TECHNISCHE UNIVERSITÄT MÜNCHEN

Institut für Chemie

An Analysis of Genetic Alterations of the Epidermal Growth Factor Receptor and the Tyrosine Kinase 2 in Human Cancer Cell Lines

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Vollständiger Abdruck der von der Fakultät für Chemie der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften (Dr. rer. nat.)

genehmigten Dissertation.

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Die Dissertation wurde am 14.04.2011 bei der Technischen Universität München eingereicht und durch die Fakultät für Chemie am 08.03.2012 angenommen.

“It always seems impossible until it is done!”

– Nelson Mandela

Acknowledgement

This thesis is part of a larger effort of the SOG Programme to identify and categorize genetic alterations in both receptor and cytoplasmic PTKs across a panel of 254 human cancer cell lines of diverse origins frequently used in scientific research. I owe all members of the SOG group a big thank you for the help you have rendered to me for the completion of this thesis.

Foremost, I would like to express my gratitude to Prof. Axel Ullrich, who has given me the opportunity to advance my career and has done so much at the university, the MPI and IMB to ensure everything runs smoothly for me. I also remember his words of wisdom and encouragement, and am grateful for him being forgiving on the mistakes I have made.

I would also like to thank Prof. Dr. Nediljko Budisa for agreeing to represent me at the university, for taking care of academic as well as administrative issues and for the many kind words of support.

I would like to thank Dr. Jens Ruhe for his contribution in conceptualizing and laying the foundation of the thesis, ensuring that many administrative issues in both Singapore and Germany are well taken care of, checking the various English-to-German translations of documents, and making my visit to Germany a wonderful experience.

The thesis has certainly improved with the many intuitive remarks from Dr. Han-Kiat Ho, who has diligently and carefully read my manuscript. More so, I am grateful for his advices on experimental design, the art of thesis writing and career guidance.

Similarly I would also like to thank Dr. Boon-Tin Chua for helping to streamline the thesis, for giving many useful advices and reminders at work, and together with Dr. Ho, for ensuring sufficient funding and time to carry out my work and to write.

I would also like to thank Dr. Stefan Hart and Dr. Sylvia Streit for useful comments on the thesis and words of encouragement.

Importantly, I am very grateful for the generosity everyone has given me, especially from Prof. Ullrich, Dr. Ho and Dr. Chua, on the amount of flexibility in the designing and performing of the experiments and the amount of time for writing. I would also like to thank Prof. Ullrich, Dr. Ho, Dr. Chua, Dr. Ruhe and Dr. Hart for the numerous insightful comments that have greatly improved the thesis.

I would like to give special thanks to Miss Winnie Wong, who has provided me with a lot of useful work-related information and helped me in numerous experiments. I would also like to thank Miss Priscilla Foo for performing some initial cloning work on the Tyk2 project.

My time in the lab has been wonderful and to a large extent stress-free, thanks to all the help, advices, good food, jokes, soccer talk and laughter from all present and previous members of the group. I am blessed to have met all of you.

I would like express my appreciation to all others at IMB, Singapore and at MPI, Germany who have, in one way or another, helped me and given me advice. I would also like to apologize to those whom I have unknowingly and unintentionally missed out in this acknowledgement.

Lastly I would like to thank my wife Hui-Hoon Chua for the helpful work-related discussions and more importantly, for her support and encouragement. I am very grateful that she has taken care of most issues within the family so that I can concentrate on my work, especially during the difficult period of my mother's chemotherapy sessions.

Abbreviations

#	Number
%	Percent
bp	Base pairs
°C	Degree Celsius
cm^2	Square centimetre
C_t	Cycle threshold
DNA	Deoxyribonucleic acid
g	Gravitational force
g / mg / μg / ng	Gram / milligram / microgram / nanogram
h	Hour
IB	Immuno-blotting
IC_{50}	Half maximal inhibitory concentration
IU	International unit
kb	Kilo-base pair
kDa	Kilodalton
l / ml / μl	Litre / milli-litre / micro-litre
mA	Milli-Ampere
mm / μm / nm	Millimetre / micrometre / nanometre
M / mM / μM / nM	Molar / millimolar / micromolar / nanomolar
min	Minute
OD	Optical density
pH	pH value [the negative logarithm (base 10) of the molar concentration of dissolved hydrogen ions in a solution]
pmol	Pico-mole
RNA	Ribonucleic acid
rpm	Rotations per minute
s	Second
Taq	*Thermus aquaticus*
UV	Ultra-violet

v/v	Volume per volume (percentage of a solution)
WT	Wild type
w/v	Weight per volume (percentage of a solution)
X	Times (equivalent to “fold increase”, usually used to indicate strength of a reagent or magnification)

Abstract

Signalling via protein tyrosine kinases (PTKs) is an essential component of many physiological processes. Its deregulation, sometimes a result of genetic alterations of PTK genes, is often associated with disease states including cancer. Here, genetic alterations of the *egfr* and the *tyk2* genes found in cancer cell lines were investigated for oncogenic properties. In the EGFR study, 10 cell lines, each carrying a genetic alteration of EGFR, were screened for sensitivity towards the EGFR tyrosine kinase inhibitor (TKI) gefitinib as a means to identify drug-sensitizing alterations. One cell line, RL95-2 (EGFR-A289V), displayed gefitinib sensitivity. Subsequently additional studies were performed on the cell lines SW-48 (G719S), RL95-2 and the P753S mutation detected in SK-MEL-28. SW-48 is resistant to gefitinib despite carrying the established EGFR TKI-sensitizing mutation G719S. Results presented here suggest that the resistant phenotype is due to a lack of EGFR protein expression but not activating mutations of *braf* and *kras* genes. Next, a delayed resistance phenotype of RL95-2 towards gefitinib was observed, which is more pronounced at low cell densities and is not associated with secreted autocrine / paracrine factors. Thirdly, stably expressed EGFR-P753S protein displayed increased auto-phosphorylation at major phosphorylation sites Y1068 and Y1173, indicating that P753S is an activating mutation. However, SK-MEL-28 is resistant to gefitinib, which may be attributed to a lack of EGFR protein expression and/or the activating BRAF-V600E mutation. On the other hand, the transforming capability of Tyk2 and its involvement in the invasion of cancer cells have been recently demonstrated. It is envisaged that an activating genetic alteration of Tyk2 may be oncogenic, and thus in the Tyk2 study an attempt was made to identify such alterations among the 12 found in various cancer cell lines earlier. One substitution, R901Q, confers constitutive activity to Tyk2 and over-expression of this protein results in increased basal phosphorylation of ERK1/2 and STAT5, all of which are properties of activating onco-proteins. siRNA-mediated knock-down of Tyk2 in the ovarian cancer cell line IGROV-1, where the mutation was detected, abolishes STAT3 phosphorylation and impairs migration. Furthermore, Tyk2-mediated STAT3 activity in IGROV-1 is not associated with the expression of 6 previously reported downstream target genes of STAT3 signalling. In all, the current study has investigated potential mechanism(s) undermining drug sensitivity conferred by EGFR TKI-sensitizing mutations in 3 cancer cell lines and has established novel, oncogenic

properties of the EGFR-P753S and the Tyk2-R901Q proteins. Moreover, the Tyk2-R901Q protein may play a role in the migratory responses of cancer cells. Therefore, these 2 proteins represent potential targets for anti-cancer therapeutic interventions.

Zusammenfassung

Deregulierte Signaltransduktion infolge abnormer Aktivität von Proteintyrosinkinase(PTK)-Varianten wird bei Krebs oft beobachtet. Hier wurden Varianten zweier PTKn, EGFR und Tyk2, auf onkogene Eigenschaften untersucht. Zwei mutierte Formen, EGFR-P753S und Tyk2-R901Q, zeigten erhöhte Autophosphorylierung an mehreren für die Aktivität wichtigen Tyrosinen. Ektopische Expression von Tyk2-R901Q erhöht ERK1/2- und STAT5-Aktivität, siRNA-vermittelte Reduktion endogenen Tyk2-R901Q-Proteins in IGROV-1 Zellen blockiert STAT3-Phosphorylierung und verringert Zellmigration. Diese Tyk2-vermittelte STAT3-Aktivität beeinflusst nicht die Expression 6 bekannter STAT3-Zielproteine und deutet die Existenz neuer Zielmoleküle an. SW-48-Zellen mit der Gefitinib-sensibilisierenden Mutation G719S erwiesen sich als gegen den Wirkstoff resistent, was wohl auf geringe EGFR-Expression und nicht auf aktivierende *braf*- und *kras*-Mutationen zurückzuführen ist. Diese Studie hat neue onkogene Eigenschaften von 2 mutierten PTKn gezeigt und Mechanismen hinterfragt, die dem wirkstoffsensibilisierenden Effekt von EGFR-Mutationen entgegenwirken.

Contents

I. Introduction

Cancer is one of the world's leading causes of non-accident fatality, especially in the developed countries. In 2008, an estimated 12.7 million individuals were newly diagnosed with cancer worldwide, based on data collected by the World Health Organization (WHO) (Ferlay *et al.*, 2010). In the same year, 7.6 million cancer-related deaths were recorded, of which approximately half of them were attributed to malignancies of the lung, stomach, liver, colon and breast. Despite advances in diagnostic technologies (imaging and bio-marker screening) and an increased understanding of the aetiologies of some cancers that lead to the development and usage of preventive vaccines [against hepatitis B virus (HBV) and human papillomavirus (HPV)] as well as both cytotoxic and targeted anti-cancer drugs, the number of cancer deaths worldwide continues to increase annually, extending an uprising trend that was first observed when data became available in the 1930s and has persisted since then. It is projected that in 2030, this figure would reach a staggering 11.9 million. The tremendous loss of lives and the disease burden to the patient (potential loss of years of life due to poor health / disability and premature death), to their families as well as to national healthcare systems strongly justify the need to quickly develop even more effective anti-cancer therapeutics. In line with the view epitomized by the Chinese proverbial idiom that can be roughly translated as "in order to fight one's enemy, one has to first understand the enemy well", deciphering the underlying mechanisms leading to a cancer state is an essential step in finding an effective cure. Indeed, the lack of understanding of the anomalies that drive many cancers is one of the reasons why no effective cure is available for these cancers, which in turn explains in part why the number of cancer deaths keeps on increasing despite aggressive intervention. Therefore much work is still required before we are able to unveil the secrets behind these not so well-elucidated cancers and identify new targets for anti-cancer therapies.

Based on decades of research, the currently accepted model on the origin of cancer centres on the sequential accumulation of alterations in the genetic material of a cell as a result of events that may be of "internal" (for example, a failure to repair errors made during DNA replication) and/or "external" (for example, presence of carcinogens) origins. These genetic alterations confer the cell various capabilities to proliferate and to thrive, and at the same time escape surveillance and control mechanisms from the immune system of the host.

These capabilities were summarized by Hanahan and Weinberg (2000) as the 6 "hallmarks of cancer", namely the abilities to proliferate indefinitely, to produce its own proliferative signals, to become insensitive to anti-proliferative signals, to evade apoptosis, to promote blood vessel formation (to obtain oxygen and nutrients), and finally to migrate to distant sites. Recently, additional hallmarks associated with immune evasion and stress phenotypes were proposed (Figure 1) (Luo *et al.*, 2009). In all, these capabilities displayed by cancer cells lead to a disruption of homeostatis, a critical component in the maintenance of the integrity of the organism. The emergence of these capabilities is often a result of deregulation of the activities of protein tyrosine kinases (PTKs). Therefore PTKs represent important targets for the development of anti-cancer therapeutics.

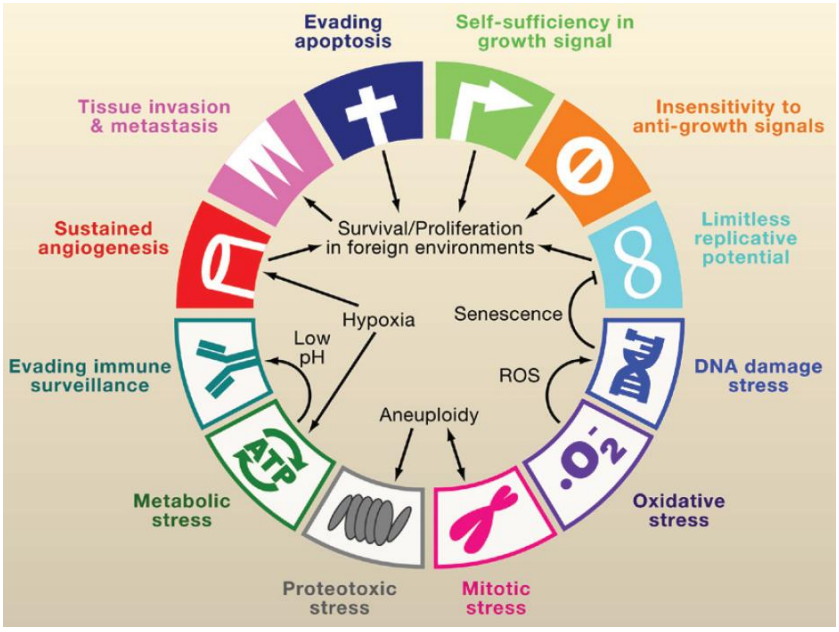

Figure 1. **An updated list of the hallmarks of cancer.** In addition to the 6 original hallmarks of cancer coined by Hanahan and Weinberg (2000) (top half of the diagram; white colour signs on coloured background), new hallmarks are recently proposed (bottom half; coloured signs on white background). They include altered metabolism of cancer cells, leading to the ability to escape immune surveillance (Kroemer and Pouyssegur, 2008) and 5 stress phenotypes and the interplay between these phenotypes. Multiple hallmarks may be affected by a single physiological condition. For example, hypoxia causes a switch to a less efficient, lactic acid-producing mode of glycolysis, leading to metabolic stress. It also promotes angiogenesis, which provides the cancer cells access to new sources of oxygen. ROS denotes reactive oxygen species. (from Luo *et al.*, 2009).

1.1 Protein tyrosine kinases

To maintain the homeostatic state, and thus the integrity of a cell, an organ or an organism, a tightly-regulated communication network between different components within each system is required. Deregulation of these networks often contributes to a wide spectrum of disease states, including developmental defects, infectious diseases, auto-immunity, metabolic diseases and cancer. In a disease-free cell, a myriad of physiological processes are regulated simultaneously by a choreographed effort of both spatial and temporal activation and inactivation of proteins, the major workhorses of cellular machinery. One of the mechanisms responsible for protein activation and inactivation is the reversible addition and removal of phosphate groups at specific tyrosine, serine or threonine residues of target proteins by kinases and phosphatases respectively. To date, there are 518 known kinases and 147 phosphatases (Manning *et al.*, 2002; Moorhead *et al.*, 2007). The kinases are further separated into 7 families, of which one of them is the tyrosine kinase family.

1.1.1 Structural characteristics

PTKs are enzymes that catalyze the transfer of the terminal γ-phosphate group of adenosine triphosphate (ATP) to the hydroxyl group of a tyrosine residue on target proteins. They are broadly separated, based on their cellular location, into 58 receptor and 32 non-receptor (cytoplasmic) PTKs (Robinson *et al.*, 2000). A receptor PTK consists of an extensively-glycosylated amino-terminal (N-terminal) extra-cellular domain, a short single-pass trans-membrane helix and a carboxyl-terminal (C-terminal) intra-cellular domain where the kinase activity resides (Figure 2). It primarily serves as a starting point for the conveyance process of the biological activities of ligands from outside the cell to the cell nucleus. As ligand binding occurs at the extra-cellular domain, it is logical to expect that the structural organization of the extra-cellular domains of individual receptor PTKs are highly variable, so as to provide specificity for binding to different ligands. In contrast, the basic structural organization of the cytoplasmic domains of all receptor PTKs are highly similar. This is also expected, as the reaction performed by all these domains is identical. The level of structural similarity is even more pronounced at the ATP binding pocket, the region where the transfer of the phosphate group to the substrate molecule actually occurs. The highly invariant

nature and the critical importance of this area for the function of the PTKs have been exploited in the design of numerous small molecule inhibitors against the proteins.

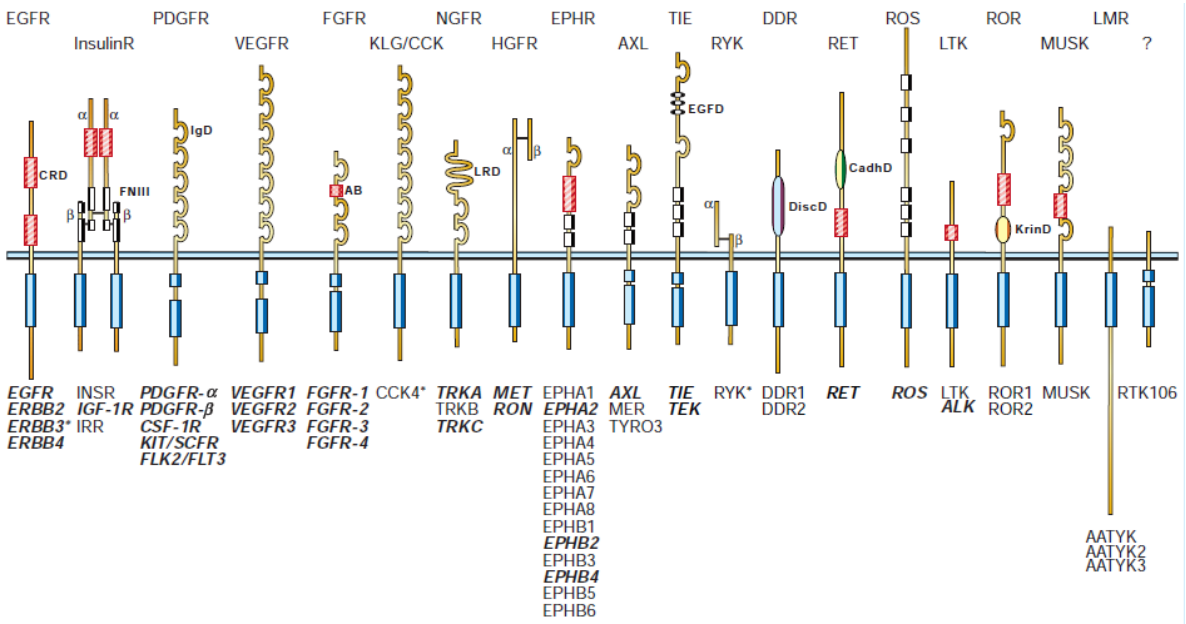

Figure 2. Domain structure of receptor proteins tyrosine kinases (PTKs). The domain arrangement of all known receptor PTK families is shown. While the cytoplasmic domains [containing the juxta-membrane domain, kinase domain (light blue box) and cytoplasmic tail] of various receptor PTK families are highly similar, the structural components comprising the extra-cellular domains are much more divergent. The insulin receptor family is the only receptor PTK family that forms dimers in the absence of ligand binding. Other family-specific features at the extra-cellular domain are indicated. The prototype receptor and all members of each receptor PTK family are shown at the top and the bottom of the Figure respectively. The symbols α and β denote distinct receptor subunits. Kinase-dead receptors are labelled with an asterisk, and receptors associated with cancers are in bold and italics. (from Blume-Jensen and Hunter, 2001).

The most significant difference between a receptor and a non-receptor PTK is the lack of a trans-membrane domain in the latter. As a result, a non-receptor PTK is not membrane-bound and thus does not have an extra-cellular domain. All non-receptor PTKs contain a kinase domain plus other modules required for inter-molecular interactions (Figure 3). These modules confer to non-receptor PTKs the capability of interacting with cytoplasmic proteins, lipids and/or DNA. This is in contrast to their counterparts in receptor PTKs, which interact with ligands. As these modules determine specificity for interaction partners, most families of non-receptor PTKs harbour unique combinations of these modules. Another feature of the non-receptor PTK worth mentioning is the way the kinase domain and the various modules required for interactions are organized. In contrast to the invariant

localization of the kinase domains of all receptor PTKs between the trans-membrane domains and the cytoplasmic tails, the kinase domains of some non-receptor PTKs are located in the middle portion [for example, the *c-abl* oncogene 1 (ABL) and the focal adhesion kinase (FAK) families] or near the N-terminal end [for example, the activated Cdc42-associated kinase 1 (ACK) family] of the proteins (Figure 3). Modules involved in inter-molecular interactions may be found at regions either proximal or distal to the kinase domain of various non-receptor PTKs.

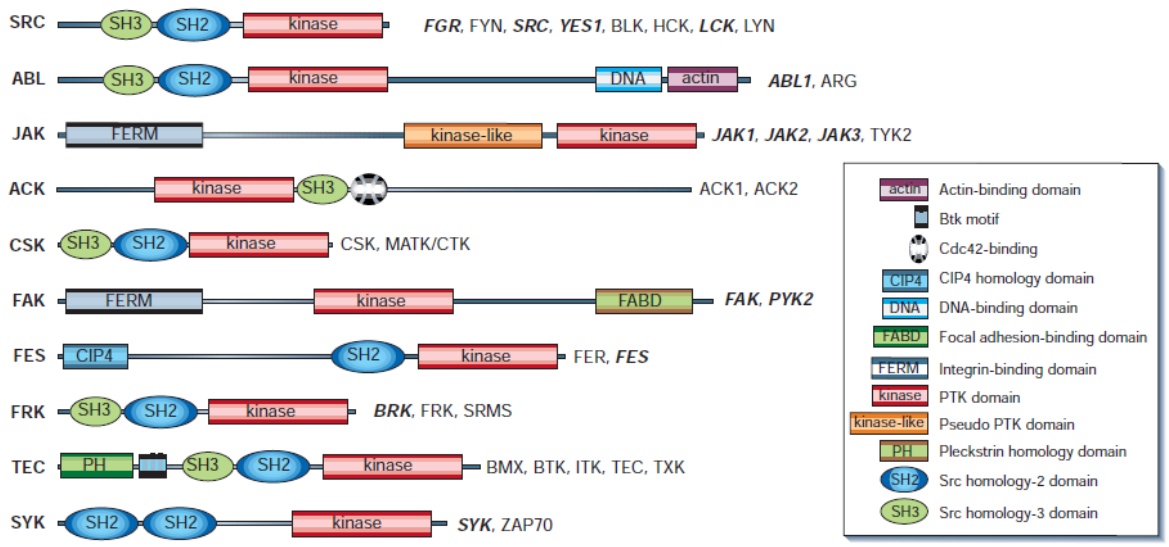

Figure 3. Domain structure of non-receptor proteins tyrosine kinases (PTKs). Besides the kinase domain (red bar), most non-receptor PTKs contain *src*-homology (SH)-2 and SH3 domains (blue and green ovals), which mediate interactions with other proteins through binding to phospho-tyrosines on these proteins. Janus kinases (JAKs) are the only non-receptor PTKs with a pseudo-kinase (kinase-like) domain (orange bar). The names of the non-receptor PTK families are listed on the left, and all members of each family are shown on the right. PTKs associated with cancers are in bold and italics. (from Blume-Jensen and Hunter, 2001).

1.1.2 Mechanism of activation

Receptor PTKs become activated upon ligand binding to the extracellular domain of a receptor PTK subunit, bringing the subunits into vicinity for dimerization to occur, forming a receptor complex. Depending on the structures and the binding characteristics of the ligand, a ligand-bound receptor PTK complex may contain 2 receptor subunits with (i). 2 mono-valent ligands, each bound to a receptor subunit [for example, the receptor for epidermal growth factor (EGF)] (Lemmon *et al.*, 1997; Ogiso *et al.*, 2002), or (ii). 1 bivalent ligand [for

example, the receptor for growth hormone (GH)] (de Vos *et al.*, 1992), or (iii). 1 naturally-occurring dimer of a mono-valent ligand [for example, the B-type receptor for platelet-derived growth factor (PDGF)] (Heldin *et al.*, 1989). Sometimes, a receptor PTK complex may contain 2 distinct receptor subunits of the same family, as seen in the *v-erb-b2* erythroblastic leukaemia viral oncogene (ErbB) family of receptor PTK hetero-dimers (Figure 4).

Ligand-induced dimerization of a receptor PTK results in receptor activation when 1 or more tyrosine residue(s) within the activation loop (A-loop) of the protein undergo auto-phosphorylation. It was suggested that receptor dimerization may promote auto-phosphorylation because the receptor subunits have a higher chance to trans-phosphorylate each other when they are aggregated. Alternatively, dimerization may stabilize the activated A-loop (reviewed in Hubbard *et al.*, 1998). This activated A-loop takes up an "open" conformation that allows trans-phosphorylations of multiple tyrosine residues at the cytoplasmic tails of the receptors. These phospho-tyrosines become targets of binding by *src* [*v-src* sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)]-homology 2 (SH2) domain-containing intra-cellular signalling molecules, which in turn relay the stimulatory signals towards the nucleus via signalling cascades (reviewed in Schlessinger, 2000).

