Rubin et al., 2002). HES/CEL patients with a constitutively active PDGFRα have shown very good response to
imatinib often resulting in hematological remission (Cools et al., 2003; Jovanovic et al., 2007). In patients with HES/CEL containing *PDGFR* fusion genes complete hematologic imatinib response was seen in 95% (Metzgeroth et al., 2008). Also GIST patients with PDGFRα mutations treated with imatinib have shown partial responses (Heinrich et al., 2003a). In two separate clinical trials of imatinib, in combination with hydroxyurea, good response was seen in a number of glioblastoma patients (Dresemann, 2005; Reardon et al., 2005).

1.4 TUMOR FIBROBLASTS

1.4.1 Origin of tumor fibroblasts

Tumor fibroblasts seem to be derived from different sources given that the different fibroblast markers are heterogeneously expressed in tumor models (Sugimoto et al., 2006). Either they are recruited from the host organ or are epithelial cells that have undergone epithelial to mesenchymal transition (Kalluri and Zeisberg, 2006). It has also been shown that endothelial cells can undergo transition (endothelial to mesenchymal transition) and contribute to a part of the fibroblast population (Zeisberg et al., 2007). Bone marrow cells have also been found in experimental models to contribute to the tumor myofibroblasts and fibroblasts (Direkze and Alison, 2006).

1.4.2 Genetic alterations of tumor fibroblasts

Recent studies of the tumor stroma in human tumors have found that also the fibroblasts, which were thought to be normal, might be genetically altered. Two studies of breast tumors which used laser capture microdissection (LCM) to look at the stroma compartment found that mutations in stromal fibroblasts correlated better with clinical outcome than changes in the epithelial compartment (Fukino et al., 2007; Patocs et al., 2007). Genetic alterations were also found in primary breast cancer associated fibroblasts (CAFs) when compared to normal fibroblasts (Hawsawi et al., 2008). However, some later studies have suggested methodological problems with these studies (Qiu et al., 2008).

1.4.3 Interactions with other cell types

Fibroblasts express growth factors like VEGF, fibroblast growth factor (FGF), hepatocyte growth factor (HGF), transforming growth factor β (TGF-β), EGF, and insulin like growth factor (IGF) which are needed to recruit different cell types to the tumor like immune cells and endothelial cells (Ostman and Augsten, 2009). Many tumor epithelial cells express the cognate receptors for some of those growth factors, and fibroblasts can thus increase the proliferation of the epithelial tumor cells (Bhowmick et al., 2004b; Ostman and Augsten, 2009).

In a breast cancer model where human CAFs were coinjected with tumor cells it was shown that CAFs increased tumor growth compared to normal fibroblasts and stimulated angiogenesis by secreting the chemokine SDF1 that recruits endothelial precursor cells (Orimo et al., 2005). In a cervical cancer model FGF derived from fibroblasts was shown to induce angiogenesis (Pietras et al., 2008). In a prostate tumor model of CXCL14 overexpressing CAFs coinjected with low malignant tumor cells, tumor growth and the infiltration of macrophages was increased (Augsten et al., 2009).

Fibroblasts have also been shown to be involved in tumor metastasis by breaking down the ECM making it easier for tumor cells to invade and migrate (Kalluri and Zeisberg, 2006). In a
breast cancer model mesenchymal stem cells increased the aggressiveness by fibroblast CCL5 stimulation of the epithelial cells (Kamoun et al., 2007).

1.4.4 Prognostic relevance of tumor fibroblasts

A breast tumor stroma 26-gene predictor of prognosis has been found to predict patient outcome (Finak et al., 2008). In head and neck squamos cell carcinoma stromal genetic alterations correlated with clinicopathological outcome (Weber et al., 2007). The genetic alterations in stromal compartments might though be due to inadequate methodology, since a study of breast and ovarian CAFs failed to detect any mutations (Qiu et al., 2008). High CAF expression of the protein SPARC, a protein involved in migration and matrix interactions, in pancreatic adenocarcinoma correlates with worse prognosis (Infante et al., 2007). In colorectal cancer the amount of stroma measured by αSMA has been shown to be a predictor of disease recurrence (Tsujino et al., 2007). In prostate cancer, the low amount of stromal androgen receptor expression correlated with worse outcome, higher Gleason score and more metastases (Wikstrom et al., 2009). Response to chemotherapy has also recently been correlated to changes in tumor stroma. A breast cancer stromal gene list predicts resistance to adjuvant chemotherapy (Farmer et al., 2009).