The activation of a non-receptor PTK also centres on tyrosine phosphorylation at the A-loop and at other locations within the protein, similar to their cell surface counterparts. However, the trigger for activation of a non-receptor PTK is not a ligand. Most non-receptor PTKs are instead activated by cell surface receptors that do not possess intrinsic catalytic activity themselves. For example, the Janus kinases (JAKs), the FAKs and the TEC kinase are activated by cytokine receptors (described in more detail in Section 1.4.1.2), integrins (Parsons, 1996) and T cell receptor (TCR) (Yang *et al.*, 1999) respectively. Thus the main function of these non-receptor PTKs appears to be the provision of a catalytic activity module for these cell surface receptors.

1.2 Anomalies in PTK signalling and cancer

Signal transduction via PTKs is an important step in the execution of a wide range of cellular activities, some leading to cell growth and others to cell death. These signals, and thus the

activities of PTKs, need to be tightly regulated so as to maintain homeostasis and integrity of the organism (Blume-Jensen and Hunter, 2001). Disruption to this balance, which in certain scenarios may be attributed to uncorrected genetic alterations, often leads to diseases including cancer. These alterations may be acquired (somatic mutation) or predisposed in the genetic make-up of an individual (polymorphism), and may come in various forms, such as over-expression, gene rearrangement, insertion / deletion and amino acid substitution. The most well-known examples of cancer-related over-expression and gene arrangement would be the over-expression of ErbB2 [also known as human epidermal growth factor receptor 2 (HER2) or Neu] in about 30 % of metastatic breast cancer patients (Slamon *et al.*, 1987) and the translocation between the long arms of chromosomes 9 and 22 [t(9;22)(q34;q11)] leading to the generation of the constitutively active breakpoint cluster region protein (BCR)-ABL fusion protein in chronic myelogenous leukaemia (CML) patients (de Klein *et al.*, 1982). While these anomalies associated with the ErbB2 and the ABL proteins represent oncogenic genetic alterations, other anomalies may hit tumour suppressor genes resulting in a loss in the capability to keep cancerous cells in check, such as the inactivating mutations of the *tp53* gene (Hollstein *et al.*, 1991). It must be noted that while some dominant genetic alterations may be able to single-handedly drive cancers, the emergence of most other cancers is apparently attributed to a collective effect caused by many mutations, each of them contributing a small effect, pushing a cell towards the cancer state. Regardless of the functional consequences of the genes they hit, all these genetic alterations contribute towards the cancer state by exerting an effect on one or more of the hallmarks of cancer mentioned earlier (Figure 1).

As genetic alterations of PTKs are frequently observed as an underlying cause of cancers, PTKs are important targets in cancer research. Genetic alterations of some PTKs, such as those of the receptor PTK epidermal growth factor receptor (EGFR, also known as ErbB1), have been intensively studied. But even so, it appears that some aspects related to these alterations are still poorly understood [for example, secondary resistance against anti-EGFR tyrosine kinase inhibitors (TKIs) (section 1.3.1.3.6.2)]. On the other hand, genetic alterations of some other PTKs, including those of the non-receptor PTK tyrosine kinase 2 (Tyk2), are under-researched. Here, 1 receptor PTK and 1 non-receptor PTK were studied. In particular, alterations of the *egfr* and *tyk2* genes that may have properties related to cancer will be identified and investigated. It is envisaged that studying these genetic alterations will

broaden the understanding of the role of these 2 PTKs in cancer. In addition, some of these alterations may represent novel targets for the development of anti-cancer chemotherapeutics.

1.3 The ErbB family of receptor PTKs

1.3.1 Discovery

EGFR is the receptor PTK investigated in this study, and is also the first receptor PTK ever identified (Ullrich *et al.*, 1984). It is the component within cell membrane extract responsible for the *in vitro* EGF-induced protein phosphorylation in the presence of radioactive $^{32}\gamma$P-labelled ATP (Carpenter *et al.*, 1978). Further work led to the discovery that EGFR phosphorylation upon EGF stimulation occurs on tyrosine residues (Ushiro and Cohen, 1980), and that this tyrosine phosphorylation leads to the (also) tyrosine phosphorylation of cytoplasmic proteins downstream of the receptor (Hunter and Cooper, 1981). These discoveries on the mechanisms behind ligand-induced activation and downstream signalling of EGFR, together with similar observations made on the platelet-derived growth factor receptor (PDGFR) and insulin receptor (INSR) at about the same time, form the basis of the current understanding of signal transduction via receptor PTKs.

1.3.2 Basic characteristics

The sequence of the human EGFR protein is highly similar to that of the v-erbB protein of the avian erythroblastosis virus (Downward *et al.*, 1984). Since the avian v-erbB protein is responsible for the formation of fibrosarcomas in birds (Frykberg *et al.*, 1983), this observation provides the initial data supporting a potential role of EGFR in oncogenesis. Later, 3 other proteins that share a high sequence homology with EGFR were identified, namely ErbB2 (HER2 or Neu) (Coussens *et al.*, 1985), ErbB3 (HER3) (Kraus *et al.*, 1989) and ErbB4 (HER4) (Plowman *et al.*, 1993). Together, these 4 proteins formed the ErbB family of receptor PTKs.

The 4 human *erbB* genes are located on different chromosomes. The *egfr* gene was initially mapped to a region encompassing chromosome 7p13-q22 (Kondo and Shimizu, 1983) but is

now assigned to 7p12.3-p12.1. On the other hand, the *erbB2*, *erbB3* and *erbB4* genes are mapped to chromosomes 17q21.1 (Muleris *et al.*, 1997), 12q13 (Kraus *et al.*, 1989) and 2q33.3-q34 (Zimonjic *et al.*, 1995) respectively. Each of the 4 *erbB* genes is located near a distinct cluster of homeobox (Hox) and collagen genes (Zimonjic *et al.*, 1995). This observation suggests the presence of an ancient gene duplication event.

The 4 ErbB proteins are synthesized as polypeptides of approximately 1200 to 1350 amino acids in length, which undergo extensive glycosylation before translocation to the cell surface. Based on the length of the polypeptides, the mature ErbB proteins are predicted to be about 130 to 150 kDa in sizes. However, on Western blots they appear as approximately 170 to 180 kDa bands, which is likely a result of glycosylation. As the name indicated, ErbB receptors are primarily expressed in epithelial cells that line up, for example, the skin, the lung as well as gastro-intestinal (GI), respiratory, reproductive and urinary tracts. These receptors are also expressed in internal organs containing epithelial and endothelial cells, including the blood vessels, kidney, liver, muscles, neurons and the developing brain and heart (Kraus *et al.*, 1989; Real *et al.*, 1986; Srinivasan *et al.*, 1998). The presence of ErbB receptors in an extensive range of tissue types indicates that they are required for the proper functioning of many organs, which in turn underlie their importance for the survival and well-being of an individual. This view is further enhanced by the lethality displayed by single gene knock-outs of various *erbB* genes in mice. Dependent on the genetic background of the mice, an EGFR knock-out in mice confers either gestational or postnatal lethality (Miettinen *et al.*, 1995; Sibilia and Wagner, 1995; Threadgill *et al.*, 1995). EGFR-deficient mice that are born alive die within a week from a lack of proper development of epithelia at multiple organs, including the GI tract, lung and skin. On the other hand, mice with single knock-outs of either *erbB2*, *erbB3* or *erbB4* gene all die halfway during gestation from abnormal development of the heart and/or the central nervous system (CNS) (Gassmann *et al.*, 1995; Lee *et al.*, 1995; Riethmacher *et al.*, 1997).

The ErbB receptors form homo- and hetero-dimers, usually upon binding of ligands, in order to carry out their supposed activities (reviewed in Alroy and Yarden, 1997; Hynes and Lane, 2005; Olayioye *et al.*, 2000) (Figure 4). Both ErbB2 and ErbB3 possess unique features that contribute to their distinctive activities. Firstly, in contrast to the other ErbB proteins, ErbB2 does not have a natural ligand. This observation may be attributed to the fact that the extra-cellular domain of ErbB2 is organized differently as compared to the other 3 ErbB receptors,

which makes ligand binding impossible (Hynes and Lane, 2005). Moreover, the distinct conformation at the extra-cellular domain of ErbB2 appears to result in the protein assuming constitutive activity. This may explain why ErbB2 appears to be the favourite partner of dimerization for the other ErbB receptors, especially ErbB3 (Graus-Porta *et al.*, 1997; Tzahar *et al.*, 1996). Secondly, ErbB3 contains 2 unique alterations that affect residues critical for ATP binding and catalytic activity of other ErbB proteins (Kraus *et al.*, 1989). Therefore ErbB3 was initially thought to be kinase-dead, in spite of its ability to bind to neuregulins (Sierke *et al.*, 1997) (Figure 4). Surprisingly, very recently it was discovered that despite these 2 alterations, ErbB3 is still able to bind to ATP and also retains a low level of kinase activity (Shi *et al.*, 2010). In addition, ErbB3 is the only ErbB receptor that contains multiple recognition sites for the p85 subunit of the phosphatidylinositol 3 kinase (PI3K) (Kim *et al.*, 1994). In essence, ErbB2 needs to couple to other ErbB receptors to perform their activities and ErbB3 is a weakly active PTK that is capable of directly activating the PI3K-AKT pathway.

1.3.3 EGFR

1.3.3.1 Functional domains of EGFR

As the archetypal receptor PTK, EGFR is a single-pass trans-membrane glycoprotein that contains the extra-cellular ligand-binding domain, the trans-membrane domain, the catalytic domain and the serine-rich cytoplasmic tail (Figure 2) (reviewed in Jorissen *et al.*, 2003). The extra-cellular domain is further separated into 4 sub-domains (I to IV). The leucine-rich sub-domains I and III (especially sub-domain III) are required for ligand binding while the cysteine-rich sub-domains II and IV are involved in receptor dimerization (Garrett *et al.*, 2002; Ogiso *et al.*, 2002). In the absence of a ligand, sub-domains II and IV interact with each other resulting in a closed, inactive conformation that prevents the formation of the ligand binding site (which is made up of sub-domains I and III).

While the extra-cellular domain of the EGFR protein is involved in ligand binding and dimerization, the sequences surrounding the trans-membrane domain carry extra information on receptor dimerization as well as determinants on receptor trafficking between cell surface and cytoplasmic components. A region at the distal end of sub-domain

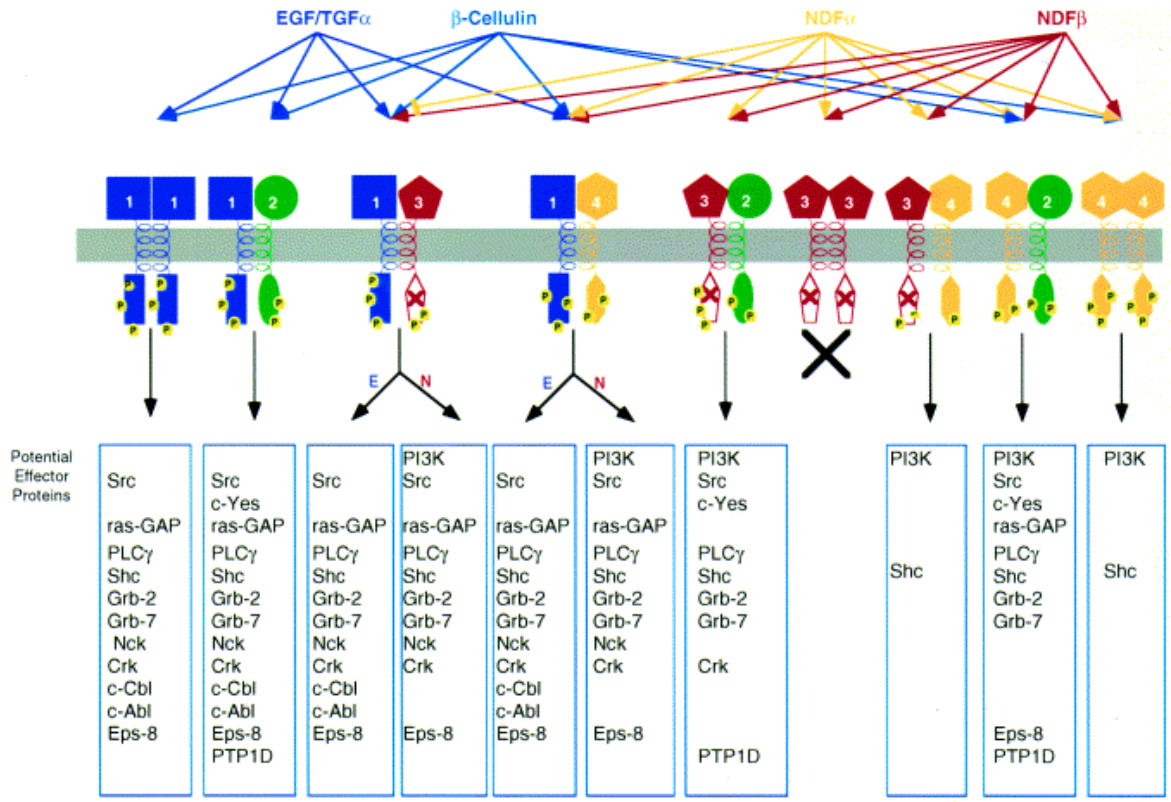

Figure 4. Stimulation of ErbB receptor homo- or hetero-dimers with epidermal growth factor (EGF)-related ligands. The 4 members of the ErbB family of receptor PTKs (denoted as receptors 1 to 4 in different shapes and colours) are activated by various ligands of similar structures, including EGF, transforming growth factor-alpha (TGF-α), betacellulin (and related ligands) as well as alpha and beta neu differentiation factor (NDF) (also known as neuregulins). Activation of a particular receptor dimer by a distinct combination of ligands results in receptor phosphorylation (denoted as yellow circles with the letter P) and subsequent activation of different combinations of downstream effector proteins. Moreover, stimulation of the same receptor dimer (of ErbB1+3 or ErbB1+4 combinations) by different ligands ("E" denotes EGF and "N" denotes NDF group of ligands) also generates different responses. The kinase-dead ErbB3 is labelled with an "X" at its kinase domain. Crk and c-Yes are the human homologs of the "*v-crk* sarcoma virus CT10 oncogene (avian)" and the "*v-yes-1* Yamaguchi sarcoma viral oncogene 1" respectively; Eps-8 denotes "EGFR pathway substrate 8"; Nck denotes the NCK adaptor protein 1; and PTP1D is also known as protein tyrosine phosphatase, non-receptor type 11 (PTPN11). The official, full-length names of the other adaptor proteins listed in this Figure are already mentioned in the main text. (from Alroy and Yarden, 1997).

IV (codons 557 to 617) is required for the aggregation of inactive EGFR receptors [which may account for approximately 65 % of all EGFR receptors on the cell surface (Mineo *et al.*, 1999)] to the caveolae / raft component of the cell membrane (Yamabhai and Anderson, 2002). On the other hand, the trans-membrane domain is required for the formation and stabilization of EGFR dimers (Chantry, 1995; Tanner and Kyte, 1999) and the di-leucine (LL) motif (codons 679 and 680) at the juxta-membrane region are necessary for receptor down-regulation but not internalization (Kil and Carlin, 2000).

Further downstream from the trans-membrane domain of EGFR is the kinase domain, which adopts a bi-lobar structure characteristic of the kinase domain of all PTKs. The smaller N-terminal lobe is made up of mainly β-sheets and 1 α-helix (the αC-helix), while the larger C-terminal lobe comprises of mostly α-helices. The kinase domain houses the ATP-binding pocket (at the interface between the 2 lobes) as well as other highly conserved elements critical for catalytic activity, such as the glycine-rich phosphate-binding loop (P-loop) (codons 695 to 700), the DFG motif (codons 831 to 833) and the A-loop (codons 831 to 852) (Stamos *et al.*, 2002). Finally, proximal to the kinase domain is the cytoplasmic tail region where the 6 major auto-phosphorylation sites of EGFR are located. Phosphorylations of these tyrosine residues provide docking sites for various downstream signalling proteins.

1.3.3.2 Signalling via EGFR

1.3.3.2.1 EGFR activation by EGF family of ligands

The major ligand of EGFR is, as the name of the receptor suggests, EGF. An EGFR dimer is bound by 2 EGF monomers (Ogiso *et al.*, 2002). This 2:2 stoichiometry is a characteristic unique to the EGF family of ligands. Binding of EGF results in receptor dimerization, which is either homo-dimerization or hetero-dimerization with ErbB2. EGFR also forms hetero-dimers with ErbB3 and ErbB4, albeit less frequently (Alroy and Yarden, 1997). ErbB receptor subunits brought together by EGF activate each other via auto- and trans-phosphorylation of multiple tyrosine residues, thus creating binding sites for SH2 domain-containing downstream signalling molecules (reviewed in Schlessinger, 2000). The major signalling pathways activated by EGFR include the MAP (mitogen-activated protein) kinase, PI3K-AKT (*v-akt* murine thymoma viral oncogene homolog 1) and JAK-STAT (signal transducers and activator of transcription) pathways (reviewed in Olayioye *et al.*, 2000 and Schlessinger, 2004; Olayioye *et al.*, 1999; Zhong *et al.*, 1994). Activation of these pathways leads to a myriad of cellular activities, ranging from proliferation and survival to apoptosis (Figure 5).

There are 6 major tyrosine residues within the kinase domain of EGFR that may be phosphorylated upon activation. Each of them is preferentially bound by different downstream signalling molecules (Figure 6). For example, tyrosine-1045 (Y1045) is recognized and bound by the RING-type E3 ligase called Cas-Br-M (murine) ecotropic

retroviral transforming sequence (c-CBL, named after Casitas B-lineage Lymphoma) protein, which tags the protein for ubiquitin-mediated degradation (Levkowitz *et al.*, 1999). On the other hand, Y1148 and Y1173 are binding targets of the *src*-homology 2 domain containing (SHC) transforming protein 1 (Okabayashi *et al.*, 1994; Rojas *et al.*, 1996), which lies upstream of the MAP kinase pathway. Y1173 is also recognized by phospholipase C – gamma (PLCγ) (Chattopadhyay *et al.*, 1999) and the negative regulator protein tyrosine phosphatase, non-receptor type 6 (PTPN6) (also known as SHP-1 and PTP-1C) (Keilhack *et al.*, 1998). Interestingly, Y1068 and Y1086 are able to bind to both STAT3 (Shao *et al.*, 2003) and the adaptor protein growth factor receptor-bound protein 2 (GRB2) (Batzer *et al.*, 1994). EGFR-bound GRB2 is able to recruit GRB2-associated binding protein 1 (GAB1) (Holgado-Madruga *et al.*, 1996), which in turn activates the PI3K-AKT pathway (Mattoon *et al.*, 2004).

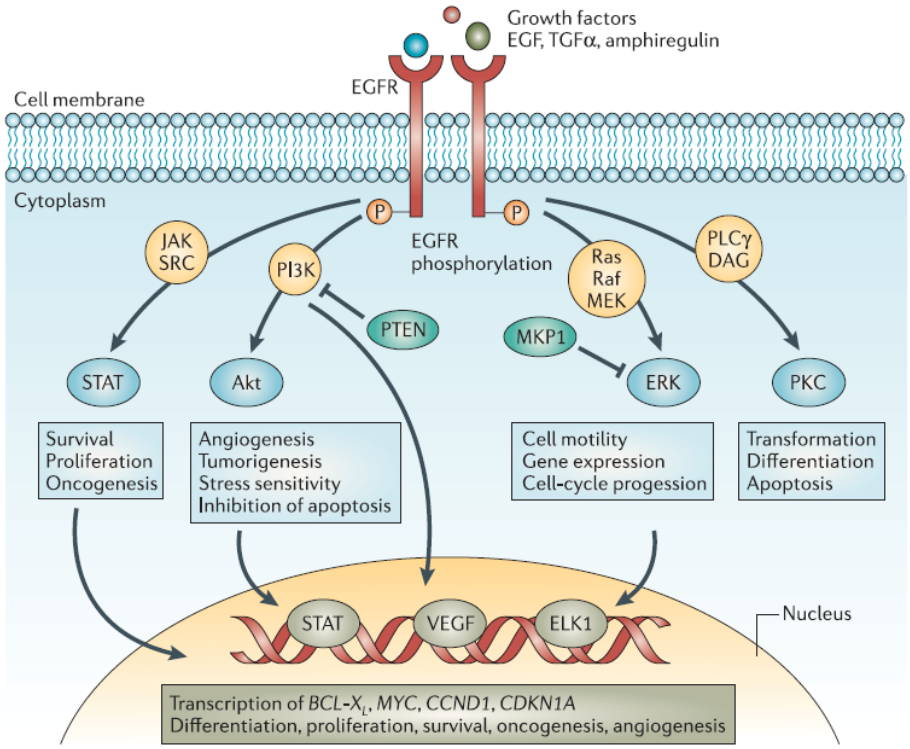

Figure 5. The major downstream signalling pathways of epidermal growth factor receptor (EGFR). Activation of EGFR by various ligands leads to increased activity of one or more of the following signalling pathways: MAP kinase, PI3K-AKT and JAK-STAT. EGFR activation also up-regulates PKC activity. Signalling activities of each pathway result in transcription of different sets of target genes, thus each pathway serves different functions (listed in light blue boxes). Negative regulators of the MAPK and AKT pathways (MKP1 and PTEN) are also shown. DAG denotes diacylglycerol, MEK denotes MAPK/ERK kinase 1, MKP1 denotes MAP kinase phosphatase 1 and CDKN1A denotes cyclin-dependent kinase inhibitor 1A (commonly known as p21 / Cip1). The full names of the other proteins included in this Figure are already mentioned in the main text. (from Nyati *et al.*, 2006).

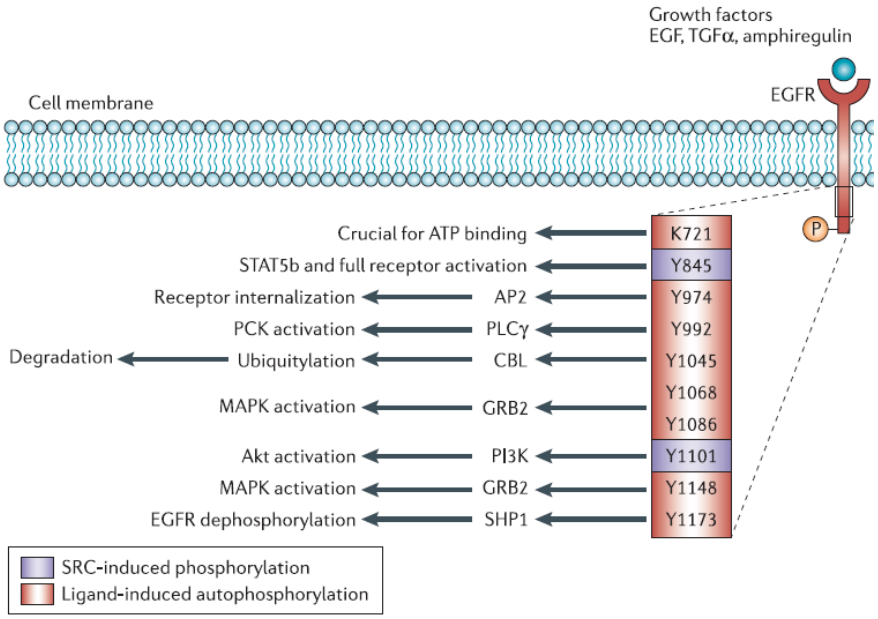

Figure 6. The major phosphorylation sites of epidermal growth factor receptor (EGFR) are binding sites for various effector proteins. Depending on the identity of the bound ligands and receptor dimerization partners, ligand-induced EGFR activation results in phosphorylation of various combinations of tyrosine (Y) residues at codons 992, 1045, 1068, 1086, 1148 and 1173. Each phospho-tyrosine residue provides a docking site for distinct adaptor protein(s), leading to the activation of various signalling pathways. Additional residues required for receptor activity and other functions are included. The tyrosine residues at codons 845 and 1101 become phosphorylated upon activation by cytoplasmic SRC-family kinases. AP2 denotes activating enhancer binding protein 2 alpha. The full names of the other adaptor proteins included in this Figure are already mentioned in the main text. (from Nyati *et al.*, 2006).

EGFR can also be activated by other members of the EGF family of ligands. Based on receptor specificity, they can be divided into 2 groups, namely (i). ligands that binds to EGFR only, including transforming growth factor-α (TGF-α), amphiregulin and epigen (and EGF), and (ii). ligands that bind to both EGFR and ErbB4, such as betacellulin, epiregulin and heparin-binding EGF (summarized in Carpenter and Cohen, 1990) (Figure 4). The primary activity of these ligands is identical to that of EGF, which is to promote epithelial cell growth (Dunbar and Goddard, 2000; Shirakata *et al.*, 2000; Shoyab *et al.*, 1989). This redundancy in ligand function is further demonstrated by the observation that the knock-out of one or more of these ligands results in healthy and fertile mice with only mild phenotypic differences (Luetteke *et al.*, 1999). Overall, the ability of EGFR to modulate multiple cellular activities stems from its ability to be stimulated by multiple ligands, to form combinations of dimers, and to recruit numerous adaptor proteins for signalling (Olayioye *et al.*, 2000). Further regulation of the strength and duration of stimulatory signals generated by EGFR is

provided by ligand-induced receptor internalization (Carpenter and Cohen, 1976) and recycling.

1.3.3.2.2 Trans-activation of EGFR

In addition to EGF and related ligands, activation of G-protein-coupled receptors (GPCRs) by various agonists also lead to activation of EGFR and the MAP kinase pathway, strongly indicating the presence of cross-talk between 2 different families of surface receptors (Daub *et al.*, 1996). Data from initial investigations suggested that some cells transmit signals generated from GPCRs to EGFR via cytoplasmic signalling molecules such as SRC-family kinases or protein kinase C (PKC) (reviewed in Fischer *et al.*, 2003). Later, Prenzel and colleagues (1999) identified cell surface zinc-dependent metallo-proteinases of the matrix metalloproteinase (MMP) and a disintegrin and metalloproteinase (ADAM) families as mediators between GPCRs and EGFR. The currently accepted sequence of events between GPCRs and EGFR is that agonist stimulation of GPCRs leads to activation of metallo-proteinases, which in turn cleave some of the membrane-bound precursors of EGF and related ligands (it should be noted here that all but one ligands of EGFR are initially produced as precursors). This cleavage, called ecto-domain shredding, releases the N-terminal soluble ligands, which subsequently bind to EGFR (reviewed in Fischer *et al.*, 2003 and Harris *et al.*, 2003). Overall, this indirect activation of EGFR by GPCRs is termed trans-activation. It is a process commonly observed in physiological processes such as mucin synthesis, as well as pathological scenarios, including bacterial infections, cardiac hypertrophy and cancers (reviewed in Fischer *et al.*, 2003).

1.3.3.2.3 Other means of EGFR activation

Besides EGF family ligands and trans-activation, EGFR may be activated by alternative physiological events including hyper-osmotic shock and calcium influx. JAK2 (upon stimulation by GH) and integrins are also able to activate EGFR (summarized in Gschwind *et al.*, 2004).

1.3.3.3 Genetic anomalies leading to a deregulation in EGFR signalling may lead to cancer

EGFR is the first receptor PTK in which deregulation of its signalling activity is associated with the cancer state (reviewed in Gschwind *et al.*, 2004). Anomalies leading to defective EGFR signalling can be roughly grouped into gene amplification leading to over-expression, excessive ligand stimulation, activating genetic alterations (including splice variants) and defective receptor down-regulation (Gschwind *et al.*, 2004). A cell may be driven towards a cancer state due to the generation of excessive amount of proliferative signals as a result of the presence of too many receptors and/or ligand molecules. These ligands may be released by the cell itself or from neighbouring stromal cells, forming autocrine or paracrine loops respectively (Salomon *et al.*, 1995). In support of this view, over-expression of the metallo-proteinase ADAM17 [also known as tumour necrosis factor-alpha (TNF-α) converting enzyme (TACE)] is associated with increased EGFR activity in primary breast cancers (Borrell-Pagès *et al.*, 2003). Furthermore, lysophosphatidic acid (LPA)-mediated EGFR trans-activation via ADAM17 activity leads to EGFR trans-activation and subsequent DNA synthesis, migration and cell cycle progression in head and neck squamous cell carcinoma (HNSCC) cells (Gschwind *et al.*, 2002). Additional examples of EGFR trans-activation in other cancer types were summarized by Fischer and colleagues (2003).

Another group of genetic anomalies that may also lead to deregulated EGFR signalling is activating genetic alterations, which empower EGFR to generate proliferative signals in the absence of ligand stimulation. These alterations can be further grouped into in-frame deletions of various lengths at the extra-cellular domain, as well as point mutations, small deletions and/or insertions at the kinase domain of the receptor. The most common naturally-occurring extra-cellular domain deletion of EGFR is the variant III (EGFRvIII) (Pedersen *et al.*, 2001). This variant, as well as other EGFR ecto-domain deletions have been detected in multiple cancer types, mainly in glioblastomas (Libermann *et al.*, 1985; Wong *et al.*, 1992; Yamazaki *et al.*, 1988) but also in non-small cell lung carcinomas (NSCLCs) and cancers of colon and ovary (Moscatello *et al.*, 1995). The messenger RNA (mRNA) of EGFRvIII is generated from the skipping of exons 2 to 7 of the *egfr* gene during transcription. The resultant truncated protein lacks most of the extra-cellular domain and is thus unable to bind to any ligand. Despite this inability, EGFRvIII is constitutively active (Yamazaki *et al.*,

1988). This phenomenon may be attributed to the fact that the deletion responsible for EGFRvIII removes domain II at the extra-cellular domain, and at the same time the ability to assume an inactive conformation in the absence of ligand (Hynes and Lane, 2005). Over-expression of EGFRvIII in NIH-3T3 cells results in oncogenic transformation and constitutive activation of Jun oncogene (c-Jun) product via the PI3K-AKT pathway (Antonyak *et al.*, 1998). In contrast, the MAP kinase pathway is not activated by EGFRvIII (Moscatello *et al.*, 1996). Therefore the profile of signalling proteins activated by EGFRvIII is qualitatively (and quantitatively) different from the signals generated from wild-type EGFR (Pedersen *et al.*, 2001).

The other group of cancer-related activating EGFR alterations mentioned above affect residues within the kinase domain of the protein. These mutations are almost exclusively found in NSCLC, which contributes to about 80 % of all cases of lung cancers. They were first discovered as mutations predictive of a favourable clinical response towards anti-EGFR small molecule TKI treatment in NSCLC patients (Lynch *et al.*, 2004; Paez *et al.*, 2004; Pao *et al.*, 2004), and they would be described in more detail later (Section 1.3.3.5).

1.3.3.4 Targeted chemotherapeutics against EGFR

1.3.3.4.1 A need to develop targeted cancer chemotherapeutics

Currently, the standard treatment regime for many types of cancer is surgical resection (of resectable solid tumours only) followed by ionizing irradiation and/or conventional cytotoxic chemotherapeutics. Based on their chemical structures, modes of action and/or molecular targets, conventional chemotherapy drugs are divided into the following categories under the WHO's Anatomical Therapeutic Chemical Classification System: (i). alkylating agents (that covalently modify guanine bases and thus directly damage DNA), (ii). anti-metabolites (nucleotide analogues that are incorporated into newly synthesized DNA and/or RNA molecules, causing damage to genetic material), (iii). topoisomerase inhibitors (that interfere with DNA super-coiling), (iv). anthracyclines (that intercalate DNA, inhibit topoisomerase and generate free radicals) and (v). mitotic inhibitors (that prevent microtubule function). These compounds act on fundamental cellular processes central to the survival of both healthy and cancerous cells. As a result of a loss of healthy cells, and

thus vital physiological functions, these cytotoxic drugs often cause systemic and severe side effects (including myelo-suppression, toxicity to internal organs and distress to the lining of the GI tract, leading to vomiting and diarrhoea). The clinical benefit brought by the drugs is often accompanied by unpleasant, sometimes even life-threatening, experience. Unfortunately, such a regime is still in use for most cancers because a superior treatment option with fewer and milder side effects is not available. Thus similarly effective chemotherapeutics but with more tolerable side effects are urgently needed. Since the killing of healthy cells by cytotoxic drugs underlies the severe side effects associated with the drugs, drugs that target only the cancerous cells but spare the healthy ones represent the logical solution to this issue. Cancer cells are distinguishable from healthy cells by the molecular defects they display as a result of genetic alterations they have gathered throughout their development. Drugs that specifically target and abolish these defects should therefore affect cancer cells only. Based on this, novel targeted chemotherapeutics have been developed for the treatment of cancers, including neutralizing antibodies and small molecule TKIs, among which, TKIs have made the most significant clinical breakthroughs. Encouragingly, the side effects of these targeted drugs are, as predicted, more tolerable than those of conventional drugs, thus validating the approach of targeted chemotherapies.

1.3.3.4.2 Specific anti-EGFR antibodies

Since constant up-regulation of EGFR signalling is a feature in many cancer types, it appears logical to remove signals generated from the receptor as a form of therapeutic intervention. One strategy to remove these signals is to use anti-EGFR neutralizing antibodies to block the ligand binding domain of the receptor, thus disrupting EGF / TGF-α autocrine loops (Mendelsohn, 1992). Accordingly, 2 groups have generated specific anti-EGFR monoclonal antibodies from the EGFR over-expressing epidermoid carcinoma cell line A-431 (Schreiber *et al.*, 1981) and from partially purified EGFR protein (Sato *et al.*, 1983). Further modifications of the mAb225 antibody generated by Sato and colleagues lead to cetuximab (IMC-C225 or Erbitux®, ImClone Systems / Merck, New York, USA), a chimeric antibody that consists of human IgG_1 constant domain joined to a murine variable domain. It is currently one of the 2 anti-EGFR antibodies approved by the United States Food and Drug

Administration (USFDA) for the treatment of cancer, and is indicated for (i). colorectal carcinoma unresponsive to previous treatment with irinotecan- and oxaliplatin-based compounds and (ii). HNSCC [US National Cancer Institute (NCI) website]. The other approved antibody is panitumumab (ABX-EGF or Vectibix®, Amgen, Thousand Oaks, California, USA), a fully humanized IgG_2 monoclonal antibody indicated for EGFR-positive metastatic colorectal carcinoma. These antibodies abolish EGFR activation primarily by preventing ligand binding. In particular, cetuximab binds exclusively to domain III at the extra-cellular region of EGFR with an affinity approximately 50-fold higher than EGF (Li *et al.*, 2005). Additionally, EGFR-bound antibodies may promote receptor internalization and degradation (Sunada *et al.*, 1986) and trigger an immune response when immune cells [such as natural killer (NK) cells] recognise and bind to the constant region of these antibodies (Clynes *et al.*, 2000). Currently, more anti-EGFR antibodies are under development and/or clinical evaluation for USFDA approval. Examples of these candidate drugs include necitumumab, nimotuzumab and zalutumumab [US National Institute of Health (NIH) clinical trials website]. Some of them are already in clinical use in some countries outside USA and Europe though.

1.3.3.4.3 Specific anti-EGFR TKIs

Besides blocking ligand binding, another strategy taken by researchers and companies in the development of EGFR-specific chemotherapeutics is to inhibit the catalytic activity of the receptor. In 1994, 4-(3-chloroanilino)-quinazoline was identified as a lead compound after searching through a large chemical structure database (Ward *et al.*, 1994). This class of compounds (4-anilinoquinazolines) compete with ATP for binding to the lysine-745 (K745) residue within the ATP-binding pocket of the EGFR protein (Barker *et al.*, 2001; Wakeling *et al.*, 2002; Ward *et al.*, 1994) and thus are reversible inhibitors. Further development resulted in the creation of 2 USFDA-approved, orally available EGFR TKIs for the treatment of cancer, namely gefitinib (ZD1839 or Iressa®, AstraZeneca, London, UK) and erlotinib (OSI-774 or Tarceva®, Genentech, San Francisco, California, USA) (Figure 7).

Gefitinib was initially approved in May 2003 as a third-line mono-therapy for locally advanced or metastatic NSCLCs that have failed previous first-line platinum-based and second-line TAXOL® (Bristol-Myers Squibb, New York, USA)-based chemotherapies (Cohen

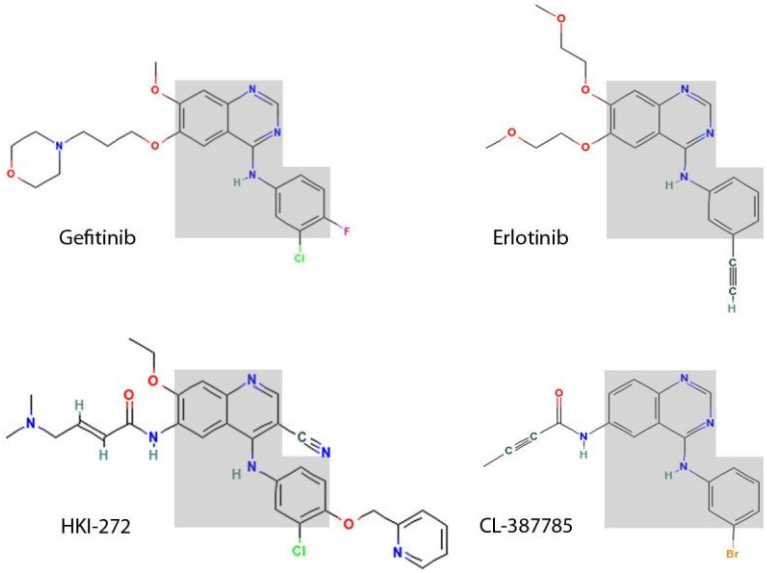

Figure 7. Chemical structures of selected small molecule EGFR tyrosine kinase inhibitors (TKIs). The structures of the reversible inhibitors of EGFR gefitinib and erlotinib, as well as that of the irreversible inhibitors CL-387785 and HKI-272 are shown. Note the quinazoline moiety central to all 4 structures (shaded area). (images were taken from the NCBI PubChem website).

et al., 2004), based on an overall average of approximately 10 % objective response rate in clinical trials (Fukuoka *et al.*, 2003; Kris *et al.*, 2003). However, addition of the drug to the combinatory treatment regime of cisplatin plus gemcitabine (Giaccone *et al.*, 2004) and paclitaxel plus carboplatin (Herbst *et al.*, 2004) did not result in any improvement in multiple clinical endpoints. Therefore gefitinib was not indicated by USFDA for combinatorial chemotherapy with currently available cytotoxic anti-cancer drugs. Later in November 2004, erlotinib was approved as a mono-therapy for the treatment of locally advanced or metastatic NSCLCs that did not respond to at least 1 round of chemotherapy (Johnson *et al.*, 2005), based on increased overall survival (Pérez-Soler *et al.*, 2004; Shepherd *et al.*, 2005). Additional approvals to erlotinib were subsequently granted for pancreatic cancer in November 2005 (Moore *et al.*, 2007) and maintenance therapy for NSCLCs in April 2010 (US NCI website). The side effects of these TKIs, which mainly consist of rashes and diarrhoea, are more tolerable than conventional cytotoxic chemotherapies. The types of side effects associated with these TKIs correspond to the physiological role of the receptor in epithelial cells of the skin and the GI tract.

The initial euphoria on the effectiveness of the above-mentioned EGFR TKIs on selected patients (described in more detail in the next section) was later replaced by the emergence of secondary resistance in treated patients who suffered from a relapse (Kobayashi *et al.*, 2005; Kwak *et al.*, 2005; Pao *et al.*, 2005a). This consequently kick-started the development of novel inhibitors that irreversibly bind to the ATP-binding pocket of EGFR, based on the belief that they are able to circumvent the effects of the secondary mutations (Kwak *et al.*, 2005). To date, many candidate irreversible inhibitors are undergoing clinical evaluation (summarized in Sharma *et al.*, 2007), including the EGFR inhibitor CL-387785 (Discafani *et al.*, 1999) and the dual EGFR / ErbB2 inhibitor HKI-272 (also known as neratinib) (Rabindran *et al.*, 2004) (both by Wyeth / Pfizer, Madison, New Jersey, USA) (Figure 7).

1.3.3.5 EGFR-TKI sensitizing genetic alterations

Within a few months in 2004, several groups have reported the discovery of a small subset of lung cancer patients who displayed dramatic response to treatment with gefitinib or erlotinib and the presence of somatic genetic alterations within the kinase domain of the EGFR protein of most of these EGFR TKI responders (Lynch *et al*., 2004; Paez *et al.*, 2004; Pao *et al.*, 2004). These mutations tend to be more frequently found in patients who have adenocarcinomas, never smoke, are of female sex or are of East Asian ethnicity (Kosaka *et al.*, 2004; Pao *et al.*, 2004; Shigematsu *et al.*, 2005). On the contrary, their presence is not associated with age and tumour stage at the moment of first diagnosis. The lower prevalence of these EGFR kinase domain mutations in Caucasian patients (Paez *et al.*, 2004; Pao *et al.*, 2004; Shigematsu *et al.*, 2005) appears to correlate to the lower response rate (about 10 %, versus almost 30 % in Japanese patients) in clinical trials of gefitinib in these populations (Fukuoka *et al.*, 2003; Kris *et al.*, 2003). This may have contributed in part to the subsequent decision by USFDA in June 2005 to re-label the indication of gefitinib, which limits its usage to patients who have previously or are currently benefiting from the drug only.

The most common genetic alterations observed in these patients include small deletions of various lengths of amino acids around codons 746 to 750 encompassing the highly conserved leucine-arginine-glutamic acid-alanine (LREA) motif (detected in approximately 48 % of EGFR TKI responders), the substitution of lysine-858 to arginine (L858R) at exon 21

of the gene (~40 %), and the substitution of glycine-719 to alanine, cysteine or serine (G719A/C/S) at exon 19 (~5 %) (Jänne *et al.*, 2005; Shigematsu *et al.*, 2005). Although these affected residues are located far away from each other at the primary structure (that is, the amino acid sequence) level, at the secondary structure level they are actually situated very close to each other: they are all positioned within or near various elements that form part of the ATP-binding pocket, namely the P-loop (G719), the αC-helix (codons 746-750) and the A-loop (L858). Alterations at these critical regions are expected to alter the 3-dimensional structure of the ATP-binding pocket, and thus the activity of the protein. Indeed, mutant EGFR proteins carrying these point mutations and deletions are constitutively active (Greulich *et al.*, 2005; Jiang *et al.*, 2005; Sordella *et al.*, 2004). These alterations prevent the kinase domain from adopting a resting, auto-inhibitory conformation (Yun *et al.*, 2007). Further downstream, these mutant proteins constantly activate multiple signalling proteins, including extra-cellular signal-regulated kinase (ERK), AKT, STAT3 and STAT5, which in turn modulate various cellular activities leading to oncogenic phenotypes such as anchorage and ligand-independent growth, focus formation and the ability to form tumours in mice. These cells have become dependent [or "addicted", as coined in the term "oncogenic addiction" (Weinstein, 2002)] on the activities of the mutant proteins for growth and survival, and it is this dependence that makes the cells hyper-sensitive to EGFR inactivation (Sordella *et al.*, 2004). Importantly, each of the above-mentioned mutations alters the basal phosphorylation levels of distinct combinations of the major phosphorylation sites of EGFR (Greulich *et al.*, 2005; Sordella *et al.*, 2004). This differential basal phosphorylation pattern was also observed in other clinically relevant EGFR mutant proteins (Chen *et al.*, 2006). These data suggest that the phenotype conferred by each mutation is distinct. In line with this view, distinct mutant EGFR proteins display different levels of catalytic activities, affinities to ATP and EGFR TKIs, as well as sensitivities to these inhibitors (Carey *et al.*, 2006; Chen *et al.*, 2006; Jiang *et al.*, 2005; Yun *et al.*, 2007).

In contrast to the dramatic drug sensitivities displayed by cancer cells expressing EGFR proteins with EGFR TKI-sensitizing kinase domain mutations, these cells are resistant to anti-EGFR antibodies (Amann *et al.*, 2005; Mukohara *et al.*, 2005). These mutant proteins, being able to generate proliferative / pro-survival signals constitutively, would continue to do so when ligand binding is blocked by the antibodies. In contrast, cancer cells that thrive on excessive signal generated by excessive number of EGFR receptors on cell surface (over-

expression) are often badly affected by anti-EGFR antibodies. In all, these observations support the determination of EGFR protein expression level and/or mutational status in patients prior to deciding on the most suitable treatment regime. The advantage of this "pre-screening", as seen in an increase in response rate in patients, has already been demonstrated in the clinics (Asahina *et al.*, 2006; Mitsudomi *et al.*, 2010). Furthermore, the "personalized" usage of EGFR TKIs based on EGFR mutational status leads to savings in cost to both the patient and national healthcare systems.

The EGFR kinase domain mutations mentioned here are almost exclusively found in NSCLCs, but are rarely found in other solid tumours, such as HNSCCs (Lee *et al.*, 2005), breast cancers (Reis-Filho *et al.*, 2006), colon cancers and glioblastomas (Barber *et al.*, 2004; Fumagalli *et al.*, 2010; Moroni *et al.*, 2005). In 2006, erlotinib-sensitizing EGFR mutations that do not affect residues at the kinase domain of the protein were identified in gliomas biopsies and a glioma cell line (Arjona *et al.*, 2005; Ikediobi *et al.*, 2006; Lee *et al.*, 2006; Thomas *et al.*, 2007). These single amino acid substitutions (R108K, T263P, A289V and G598V) are instead located at the extra-cellular domain of the protein. Interestingly, these point mutations also confer constitutive activity, likely by altering the extra-cellular domain into conformations that resemble a ligand-bound, active state (Huang *et al.*, 2009).

1.3.3.6 EGFR-TKI resistance-conferring genetic alterations

1.3.3.6.1 Primary resistance

Not all primary (alterations that are already present prior to treatment) EGFR kinase domain mutations identified in NSCLC patients to date confer sensitivity to EGFR TKIs. On the contrary, some confer resistance to these drugs. These alterations include the activating small insertion D770-N771insNPG within exon 20 of the gene (Greulich *et al.*, 2005) and substitutions such as E709G and S768I (Chen *et al.*, 2006). Another substitution, E884K located within exon 22, confers resistance to erlotinib but not gefitinib (Choong *et al.*, 2006). This indicates differential activity profiles of the 2 compounds, despite their similar chemical structures (Figure 7).

Primary resistance towards EGFR TKIs can also be contributed by genetic alterations in other genes within the EGFR signalling pathway. Two prominent examples of such mutations are

the activating point mutations within exons 1 and 2 (especially at glycine-12) of the *v-Ki-ras2* Kirsten rat sarcoma viral oncogene homolog (*kras*) gene (Mitsudomi *et al.*, 1991; Rodenhuis *et al.*, 1988) and within exons 11 and 15 (especially valine-600) of the *v-raf* murine sarcoma viral oncogene homolog B1 (*braf*) gene (Davies *et al.*, 2002). These mutations tend to appear in cancers associated with cigarette smoking (Ahrendt *et al.*, 2001), whereas EGFR kinase domain mutations are more common in non-smokers (Kosaka *et al.*, 2004; Pao *et al.*, 2005b). As members of the MAP kinase pathway, mutated KRAS and BRAF proteins are able to constitutively generate proliferative signals regardless of the activity status of the EGFR protein upstream. Interestingly, activating *kras* and *braf* mutations appear to be almost mutually exclusive to activating EGFR mutations (Eberhard *et al.*, 2005; Han *et al.*, 2006; Kosaka *et al.*, 2004; Pao *et al.*, 2005b), probably reflecting the "economy" in the development of a cancer clone, whereby there is no incentive – on the aspect of evolution – to keep "redundant" mutations that activate the same signalling pathways in the same cancer cell.

1.3.3.6.2 Secondary resistance

In addition to primary resistance, secondary "acquired" (mutation emerges after treatment) EGFR TKI-resistance conferring mutations were also reported. The most prominent example of such a mutation is the substitution of threonine-790 of the EGFR protein with methionine (T790M) (Kobayashi *et al.*, 2005; Kwak *et al.*, 2005; Pao *et al.*, 2005a). This mutation is structurally analogous to the imatinib (Gleevec®, Novartis International AG, Basel, Switzerland)-resistance conferring T315I mutation of the BCR-ABL fusion protein found in CML patients treated with this drug (Gorre *et al.*, 2001; Shah *et al.*, 2002). EGFR threonine-790 is called the "gate-keeper" residue because it is located at the entrance of a hydrophobic pocket at the back of the ATP-binding pocket. Replacement of this residue was initially thought to alter substrate specificity of the ATP-binding pocket via steric hindrance (Kobayashi *et al.*, 2005; Kwak *et al.*, 2005; Pao *et al.*, 2005a). However, Yun and colleagues (2008) demonstrated that the T790M mutation alone does not alter the affinity of EGFR to gefitinib. Surprisingly, when T790M is introduced to an EGFR-L858R background, the double mutant protein displays a 10-fold increase in affinity to ATP but no change in affinity to gefitinib. Thus the T790M mutation confers EGFR TKI resistance only in the presence of

L858R by making the mutant protein more inclined to bind to ATP over the competitive inhibitors. Additionally, the EGFR T790M mutation is activating (Yun *et al.*, 2008) and is able to form tumours in mice (Regales *et al.*, 2007). Ectopic expression of an EGFR protein carrying the T790M/L858R double mutation results in enhanced transformation and also tumour formation in nude mice (Godin-Heymann *et al.*, 2007). Furthermore, while it is often detected in patients treated with EGFR-TKIs, the EGFR T790M mutation has been reported as a germ-line alteration in a family with a hereditary susceptibility to NSCLC (Bell *et al.*, 2005). Together, these data represent EGFR T790M as an oncogenic mutation capable of conferring resistance to reversible EGFR TKIs.