1.4.5 Therapeutic targeting of tumor stroma

Experimental tumor models have made use of the FDA approved drug imatinib to target the tumor stroma by inhibiting PDGFR. In a colon cancer model, imatinib inhibited the activation of PDGFR and in combination with chemotherapy inhibited both tumor growth and metastases (Kitadai et al., 2006). In an experimental cervical cancer model with PDGFR positive stroma imatinib also slowed down tumor progression (Pietras et al., 2008). Imatinib in combination with anti-angiogenic treatment after castration significantly inhibited tumor growth compared to castration alone in a prostate tumor model (Johansson et al., 2007). A study by Bergers et al. showed that targeting both endothelial cells and pericytes in the RIP1Tag2 model of pancreatic cancer was beneficial compared to monotherapy (Bergers et al., 2003).

TGF-β signaling has been implicated to be both anti- and pro-tumorigenic. In a study of fibroblast specific TGF-β type II receptor knockout mice, epithelial neoplasias was observed in prostate and forestomach suggesting an anti-proliferative role of TGF-β signaling in these organs (Bhowmick et al., 2004a).

Hedgehog signaling has recently been shown to be required for tumor growth through stimulation of tumor stroma, suggesting its role as a candidate drug target (Yauch et al., 2008).
2 PRESENT INVESTIGATION

2.1 AIMS OF THIS THESIS

- To develop reliable methodology for detection of activated PDGFR and down stream signaling molecules like Akt
- To characterize the expression and activation of PDGFR in human tumors and investigate the potential prognostic and response predictive significance of PDGFR in tumors

2.2 RESULTS AND DISCUSSION

2.2.1 Paper I

Gyrolab Bioaffy – a novel method for signal transduction profiling of tumor tissue

Tumors often have genetic alterations in enzymes involved in signal transduction which are responsible for the tumor growth. The aim of this study was to develop Gyrolab bioaffy for detection of signal transduction pathway status. Gyrolab bioaffy is an ELISA-like method using a column immobilized capture antibody and a fluorescently conjugated detection antibody. All is done on a compact disc containing about 100 columns, testing either different antibodies or different samples. To test if the method was able to analyze signal transduction pathways we decided to look at Akt phosphorylation. Activation of Akt is both a candidate poor prognostic marker in prostate cancer and has also been suggested to be a response predictor to targeted therapies in gliomas and breast cancer (Haas-Kogan et al., 2005; Kreisberg et al., 2004).

To establish the method we used glioma cells with known high levels of phosphorylated Akt (pAkt) either untreated or treated with a PI3K inhibitor, to block the activation, and compared with SDS-PAGE/immuno-blotting. In glioma cells both Akt and pAkt was detected using Gyrolab bioaffy.

To further test the sensitivity of the method we used human prostate tumor lysates. Akt was detected in all tumor samples and pAkt was also well above background in Gyros bioaffy with only borderline detection using SDS-PAGE/immuno-blotting.

To establish if the low signals that are detected by Gyrolab bioaffy can be trusted we performed additional titration experiments of tumors where more material was available. First we checked the Akt and pAkt expression in the twelve additional tumors with SDS-PAGE/immuno-blotting. In all tumors Akt expression was detected and in seven pAkt was also detected by immuno-blotting. Gyrolab bioaffy could detect Akt and pAkt in all tumors. Three tumor samples were selected for the titration experiments. Four different lysate dilutions, 1:3, 1:10, 1:30 and 1:100, were used. Reliable Akt and pAkt signals were detected down to ten times dilution. Together this indicates that the signal from Gyrolab bioaffy is proportional to the input amount.

In a set of 27 matched normal and prostate tumor samples Akt was detected in all but one tumor and pAkt in 21 matched tumor and normal samples. Tumor pAkt/Akt was then correlated to Gleason score (GS). A trend towards worse outcome was seen when tumors had a high pAkt/Akt.
The results from this ongoing study demonstrate that the Gyrolab platform can be used for analyses of activation status of signal transduction proteins derived from cultured cells. Also in archival frozen tumor tissues we were able to detect both Akt and pAkt. Titration experiments confirmed that the assay system could be used for quantitative purposes, since signal were proportional to input amounts.