1.4 The JAK family of non-receptor PTKs

1.4.1 History and general characteristics

The JAK family of cytoplasmic PTKs, which was first ingeniously called "just another kinase" when it was first detected (Wilks, 2008), consists of 4 members, namely JAK1, JAK2, JAK3 and Tyk2. Tyk2 is the founding member of this family, and is also the non-receptor PTK to be investigated in this study. Tyk2 was identified 20 years ago from the screening of complementary DNA (cDNA) libraries using a probe with a sequence based on the colony stimulating factor 1 receptor (*csf1r*) gene (Krolewski *et al.*, 1990). This is quickly followed by the identification of JAK1 and JAK2, together with the first mention of the current name of this PTK family (Harpur *et al.*, 1992; Wilks *et al.*, 1991), and finally the identification of JAK3 (Kawamura *et al.*, 1994; Rane and Reddy, 1994). The *jak1*, *jak2*, *jak3* and *tyk2* genes are located at chromosomes 1p31.3, 9p24, 19p13.1 and 19p13.2 respectively (Firmbach-Kraft *et al.*, 1990; Pritchard *et al.*, 1992; Riedy *et al.*, 1996). Simply based on the proximity of the *jak3* and *tyk2* genes on chromosome 19, it is tempting to suggest that they may have arisen from an ancient duplication event. In fact, phylogenetic analyses of JAK proteins are in support of this speculation, in which these 4 proteins arise from 2 rounds of duplications from a common ancestral gene (Gu *et al.*, 2002).

The JAK1, JAK2 and Tyk2 proteins are ubiquitously expressed in all tissues, whereas JAK3 is mainly found in NK cells, activated or transformed T cells (Kawamura *et al.*, 1994). This cell type-specific expression explains why JAK3 was initially called the leukocyte Janus kinase (L-

JAK). Later, JAK3 was also detected in endothelial and smooth muscle cells (Verbsky *et al.*, 1996). All 4 JAK proteins are approximately 1150 amino acids in length and 130 kDa in size (Leonard and O'Shea, 1998). These proteins are highly similar in terms of the presence and the organization of 7 JAK-homology (JH) domains, which are termed JH1 through JH7 (Figure 11 in the Results section), and the absence of a trans-membrane domain, which explains their localization at the cytoplasm and supports their categorization as non-receptor PTKs.

1.4.2 JAK proteins primarily serve as cytoplasmic partners of cytokine receptors

Since JAK proteins do not possess a trans-membrane domain, they are unable to interact with extra-cellular ligands directly. Instead, they often participate in the transmission of extra-cellular signals by providing the tyrosine kinase activity module to various cell surface receptors that do not possess cytoplasmic kinase domains, particularly the cytokine receptors. These receptors and the constitutively C-terminal bound JAKs together form "fully functional" complexes capable of relaying extra-cellular signals to the cell nucleus. There are more than 30 receptors for cytokines that employ various combinations of JAK proteins for signal transduction (summarized in Murray, 2007) (Figure 8). For example, the interferon (IFN)-$\alpha/\beta/\omega$ receptor 1 (IFNAR1) employs JAK1 and Tyk2; the IFN-γ receptor is bound to JAK1 and JAK2; the receptors for interleukin (IL)-6, IL-10, IL-11, IL-22 and oncostatin M (OSM) are associated with 2 JAK1 proteins, and common gamma c (γc) chain-containing receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 all use the JAK1 / JAK3 combination. Therefore JAK activity is required for a myriad of critical cellular functions, especially the development, proliferation and maintenance of various lineages of both T and B lymphocytes (reviewed in Ghoreschi *et al.*, 2009). The usage of different combinations of JAK proteins as partners for various cytokine receptors represents an evolutionarily efficient system that allows the signalling of numerous ligands by a much smaller number of JAK proteins. On the other hand, the fact that a single combination of JAK proteins is used by multiple cytokine receptors suggests that additional regulatory mechanisms must exist, so that the cell can distinguish signals generated from identical sets of JAK proteins. One such mechanism is provided by the specific tyrosine-based motifs located at the C-terminal ends of cytokine receptors, as individual motifs within different receptors can only bind to certain

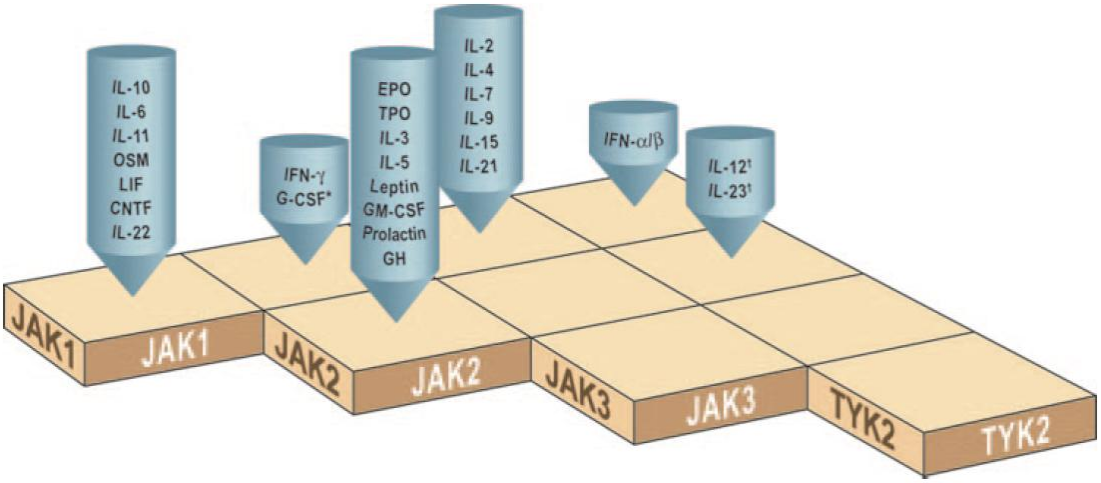

Figure 8. **Cytokine receptors employ various combinations of Janus kinases (JAKs) for signalling.** Most cytokine receptors are associated with only 3 combinations of JAKs, namely 2 JAK1 proteins, 2 JAK2 proteins and JAK1+JAK3. Tyk2, on the other hand, is less frequently used by cytokine receptors. It should be noted that Tyk2 is actually a component of the receptor complexes of IL-6 and LIF (Stahl *et al.*, 1994), IL-10 (Finbloom and Winestock, 1995), G-CSF (Shimoda *et al.*, 1997) and TPO (Ezumi *et al.*, 1995). However, these receptor complexes appear to retain their activities in cells devoid of Tyk2, indicating either redundancy or an ancillary role of Tyk2 (Drachman *et al.*, 1999; Shimoda *et al.*, 1997, 2000). Some JAK combinations, such as JAK2+JAK3, have never been observed in any cytokine receptor complex (empty squares). CNTF denotes ciliary neurotrophic factor. The full-length names of the other ligands listed in this Figure are already mentioned in the main text. (from Murray, 2007).

set of signalling partners (Stahl *et al.*, 1995). Another level of regulation may be provided by the differential expression of receptors / JAKs / ligands / negative regulators involved in signalling via JAKs in distinct cell types (spatial regulation) and/or at different times (temporal regulation), which is in turn dependent on the micro-environment surrounding the cells (Murray, 2007). Finally, the cytokine receptors are not the only receptors that employ JAK proteins for signalling. For example, PDGFR is also associated with JAK1, JAK2 and Tyk2 and is able to phosphorylate these JAKs upon ligand stimulation (Vignais *et al.*, 1996). Moreover, the receptors for the serine protease called urokinase-type plasminogen activator (uPA) and the phospholipid called platelet-activating factor (PAF) transmits their signals via combinations of JAK1 / Tyk2 (Dumler *et al.*, 1998) and JAK2 / Tyk2 (Lukashova *et al.*, 2003) respectively.

1.4.3 Functional domains of JAKs

1.4.3.1 JH1: kinase domain

As mentioned earlier, JAK proteins contain 7 JH domains. The C-terminal JH1 domain of JAKs is solely responsible for the catalytic activity of JAK proteins and has a sequence that generally resembles the sequences of their counterparts from other PTK families. However, this domain also contains features unique to JAKs. The highly conserved (K/R)W(M/T)APE motif in the sub-domain VIII of the kinase domain of all other PTK families is presented instead as FWYAPE in the JH1 domains of JAKs (Wilks, 1989). In addition, the major phosphorylation sites within the A-loop of JAKs consist of 2 adjoining tyrosine residues (Y1034 / Y1035 for JAK1, Y1007 / Y1008 for JAK2, Y980 / Y981 for JAK3 and Y1054 / Y1055 for Tyk2), instead of a single tyrosine residue in other PTKs. Interestingly, each member of the double tyrosines in the kinase domain of each JAK appears to have different functional importance. It appears that only one of the 2 tyrosine residues at the major phosphorylation sites of JAK1 (Y1034), JAK2 (Y1007) and Tyk2 (Y1054) are essential for the catalytic activities of the respective JAK proteins (Feng *et al.*, 1997; Korniski *et al.*, 2010; Liu *et al.*, 1997). On the other hand, replacement of Y980 of JAK3 with phenylalanine increases the kinase activity of the protein, whereas a similar replacement at Y981 decreases catalytic activity (Zhou *et al.*, 1997).

At the structural level, the kinase domains of JAKs are also highly similar to the kinase domains of other PTKs. However, features specific to JAKs are also present. The currently available crystal structures indicate a bi-lobed architecture of the JH1 domains of both JAK2 (Lucet *et al.*, 2006) and JAK3 (Boggon *et al.*, 2005), akin to the kinase domains of all other PTK families. On the other hand, the “kinase insertion loop” at codons 1056 to 1068 of JAK2 and the “constricted” nature of the ATP-binding pocket are features not observed in the crystal structures of the kinase domains of other PTKs.

1.4.3.2 JH2: pseudokinase domain

Adjacent and proximal to the JH1 domain of JAK proteins is the JH2 domain, which is also known as the pseudokinase or kinase-like domain. The presence of tandem kinase domains,

a defining feature of JAKs, explains why this PTK family is named after Janus, the two-faced Roman god of doorways, and suggests that an intragenic duplication event may have occurred during the evolution of JAKs. The JH2 domain is, sequence-wise, highly homologous to JH1, and this explains why the terms “pseudo” and “-like” are used to name this domain. In contrast to JH1, JH2 is catalytically inactive (Wilks *et al.*, 1991), which is attributed to amino acid substitutions at several motifs critical for activity. These motifs include the P-loop as well as the DFG and FWYAPE motifs, which are required for the binding of ATP, magnesium (Mg^{2+}) and the substrate respectively.

Since the JH2 domains of JAKs are catalytically inactive, it has to be serving some alternative function(s) to justify its positive selection during evolution. Indeed, such a role was established when it was discovered that removal of this domain abrogates the catalytic activity of Tyk2 (Velazquez *et al.*, 1995). Various genetic alterations at the JH2 domain of JAK3 and Tyk2 also have the same effect (Chen *et al.*, 2000; Yeh *et al.*, 2000). In contrast, removal of the whole JH2 domain of JAK2 confers increased catalytic activity to the protein (Saharinen and Silvennoinen, 2002). Regardless of their catalytic capabilities, 2 notable characteristics common to all the above-mentioned JAK JH2 domain alterations were observed. Firstly, they perturb signalling of the cytokines the affected proteins are associated with. Secondly, almost all of them result in an increased basal phosphorylation of the proteins. Therefore the JH2 domain is required for maintaining the proper functioning of the cytokine signalling cascades and engaging the JH1 domain in an inactive, hypo-phosphorylated conformation. Interference of this interaction releases JH1 from this inhibition, thus allowing constant activity.

1.4.3.3 JH3 and JH4: SH2-like domain

Further upstream of the tandem kinase domains of JAK proteins is a region, encompassing the whole JH3 and part of the JH4 domains, which displays only a moderate level of sequence homology to the typical SH2 domain of other PTKs (Kampa and Burnside, 2000; Radtke *et al.*, 2005). Several amino acid residues highly conserved among the SH2 domains of most PTKs, such as the tryptophan and tyrosine residues within the βA strand (altered in all 4 JAKs) and the phospho-tyrosine-interacting arginine residue at the position βB5 (altered in Tyk2) are altered in their counterparts in JAKs (Al-Lazikani *et al.*, 2001). Such

altered SH2-like domains of JAKs are thought to be non-functional and/or unable to bind to phospho-tyrosines. Recently, an alternative function of the SH2-like domains of JAKs has emerged: they appear to be involved in the maintenance of the cell surface receptors they are attached to. For example, the SH2-like domain is required for the binding of Tyk2 to IFNAR1 (Richter *et al.*, 1998; Yan *et al.*, 1998) and for the anchoring of this receptor on the cell surface (Ragimbeau *et al.*, 2003). This structural role of the JAK SH2-like domain is also shared by the FERM domain [four point one (4.1) protein, ezrin, radixin and moesin], which lies further upstream towards the N-termini of the proteins.

1.4.3.4 JH5 to JH7: FERM domain

JAKs and FAKs are the only non-receptor PTKs carrying a FERM domain (Girault *et al.*, 1999), which is usually about 300 amino acids in size (Chishti *et al.*, 1998) (Figure 3). The FERM domain of JAKs encompasses the JH5, JH6 and JH7 domains, as well as the N-terminal half of the JH4 domain, and can be further separated into 3 sub-domains, namely the β-grasp (JH7), the helix bundle (JH6) and the pleckstrin homology (PH) (JH4 and JH5) domains. The main function of the FERM domain of JAKs is to facilitate the non-covalent attachment of JAKs to the C-terminal portion of cell surface receptors. A small region encompassing the β-grasp and the helix bundle appears to be critical for the actual interaction, as illustrated by several mutational studies on JAK1 (Haan *et al.*, 2001), JAK2 (Kohlhuber *et al.*, 1997) and JAK3 (Cacalano *et al.*, 1999; Zhou *et al.*, 2001). On the other hand, Tyk2 requires the presence of all regions from JH3 to JH7 for maximal binding to IFNAR1, with critical requirement of JH3 and a region spanning JH6 and JH7 (Richter *et al.*, 1998; Yan *et al.*, 1998).

The FERM-domain-mediated binding of JAK proteins to the distal ends of some receptors appears to ensure that the receptors stay on the cell surface, as observed in the oncostatin M receptor (OSMR), the erythropoietin receptor (EPOR) (both bound and stabilized by JAK2) (Huang *et al.*, 2001; Radtke *et al.*, 2005) and IFNAR1 (by Tyk2) (Ragimbeau *et al.*, 2003). Moreover, the FERM (and the SH2) domain(s) of Tyk2 are needed to maintain surface expression as well as prevention of both internalization and degradation of IFNAR1 (Gauzzi *et al.*, 1997; Ragimbeau *et al.*, 2003). Intriguingly, the same JAK3 FERM domain alterations that abolish binding to the γc chain mentioned in the previous paragraph also abrogate its ability to bind to ATP, and thus its catalytic activity (Zhou *et al.*, 2001). Therefore the FERM

domain of JAKs may play extra role(s) beyond the binding and anchoring of surface receptors.

1.4.4 Mediators of JAK signalling

1.4.4.1 The STAT proteins

Stimulatory signals generated by JAKs upon ligand binding to the kinase activity-deficient cytokine receptors are subsequently transmitted towards the nucleus via the STAT proteins. STATs are latent cytoplasmic proteins first isolated as members of the IFN-stimulated gene factor 3 (ISGF-3) complex bound to a DNA fragment that resembles the core sequence of the IFN-stimulated response element (ISRE) found in IFN-stimulated cells (Fu *et al.*, 1992; Darnell *et al.*, 1994). The founding members of the family of human STAT proteins, STAT1 and STAT2, are later joined by STAT3, 4, 5a, 5b and 6. The genes of these 7 proteins are clustered on chromosomes 2, 12 and 17. STATs are constitutively expressed, are about 750 to 850 amino acid in size, and contain the following domains: (i). the N-terminal oligomerization domain (John *et al.*, 1999; Vinkemeier *et al.*, 1996), (ii). the coiled-coil domain involved in interactions with other proteins such as the interferon response factor 9 (IRF9) during the formation of ISGF-3 (Horvath *et al.*, 1996), (iii). the DNA-binding domain that binds to, for example, the ISRE sequence, (iv). the linker domain that appears to maintain the correct structure of the DNA-binding domain, (v). the SH2 domain that binds to phospho-tyrosines and (vi). the trans-activation domain which contains the major tyrosine phosphorylation site.

In addition to this tyrosine residue, a serine residue within the trans-activation domain of STAT1, 3, 4, 5a and 5b may also become phosphorylated (Beadling *et al.*, 1996; Cho *et al.*, 1996; Wen *et al.*, 1995; Zhang *et al.*, 1995). Depending on the cellular context (for example, the identity of the STAT protein being serine phosphorylated and the protein carrying out this reaction), this serine phosphorylation may enhance (Wen *et al.*, 1995) or attenuate the activity of STATs (Chung *et al.*, 1997; Kovarik *et al.*, 2001). On the other hand, as a result of alternative splicing, shorter variants of STAT1, 3 and 4 are also expressed. These variants carry truncations at the C-termini, in which the above-mentioned serine residue is also removed. Since phosphorylation of this proximal serine residue alters the activity of STATs,

one would envisage that these truncated STAT proteins are functionally distinct from their full-length counterparts.

1.4.4.2 The JAK-STAT signalling pathway

The first established and the most well-known role of STAT proteins is to relay cytokine-mediated stimulatory signals from the ligand-receptor-JAK complexes to the nucleus via the JAK-STAT pathway (Darnell *et al.*, 1994; reviewed in Bromberg and Darnell, 2000 and Rawlings *et al.*, 2004). The cytokine kick-starts this pathway when it binds to the cytokine receptors on the surface of the cell, which results in the dimerization of receptor subunits. The receptor dimers then undergo ligand-induced conformational changes that lead to their activation. At the same time, the 2 inactive JAK proteins attached to the proximal ends of each receptor subunit are brought into proximity. This allows the JAKs to phosphorylate and activate each other. The view that these cytokine receptor-associated JAKs trans-phosphorylate rather than self-phosphorylate is supported by the observation that stimulation of JAK1-deficient cells with IFN-α and IFN-γ does not result in the phosphorylation of the respective JAK partners of the IFN-$\alpha/\beta/\omega$ and IFN-γ receptors, Tyk2 and JAK2 (Müller *et al.*, 1993). Similarly, when cells lacking in Tyk2 or JAK2 are stimulated by IFN-α and IFN-γ respectively, JAK1 phosphorylation cannot be detected. Thus upon IFN stimulation, it is JAK1 that trans-phosphorylates Tyk2 and JAK2 and vice versa.

The above phosphorylation and activation of JAKs enable them to phosphorylate both receptor subunits at their cytoplasmic tails. This results in the generation of phospho-tyrosines sites for the SH2 domains of various STAT proteins to bind to (Greenlund *et al.*, 1994; Stahl *et al.*, 1995). Receptor-bound STAT proteins are phosphorylated by JAKs, and subsequently dissociate from the receptors, dimerize via reciprocal binding of the SH2 domains (Greenlund *et al.*, 1995; Shuai *et al.*, 1994), and translocate into the nucleus. There, the complexes bind to IFN-γ activated sequence (GAS) (Decker *et al.*, 1991) or ISRE (Dale *et al.*, 1989) sequences and up-regulate the transcription of downstream genes. Some of these STAT dimers may also associate with additional factors prior to binding to DNA. One example is the STAT1-STAT2 hetero-dimer generated upon IFN-α stimulation, which is coupled to IRF9 to form the ISGF-3 complex before binding to an ISRE (Fu *et al.*, 1992;

Schindler *et al.*, 1992). Thus, in addition to their role of relaying signals generated from ligand stimulation to the nucleus, STATs also directly mediate gene transcription by binding to regulatory elements on DNA. This defining "dual-role" feature of STATs explains why they are named as such (Schindler *et al.*, 1992).

1.4.4.3 STATs are employed by other non-JAK PTKs for signalling

Beside JAKs, STATs also serve as messengers for many receptor and non-JAK cytoplasmic PTKs, and thus are also involved in the signalling of non-cytokine growth factors and hormones. Various STATs, individually or in combinations, are directly activated by receptor PTKs such as EGFR (Olayioye *et al.*, 1999; Silvennoinen *et al.*, 1993), hepatocyte growth factor receptor (HGFR/MET) (Boccaccio *et al.*, 1998), vascular endothelial growth factor receptors (VEGFR) 1 to 3, tyrosine kinase with immunoglobulin (Ig)-like and EGF-like domains 1 (TIE1) and TEK (TIE2) (Bartoli *et al.*, 2000; Korpelainen *et al.*, 1999) as well as cytoplasmic PTKs including SRC (Chaturvedi *et al.*, 1998), feline sarcoma oncogene (FES) (Nelson *et al.*, 1998) and ABL (Danial *et al.*, 1995). In addition, cell surface G-coupled receptors (Wong and Fish, 1998) and some members of the ERK family of secondary messengers (Chung *et al.*, 1997; Lim and Cao, 2001) are also able to phosphorylate STATs. Many of the above-mentioned STAT-activating proteins apparently prefer to use STAT1, 3, and/or 5 for signalling.

1.4.4.4 Differential activities of STATs

Since the STAT proteins serve as secondary messengers for a lot of PTKs, additional mechanism(s) must exist that ensure their binding to the correct sets of promoters, and thus ensure their ability to properly regulate a wide spectrum of cellular processes. One such mechanism may be the requirement of co-activators, such as IRF9 (Fu *et al.*, 1992; Schindler *et al.*, 1992), the transcription factor Sp1 (Look *et al.*, 1995), c-Jun (Zhang *et al.*, 1999), nuclear factor IL-4 (NF-IL4) and NF-κB (Delphin and Stavnezer, 1995) for efficient binding. Interestingly, some cellular processes regulated by STATs lead to apparently contradictory consequences. On one hand, IFN-mediated STAT1 activation is associated with impaired cell growth (Bromberg *et al.*, 1996; Chin *et al.*, 1996; Shuai *et al.*, 1996), and mice

devoid of STAT1 are prone to tumour formation (Kaplan *et al.*, 1998). In contrast, constitutive activation of STAT3 is a feature associated with SRC-mediated transformation (Bromberg *et al.*, 1998; Turkson *et al.*, 1998). Furthermore, activated STAT3 and/or STAT5 up-regulate the expression of several anti-apoptotic proteins (Aoki *et al.*, 2003; Catlett-Falcone *et al.*, 1999; Epling-Burnette *et al.*, 2001; Grandis *et al.*, 2000; Socolovsky *et al.*, 1999; Zamo *et al.*, 2002) and promote cell cycle progression (Masuda *et al.*, 2002; Moriggl *et al.*, 1999). On the other hand, both activated STAT3 and STAT5 are often found in primary tumours of various origins (Bowman *et al.*, 2000). Thus STAT3 and STAT5 possess characteristics of a proto-oncogene. Taken together, STAT proteins are cytoplasmic messengers commonly employed by numerous signalling pathways for the regulation of a myriad of cellular functions, some of which are counteracting in nature.

1.4.4.5 Inactivation of JAK-STAT signalling

Cytokine-mediated activation of the JAK-STAT pathway is a transient event that lasts for up to several hours. The return of activated STATs to their resting state after ligand stimulation is mediated by 3 groups of negative regulators, namely the suppressor of cytokine signalling (SOCS), protein inhibitor of activated STAT (PIAS), and tyrosine phosphatase (reviewed in Hilton, 1999). These regulators down-regulate different components within the JAK-STAT pathway. The 8-membered family of SOCS proteins, which is only expressed upon cytokine stimulation (and thus this system works like a classical feedback loop), attenuates JAK-STAT activity by directly binding to phospho-tyrosine residues on both activated JAKs and receptors via their SH2 domains and tagging the target proteins for proteasomal degradation (reviewed in Croker *et al.*, 2008 and Kile *et al.*, 2002). On the other hand, the 4 constitutively expressed PIAS proteins (reviewed in Shuai, 2005) primarily prevent STATs from binding to DNA. Additionally, PIAS proteins are able to block signals generated from the JAK-STAT pathway by SUMOylation [tagging proteins with small ubiquitin-like modifier (SUMO) proteins] or aggregation of transcription factors, so that they become unable to bind to the promoters of target genes. Lastly, JAK-STAT signalling may be down-regulated simply by de-phosphorylating the proteins involved in the pathway by protein tyrosine phosphatases (PTPs). It appears that each JAK is de-phosphorylated only by a certain combination of PTPs. This specificity is likely to be attributed to the ability of a PTP to

recognize the different sequences at the A-loop of individual JAKs. In all, these 3 groups of proteins play an essential role in modulating the activity status of the JAK-STAT pathway. Deregulation of the activities of these negative regulators leads to uninhibited activation of the JAK-STAT pathway, which is associated with various disease states including allergy, inflammation and cancer.

1.4.5 Diseases caused by genetic anomalies of JAK proteins

1.4.5.1 Deficiencies in JAKs

1.4.5.1.1 Deficiencies in JAK1 and JAK2 confer lethality

Since the JAK proteins are highly conserved throughout human evolution and have many essential roles, it is expected that catastrophic consequences would ensue in the absence of any of these proteins. Indeed, the importance of JAK1 and JAK2 is demonstrated by the fact that an individual devoid of activity of either protein has never been found. Furthermore, a knock-out of JAK1 or JAK2 in mice results in perinatal or embryonic lethality respectively (Neubauer *et al.*, 1998; Rodig *et al.*, 1998). Cells obtained from JAK1-deficient mice do not respond to both types of IFNs and to cytokines that stimulate receptors containing the γc chain and the gp130 subunit. Similarly, a JAK1-null fibro-sarcoma cell line called U4A is also resistant to stimulation by IFNs (McKendry *et al.*, 1991). On the other hand, cells obtained from JAK2-deficient mice are unresponsive to EPO, IFN-γ, IL-3 and IL-5. The lack of EPO signalling via JAK2 in these mice is likely to be responsible for the failure of the erythrocytes of JAK2-deficient mice to develop properly.

1.4.5.1.2 Deficiency in JAK3 causes SCID

Genetic alterations of JAK3 that abolish the protein's activity and/or its ability to bind to the γc chain are found in patients suffering from an autosomal recessive form of severe combined immuno-deficiency (SCID) (Candotti *et al.*, 1997; Chen *et al.*, 2000; Macchi *et al.*, 1995; Russell *et al.*, 1995). In humans, this immune deficiency is characterized by a dramatic decrease in the number of T cells with normal or increased number of non-functional B cells.

As a result, SCID patients constantly suffer from recurrent or opportunistic infections, and need to remain in a sterile environment. JAK3 knock-out mice also suffer from SCID, but these mice lack both T and B cells (Nosaka *et al.*, 1995; Park *et al.*, 1995; Thomis *et al.*, 1995). In both SCID patients and mice, the residual T cells do not respond to cytokine stimulation, and the B cells are developmentally immature. In addition, the number, level of maturation and cytokine responsiveness of other cells of lymphoid lineage, such as the NK cells and thymocytes, is also negatively affected in SCID patients and mice. In contrast, cells of myeloid and erythroid origins do not appear to be affected (Park *et al.*, 1995; Villa *et al.*, 1996). Therefore a JAK3 deficiency leads to a loss of the lymphoid arm of the immune system.

SCID may also arise from a deficiency of adenosine deaminase (ADA) or inactivating mutations of the γc chain (Noguchi *et al.*, 1993). The clinical presentation and immunological defects of X-linked SCID (due to γc chain mutations) patients are highly similar to those with JAK3 mutation-mediated SCID. Thus genetic alterations in 2 proteins involved in the same signalling pathway cause the same disease since JAK3 is exclusively used by the γc chain for signalling. Regardless of the underlying cause, SCID patients may be treated with allogeneic transplantation of hematopoietic stem cells or retroviral or lentiviral-based gene therapy (the latter is still in clinical evaluation). However, issues on graft-verses-host diseases after transplantation and on the efficacy and toxicity of gene therapy need to be resolved before these treatment options can be more widely used.

1.4.5.1.3 Deficiency in Tyk2 causes mild effects in mice but severe disease in humans

The phenotypes of deficiencies of JAK1 to JAK3 are highly similar between humans and mice. In contrast, while Tyk2 knock-out mice display relatively subtle phenotypes, the only individual ever reported to be deficient in Tyk2 suffers from severe, life-threatening immunological defects (Minegishi *et al.*, 2006).

Unlike the knock-out mice of JAK1 and JAK2, Tyk2-deficient mice are viable and fertile. They appear to have normal development of both lymphoid and myeloid cells, and do not display a profound loss of critical immunological capabilities, as observed in JAK3-deficient mice. The major phenotype of Tyk2 knock-out mice is a reduction of signalling of type I IFNs (Karaghiosoff *et al.*, 2000; Shaw *et al.*, 2003; Shimoda *et al.*, 2000). These mice are still

responsive to IFN-α/β, albeit only at a higher concentration (1000 U/ml). This observation suggests that Tyk2 is not critical for signalling of type I IFNs; its function would rather be the amplification of signals generated by these cytokines. This was a surprising result because it overturned the consensual view at that time that Tyk2 is indispensable for IFN-α/β signalling (Velazquez *et al.*, 1992).

Among the other cytokines that also signal via receptor complexes that contain Tyk2 [such as IL-6, IL-10, IL-12, IL-23, leukaemia inhibitory factor (LIF), granulocyte colony-stimulating factor (GCSF, also known as CSF3) and thrombopoietin (THPO or TPO)], Tyk2 knock-out results in an impaired IL-12 and IL-23-mediated responses, but has no observable effect on the signalling of the other cytokines listed above (Karaghiosoff *et al.*, 2000; Nakamura *et al.*, 2008; Shaw *et al.*, 2003; Shimoda *et al.*, 2000). Intriguingly, the signalling of IFN-γ is down-regulated in Tyk2 knock-out mice, even though the IFN-γ receptor requires JAK1 and JAK2 for signalling (Igarashi *et al.*, 1994). In all, Tyk2 deficiency in mice results in partial impairment of selected cytokine signalling pathways. This contrasts to the deficiencies of any of the other 3 JAKs, in which there is a total abolishment of all signalling pathways each JAK is involved in.

The impaired responses to IFNs and IL-23 in Tyk2 knock-out mice results in an absence of T helper (Th)-1 and Th17 responses, which may explain why these mice are susceptible to infections with certain viruses and bacteria (Karaghiosoff *et al.*, 2000; Nakamura *et al.*, 2008; Shimoda *et al.*, 2000). The importance of murine Tyk2 in fighting infections is also demonstrated by its requirement in the presentation of infectious agent-derived antigens on the surface of dendritic cells, which is needed for the priming of T cells (Aizu *et al.*, 2006). In addition to T cells and dendritic cells, Tyk2 also affects NK cells. Impaired IL-12 signalling in Tyk2 knock-out mice results in a lower expression level of the IL-18 receptor, and IL-18 signalling is needed for the activation of NK cells (Shimoda *et al.*, 2002). Furthermore, Tyk2 knock-out mice display an increased IL4-mediated Th2 response (Seto *et al.*, 2003) because the lack of IL-12 and IFN activities in these mice promotes Th2 cell differentiation and tilt the balance of the immune response away from Th1. Lastly, the defective NK and CD8+ T cell responses seen in Tyk2 knock-out mice is likely to be behind the inability of these mice to prevent tumour formation and progression after the introduction of oncogenic viruses or after the ectopic expression of constitutively active onco-proteins (Simma *et al.*, 2009;

Stoiber *et al.*, 2004). Thus in mice, Tyk2 also displays properties characteristic of tumour suppressor genes.

To date, there is only a single report on Tyk2 deficiency in humans in the literature (Minegishi *et al.*, 2006). Since birth, the male Japanese patient has experienced recurrent opportunistic infections by bacteria, viruses and fungi. He also suffers from atopic dermatitis and has a greatly elevated serum IgE level. Based on these clinical presentations, he was diagnosed as suffering from hyper IgE syndrome (HIES). Detailed investigation revealed that the patient carries a homozygous deletion of 4 bp in the Tyk2 gene that translates to a frame-shift at the FERM domain of the protein. As a result the Tyk2 protein he expresses is a peptide of only 89 amino acids in length (full-length Tyk2 has 1187 amino acids), which lacks the kinase, pseudokinase, SH2-like domains as well as most of the FERM domain. Thus this truncated protein is not expected to be functional. Cells obtained from the patient do not respond to stimulation by IFNα/β, IL-6, IL-10, IL-12 or IL-23. This phenotype is very different from that of the Tyk2 knock-out mice, which displays either partial or normal response to these cytokines (Karaghiosoff *et al.*, 2000; Nakamura *et al.*, 2008; Shimoda *et al.*, 2000). One thing similar between cells taken from this patient and those from Tyk2-deficient mice is that both types of cells tend to differentiate into the Th2 lineage *in vitro* (Minegishi *et al.*, 2006; Seto *et al.*, 2003). Overall, it appears that there is an obligate requirement of Tyk2 in humans but not in mice. It is possible that in mice, some immunological defects caused by Tyk2 deficiency are negated by compensatory mechanisms.

1.4.5.2 Genetic alterations in JAKs in human malignancies

1.4.5.2.1 The JAK2 V617F mutation is associated with myeloproliferative diseases

While a loss of JAK activity abolishes the development, production and/or proper functioning of various cell lineages within the immune and the haematological systems (section 1.4.1.5.1), too much JAK activity, on the opposite, may lead to pathological scenarios characterized by (i). the production of excessive number of various cell lineages of the immune and haematological systems and/or (ii). disproportionate level of signalling activities from these cells. A classic example of such a scenario is seen in a majority of patients suffering from the myeloproliferative disease (MPD) polycythemia vera (PV) and in

more than half of the patients with essential thrombocythemia (ET) and primary myelofibrosis (PMF) (Baxter *et al*., 2005; James *et al*., 2005; Kralovics *et al.*, 2005; Levine *et al*., 2005). These patients carry the activating JAK2 V617F mutation, which affects a highly conserved residue within the JH2 pseudokinase domain. This mutant protein is able to confer EPO-independent growth and survival *in vitro* (James *et al.*, 2005; Kralovics *et al*., 2005; Levine *et al*., 2005; Zhao *et al.*, 2005), which corresponds to the defining clinical feature of PV [excessive autonomous erythropoiesis from myeloid progenitor cells in the absence of EPO stimulation, leading to the presence of a large number of red blood cells (RBCs) in circulation]. Moreover, expression of this mutant protein alone is sufficient for the generation of PV in mice (Bumm *et al.*, 2006; Lacout *et al.*, 2006; Tiedt *et al.*, 2008; Wernig *et al.*, 2006; Zaleskas *et al.*, 2006). The clinical manifestation and disease progression (a primary proliferation phase followed by a secondary fibrosis phase) of PV in mice are highly similar to those seen in PV patients. Based on this direct causal relationship between the JAK2 V617F mutation and MPDs, many pharmaceutical companies have developed specific inhibitors against wild-type and/or the V617F mutant JAK2 proteins. Many of these inhibitors are currently undergoing phase I/II evaluations (US NIH clinical trials website).

1.4.5.2.2 Other activating mutations of JAKs are found in various lymphoid and myeloid leukaemias

Besides the JAK2 V617F mutation seen in MPDs, other genetic anomalies of JAK2 are also associated with proliferative diseases of cells from the circulatory and lymphoid systems, including leukaemia. One of them is the TEL-JAK2 fusion protein found in some patients suffering from acute lymphocytic leukaemia (ALL). The gene that gives rise to this protein is created after a translocation event between the short arms of chromosomes 9 and 12 [t(9;12)(p24;p13)] that brings together the JH1 kinase domain of JAK2 and the oligomerization domain of the transcription factor E26 transformation specific (ets) variant 6 (ETV6, also known as TEL) (Lacronique *et al.*, 1997; Peeters *et al.*, 1997). When expressed in Ba/F3 cells, this constitutively active fusion protein up-regulates basal phosphorylation of STAT3 and STAT5, as well as confers both growth factor independence and increased proliferation (Ho *et al.*, 1999; Lacronique *et al.*, 1997; Schwaller *et al.*, 1998). Additionally, mice expressing the TEL-JAK2 fusion protein succumb to CD8+ T-cell leukaemia (Carron *et*

al., 2000) and mice injected with TEL-JAK2-expressing cells develop myelofibrosis (Kennedy *et al.*, 2006). Besides TEL, JAK2 fusion proteins with BCR [t(9;22)(p24;q11)] and pericentriolar material 1 protein (PCM-1) [t(8;9)(p22;p24)] have also been observed in AML and CML (Bousquet *et al.*, 2005; Cirmena *et al.*, 2008; Griesinger *et al.*, 2005; Reiter *et al.*, 2005). Lastly, various activating single amino acid substitutions of JAKs (such as JAK1 T478S and V623A, JAK2 T875N and JAK3 P132T, A572V and V722I) have been detected in ALL and acute megakaryoblastic leukaemia (AMKL, a subtype of AML) patients and AMKL cell lines (Mercher *et al.*, 2006; Walters *et al.*, 2006; Xiang *et al.*, 2008).

1.4.5.2.3 Genetic alterations of Tyk2 associated with cancer

Similar to JAK1 to JAK3, several clinically relevant genetic alterations of Tyk2 have been reported in literature to date. Some of them were polymorphisms associated with protection against auto-immune diseases. For example, the V362F and I684S polymorphisms of Tyk2 are less frequently detected in patients suffering from multiple sclerosis (MS) (Sigurdsson *et al.*, 2005), while the P1104A polymorphism was under-presented in systemic lupus erythematosus (SLE) patients (Mero *et al.*, 2010). On the other hand, the experimentally introduced Tyk2 V678F mutation, which is homologous to the PV-associated JAK2 V617F substitution, confers constitutive activity to Tyk2 (Gakovic *et al.*, 2008; Staerk *et al.*, 2005). Ectopic expression of this mutant protein alone in Ba/F3 cells results in cytokine-independent proliferation and activation of STAT3, STAT5, and ERK. Similarly, experimentally generated point mutations that affect other residues within the JH2 domain of Tyk2 (for example, H669P and R856G) also confer increased basal phosphorylation (Yeh *et al.*, 2000). Recently, Tomasson and colleagues (2008) reported the identification of 18 non-synonymous polymorphisms of Tyk2 in 94 AML patients of European ancestry. One alteration, the G363S substitution (cluster ID: rs2304255) is significantly under-presented in AML patients, suggestive of a protective role against leukaemias. Further biochemical characterization of the Tyk2-G363S variant protein would be able to determine if this polymorphism is a cancer-associated genetic alteration. The presence and prevalence of genetic alterations of Tyk2 in other subtypes of leukaemias awaits investigation.

1.4.5.2.4 A possible role of Tyk2 in tumour formation and invasion

In support of the notion that a cancer-related genetic alteration of Tyk2 may actually exist, recent data indicate that Tyk2 is required, at least in part, for the invasiveness of breast, prostate and kidney cancer cell lines (Caldas-Lopes *et al.*, 2009; Ide *et al*., 2008; Wu *et al*., 2007). Abrogation of Tyk2 activity by small molecule inhibitors or removal of the protein by small-interfering RNA (siRNA) results in a decrease in the number of cells moving across the Matrigel and Transwell pores. In another report, it was demonstrated that over-expression of Tyk2 can lead to oncogenic transformation (Knoops *et al.*, 2008). Upon stable expression of wild-type Tyk2, Ba/F3 cells gain the ability to proliferate in the absence of IL-3. These growth-independent cell clones display constitutive phosphorylation of Tyk2, STAT3, STAT5 and ERK. Therefore together these data hint at the possibility that anomalies of the Tyk2 gene may contribute to cancer at 2 stages, namely (i). to promote transformation of normal cells into cancerous cells and/or (ii). to enhance the capability of cancer cells to move to distant sites.

II. Specific Aims

The genetic anomalies underlying many types of cancers have remained elusive despite intense research effort. The association between activating mutations of the *egfr* gene and NSCLC is one of the few established associations between an anomaly of a PTK and cancer. The identification of EGFR TKI-sensitive mutations that affect residues at the extra-cellular domain of EGFR (Lee *et al.*, 2006) opens up the possibility that a mutation affecting a residue anywhere outside the kinase domain of EGFR may be similarly drug-sensitizing. Since the presence of currently known EGFR TKI-sensitizing mutations is a determinant for clinical response in cancers (Lynch *et al*., 2004; Paez *et al.*, 2004; Pao *et al.*, 2004), a possible identification of novel EGFR-TKI-sensitizing mutation(s) in this study would mean that a new subset of cancer patients is likely to benefit from these TKIs. With this in mind, 254 cancer cell lines were checked for somatic mutations of EGFR and were further screened for sensitivity against the EGFR TKI gefitinib, with an aim to identify novel drug-sensitizing mutations of EGFR. Initial biochemical analyses will also be carried out to establish the properties of selected mutations of interest.

On the other hand, the role of most PTKs, including Tyk2, in cancer is either unexplored or is merely emerging. Recent data indicating the transforming nature (Knoops *et al.*, 2008) and the possible role in the migratory and/or invasive properties of cancer cells (Caldas-Lopes *et al.*, 2009; Ide *et al*., 2008; Wu *et al*., 2007) portray Tyk2 as a potential proto-oncogene. It is therefore foreseeable that an activating genetic alteration of Tyk2 may be able to contribute to one or more aspects of cancer, probably in a dominant fashion. Therefore the second part of this study aims to assess the oncogenic potential of all genetic alterations of Tyk2 identified by the Singapore OncoGenome (SOG) Group. This is achieved via biochemical and functional characterizations in both transient over-expression as well as endogenous settings.

III. Material and Methods

3.1 Reagents

Phosphate buffered saline (PBS) and 0.125 % (w/v) trypsin solution were purchased in-house from Biopolis Shared Facilities (BSF) [Agency for Science, Technology and Research (A*STAR), Singapore]. Nuclease-free water was purchased from Applied Biosystems / Ambion (Austin, Texas, USA). 30 % acrylamide / bis solution (19:1), ammonium persulphate (APS), ethidium bromide (10 mg/ml solution), glycine powder and 50 X Tris-acetate-EDTA (TAE) buffer was purchased from Bio-Rad (Hercules, California, USA). Agarose, Tris (hydroxymethyl)aminomethane (Tris base), Tris hydrochloride (Tris-HCl) and Trizol® were purchased from Invitrogen (Carlsbad, California, USA). Chloroform, dimethyl sulphoxide (DMSO), ethanol, isopropanol, potassium chloride (KCl), potassium hydroxide (KOH), sodium chloride (NaCl), sodium hydroxide (NaOH), N,N,N',N'-tetramethylethylenediamine (TEMED) and all acids used in this study were purchased from Merck (Whitehouse Station, New Jersey, USA). All restriction enzymes used in this study were purchased from New England Biolabs (Ipswich, Massachusetts, USA). N,N-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid (BES), betaine, bovine serum albumin (BSA) powder, calcium chloride ($CaCl_2$), ethylenediaminetetraacetic acid (EDTA), gelatin, glycerol, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) powder, magnesium sulphate ($MgSO_4$), manganese chloride ($MnCl_2$), β-mercaptoethanol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 1,4-piperazinediethanesulphonic acid (PIPES), phenylmethanesulphonyl fluoride (PMSF), potassium phosphate monobasic (KH_2PO_4), sodium fluoride (NaF), sodium orthovanadate (Na_3VO_4), sodium phosphate dibasic heptahydrate ($Na_2HPO_4.7H_2O$) and sodium pyrophosphate ($Na_4P_2O_7$) were purchased from Sigma (St. Louis, Missouri, USA). Aprotinin, bromophenol blue, crystal violet, Ponceau S powder, sodium dodecyl sulphate (SDS), Triton-X 100, polyoxyethylene (20) sorbitan monolaurate (Tween-20) and xylene cyanol were purchased from USB Chemicals (Cleveland, Ohio, USA).

EGFR inhibitors gefitinib and erlotinib were purchased from Biaffin GmbH & Co KG (Kassel, Germany) and LC Laboratories (Woburn, Massachusetts, USA) respectively. The specific STAT3 inhibitor S3I-201 (also known as NSC 74859) was purchased from Santa Cruz

Biotechnology (Santa Cruz, California, USA). All compounds were dissolved in DMSO and stored as aliquots at -20 °C, at a concentration of 20 mM.

Human recombinant EGF (100 μg/ml) was purchased from Cell Signaling Technology (Danvers, Massachusetts, USA), while human recombinant IFN-α was purchased from BioVision (Mountain View, California, USA). IFN-α was dissolved in water to a concentration of 2.7 x 10^6 IU/ml and stored as aliquots. Tetramethylammonium (TMA) oxalate, an additive used to improve specificity and yield of polymerase chain reactions (PCRs) (Kovárová and Dráber, 2000), was kindly provided by Prof. A. Ullrich at the Max Planck Institute for Biochemistry (MPI) at Martinsried, Germany.

3.2 Media and supplements

Dulbecco's minimum essential medium (DMEM), Roswell Park Memorial Institute (RPMI)-1640 medium and 1 M HEPES solution for cell culture use were purchased in-house from A*STAR-BSF. Insulin solution (10 mg/ml) was purchased from Sigma. Other cell culture media, such as DMEM/F-12 (1:1) mixture (catalogue number 11320), Ham's F-12 medium (#11765), Leibovitz L15 medium (#11415) and minimal essential medium (MEM) (#11095) were all purchased from Invitrogen. Foetal bovine serum and supplements to culture medium were purchased from Gibco (Invitrogen, Carlsbad, California, USA).

Agar powder, Bacto-Tryptone® and yeast extract for preparation of Luria-Bertani (LB) medium (10 g/L Bacto-Tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH adjusted to 7.2 before autoclaved) or agar for culturing of *Escherichia coli* DH5αF' cells were purchased from Becton Dickinson Biosciences (San Jose, California, USA). Ampicillin and kanamycin for bacterial clone selection were purchased from Sigma and Gibco respectively. Ampicillin was dissolved in 50 % (v/v) ethanol to 100 mg/ml and kanamycin in water to 10 mg/ml and stored at -20 °C as aliquots. LB agar plates were prepared by addition of 1.5 % (w/v) agar powder to LB medium before autoclave. When necessary, ampicillin or kanamycin was added to warm autoclaved medium or molten agar to final concentrations of 100 μg/ml and 10 μg/ml respectively.

3.3 Antibodies

Antibody against β-actin (catalogue number ab6276) was purchased from Abcam (Cambridge, UK). Monoclonal antibodies against cyclin D1 (#554180), phospho-STAT5 [phospho-tyrosine (p-Tyr)-694] (#611964) and Tyk2 (#610173) were purchased from Becton Dickinson Biosciences. Antibody against the *v-myc* myelocytomatosis viral oncogene homolog (avian) (c-Myc) protein (#631206) was kindly provided by Dr. T. H. Loo (Laboratory of Developmental and Regenerative Biology, Institute of Medical Biology (IMB), A*STAR, Singapore) and was purchased from Clontech / Takara Bio USA (Madison, Wisconsin, USA). Horseradish peroxidase-conjugated anti-mouse (#31430) and anti-rabbit (#31460) secondary antibodies were purchased from Pierce / Thermo-Fisher Scientific (Rockford, Illinois, USA). Antibody against the myeloid cell leukaemia sequence 1 (Mcl-1) proteins (sc-819) was purchased from Santa Cruz Biotechnology. Antibody against the heat shock protein Hsp60 (H4149) was purchased from Sigma. Polyclonal antibodies against EGFR (#06-847) and p-Tyr (clone 4G10) (#05-321) were purchased from Upstate / Millipore (Billerica, Massachusetts, USA). All other antibodies used in this study were purchased from Cell Signaling Technology. They include antibodies against: AKT (#9272), phospho-AKT (p-Ser473) (#9271), BCL2-like 1 (Bcl-x_L) protein (#2764), baculoviral IAP repeat-containing 5 (BIRC5, also known as survivin) (#2808) (kindly provided by Dr. N. Kobayashi, Laboratory of Stem Cell Disease Models, IMB, A*STAR, Singapore), EGFR [specific for immuno-precipitation (IP)] (#2256), phospho-EGFR (p-Tyr1045) (#2237), phospho-EGFR (p-Tyr1068) (#2236), phospho-EGFR (p-Tyr1173) (#4407), ERK1/2 (#9102), phospho-ERK1/2 (p-Thr202/p-Tyr204) (#9101), phospho-STAT1 (p-Tyr701) (#9171), STAT1 (#9176), phospho-STAT3 (p-Tyr705) (#9131), STAT3 (#9132), STAT5 (#9310), α/β-tubulin (#2148) and phospho-Tyk2 (p-Tyr1054/1055) (#9321).

3.4 Genetic material used in confirmatory mutational analyses of selected genetic alterations

Biopsies of primary invasive breast carcinomas were kindly provided by Dr. H. Hoefler (Department of Pathology, Technical University of Munich, Munich, Germany) and Dr. S. Iacobelli (Department of Oncology, University of Chieti Medical School, Chieti, Italy),

whereas biopsies of kidney and prostate tumours were provided by Dr. S. Peter (Department of Urology, Klinikum Darmstadt, Darmstadt, Germany). Genomic DNA samples extracted from peripheral blood of 90 non-cancerous individuals were purchased from Coriell Institute for Medical Research (Camden, New Jersey, USA).