This study selected pAkt analyses of prostate cancer as a pilot case of clinical application. Our preliminary analyses provided preliminary support for this, by demonstrating a significant association between tumor pAkt levels and GS in prostate tumors. However it should be noted that analyses were performed with few samples and analyses will therefore be expanded to larger tumor sets.

### 2.2.2 Paper II

**In situ detection of phosphorylated platelet-derived growth factor receptor β using a generalized proximity ligation method**

The aim of this study was to develop the *in situ* proximity ligation assay (PLA) for detection of phosphorylated PDGFR.

One antibody towards the c-terminal of PDGFRβ and one site specific phospho-tyrosine (pY751) PDGFRβ antibody were used. We used secondary proximity probes containing secondary antibodies conjugated to an oligo in contrast to what were used in former PLA studies to detect protein interactions. These secondary proximity probes can be used with any pair of primary antibodies derived from the right species.

In PDGF-BB stimulated cultured fixed HEK293 cells stably overexpressing the PDGFRβ *in situ* detection of phosphorylated PDGFRβ was high. In contrast only minimal signals were seen in unstimulated cells. We performed a series of control experiments to verify the results, for example omitting one of the primary antibodies, not stimulating the cells, or using different types of non-expressing cells.

Cultured fixed cells expressing high amounts of either PDGFR-α (PAEα) or -β (PAEβ) were used to analyze the specificity. Phosphorylated PDGFRβ was detected in PDGF-BB stimulated PAEβ cells with a small amount of signals in unstimulated cells. Although some signals were seen in PAEα cells, this was seen regardless of PDGF-BB stimulation. All control experiments resulted in negligible amounts of signals.

We also detected phosphorylated PDGFRβ in cells expressing endogenous amounts of receptors and in fresh frozen scar tissue.

The amount of phosphorylated PDGFRβ molecules in individual cells was quantified using the BlobFinder software by counting the fluorescent RCPs (rolling circle amplification products).

The amount of phosphorylated receptors detected by *in situ* PLA correlated well with immuno-blotting data.

This method will be further validated for detection of pPDGFR in human tumor tissues.
2.2.3 Paper III and IV

Platelet-derived growth factor receptor expression and amplification in choroid plexus carcinomas and Functional role of platelet-derived growth factor receptor signaling in choroid plexus tumors

Tumors of the choroid plexus are rare and include only about 0.4-0.6% of all intracranial tumors, although more frequent in young children where they represent 2-3% of pediatric intracranial tumors. The tumors arise from the epithelial cells of the choroid plexus and are most often found in the lateral and fourth ventricles (Rickert and Paulus, 2001). Histologically there are three groups of choroid plexus tumors, the choroid plexus papillomas (CPP) which are WHO (World Health Organization) grade I, atypical choroid plexus papillomas (aCPP) (WHO grade II) and choroid plexus carcinomas (CPC) (WHO grade III) (Jeibmann et al., 2006).

CPPs, which are benign tumors, are commonly cured by surgery whereas the very malignant form CPC has a worse outcome. It is sometimes hard to distinguish between the two forms although CPP lack necrosis and is not invasive compared to CPC. The treatment is surgery followed by adjuvant chemotherapy in children or radiotherapy in adult patients. The best prognostic determinant is total resection which has proven difficult.

PDGFR signaling has been found in brain malignancies like glioma and medulloblastoma. In the first study we checked the PDGFR-α and -β expression by IHC and also the amplification of the corresponding genes by chromogenic in situ hybridization (CISH) in 22 choroid plexus carcinomas. We found that the majority expressed PDGFR. PDGFRβ was more frequently expressed than PDGFRα. Gene amplification was also more frequent for the PDGFRB and this was significantly correlated to protein expression.

In the second study we extended the samples to also look at the WHO grade I choroid plexus papillomas and the WHO grade II atypical choroid plexus papillomas. We also checked the activation status of both PDGFα and β receptors by in situ PLA in formalin fixed paraffin embedded tumor tissue using a general phosphotyrosine antibody.