3.5 Primers

All primers were designed at the Primer3 website (http://fokker.wi.mit.edu/primer3/input.htm), using reference sequences from NCBI GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) (NM_005228.2 for EGFR mRNA, NG_007726.1 for EGFR genomic DNA, NM_003331.3 for Tyk2 mRNA and NG_007872.1 for Tyk2 genomic DNA) unless otherwise stated. Primers were purchased as 100 μM stock from Sigma-Proligo (Singapore) and diluted to a 10 μM working concentration with nuclease-free water. The list of primers used in PCR amplifications from cDNA from cancer cell line mRNAs and subsequent sequencing experiments for the identification and verification of genetic alterations are as follows:

Primer name	Sequence (5’ to 3’)
EGFR-1	CTCCGTCCAGTATTGATCG
EGFR-2	CGCAGGTGGCACCAAAGC
EGFR-3	GCTCTACAACCCCACCACG
EGFR-4	CTGTGCAGGTGATGTTCATGG
EGFR-5	GTGCCACCCAGAGTGCCTG
EGFR-6	CCGCACCCAGCAGTTTGGC
EGFR-7	CCTGGCAGCCAGGAACG
EGFR-8	GGGCTCATACTATCCTCCG
Tyk2-1	CTGAGGCCCAGAATTGCTAAGT
Tyk2-2	CACTGTCCCGGATGTAGCAG
Tyk2-3	TGGTCATGGTCAAATACCTAGCC
Tyk2-4	CATGATGATGAGATTGGAGGTTTC
Tyk2-5	GATGACTGCTTCTCTCTGCGTC

Primer name	Sequence (5’ to 3’)
Tyk2-6	CGGAGGGACTGCGGCTCTGC
Tyk2-7	GGGAGGAGCGGGTGGAGAGG
Tyk2-8	GTCCAGCAGCACGTTGCGCGC
Tyk2-9	ACCAAGGCGAGAAGTCGCTGC
Tyk2-10	TGGAGCAGGGAGCAGGAGGC

The list of primers used in PCR amplifications from genomic DNA of cancer cell lines for the verification of genetic alterations is as follows:

Primer name	Sequence (5’ to 3’)
EGFR-exon3-F	CATTATCACAGGGGTCAAAGG
EGFR-exon3-R	TGCCTACACACATAGATTCACG
EGFR-exon7-F	GGGCTTTCTGACGGGAGT
EGFR-exon7-R	GCAAGGCAAACACATCCAC
EGFR-exon8-F	TTTCCATCACCCCTCAAGA
EGFR-exon8-R	CCTTCCCATTGCCTAACCTA
EGFR-exon20-F	GACTGGGGAGAGCTTGAGAA
EGFR-exon20-R	TCCCTCCTATGCACACACAG
EGFR-exon26-F	TGGGTCCTTACAGCAATCCT
EGFR-exon26-R	GGGCAACTCGGTATTTCATT
EGFR-exon31-F	GCTCCTGCTCCCTGTCATAA
EGFR-exon31-R	ATTTGGCTTGGCTTCCTTG
Tyk2-exon17-F	CTGTGGCTGGCTTTGTGAC
Tyk2-exon18-R2	CTCACCCAGATGCCAAGAAC
Tyk2-exon21-F	GCTGGGGTCACTTGGAAC
Tyk2-exon21-R	TCTCCGTCCTGTCCTGTCTT
Braf-exon11-F*	TCCCTCTCAGGCATAAGGTAA
Braf-exon11-R*	CGAACAGTGAATATTTCCTTTGAT
Braf-exon15-F*	TCATAATGCTTGCTCTGATAGGA
Braf-exon15-R*	GGCCAAAAATTTAATCAGTGGA

Primer name	Sequence (5' to 3')
Kras-exon1-F*	GGTGGAGTATTTGATAGTGTATTAACC
Kras-exon1-R*	AATGGTCCTGCACCAGTAATATG
Kras-exon2-F*	TCTTTGGAGCAGGAACAATG
Kras-exon2-R*	TGCATGGCATTAGCAAAGAC

{*These primers were kindly provided by Miss Winnie Wong [SOG Group, IMB, A*STAR, Singapore]. The references of the *kras* and *braf* primers are Yamamoto *et al*., (2007) and Immervoll *et al.*, (2006) respectively.}

The list of primers used in the site-directed mutagenesis reactions is as follows:

Primer name	Sequence (5' to 3')
EGFR-G719Smut-F	caaaaagatcaaagtgctgAgctccggtgcgttcggc
EGFR-G719Smut-R	gccgaacgcaccggagcTcagcactttgatctttttg
EGFR-P753Smut-F	ggaattaagagaagcaacatctTcgaagccaacaaggaaatcc
EGFR-P753Smut-R	ggatttccttgttggctttcgAagatgttgcttctcttaattcc
EGFR-L858Rmut-F	gatcacagattttgggcGggccaaactgctgggtg
EGFR-L858Rmut-R	cacccagcagtttggccCgcccaaaatctgtgatc
EGFR-del747-753insSmut-F	cccgtcgctatcaaggaatcgaaagccaacaaggaaatcctcg
EGFR-del747-753insSmut-F	cgaggatttccttgttggctttcgattccttgatagcgacggg
Tyk2-A53Tmut-F	cagtgagtcatcgctgacaActgaggaagtctgcatccac
Tyk2-A53Tmut-R	gtggatgcagacttcctcagTtgtcagcgatgactcactg
Tyk2-V362Fmut-F	gccaaggctcacaaggcaTtcggccagccggcagacag
Tyk2-V362Fmut-R	ctgtctgccggctggccgaAtgccttgtgagccttggc
Tyk2-G363Smut-F	aaggccaaggctcacaaggcagtcAgccagccggcagacagg
Tyk2-G363Smut-R	cctgtctgccggctggcTgactgccttgtgagccttggccttc
Tyk2-I684Smut-F	tgtgcgcggccctgaaaataGcatggtgacagagtacgtg
Tyk2-I684Smut-R	cacgtactctgtcaccatgCtattttcagggccgcgcac
Tyk2-R701Tmut-F	tggatgtgtggctgcggaCggagcggggccatgtgcc
Tyk2-R701Tmut-R	gcacatggccccgctccGtccgcagccacacatccag

Primer name	Sequence (5’ to 3’)
Tyk2-D883Nmut-F	tcttgactgtgaacccgAactcaccggcgtcggaccctac
Tyk2-D883Nmut-R	gtagggtccgacgccggtgagtTcgggttcacagtcaag
Tyk2-R901Qmut-F	gctatttgaaaaagatccAagatctgggcgagggtcacttc
Tyk2-R901Qmut-R	gaagtgaccctcgcccagatctTggatctttttcaaatagc
Tyk2-A928Vmut-F	ggcactggcgagatggtggTggtgaaagccctcaaggcag
Tyk2-A928Vmut-R	ctgccttgagggctttcaccAccaccatctcgccagtgcc
Tyk2-K930Rmut-F	ggcgagatggtggcggtgaGagccctcaaggcagactgc
Tyk2-K930Rmut-R	gcagtctgccttgagggctCtcaccgccaccactcgcc
Tyk2-P1104Amut-F	ctgtgactccagccagagcGccccccacgaaattccttgagc
Tyk2-P1104Amut-R	gctcaaggaatttcgtgggggCgctctggctggagtcacag
Tyk2-Del340mut1-F	gaggaggtgaacaaggagACCggttctagtggcagcagtg
Tyk2-Del340mut1-R	cactgctgccactagaaccGGTctccttgttcacctcctc
Tyk2-Del340mut2-F	gggatccacggacccctAcCggTgccatttgtgcaggcc
Tyk2-Del340mut2-R	ggcctgcacaaatggcAccGgTaggggtccgtggatccc
Tyk2-Del340mut3-F	gaggaggtgaacaaggagGAGggAgccatttgtgcaggcc
Tyk2-Del340mut3-R	ggcctgcacaaatggcTccCTCctccttgttcacctcctc
Tyk2-Del971mut1-F	ctgctgcgaggaccaagCTTagaagtcgctgcagctg
Tyk2-Del971mut1-R	cagctgcagcgacttctAAGcttggtcctcgcagcag
Tyk2-Del971mut2-F	cgcccagcagatctgcAagCTTatggcctatctgcacgcgc
Tyk2-Del971mut2-R	gcgcgtgcagataggccatAAGctTgcagatctgctgggcg
Tyk2-Del971mut3-F	ctgctgcgaggaccaagGGCatggcctatctgcacgcgc
Tyk2-Del971mut3-R	gcgcgtgcagataggccatGCCcttggtcctcgcagcag

The list of primers used in the real-time PCR reactions is as follows:

Primer name	Sequence (5’ to 3’)
Bclxl-rt-F	GCGTGGAAAGCGTAGACAAG
Bclxl-rt-R	GCTGCATTGTTCCCATAGAG
Birc5-rt-F	TTCAGGTGGATGAGGAGACA
Birc5-rt-R	CAGCAGTGGCAAAAGGAGTA

Primer name	Sequence (5' to 3')
Ccnd1-rt-F	CCCTCGGTGTCCTACTTCAA
Ccnd1-rt-R	GAAGCGGTCCAGGTAGTTCA
GAPDH-rt-F	ATGTTCGTCATGGGTGTGAA
GAPDH-rt-R	TGTGGTCATGAGTCCTTCCA
Mcl-1-rt-F	TAAGGACAAAACGGGACTGG
Mcl-1-rt-R	CATTCCTGATGCCACCTTCT
Myc-rt-F^	TCAAGAGGCGAACACACAAC
Myc-rt-R^	GGCCTTTTCATTGTTTTCCA
Tyk2-rt-F	AGCTGGTCATGGAGTACGTG
Tyk2-rt-R	TCTCGGTGGATGTAGTGCTG
Vegf-rt-F	AGTCCAACATCACCATGCAG
Vegf-rt-R	TTTCCCTTTCCTCGAACTGA
18S-rRNA-rt-F	CGGCTTAATTTGACTCAACACG
18S-rRNA-rt-R	TTAGCATGCCAGAGTCTCGTTC

[^The reference of the Myc-rt-F and Myc-rt-R primers is Wang *et al*., (2008).]

3.6 Cell lines

A-427 cells (Giard *et al.*, 1973) were purchased from German Collection of Microorganisms and Cell Cultures (DMSZ) (Braunschweig, Germany). FTC-133 and FTC-238 cells were purchased from European Collection of Cell Cultures (ECACC) (Porton Down, Salisbury, UK). 818-4 cells were kindly provided by W. Schmiegel at the Universitätskrankenhaus Eppendorf, Medizinische Klinik, Hamburg, Germany (Schmiegel *et al*., 1993). MM-Arn cells were provided by Koerner. U-1240 cells were given by M. Nistér at the University of Uppsala, Sweden (Nistér *et al*., 1988). UM-SCC-17B cells were kindly provided by D. R. Vlock at the Pittsburgh Cancer Institute, University of Pittsburgh, Pennsylvania, USA (Vlock *et al*., 1989). The ovarian carcinoma cell line IGROV-1 was a generous gift from J. Bénard at the Institut de Cancérologie Gustave Roussy, Villejuf, France (Bénard *et al*., 1985). All other human cancer cell lines used in this study were purchased from American Type Culture Collection (ATCC) (Manassas, Virginia, USA).

3.7 Propagation of mammalian cell lines

All cell lines were maintained in suitable media added with necessary supplements in a humidified incubator (Heraeus, Thermo-Fisher Scientific, Waltham, Massachusetts, USA) at 37 °C in the presence of 5 % (v/v) carbon dioxide (CO_2), except for those cultured in Leibovitz L15 medium, which were maintained in air. DMEM and RPMI media were routinely supplemented with 1 mM sodium pyruvate and 2 mM L-glutamine before use, while MEM was supplemented with 1 mM sodium pyruvate and 1 X non-essential amino acids (NEAA) mixture. All cell lines were discarded after 15 passages or 2 months of active culturing, whichever was reached first, to prevent possible accumulation of extra genetic alterations and cross-contamination which may affect subsequent work.

Culture conditions of all cell lines used in this study are summarised in the following table:

Cell line	Description	Medium	Split ratio
818-4	Human pancreas carcinoma	RPMI + 10 % serum	1:2 to 1:5
A-427	Human lung carcinoma	MEM + 10 % serum	1:2 to 1:6
A-431	Human epidermoid carcinoma	DMEM + 10 % serum	1:3 to 1:8
FTC-133	Human follicular thyroid carcinoma	DMEM/F12 + 10 % serum	1:8 to 1:12
FTC-238	Human follicular thyroid carcinoma	DMEM/F12 + 5 % serum	1:10 to 1:20
HEK-293	Human embryonic kidney fibroblasts	DMEM + 10 % serum	1:4 to 1:10
HeLa S3	Human cervix adenocarcinoma	Ham's F12 + 10 % serum	1:4 to 1:10
IGROV-1	Human ovary adenocarcinoma	RPMI + 10 % serum	1:3 to 1:10
MDA-MB-361	Human mammary gland adenocarcinoma	Leibovitz L15 + 10 % serum	1:2 to 1:6
MM-Arn	Human malignant melanoma	RPMI + 10 % serum + 5 μg/ml insulin	1:2 to 1:5

Cell line	Description	Medium	Split ratio
RL95-2	Human endometrium adenocarcinoma	DMEM/F12 + 10 % serum + 10 mM HEPES + 5 μg/ml insulin	1:2 to 1:5
SK-MEL-28	Human malignant melanoma	MEM + 10 % serum	1:3 to 1:8
SW-48	Human colon adenocarcinoma, Duke's type C, grade IV	Leibovitz L15 + 10 % serum	1:2 to 1:6
U-1240	Human glioblastoma	DMEM + 10 % serum	1:2 to 1:5
UM-SCC-17B	Human larynx squamous cell carcinoma	MEM + 10 % serum	1:2 to 1:5

3.8 Extraction of total RNA and genomic DNA

Approximately 5 x 10^6 cells were harvested by trypsinization or physical removal using a cell scraper. Total RNA was extracted from cell pellets using a RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Separately, total RNA used to generate cDNA for the mutational screening of genetic alterations of the cancer cell lines were extracted by Tatjana Knyazeva (MPI, Martinsried, Germany) using previously described protocols (Puissant and Houdebine, 1990).

To extract genomic DNA from human cancer cell lines, approximately 4 x 10^6 cells were harvested and resuspended in ice-cold PBS to a final volume of 200 μl. From here genomic DNA was extracted from the cell suspension by a QIAamp DNA mini kit (Qiagen), according to manufacturer's instructions.

Quality and quantity of the RNA and DNA obtained were determined using a Nanodrop UV spectrophotometer (Thermo-Fisher Scientific) and/or visualised by agarose gel electrophoresis.

3.9 Generation of cDNA by reverse transcription

cDNA used to identify genetic alterations of the cancer cell lines in the SOG project was kindly reverse transcribed from total RNA by T. Knyazeva (MPI, Martinsried, Germany) using

established protocol (Sambrook *et al.*, 1989). In Singapore, an additional procedure was performed prior to cDNA synthesis. Genomic DNA contaminant in total RNA preparations was removed using a TURBO DNA-free™ kit (Ambion) (the kit was a generous gift from Ms. H. H. Chua, Genome Institute of Singapore (GIS), A*STAR, Singapore) according to manufacturer's protocol.

First-strand cDNA was generated from cleaned-up total RNA using a Super Script III first-strand synthesis system for RT-PCR (Invitrogen). In a typical reaction, approximately 2 μg total RNA was mixed together with a deoxyribonucleotide triphosphate (dNTP) mix and either (1). 50 pmol oligo-dT_{20} primer or (2). 50 ng random hexamer primer mix. The total volume was adjusted to 10 μl with water. This mixture was heated to 65 °C for 5 min to remove secondary structures and to allow annealing. Subsequently the mixture was cooled on ice for 5 min.

The RNA / primer / dNTP mixture was added to 10 μl of a cDNA synthesis mix. The final mixture (20 μl) contains 1 X reaction buffer, 5 mM magnesium chloride ($MgCl_2$), 0.5 mM dNTP, 10 mM dithiothreitol (DTT), 40 units of RNaseOUT™ ribonuclease (RNase) inhibitor and 200 units of reverse transcriptase. cDNA synthesis was carried out at 50 °C for 50 min, and stopped by heating at 85 °C for 5 min. The RNA template was subsequently removed by incubation with 2 units of RNase H at 37 °C for 20 min.

3.10 Polymerase chain reaction

DNA fragments used for identification of genetic alterations and for cloning purposes were generated by PCR using a MJ Research PTC-100 Tetrad thermal cycler (Bio-Rad). Optimum PCR reaction conditions for individual primer pairs were determined using pooled cDNA or genomic DNA from human cancer cell lines and one of the following DNA polymerases: (1) KOD Hot Start polymerase (Novagen® / Merck), (2) AccuPrime™ *Taq* polymerase (Invitrogen), and (3) FastStart *Taq* DNA polymerase (Roche, Basel, Switzerland). When necessary, one of the following additives was added to the reaction mixture to improve yield and / or specificity: (I). betaine (to a final concentration of 1.2 M), (II). DMSO [final 10 % (v/v)] or (III). TMA oxalate (final 2 mM). The best set of parameters [a combination of (i). the choice of polymerase, (ii). additive, (iii). annealing temperature and (iv). number of

cycles] that produce the optimum product [as determined by (i) substantial yield and (ii) absence of non-specific products] for each primer pair was then applied to actual reactions. For other parameters, such as the concentrations of each component in the reaction mixture and the duration and temperature of each cycle step, recommended values provided by the manufacturers of the respective polymerases were used.

If the PCR products were to be used in salt-sensitive downstream applications, they were purified using a Montáge PCR_{96} clean up kit (Millipore) before used in subsequent experiments.

3.11 Agarose gel electrophoresis

PCR product, genomic or plasmid DNA was mixed with a 6 X loading dye [0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol, 30 % (v/v) glycerol, 0.1 M EDTA, pH 8.0] to a 1 X final dye concentration before loaded onto an agarose gel [0.7 %, 1 % or 3 % (w/v) in 1 X TAE buffer, and added with ethidium bromide to 0.15 μg/ml] for separation. DNA fragments were visualised under a UV light trans-illuminator (Syngene, Cambridge, UK). The size of a fragment of interest was estimated by comparing the distance travelled by the fragment with the distance travelled by reference fragments of known sizes in the 100 bp or 1 kb EZ load molecular ladders (Bio-Rad).

3.12 Dideoxy-sequencing and sequence analysis

Nucleotide sequences of PCR products and plasmid inserts were determined by Sanger dideoxynucleotide sequencing (Sanger *et al*., 1977). Sequencing reactions were performed using a BigDye™ Terminator v3.1 cycle sequencing kit (Applied Biosystems) according to manufacturer's protocol. Un-incorporated nucleotides and other unwanted reaction components were removed from sequencing products using a Montáge SEQ_{96} clean up kit (Millipore) prior to loading to an ABI 3730xl DNA analyzer (Applied Biosystems). Sequence chromatograms were visualised using a freely-available software FinchTV, version 1.4 (Geospiza, Seattle, Washington, USA).

Sequences from multiple samples were aligned together with reference sequences using the Mutation Surveyor™ analysis software, version 2.60 (SoftGenetics LLC, State College,

Pennsylvania, USA). Genetic alterations observed in both directions would be reported by the program. Dubious calls (indicated by low confidence level scores) were verified by visual inspection of chromatograms. When necessary, re-sequencing using internal primers were performed.

3.13 Restriction digestion of DNA

Up to 1 μg of plasmid DNA was mixed with 5 units of restriction enzyme AgeI or 20 units of HindIII in a 20 μl reaction mixture containing recommended reaction buffer and BSA, both at a 1 X final concentration. Digestion was carried out at 37 °C for 1 h. Reactions using AgeI were terminated by incubation at 65 °C for 20 min.

In the event that one of the digested DNA fragments was to be used for cloning experiments, the desired fragment was first separated from other fragments by agarose gel electrophoresis, and then excised from the gel with a blade under UV illumination. Finally the fragment was purified from the agarose using a QIAquick gel extraction kit (Qiagen).

3.14 Ligation of vector and insert

Plasmid vector and DNA fragments, digested with suitable restriction enzymes and purified, were joined together by T4 DNA ligase (Fermentas, Burlington, Ontario, Canada). Insert and vector DNA, usually at either 2:1 or 3:1 molar ratio, were mixed with 1 unit of T4 DNA ligase, ligase buffer [40 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT and 0.5 mM (ATP)] (to a 1 X final concentration) and water to a final volume of 10 μl. The reaction mix was incubated at 16 °C overnight.

3.15 Preparation of competent *E. coli* cells

E. coli DH5αF' cells [F′/ *end*A1 *hsd*R17 (r_K^- , m_K^+) *gln*V44 *thi*-1 *rec*A1 *gyr*A (Nal^r) *rel*A1 Δ(*lac*ZYA-*arg*F) U169 *deo*R (ϕ80d*lac*Δ(*lac*Z)M15] (Genentech) cultured on LB agar plates were inoculated into SOB medium (5 g/L yeast extract, 20 g/L Bacto-Tryptone, 0.584 g/L

NaCl, 0.186 g/L KCl and 2.4 g/L $MgSO_4$, pH adjusted to 7.5 with NaOH) and incubated at 18 °C with shaking at 250 rpm for about 35 to 40 h until the OD_{600} value reached 0.6.

Cells were chilled on ice for 10 min and then pelleted by centrifugation at 3220 x *g* at 4 °C for 10 min. Cells were resuspended in ice-cold buffer TB [18.65 g/L KCl, 2.2 g/L $CaCl_2$, 10 mM PIPES, 10.88 g/L $MnCl_2$, pH adjusted to 6.7 with KOH, filter (Sartorius AG, Goettingen, Germany)-sterilized], allowed to stand for 10 min on ice before centrifuged again. The resultant pellet was resuspended in ice-cold buffer TB supplemented with 7 % (v/v) DMSO and allowed to stand for another 10 min on ice. Finally the cells were snap-frozen in liquid nitrogen before stored at -80 °C as aliquots.

3.16 Transformation of plasmid DNA

Plasmid DNA was introduced into chemically-competent *E. coli* DH5αF' cells by a "heat-shock" method. 50 µl of thawed competent cells were incubated with DNA for 30 min on ice. The mixture was put into a 42 °C water-bath for 45 s and immediately placed back on ice for 2 min. The cells were allowed to recover, after addition of 1 ml pre-warmed LB medium, at 37 °C for 1 h with shaking at 250 rpm before spread on LB agar plates supplemented with a suitable antibiotic.

For long-term storage (more than 1 month) of transformed *E. coli* cells, a single colony was inoculated into 2 ml LB medium supplemented with a suitable antibiotic, and incubated at 37 °C overnight with shaking at 250 rpm. Overnight culture was added with glycerol to a final concentration of 15 % (v/v), snap-frozen in liquid nitrogen and stored at -80 °C as aliquots.

3.17 Large-scale production of plasmid DNA

To generate plasmid DNA for storage purpose or for experiments, a single colony of transformed *E. coli* DH5αF' cells were inoculated into 2 ml LB medium with antibiotic, and incubated at 37 °C for 6 to 8 h with shaking at 250 rpm. At this point, plasmid DNA might be extracted using a QIAprep spin miniprep kit (Qiagen) if the desired yield is low (up to 20 µg). Alternatively, the culture might be inoculated into a larger volume of LB medium with

antibiotic and incubated at 37 °C overnight with shaking. Plasmid DNA was extracted from the overnight culture using a QIAGEN plasmid midi or maxi kit (Qiagen), with a higher yield of up to 100 or 500 μg respectively.

3.18 Site-directed mutagenesis and cloning

Creation of expression vectors carrying genetic alterations-of-interest was carried out by site-directed mutagenesis using a QuikChange® site-directed mutagenesis kit (Stratagene, La Jolla, California, USA) according to manufacturer's recommendations.

All expression vectors harbouring genetic alterations to the EGFR coding sequence were created by Miss Winnie Wong (SOG Group). First, site-directed mutagenesis was performed to generate pcDNA3-ESK-EGFR-WT (pcDNA3 backbone with wild-type EGFR coding sequence cloned into the multiple cloning site of the plasmid) from the plasmid pcDNA3-ESK-EGFR-KA [pUC and Simian virus 40 (SV40) origin of replication (ori), human cytomegalovirus (CMV) immediate-early promoter, *neo*^{R}, *bla*, BGH and SV40 poly-A sequences, f1 intergenic region, modified with the insertion of the multiple cloning site of pBlueScript II SK+] (provided by Dr. J. E. Ruhe, SOG Group). The starting plasmid contains the 2399-2400 AA→GC and 2436 A→T nucleotide substitutions of the EGFR coding sequence. As a result, ectopic protein expression from this plasmid would generate a kinase-dead EGFR protein (with amino acid substitutions of lysine at position 745 to alanine and lysine at position 757 to methionine).

After confirmation of successful generation of wild-type EGFR expression vector by sequencing, the plasmid was used as a template to generate a series of expression vectors carrying various EGFR mutations, also by site-directed mutagenesis.

Plasmids carrying genetic alterations of Tyk2 were similarly generated by site-directed mutagenesis starting from the Human Ultimate™ Full ORF Gateway® Shuttle plasmid pENTR-Tyk2-WT (RZPD, ImaGenes GmbH, Berlin, Germany). This plasmid was originally generated by the insertion of the full-length coding sequence of the Tyk2 transcript into the multiple cloning site of the Gateway® pENTR™-221 vector (pUC ori, *kan*^{R}, *attL1* and *attL2* recombination sites) (Invitrogen).

Generation of the Tyk2-E971fsX67 deletion (removal of Tyk2 exon 19 from pENTR-Tyk2-WT) was initiated by creation of HindIII restriction sites, by site-directed mutagenesis, at positions on the plasmid that correspond to the boundaries of exon 18-exon 19 (using primers Tyk2-Del971mut1-F and 1-R) and exon 19-exon 20 of the gene (using primers Tyk2-Del971mut2-F and 2-R). The resultant plasmid was restriction digested by HindIII. The approximately 7 kb fragment from the digestion was gel-purified using a QIAquick gel extraction kit, and self-ligated by T4 DNA ligase. As a result a 119 bp fragment corresponding to Tyk2 exon 19 was excised from the plasmid. This plasmid was then subjected to a second site-directed mutagenesis reaction to convert the HindIII site that corresponds to the boundary of exon 18-exon 20 back to Tyk2 WT sequence (using primers Tyk2-Del971mut3-F and 3-R).