Both PDGFR-α and -β were frequently expressed in the choroid plexus tumors. The α-receptor was less expressed in the carcinomas compared to the two papillomas. Also the β-receptor was significantly less expressed in the carcinomas. In contrast the activated β-receptor was found more frequently in the carcinomas whereas the activated α-receptor did not differ between the tumor types.

To evaluate the effect of PDGFR activation we did proliferation assays in the immortalized rat epithelial choroid plexus cell line Z310 expressing the PDGF β-receptor. PDGF-BB stimulated cells showed increased proliferation compared to untreated cells which could be blocked by addition of imatinib.

These studies identify PDGF receptors as novel candidate drug targets for aCPP and CPC. It also shows, for the first time, that the in situ PLA can be used in archival formalin fixed paraffin embedded tissues.
2.2.4 Paper V

Predictive role of PDGF receptors in patients with recurrent glioblastoma treated with hydroxyurea monotherapy or with hydroxyurea and imatinib

Prognosis of patients diagnosed with glioblastoma remains poor despite recent advances in radiation therapy and chemotherapy. A biological heterogeneity of these highly malignant brain tumors exists that involves perturbation of signaling pathways of various growth factors and regulators of the cell cycle and apoptosis.

We have analyzed 101 glioblastoma samples from patients who participated in a randomized clinical trial that included 240 patients with recurrent glioblastoma treated with either hydroxyurea alone or in combination with imatinib.

The rationale in the present study was to examine the expression and amplification status of PDGFR-α and –β in glioblastoma tissue samples to assess the relationships between PDGF receptor status and the molecular features of glioblastoma, and the clinical outcome of the patients. In particular, an attempt was made to identify subsets of patients who might benefit from imatinib.

We have analyzed protein expression of PDGFR-α and -β, p53, EGFR and PTEN and gene amplification of PDGFRA.

Sixty-five (64%) of the 101 study patients were male and 36 (36%) female. Their median age at the time of randomization was 54 years, 58 (57%) were older than 50. A total of 44 patients (44%) received combination treatment with imatinib and hydroxyurea and 57 (56%) received hydroxyurea alone. The groups were well balanced with respect of age and gender, and expression of PDGFRα, p53, EGFR and PTEN. The overall survival of the patients treated with hydroxyurea and of those treated with the combination did not differ significantly (median, 165 days and 153 days, respectively; p= .68), closely resembling survival results from the whole population of the clinical trial.

Nuclear accumulation of p53 protein was present in 22 cases (22%), whereas loss of PTEN protein expression was encountered in 47 cases (47%). EGFR over-expression was observed in 19 cases (19%). No significant associations were present in pair-wise comparisons of these proteins.

PDGFRα was associated with male sex (p= .004), young age (p= .009) and loss of PTEN expression (p= .034).

Patients whose tumor lacked PDGFRα had more favorable survival than those whose tumor expressed PDGFRα (median survival 187 days vs. 135 days, p= .034), whereas age at diagnosis, gender, the type of systemic treatment administered, p53, EGFR, and PTEN were not significantly associated with survival.

When age at diagnosis (≤50 vs. > 50), gender (male vs. female), treatment (hydroxyurea and imatinib vs. hydroxyurea), p53 expression (positive vs. negative), PTEN expression (positive vs. negative), EGFR expression (positive vs. negative) and PDGFRα expression (positive vs. negative) were entered as cofactors in a Cox multivariate model, PDGFRα expression turned
out to be independently associated with survival \((p = .037, \text{ hazard ratio } 1.69, 95\% \text{ confidence interval } 1.03-2.78)\), whereas none of the other variables were significantly associated with survival in this model \((p > .20 \text{ for each variable})\).

Taken together, these data suggest a previously unrecognized role of PDGFR\(\alpha\) expression in predicting outcome of patients diagnosed with recurrent glioblastoma.

We next analyzed the effect of the PDGFR\(\alpha\) expression status on outcome within the subset of patients treated with hydroxyurea monotherapy and within the subset treated with the combination of hydroxyurea and imatinib. Patients whose tumor expressed PDGFR\(\alpha\) had unfavorable survival as compared to those whose glioblastoma did not express PDGFR\(\alpha\) in the subset of patients who were treated with hydroxyurea monotherapy (median survival time 142 days vs. 202 days, respectively; \(p = .006\)). No such association with PDGFR\(\alpha\) expression and survival was found in the subset of patients treated with the combination of hydroxyurea and imatinib \((p = .823, \text{ Figure 3})\).