Using a similar strategy, the Tyk2-S340fsX26 deletion (removal of Tyk2 exons 6 and 7) was generated by creation of AgeI sites at the boundaries of exon 5-exon 6 and exon 7-exon 8, followed by restriction digestion, gel extraction, ligation and second site-directed mutagenesis, as mentioned above.

Since the pENTR™-221 backbone does not contain a promoter that would allow protein expression in mammalian cells, all pENTR™-221 plasmids carrying various Tyk2 genetic alterations were transferred to a pcDNA-DEST40 Gateway™ backbone (pUC ori, CMV and SV40 promoters, *Cm*^{R}, *ccdB*, *attR1* and *attR2* recombination sites, V5 epitope, 6 X His-tag, BGH poly-A sequence, f1 intergenic region, *neo*^{R}, *bla*) by homologous recombination using a Gateway® LR clonase™ II enzyme mix (Invitrogen). In a typical reaction, 150 ng each of the pENTR™-221 (donor) and the pcDNA-DEST40 (recipient) vectors were incubated with 2 µl of the enzyme mix at 25 °C for 1 h. The reaction was stopped by incubation with proteinase K at 37 °C for 10 min. Finally, all EGFR and Tyk2 expression constructs were checked by sequencing before use in subsequent experiments.

3.19 Transfection of plasmids

Transient expression of EGFR and Tyk2 variant proteins in HEK-293 cells was performed using a DNA-calcium phosphate co-precipitation method (Chen and Okayama, 1987). 4 x 10^5 cells/well were seeded to a 6-well plate (Nunc / Thermo-Fisher Scientific, Roskilde,

Denmark) and incubated overnight to allow attachment. On the next day, a transfection mixture containing 1 μg plasmid DNA and 0.25 M $CaCl_2$ in 1 X BBS buffer (50 mM BES, 280 mM NaCl, 1.5 mM $Na_2HPO_4.7H_2O$, pH adjusted to 6.92 to 6.98) in a total volume of 200 μl was added to each well. Cells were incubated overnight to allow transfection to occur before medium change.

Stable HEK-293 cell lines expressing various EGFR variant proteins were generated by Miss Winnie Wong (SOG Group). First, cells were transfected with pcDNA3-ESK-EGFR mutant constructs using the above-mentioned calcium phosphate protocol. 2 days after transfection, cells were sub-cultured and grown in the presence of 0.6 mg/ml G418 sulphate (Geneticin®, Gibco). Single colonies were trypsinized and transferred to individual wells by the means of cloning-discs (3 mm) (Sigma) and allowed to propagate separately in the continued presence of G418. Stable clones were tested for EGFR expression levels by Western blotting, and those with similar expression levels were used for subsequent experiments.

The efficiency of transfection was assessed by visual inspection of cells ectopically expressing green fluorescent protein (GFP) under a fluorescent microscope (Nikon, Tokyo, Japan). Transfection of GFP expression vectors such as pcDNA3-GFP (Dr. J. E. Ruhe, SOG Group) and pmax-GFP® (Amaxa / Lonza, Cologne, Germany) was performed in every experiment.

3.20 Cell lysis for protein detection

Cells were treated accordingly before lysed in pre-chilled lysis buffer [50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 10 % (v/v) glycerol, 1 % (v/v) Triton-X 100, 10 mM $Na_4P_2O_7$ supplemented with proteases inhibitors: 10 mM NaF, 2 mM Na_3VO_4, 2 mM PMSF and 0.1 μg/ml aprotinin] for 20 min at 4 °C.

Cellular debris was removed by centrifugation at 13000 rpm for 10 min at 4 °C. Protein content of all whole-cell lysates was determined using a BCA protein assay kit (Pierce). Protein sample-BCA reagent mixtures were incubated at 37 °C for 30 min before OD_{562} was determined using a SpectraMax M5 spectrophotometer (Molecular Devices, Sunnyvale, California, USA). Protein concentration of lysates was determined by comparing the OD_{562}

values against a standard curve generated from performing the BCA assay on a dilution series of BSA solutions of known concentrations.

3.21 Western blotting and immuno-precipitation

Equal amounts of total protein from various samples were mixed with 6 X SDS sample buffer [0.35 M Tris-HCl, pH 6.8, 0.12 mg/ml bromophenol blue, 30 % (v/v) glycerol, 10 % (w/v) SDS, 6 % (v/v) β-mercaptoethanol]. Volume of the mixture was adjusted with water so that the final concentration of the SDS buffer is 1 X. The mixture was heated at 95 °C for 10 min before separated on a 7.5 % resolving polyacrylamide gel in a gel tank containing a polyacrylamide gel electrophoresis (PAGE) buffer [5 mM Tris base, 0.192 M glycine, 0.1 % (w/v) SDS]. For separation of proteins of smaller molecular weight (about 15-30 kDa), a 10 % or 15 % resolving gel was used.

The recipe for preparation of different components of the PAGE gel is as follows:

Resolving gel buffer (4 X stock)	**Stacking gel buffer (4 X stock)**
1.5 M Tris base	0.5 M Tris base
0.4 % (w/v) SDS	0.4 % (w/v) SDS
pH adjusted to 8.8 using concentrated hydrochloric acid (HCl)	pH adjusted to 6.8 using concentrated hydrochloric acid (HCl)

Resolving gel	**Stacking gel**
1 X resolving gel buffer	1 X stacking gel buffer
Various percentages (w/v) of acrylamide / bis	4 % (w/v) acrylamide / bis
0.04 % (w/v) APS	0.08 % (w/v) APS
0.16 % (v/v) TEMED	0.16 % (v/v) TEMED

Proteins separated by PAGE were then transferred to a nitrocellulose (Bio-Rad) or polyvinylidene fluoride (PVDF) (Amersham / GE Healthcare, Chalfont St. Giles, UK) membrane using a semi-dry transfer block with a semi-dry transfer buffer [48 mM Tris base,

39 mM glycine, 0.0375 % (w/v) SDS, 20 % (v/v) methanol, pH 9.0-9.4] at a constant current of 80 mA per 100 cm^2 membrane. At this point the membrane was routinely incubated with a Ponceau S solution [2 g/L in 2 % (v/v) trichloro-acetic acid] at room temperature for 5 min to confirm successful transfer.

The membrane was washed in distilled water to remove Ponceau S and subsequently blocked with either (i) 5 % (w/v) skim milk powder (Anlene™, Fonterra Co-operative Group, New Zealand) in PBS-Tween [8 g/L NaCl, 0.2 g/L KCl, 10 mM $Na_2HPO_4.7H_2O$, 0.24 g/L KH_2PO_4, 0.05 % (v/v) Tween-20], (ii) 5 % (w/v) BSA in TBS-Tween [25 mM Tris, pH 8.0, 0.375 M NaCl, 0.125 % (v/v) Tween-20] or (iii) NET-gelatin buffer [6.766 g/L NaCl, 5 mM EDTA, pH 8.0, 50 mM Tris, pH 7.5, 0.05 % (v/v) Triton-X 100, 2.5 g/L gelatin] before incubated with appropriately diluted primary and then secondary antibodies. Chemiluminescence signal was generated using a SuperSignal West Dura ECL kit (Pierce) or Immobilon™ Western Chemiluminescent HRP substrate (Millipore). Signals were detected on Hyperfilm MP autoradiography X-ray film (Amersham / GE Healthcare) and an imaging system equipped with a charge-coupled device (CCD) camera (Fujifilm, Tokyo, Japan). Approximate sizes of proteins were estimated by comparing the distance travelled by the protein and that of the proteins in the PageRuler™ pre-stained protein ladder (Fermentas). When necessary, bound antibodies were removed from the membrane by incubation in a stripping buffer [0.1 M NaOH, 10 mM EDTA pH 8.0, 0.1 % (w/v) SDS] at room temperature for 15 min with shaking, washed and subsequently detected for another protein.

For IP, suitable volumes of whole-cell lysates (usually containing 300 to 500 μg total protein) were incubated with primary antibody and a mixture of protein A/G Sepharose beads (GE Healthcare) in ice-cold 1 X HNTG buffer [50 mM HEPES, pH 7.5, 0.15 M NaCl, 10 % (v/v) glycerol, 1 % (v/v) Triton-X 100] at 4 °C overnight with shaking. The beads were washed 3 times with ice-cold 1 X HNTG buffer to remove unbound proteins. Bound proteins were released from the beads by boiling in 6 X SDS sample buffer, and then separated on a SDS-PAGE gel.

3.22 Determination of cellular viability using MTT

Investigation of growth characteristics and sensitivity to EGFR inhibitors of human cancer cell lines were determined by means of the tetrazolium salt MTT (Plumb *et al*. 1989). The yellow MTT is reduced by mitochondrial enzymes present only in viable cells into a purple compound called formazan, which is subsequently dissolved in DMSO and quantified by spectrophotometry. The quantity of formazan produced is directly proportional to the number of viable cells in test wells, and is thus indicative of cellular toxicity brought about by the drug.

To perform this experiment, a suitable number of cells were seeded onto a 96-well flat-bottom cell culture plate (Nunc) at a volume of 200 µl/well and incubated overnight to allow attachment. Optimum seeding densities of the cell lines were empirically tested: the value was chosen based on the observation that the test wells would become approximately 90 % confluent at the end of the assay (4 days after seeding). The seeding densities are: 2500 cells/well for A-427 and A-431, 5000 cells/well for FTC-133, FTC-238, HeLa S3 and SK-MEL-28, 10000 cells/well for 818-4, MDA-MB-361, U-1240 and UM-SCC-17B, and 20000 cells/well for SW-48. For RL95-2 cells, seeding densities of 5000, 10000, 20000 and 30000 cells/well were tested.

On the next day, medium in each well was replaced with 200 µl of fresh medium containing either vehicle [0.5 % (v/v) DMSO] or various concentrations of the drug. Care was taken to minimize disturbance to the cell monolayer during medium removal and drug addition. The cells were incubated at 37 °C for a further 72 h.

To determine the number of viable cells, medium in each well was replaced with 200 µl/well pre-warmed serum-free medium freshly supplemented with 10 mM HEPES. Then 50 µl/well of a 2 mg/ml MTT solution in PBS was added. The contents were briefly mixed using a plate-shaker and incubated at 37 °C for 2 h in the presence of 5 % CO_2. All liquid in each well were then removed. The purple formazan, formed as a result of reduction of MTT by mitochondrial enzymes of living cells, was dissolved in 200 µl/well DMSO and 25 µl/well Sorenson's buffer (0.1 M NaCl, 0.1 M glycine, pH 10.5). The contents were mixed for 10 min on a plate-shaker before the plate was read at 570 nm using a SpectraMax M5 microplate reader.

OD_{570} values were converted to percentage viability by dividing the values obtained from drug-treated cells by those from vehicle-treated cells. The extent of drug-induced cellular toxicity was visualised by plotting the percentage viability values against drug concentrations to generate a dose response curve using a GraphPad Prism software, version 5 (La Jolla, California, USA).

3.23 Determination of proliferation by cell counting

Growth rate of human cancer cell lines was determined by counting the number of cells in assay wells. Cells were seeded to a 6-well plate and allowed to grow for a suitable length of time with or without treatment. A-427 and A-431 cells were seeded at a density of 7.5 x 10^4 cells/well, while IGROV-1 cells were seeded at 1.5 x 10^5 cells/well. For RL95-2 cells, 2 seeding densities were used, namely 1.5 x 10^5 and 9 x 10^5 cells/well. Cells were harvested after incubation by trypsinization and resuspended in a suitable volume of medium. The cells were further diluted in Isoton® II diluent (Beckman Coulter, Fullerton, California, USA) before counting on a Z1™ Coulter Particle Counter® (Beckman Coulter).

3.24 siRNA knock-down of Tyk2

Tyk2 mRNA level in IGROV-1 cells was transiently decreased by ON-TARGETplus™ siRNA (Dharmacon / Thermo-Fisher Scientific, Lafayette, Colorado, USA). 40000 cells/well were seeded to a 24-well plate (Nunc) and allowed to attach overnight. On the next day, 1 µl Oligofectamine™ reagent (Invitrogen) and 1.3 µl of a 20 µM siRNA solution (amount of siRNA is 26 pmol) were incubated with 7.5 µl and 40.2 µl of serum-free medium respectively at room temperature for 5 min. The 2 mixtures were combined and incubated at room temperature for a further 20 min. Meanwhile, medium in each well was replaced with 200 µl of serum-free medium. After incubation, 50 µl of the siRNA-Oligofectamine mixture was added to each well. The cells were incubated at 37 °C with 5 % CO_2 for 4 h and then added with 250 µl of medium supplemented with 20 % (v/v) serum. The cells were incubated for a further 3 days before harvest. To assess transfection efficiency, one well in each experiment was transfected with siGLO® Green transfection indicator (Dharmacon).

3.25 Transwell migration assay

Migratory capability of IGROV-1 cells upon siRNA knock-down of Tyk2 was investigated using Transwell migration chambers (Sieuwerts *et al.*, 1997) (8.0 μm pore size, Costar / Corning, New York, USA). The assay plate was set up by seeding 40000 serum-starved (for 24 h) cells, which were resuspended in 200 μl serum-free medium, to the inner chamber and then filling the outer chamber with 600 μl of medium with or without 10 % (v/v) serum. Cells were incubated for 24 h at 37 °C in the presence of 5 % CO_2.

The chambers were submerged in crystal violet [0.5 % (v/v) in 25 % methanol] for 10 min at room temperature, and excess crystal violet was washed away with distilled water. Cells that did not migrate across the pores were removed by a cotton bud. The chambers were allowed to air dry overnight before migrated cells was counted under 100 X magnification. For each chamber the total number of cells in 5 fields was recorded.

3.26 Real-time PCR

Quantitative analysis of the transcript levels of genes of interest in IGROV-1 cells after transfection with Tyk2 siRNA was achieved by real-time PCR, using a detection methodology based on the fluorescent dye SYBR Green (Zipper *et al.*, 2004).

Total RNA extracted from cells transfected with Tyk2 siRNA was first reverse transcribed into cDNA using random hexamer primer mix as mentioned in the previous section. To set up the real-time PCR reaction, cDNA generated from an equivalent of 1.5 ng total RNA was mixed with 20 pmol of each primer and 2 X Power SYBR PCR master mix (Applied Biosystems) (to a final concentration of 1 X) to a total volume of 20 μl. Amplification was carried out using a 7500 Fast Real-Time PCR System (Applied Biosystems), using default cycling parameters from the manufacturer. Specificity of the PCR reactions was confirmed by submission of the primer sequences to nucleotide BLAST (NCBI) and dissociation curve analysis of the products.

Raw data (in terms of C_t values) were first normalized against one of the following reference genes: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or 18S ribosomal RNA (rRNA). Then a second normalization was performed, against ΔC_t values obtained from assay wells

using cDNA from cells treated with scrambled siRNA, to generate $\Delta\Delta C_t$ values. Fold changes in transcript level is expressed as the value $2^{(-\Delta\Delta C_t)}$.

3.27 Statistical analysis

Significance was determined by performing 2-tailed, unpaired Student's *t* test. Differences between sets of data were considered statistically significant if the *p* value is less than 0.05.

IV. Results

4.1 Analyses of genetic alterations of the *egfr* and *tyk2* genes

4.1.1 Genetic alterations of the *egfr* gene

EGFR is a well-established oncogene involved in the aetiology of a wide spectrum of cancer types, such as lung cancer, colon cancer and glioblastoma multiforme. Recently, EGFR kinase domain mutations such as the G719A/C/S substitutions and exon 19 deletions were discovered in NSCLC patients responsive to treatment with EGFR TKIs (Lynch *et al.*, 2004; Paez *et al.*, 2004, Pao *et al.*, 2004). Here, 10 single amino acid substitution events have been identified from the screening of EGFR transcripts from 254 cancer cell lines (Table 1). They consist of 7 novel and 3 previously published (A289V reported in Thomas *et al.*, 2007; R521K in Moriai *et al.*, 1993 and P753S in Ikediobi *et al.*, 2006) alterations. 9 out of these 10 alterations are somatic in nature (R521K is a polymorphism), and they appear to be evenly distributed among various domains of the protein (Figure 9). Strikingly accordant with the earlier description of the uniqueness of the somatic mutations, 8 out of the 9 EGFR somatic mutations were each found in one cell line, while the E922K mutation was found in two cell lines.

To provide a basis for further biochemical and/or functional studies on human cancer cell lines harbouring the somatic EGFR mutations listed in Table 1, the presence of these mutations in both genomic DNA and cDNA obtained from new sources of cells was

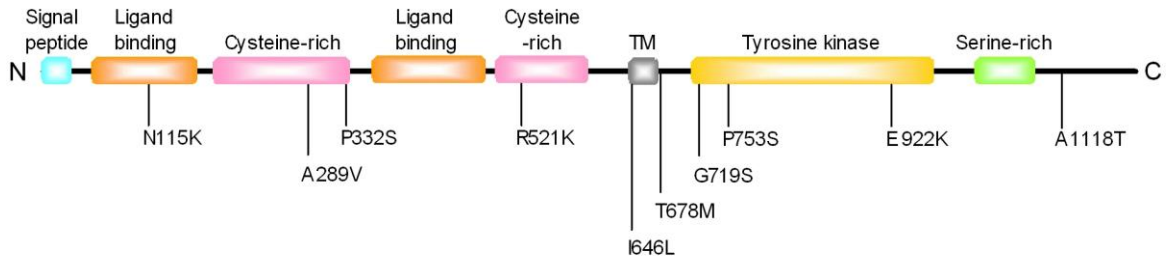

Figure 9. Spatial arrangement of the genetic alterations found in the epidermal growth factor receptor (EGFR) transcripts of human cancer cell lines and non-cancer cells. Genetic alterations identified were evenly distributed among various domains of the protein. TM denotes trans-membrane domain, while "N" and "C" denote amino- and carboxyl-terminal ends of the protein respectively.

confirmed. In all, the mutations in 10 out of the 11 cell lines could be confirmed using either type of genetic material as a template (Figure 10). The exception is the melanoma cell line MM-Arn, which yielded wild-type sequence at codon 922 when both its genomic DNA and cDNA were sequenced (Figure 10i). Based on this observation, further work on MM-Arn cells was discontinued.

Earlier during the screening project, both wild-type and P753S mutant EGFR transcripts were detected in the melanoma cell line SK-MEL-28. However, this substitution was reported as homozygous by Ikediobi and colleagues (2006). To clarify the status of this mutation, repeat sequencing of both genomic DNA and cDNA obtained from SK-MEL-28 was performed, and the results confirmed the homozygous nature of the substitution (Figure 10g).

Genetic alteration		Cell line	Cancer type	Homozygous (HO) or heterozygous (HE)	Type of alteration
Nucleotide change	**Amino acid change**				
T511A	N115K	UM-SCC-17B	Head & neck	HE	Mutation
C1032T	A289V	RL95-2	Endometrium	HE	Mutation
C1160T	P332S	U-1240	Brain	HE	Mutation
G1728A	R521K	Multiple	Multiple	Mixture (7 HO, 73 HE)	Polymorphism
A2102C	I646L	HeLa S3	Cervix	HE	Mutation
C2199T	T678M	MDA-MB-361	Breast	HE	Mutation
G2321A	G719S	SW-48	Colon	HE	Mutation
C2423T	P753S	SK-MEL-28	Skin	HO	Mutation
G2930A	E922K	818-4 MM-Arn	Pancreas Skin	HO HE	Mutation
G3518A	A1118T	FTC-133 FTC-238	Thyroid Thyroid	HE HE	Mutation

Table 1. List of genetic alterations in epidermal growth factor receptor (EGFR) transcripts identified by the mutational screening project.

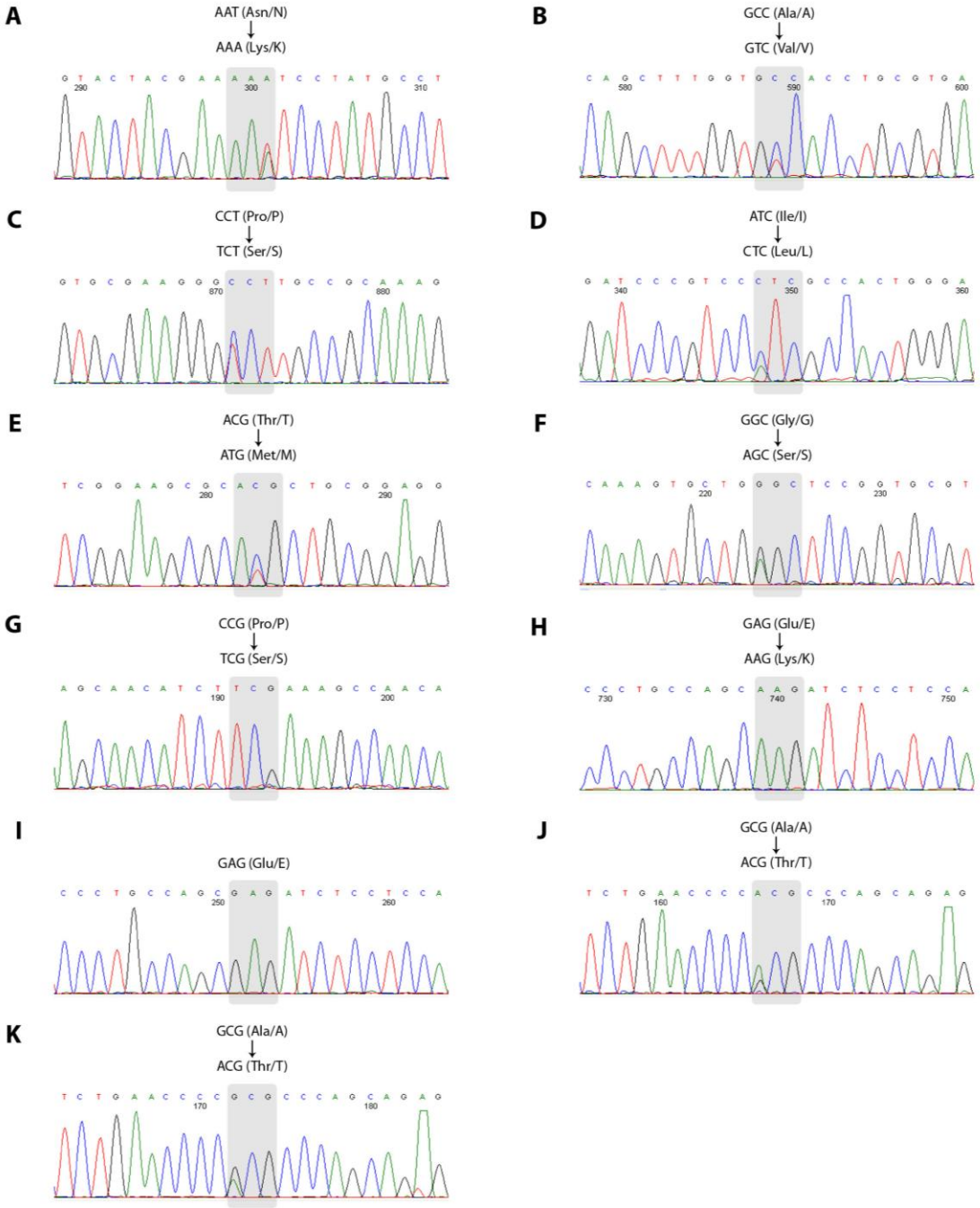

Figure 10. Somatic mutations of the epidermal growth factor receptor (*egfr*) gene are confirmed in 10 human cancer cell lines. Genomic DNA of (a). UM-SCC-17B (with N115K substitution), (b). RL95-2 (A289V), (c). U-1240 (P332S), (d). HeLa S3 (I646L), (e). MDA-MB-361 (T678M), (f). SW-48 (G719S), (g). SK-MEL-28 (P753S), (h). 818-4 (E922K), (i). MM-Arn (E922K), (j). FTC-133 (A1118T) and (k). FTC-238 (A1118T) was sequenced using suitable primers flanking the expected location of the mutations. In all, 9 somatic EGFR mutations previously reported by the Singapore OncoGenome (SOG) Group were confirmed in 10 cell lines. Sequencing reactions using DNA of MM-Arn cells returned with a wild-type sequence at the position corresponding to codon 922. Similar reactions using DNA of SK-MEL-28 cells returned with a homozygous P753S substitution. Identical results were obtained in sequencing reactions using complementary DNA (cDNA) generated from these cell lines as starting material.

4.1.1.1 Prevalence of somatic EGFR mutations in primary tumours

To establish the clinical relevance of the EGFR mutations identified from the cancer cell line screen (Table 1), cDNAs generated from primary samples, including 55 primary tumours each of breast, kidney and prostate origins and non-cancerous kidney tissues matching to 50 of the above-mentioned kidney tumours, were analyzed for EGFR mutations. A section of the EGFR transcript corresponding to codons 575 to 845, which encompasses the trans-membrane domain, the P-loop, the αC-helix and part of the A-loop, was amplified and sequenced. All clinical samples screened here displayed wild-type sequences in this section of the EGFR transcript. Somatic mutations previously identified in cancer cell lines that affect residues within this section (I646L, T678M, G719S and P753S) were not detected in these samples. Moreover in contrast to their relatively frequent detection in EGFR TKI-responsive NSCLC tumours, mutations such as G719A/C/S substitutions and exon 19 deletions are absent in the collection of primary breast, kidney and prostate tumours analyzed here.