These data suggests that imatinib may have altered the clinical behavior of glioblastomas that express PDGFR\(\alpha\).

### 2.2.5 Paper VI

**Prognostic significance of stromal PDGF \(\beta\)-receptor expression in human breast cancer**

This study aimed at characterizing PDGF receptor expression in six types of common tumors; lymphoma, breast, colon, lung, ovarian and prostate. In breast tumors the prognostic significance of PDGFR expression was also analyzed.

We used IHC with antibodies that have been characterized using formalin fixed paraffin embedded cells expressing either of the receptors (PAE\(\alpha\) and PAE\(\beta\)).

Both PDGF receptor \(\alpha\) and \(\beta\) were commonly expressed on fibroblasts and pericytes of all tumors but varied between tumors. The \(\alpha\)-receptor was less expressed than the \(\beta\)-receptor varying between 2\% in prostate tumors to 65\% in colon tumors. Fibroblast PDGFR\(\beta\) expression was most common in lung and colon tumors with 58\% and 68\% expression respectively. Ovarian tumors showed the least expression (11\%) whereas prostate and lymphomas had about 20\% PDGFR\(\beta\) expression.

Analyses of the PDGF \(\alpha\)- and \(\beta\)-receptors in the different tumor types failed to identify a significant association between the PDGF \(\alpha\)- and \(\beta\)-receptor expression in tumor fibroblasts. Among all the different tumor types analyzed, individual tumors were found that expressed either none of the receptors, the \(\beta\)-receptor alone or the \(\beta\)-receptor together with the \(\alpha\)-receptor.

Subsequent analyses focused on the relationship between PDGF \(\beta\)-receptor status of pericytes and fibroblasts in individual tumors. Both in colon and prostate tumors significant positive associations were observed between the PDGF \(\beta\)-receptor status of perivascular cells and stromal fibroblasts in individual tumors.

These observations thus demonstrate that PDGF \(\alpha\)- and \(\beta\)-receptors are independently regulated. Furthermore, the relationship between the expression of PDGF \(\beta\)-receptors in
fibroblasts and perivascular cells suggest a functional link between the PDGF receptor status of fibroblasts and perivascular cells.

In breast tumors we found associations between strong PDGFRβ in fibroblasts and high histological grade, ER and PR negativity, HER2 expression and proliferation rate. High stromal PDGFRβ expression was also associated with shorter recurrence free survival and breast cancer specific survival. This finding was most pronounced in premenopausal patients (multivariate analysis: RR=5.49 [1.33-22.74] 95% CI; p=0.02). This indicates a possible relationship between hormone receptor signaling via ER and stromal PDGFR.

These findings highlight the prognostic significance of stromal markers, and should be considered in ongoing clinical development of PDGF receptor inhibitors.

### 2.3 CONCLUSION AND FUTURE PERSPECTIVES

In summary, we have developed methodology for detection of cancer relevant proteins analyzed both in solution (Gyrolab bioaffy), and in tissue sections by *in situ* PLA.

Furthermore, by analyzing PDGF receptor expression in human tumors we have observed previously unrecognized clinically relevant associations between PDGF receptor expression and survival in GBM and breast cancer.

The results from the ongoing study analyzing Gyrolab bioaffy demonstrates that the Gyrolab platform can be used for analyses of activation status of signal transduction proteins derived from cultured cells. Also in archival frozen tumor tissues we were able to detect both Akt and pAkt. Titration experiments confirmed that the assay system could be used for quantitative purposes, since signal were proportional to input amounts. Our next step is to analyze more prostate tumors for pAkt and see if there is a prognostic relevance of pAkt. In the future, obviously the Gyrolab bioaffy format can be expanded to multiple other endpoints than pAkt. Some potentially interesting applications include analyses of receptor tyrosine kinases such as EGF-, PDGF- and VEGF-receptors which are now well validated cancer drug targets.

We have demonstrated that *in situ* PLA is a suitable technique for detection of phosphorylation of PDGFR in individual cells with high selectivity and sensitivity. Reliable and sensitive detection of protein tyrosine kinase receptor phosphorylation by *in situ* PLA in cultured cells and on tissue sections is a new tool of great potential value in basic research and in histopathology. The *in situ* PLA method should be suitable to investigate pathophysiological processes in inflammatory and neoplastic diseases, and it may be of value in the development of PDGFR inhibitors and for predicting the clinical response to tyrosine kinase inhibitors in patients. Moreover the *in situ* PLA technique could be used to visualize almost any functional protein state in a cell, including other protein modifications that can be recognized with binder molecules and protein-protein interactions.

Choroid plexus tumors were shown to frequently express PDGF receptors and using the *in situ* PLA we have also shown that these proteins are activated. This is the first study to show the value of the methodology in formalin fixed paraffin embedded tissues. Choroid plexus carcinomas usually have a poor outcome if surgery is not feasible since there is no good
treatment. We now have shown that these tumors express activated PDGFRβ. This implies that PDGF receptors might be targets for treatment of choroid plexus tumors.

PDGFRα is frequently expressed in progressive glioblastomas and associated with loss of PTEN, male sex and younger age. It is associated with unfavorable survival among patients treated with hydroxyurea, but not among those treated with the PDGFRα inhibitor imatinib plus hydroxyurea. In The overall study PDGFRα was associated with worse outcome. The next step will be to further analyze the activation status of the PDGFRα by in situ PLA. We have indications of worse outcome when tumors express activated PDGFRα. More samples are needed to get relevant statistics. We will try to collect more samples from the study and analyze expression and activation of PDGFRα.

Stromal PDGF α- and β-receptor expression is common, but variable and independent, properties of solid tumors. In breast cancer, high stromal PDGF β-receptor expression significantly correlates with less favorable clinico-pathological parameters and shorter survival. The prognostic significance of stromal PDGF β-receptor expression was particularly prominent in tumors from pre-menopausal women. These analyses of breast cancer revealed previously un-recognized associations between stromal PDGF β-receptor expression and tumor characteristics such as histological grade, tumor cell proliferation, ER status and HER2 status. The mechanisms underlying these relationships remain to be clarified in future studies. Since we saw a correlation between high fibroblast PDGFRβ expression and worse survival in premenopausal women the mechanism behind it would be interesting to explore. It would also be interesting to investigate if any relationships exist between stromal expression of PDGF receptors and outcome in other hormone dependent tumors like prostate cancer. We will also analyze the potential response predictive significance of PDGFR in breast cancer. In this context we have noted an association between stromal PDGFRβ expression and response to tamoxifen treatment, which should be further explored.
3 ACKNOWLEDGEMENTS

I would like to thank all people involved in the projects and other colleagues;

First I would like to thank my supervisor Arne Östman, first for letting me do my masters thesis is his lab at the Ludwig Institute for Cancer Research in Uppsala and then for taking me on as a PhD student in the move to CCK. Thanks for being an excellent supervisor, sharing your scientific knowledge and for always having time to help.

Tobias Sjöblom who helped me a lot in the lab the first three weeks of my masters project.

All the present members of the Östman group: Akira, Sabine, Betzy, Markus, Jeroen, Martin, Maja, Alessandra, Cristina
Especially: Åsa and Christina, we started in the lab at almost the same time and have shared almost everything these last six years, both good and bad in and out of the lab.

Past members of the group; Daniel, Patrick, Kai, Sefanjia, Carina, Masao, Irene, Frank, Annette, Mitchan, Claudia, Francesca, Kristian

All my collaborators; Mats Inganäs and Josefin Bolik at Gyros, Malin Jarvius, Irene Weibrecht, Ola Söderberg, Ulf Landegren, Fredrik Pontén, Patrick Micke and Tobias Sjöblom at Rudbeck, Martin Hasselblatt, Werner Paulus and Björn Koos in Munster, Karin Jirström and Göran Landberg in Malmö, Calle Heldin at LICR, Nina Nupponen and Heikki Joensuu in Helsinki, Gregor Dresermann in Dulmen, Jonas Bergh, Monica Nistér, Lars Egevad and Fredrik Petersson at the Department of Oncology-Pathology

The chairman of CCK, Tina Dalianis for creating a great working environment

Doktorandgruppen; Maria B, Maria L, Maya, Linn, Mira, Emma, Christina and Åsa for many nice dinners and chats!

People who have worked or are working on the 3rd floor, especially; Tanja, Katja, Mikael, Emma, Lars, Nathalie, Mahdi, Cheng, Liping, Simona, Dan, Eva B, Masako, Per, Mimmi, Eva, Marianne, Marja, Katarina, Rainer, Marcela, Anna-Lena, Johanna, Braslav, Tao, Lotte, Ulrika, Pinelopi, Bertrand

Other people at CCK, especially; Jeremy for being a great JC organizer, Maria Hägg, Rona, Inga, Ulrika, Tamador, Fredrik, Anna, Kalle, Annki, Stig, Bertha, Pålraig, Walid, Anna

People who have been helping a lot with different things, Ann-Gitt, Eva-Lena, Marie, Joe, Sören, Elle, Elisabeth, Emily, Anders, Evi, Anki and Monica

Margareta, Liss and Inger for all sectioning help

Till alla nära och kära, ni vet vilka ni är och att ni betyder mycket för mig!

En tumör uppstår på grund av genetiska förändringar i en cell som gör att cellen kan växa oberoende av tillväxtsignaler. Två olika genetiska förändringar är avgörande för tumörtillväxt; antingen har onkogener blivit aktiverade eller så har cellen förlorat tumörsuppresorgener.

Solida tumörer i organ som bröst, prostata, lung och colon består inte endast av tumörceller utan även stödjeceller såsom fibroblaster, immunceller och celler som bygger upp tumörblodkärl. Tumörer kan vara både godartade och maligna. Godartade eller beninga tumörer är lokaliserade till ett organ. Maligna tumörer, å andra sidan, invaderar oftast närliggande organ och kan även förflytta sig via blodkärl till avlägsna organ, och bilda dottertumörer, så kallade metastaser.

Den nyaste formen av läkemedel för behandling av cancer är specifika, dvs riktade mot ett speciellt målprotein till skillnad från cytostatika. Dessa läkemedel, tyrosinkinas hämmare, används nu som behandling för många tumörformer. I takt med att dessa nya läkemedel utvecklas behövs nya metoder för att analysera uttryck och aktiveringsstatus av dessa läkemedels målproteiner i tumören.

Syftet med detta projekt var att utveckla nya metoder för att titta på förekomsten av olika cancerrelevanta proteiner i olika tumörer och även analysera om ett specifikt protein, PDGF receptorn, har någon prognostisk relevans i tumörer. Detta protein är, i de flesta tumörer, inte uttryckt i de maligna cellerna utan i cellerna som omger tumörcellerna, fibroblasterna, och i celler som är del av tumörblodkärlen.

Vi har utvecklat två olika metoder för att titta på relevanta proteiner i humana tumörer, både i lösning och på vävnadsnitt, Gyrolab bioaffy och in situ Proximity Ligation Assay. Dessa metoder kan förhoppningsvis i framtiden användas för bättre prognosbedömning och för att identifiera patienter som har nytta av specifika behandlingar.

I analyser av en stor serie brösttumörer fann vi att högt PDGF receptoruttryck var förknippat med sämre överlevnad. Detta samband var särskilt starkt bland yngre kvinnor med bröstcancer.

I analyser av en ovanlig form av barnhjärntumörer, choroid plexus tumörer, kunde vi visa att aktiverad PDGF receptor förekom i de mer maligna formerna av denna tumörtyp. Cellodlingsförsök antydde att växten av dessa tumörceller drivs av PDGF receptorer. Sammantaget föreslår dessa studier att hämmare av PDGF receptorer kan vara till nytta som behandling för dessa tumörer.

Analyser av mer än hundra glioblastom, en elakartad hjärntumör, visade också ett samband mellan högt PDGF receptoruttryck och dålig prognos. Studierna antydde också att patienter med högt PDGF-uttryck kan ha nytta av behandling med PDGF receptor hämmare.
Sammanfattningsvis; vi har utvecklat metoder som kan användas för analys av tumörbiologiskt relevanta proteiner, Gyrolab bioaffy och *in situ* PLA. Dessutom har tumöranalyser visat kliniskt relevanta, förut okända associationer mellan PDGF receptorer och överlevnad i GBM och bröst cancer.
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