4.1.2 Genetic alterations of the *tyk2* gene

As the mutational analysis of EGFR across selected cancer cell lines and primary tumors has been expounded above, a similar approach was adopted for another oncogene, Tyk2. The interest in this target gene stems from recent observations of the involvement of Tyk2 activity in cancer cell invasion (Caldas-Lopes *et al.*, 2009; Ide *et al*., 2008; Wu *et al*., 2007). Based on these results, it was hypothesized that de-regulation of Tyk2 activity due to events such as activating mutations may be responsible for an increased metastatic capability of a cancer cell. Therefore an attempt was made to identify Tyk2 genetic alterations that may have a role in cancer, and thereby, carry the promises of a novel drug target against affected cancer types.

In order to address this question, the Tyk2 transcripts from the same panel of 254 cancer cell lines were sequenced and 10 non-synonymous single amino acid substitutions were identified (Table 2). In addition, 2 out-of-frame deletions were detected. The deletions result in variant Tyk2 proteins with truncations at the carboxyl-terminus. At the genomic level, the S340fsX26 deletion is mediated by the omission of exons 6 and 7 during

transcription, while the E971fsX67 deletion is a result of skipping of exon 19. A detailed description on the nomenclature used to generate the names of these 2 deletions is provided in the Appendix.

These 12 alterations are distributed among the FERM, the pseudokinase and the kinase domains (Figure 11). No alteration was found at the SH2 domain. Interestingly, one third of these alterations are located within a short stretch of 50 amino acids within and near the N-terminal end of the kinase domain. The alterations are classified into 6 somatic mutations and 6 polymorphisms (5 substitutions and 1 deletion for each category) (Table 2). From here, one could observe the rare occurrence of Tyk2 somatic mutations. Even the most frequently observed one, the S340fsX26 deletion, is found in only 3 out of the 254 cell lines screened, or 1.2 %. On the other hand, polymorphisms exhibit frequencies ranging from 0.4 % for A928V to 23.2 % for V362F. All Tyk2 somatic mutations reported here are heterozygous in nature.

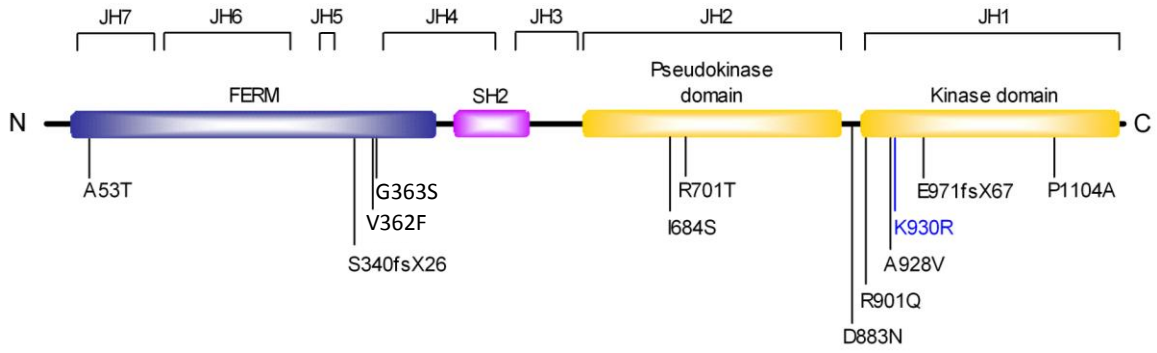

Figure 11. Spatial arrangement of the genetic alterations found in tyrosine kinase 2 (Tyk2) transcripts of human cancer cell lines as reported in Ruhe *et al*., (2007). Genetic alterations were reported in various domains of the protein, with a possible "mutational hot-spot" near the proximal end of the kinase domain. The substitution of lysine at residue 930 to arginine (K930R, labeled in blue) was identified in a kidney tumour biopsy. The term FERM is a short form of the following proteins: four point one (4.1) protein, ezrin, radixin and moesin; SH2 denotes Src-homology 2; JH denotes JAK-homology; and "N" and "C" denote amino- and carboxyl-terminal ends of the protein respectively.

4.1.2.1 Prevalence of Tyk2 genetic alterations in primary tumours

To expand the understanding on the prevalence of Tyk2 genetic alterations in cancers, the same cDNAs generated from the primary tumours and non-cancerous tissues used for the screening of EGFR genetic alterations were screened for selected Tyk2 alterations. This

effort focused on identifying genetic alterations located within the kinase domain, as this domain is critical for the catalytic activity of the protein, and alterations here are likely to result in significant functional consequences. Indeed, replacement of the ATP-binding lysine residue, which resides in the kinase domain, to arginine (K930R) abrogates basal phosphorylation of the Tyk2 protein (Gauzzi *et al.*, 1996). In particular, the frequencies of occurrence of the 4 single amino acid substitutions and 1 deletion previously identified from the mutational screening of the cancer cell lines were determined, and results are summarized in Table 3.

The Tyk2 R901Q mutation was neither found in any of the 165 primary tumours nor in the 50 non-cancerous tissues. The absence of this mutation in primary tumours mirrors its rare occurrence in cancer cell lines. Similarly the prevalence of both A928V and K930R

Genetic alteration		Cell line	Cancer type	Homozygous (HO) or heterozygous (HE)	Type of alteration
Nucleotide change	**Amino acid change**				
G498A	A53T	NCI-H146 TT	Lung Thyroid	HE HE	Mutation
G1425T	V362F	Multiple	Multiple	Mixture (23 HO, 41 HE)	Polymorphism
G1428A	G363S	Multiple	Multiple	HE (10 samples)	Polymorphism
T2392G	I684S	Multiple	Multiple	Mixture (6 HO, 20 HE)	Polymorphism
G2443C	R701T	Kasumi-1	Blood	HE	Mutation
G2988A	D883N	MeWo3	Skin	HE	Mutation
G3043A	R901Q	IGROV-1	Ovary	HE	Mutation
C3124T	A928V	MM-Alt	Skin	HE	Polymorphism
A3130G	K930R	(Tumour biopsy)	Kidney	HE	Mutation
C3651G	P1104A	Multiple	Multiple	Mixture (1 HO, 12 HE)	Polymorphism
Deletion 1354-1708	S340fsX26	BOW-G G-361 RPMI-7951	Skin Skin Skin	HE HE HE	Mutation
Deletion 3251-3369	E971fsX67	Multiple	Multiple	Mixture (1 HO, 14 HE)	Polymorphism

Table 2. List of genetic alterations in tyrosine kinase 2 (Tyk2) transcripts identified by the mutational screen.

alterations in these primary tumours is also very low. The A928V polymorphism, found once among the 254 cancer cell lines, was identified in 1 breast tumour biopsy. The kinase-dead K930R substitution was also identified in 1 tumour biopsy, but of kidney origin (Figure 11, Table 2 and Table 3). This substitution was not found in the corresponding non-cancerous tissue taken from the same patient, which indicates that it should be regarded as a somatic mutation (Table 3).

On the contrary, the P1104A polymorphism was detected more frequently in both cancer cell lines (5.3 %; 13 out of 246 cDNAs) as well as primary tumours (5.4 %; 8 out of 149 cDNAs). Remarkably the prevalence of this polymorphism is highly similar between cell lines and primary tumours. On the other hand, the single kidney tumour and the single non-cancerous kidney tissue in which P1104A were identified are actually a matching pair of genetic material obtained from the same patient. Thus this observation supports the assignment of this substitution as a polymorphism.

Tyk2 Altera-tion	**Breast Tumour cDNA**			**Prostate Tumour cDNA**			**Kidney Tumour cDNA**			**Non-cancerous Kidney tissue cDNA**			**Peripheral Blood genomic DNA**		
	Hit	**n**	**%**	**Hit**	**n**	**%**	**Hit**	**n**	**%**	**Hit**	**n**	**%**	**Hit**	**n**	**%**
R901Q	0	30	**0**	0	42	**0**	0	45	**0**	0	49	**0**	–	–	–
A928V	1	51	**2.0**	0	49	**0**	0	53	**0**	0	49	**0**	3	89	**3.4**
K930R	0	51	**0**	0	49	**0**	1	53	**1.9**	0	49	**0**	0	89	**0**
P1104A	5	53	**9.4**	2	47	**4.3**	1	49	**2.0**	1	49	**2.0**	6	90	**6.7**
E971fs-X67	15	55	**27.3**	17	55	**30.9**	1	55	**1.8**	–	–	–	–	–	–

Table 3. Prevalence of tyrosine kinase 2 (Tyk2) kinase domain genetic alterations in primary tumours and non-cancerous genetic material. Frequencies of occurrence of 4 Tyk2 single amino acid substitutions and 1 deletion in primary tumours of breast, prostate and kidney origins (55 samples each), non-cancerous kidney tissues matching to the kidney tumours (50 samples) and peripheral blood genomic DNA of healthy individuals (90 samples) were determined by sequencing. “Hit” denotes the number of samples carrying a particular genetic alteration and “%” denotes its frequency of occurrence. The term “n” denotes the number of samples of which a good quality sequence was generated. This information is shown here because a small number of PCR and sequencing reactions did not generate PCR products and sequences of reasonable quality or read length. Thus sometimes the number of samples in which the mutational status of the alteration in question can be confidently determined is lower than the total number of samples screened. Dashes in selected boxes of the table indicate that the prevalence of a particular alteration of interest is not determined for that type of genetic material.

The E971fsX67 deletion occurs even more frequently (20.0 %; 33 out of 165 cDNAs) than the P1104A substitution. Thereby, the prevalence of this deletion appears to be highly variable among cancer types. It is identified in approximately 6 % of the 254 cancer cell lines (6.1 %; 15 out of 246 cDNAs) and about 2 % of the kidney tumours but in about 27 % of breast and 31 % of prostate tumours. Finally this screening effort did not identify any genetic alteration that is not already identified in the 254 cancer cell lines.

To ascertain the origin of Tyk2 A928V, K930R and P1104A substitutions, their prevalence in 90 genomic DNA samples isolated from non-cancerous individuals were determined. K930R was not detected in these genomic DNA samples, thus confirming the somatic status of this alteration. In contrast, A928V and P1104A were found in 3 and 6 genomic DNA samples respectively. Therefore they should be regarded as polymorphisms. Interestingly, one genomic DNA sample carries both A928V and P1104A substitutions.

In summary, the current effort established the prevalence of Tyk2 kinase domain genetic alterations in primary breast, prostate and kidney tumours. Furthermore, these results provide supporting evidence for the assignment of Tyk2 R901Q and K930R substitutions as somatic mutations, and the Tyk2 A928V and P1104A substitutions as polymorphisms.

4.2 Biochemical and functional analyses of genetic alterations of the *egfr* gene

4.2.1 Response of human cancer cell lines carrying somatic EGFR mutations towards the EGFR TKI gefitinib

Next, EGFR TKI sensitivity of the 10 cell lines carrying various EGFR alterations was determined as a means to quickly identify novel EGFR TKI-modulating mutations. To ensure consistency of the MTT assay between various test runs, the epidermoid carcinoma cell line A-431 was tested alongside test cell lines as a positive control. A-431 cells, which over-express wild-type EGFR protein (Haigler *et al.*, 1978; Janmaat *et al.*, 2003), are highly sensitive to gefitinib, with an IC_{50} value of about 50 nM after 72 h of treatment (Gendreau *et al.*, 2007). The average IC_{50} value of A-431 cells observed here is higher (185 nM) (Figure 12c) but nonetheless considered sensitive, based on the cut-off value of 1 μM, which was used in several earlier studies (Bianco *et al.*, 2003; Engelman *et al.*, 2005; Janmaat *et al.*, 2003; Tracy *et al.*, 2004).

As a negative control, the NSCLC cell line A-427 expressing normal level of wild-type EGFR was also included in these experiments. These cells are weakly sensitive towards gefitinib, with an IC_{50} value of approximately 6 μM (Figure 12c). Out of the 10 test cell lines, 9 displayed resistance to gefitinib, even at a concentration of 10 μM (Figures 12a and 12b). Among the resistant cell lines treated with 1 μM gefitinib, the most noticeable inhibition in proliferation was observed in UM-SCC-17B cells, which is at an extent of a mere 15 %. More cell lines (MDA-MB-361, SK-MEL-28 and SW-48) displayed substantial inhibition of proliferation in the presence of 10 μM gefitinib, but none of them displayed more than 50 % inhibition of proliferation at this concentration.

The uterine endometrial cell line RL95-2 (with the EGFR A289V mutation) (Way *et al.*, 1983) was found to be sensitive to gefitinib (Figure 12d). Interestingly, at a lower seeding concentration of 5000 cells/well, these cells have an IC_{50} value of 35 nM against gefitinib. In contrast, in test wells initially seeded with 30000 cells/well, the IC_{50} value rises more than 30-fold to about 1.2 μM (Figure 12d). Thus RL95-2 cells appear to be more sensitive to the drug when the initial seeding density is lower. To confirm that proliferation of RL95-2 cells is dependent on EGFR signalling, RL95-2 cells, together with 3 other cell lines, were cultured in the presence of erlotinib (Figure 12f). Since these 2 EGFR TKIs are similar in both chemical structure (Figure 7) and pharmacological activity (Fabian *et al.*, 2005; Pao *et al.*, 2005a), the 4 cell lines are expected to react similarly against both drugs. Indeed, based on the pharmacologically relevant concentration of 2.5 μM as a cut-off value (Sharma *et al.*, 2007), RL95-2 cells are considered to be sensitive towards erlotinib (Figure 12f). Furthermore, the gefitinib-sensitive A-431 cells, the weakly-sensitive A-427 cells and the resistant SK-MEL-28 cells displayed extensive sensitivity, partial sensitivity and total resistance to erlotinib respectively. The IC_{50} values of all 4 cell lines against erlotinib are within 1.5-fold of those against gefitinib (compare Figure 12f with Figures 12c, 12d and 12e). Therefore, these cell lines displayed comparable sensitivity profiles towards the 2 EGFR TKIs.

To further confirm the drug sensitivity, an alternative measure of cell viability was performed by testing selected cell lines based on cell counting after drug treatment (Figure 12e). Such confirmatory experiments are necessary because firstly cell counting is the most direct method of demonstrating cell growth. Furthermore, extra consideration is required when proliferation is determined by measuring changes in a surrogate marker such as

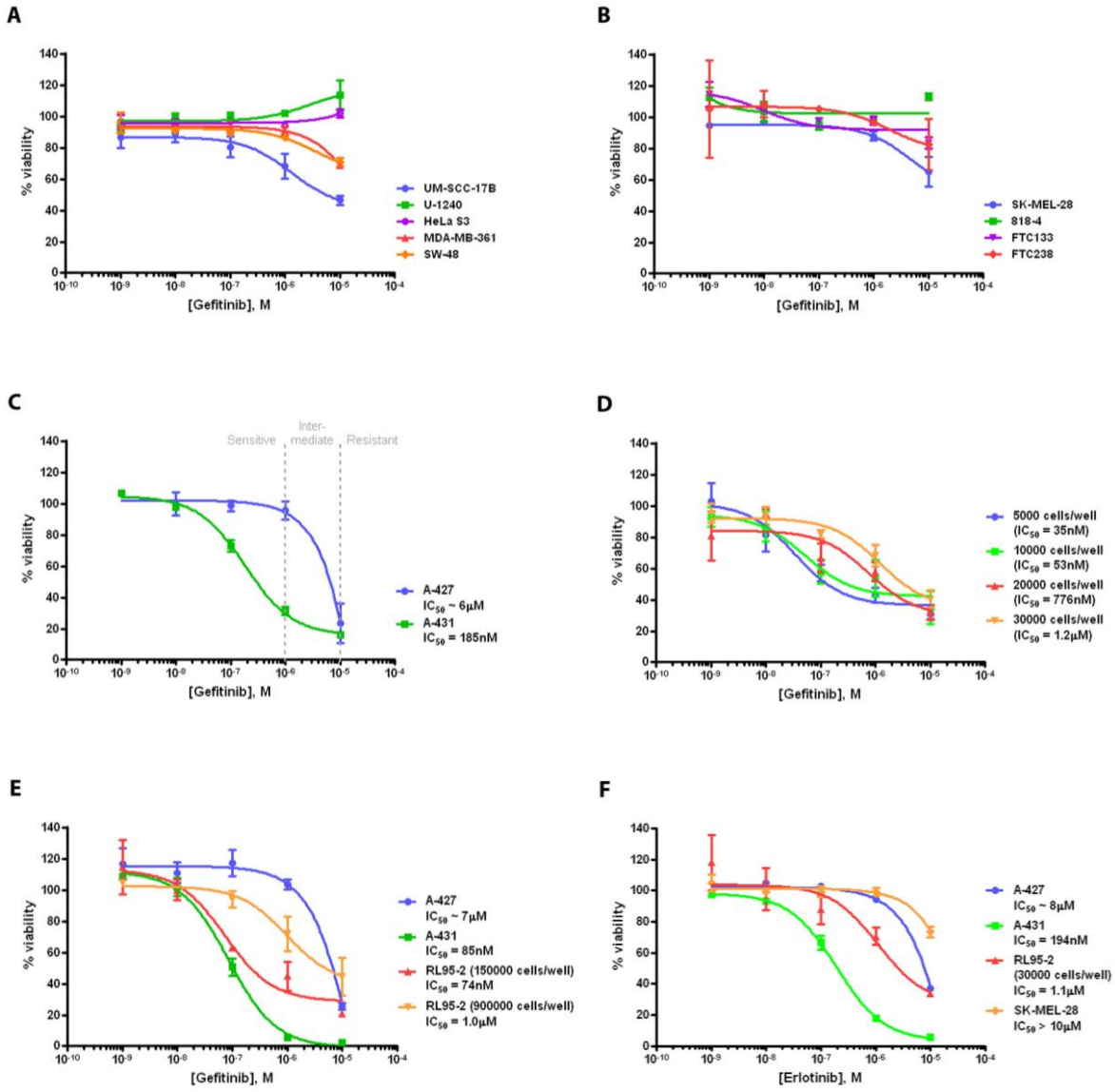

Figure 12. Sensitivity of human cancer cell lines with somatic epidermal growth factor receptor (EGFR) mutations against EGFR tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib. Cells were seeded and allowed to settle for 24 h before incubated with gefitinib [(a) to (e)] or erlotinib [(f)] at concentrations ranging from 1 nM to 10 µM at 37°C for 72 h. Cellular viability, relative to vehicle-treated cells (arbitrarily set at 100 %), was measured by an MTT assay [(a) to (d) and (f)] or by cell counting [(e)], and plotted as dose-response curves. (a). Dose-response curves of UM-SCC-17B, U-1240, HeLa S3, MDA-MB-361 and SW-48 cells. (b). Dose-response curves of SK-MEL-28, 818-4, FTC-133 and FTC-238 cells. (c). Dose-response curve of A-427 and A-431 cells. (d). Dose-response curves of RL95-2 cells, tested at different initial seeding concentrations. (e). Dose-response curves of A-427, A-431 and RL95-2 cells, using data generated from the cell counting method. (f). Dose-response curves of A-427, A-431 and RL95-2 cells against erlotinib. In all assays, cells demonstrating more than 50 % inhibition in proliferation in the presence of 1 µM of gefitinib [left vertical dotted line in (c)] are considered sensitive. Based on this criterion, A-431 and RL95-2 cells are sensitive. The dose-response curves and IC_{50} values of cell lines using data from the MTT and cell counting methods are very similar. MTT assays were performed in 8 replicates for each drug concentration, whereas cell counting assays were performed in triplicates. Each data point on all dose-response curves represents average values of relative viability from 2 independent experiments for resistant cell lines and at least 3 experiments for sensitive cell lines. Error bars denote standard error mean (SEM).

mitochondrial activity (as in the case of the MTT assay). For example, formazan formation in the MTT assay can be affected by the amount of glucose in the culture medium (Vistica *et al.*, 1991). Moreover, some cell lines do not metabolize MTT efficiently (Scudiero *et al.*, 1988). Absolute cell numbers were converted to relative viability by comparing with cell numbers in vehicle-treated wells. By comparing Figure 12e with Figures 12c and 12d, it was observed that the dose-response curves and IC_{50} values of the 4 cell lines against gefitinib obtained using this cell-counting method are very similar to those obtained using the MTT method, thus confirming drug responses of these cell lines.

In all, out of the 10 cancer cell lines tested against the EGFR TKI gefitinib, RL95-2 cells displayed sensitivity. In the following sections, 3 selected EGFR mutations of interest will be further studied, namely the G719S mutation in the colon carcinoma cell line SW-48, the A289V mutation in the uterine endometrial cancer cell line RL95-2 and the P753S mutation in the melanoma cell line SK-MEL-28. Interest is shown in the EGFR G719S mutation identified in SW-48 cells because firstly, prior to the discovery in this cell line, this mutation has never been described in a cancer type other than NSCLC. Secondly, despite the presence of the gefitinib-sensitizing EGFR mutation G719S, SW-48 cells are resistant to the drug. Therefore it will be of interest to decipher the mechanism(s) responsible for this resistance. Thereafter, further EGFR TKI sensitivity tests on RL95-2 cells, the positive cell line among the 10 cell lines tested for gefitinib sensitivity, will be performed to look at the behaviour of the activating and erlotinib-sensitive (Lee *et al.*, 2006) EGFR-A289V protein in an endogenous setting in more detail. Finally, biochemical properties of the EGFR-P753S protein will be determined. The proline residue at codon 753 is situated at the 5' end of the αC-helix, very near the ATP-binding pocket of the EGFR protein. A mutation at this location may be functionally important because it may alter the shape of the pocket.

4.2.2 Mutational profiling of the *kras* and *braf* genes of human cancer cell lines carrying somatic EGFR mutations

In all, except for RL95-2 (EGFR A289V), the other 9 cancer cell lines demonstrate resistance to gefitinib. Here the mechanism(s) underlying drug resistance was investigated. When a cancer cell harbouring an EGFR mutation displays such resistance, it could simply indicate that the EGFR mutation does not sensitize the cells to the drug. However, this scenario does

not apply to SW-48 because the mutation it carries is already well-recognized for being gefitinib-sensitizing (Lynch *et al.*, 2004; Paez *et al.*, 2004). Alternatively, in SW-48 cells the supposed gefitinib-sensitizing effects conferred by the EGFR G719S mutation could be overwhelmed by a dominant counteracting effect conferred by, for example, a resistance-conferring mutation. Activating mutations of the *kras* (Mitsudomi *et al.*, 1991; Rodenhuis *et al.*, 1988) and *braf* genes (Davies *et al.*, 2002) are 2 frequently described EGFR TKI resistance-conferring events (Section 1.3.3.6.1). These 2 genes encode for proteins that form part of the MAP kinase pathway downstream of EGFR. Activating mutant KRAS and BRAF proteins are able to constantly activate the MAP kinase pathway irrespective of the activity status of EGFR. Thus, these mutations confer to a cell an ability to ignore the effects of EGFR TKI-mediated inhibition of EGFR further upstream. To extend this thought, it is possible that the underlying cause for gefitinib resistance of the other 8 cancer cell lines tested here is also the activating *kras* or *braf* mutations. To investigate this, the mutational status of both *kras* and *braf* genes in all cell lines tested for gefitinib sensitivity was established by sequencing.

To date, almost all activating *kras* and *braf* mutations occur at "hot-spots" within exon 1 (that correspond to glycines-12 and 13) and exon 2 (glutamine-61) of the *kras* gene and exon 15 (valine-600) of the *braf* gene. These mutations result in increased activities of AKT and ERK1/2. Therefore, the current sequencing effort targets regions encompassing these 4 exons. Results were summarized in Table 4.

Out of the 10 cell lines tested here, 3 carry genetic alterations at the established mutational "hot-spots" of the *kras* or the *braf* genes. Alterations responsible for the activating KRAS-G12V and KRAS-G12R mutant proteins were detected in MDA-MB-361 and 818-4 cells, respectively. On the other hand, SK-MEL-28 cells carry an alteration corresponding to the constitutively active BRAF-V600E mutant protein. These results suggest that the EGFR mutations carried in these 3 cell lines may be gefitinib-sensitizing, but this effect may be counteracted by the activating *kras* or *braf* mutations. In addition, this is the first time the KRAS G12V mutation is found in MDA-MB-361 cells. Further testing on multiple sources of the same cell line may be required to ascertain the mutational status of the *kras* gene in MDA-MB-361. Finally, the remaining 7 cell lines did not harbour any non-synonymous genetic alteration at the mutational "hot-spots" of both *kras* and *braf* genes. Thus, activities

of these 2 genes do not seem to interfere with the response (or the lack of it) of these cell lines against gefitinib.

Genetic altera-tion	Cell line	Mutational status		Previously published mutational status	References
		KRAS	BRAF		
N115K	UM-SCC-17B	WT	WT	Not available	---
A289V	RL95-2	WT	WT	BRAF WT KRAS WT	Sasaki *et al.*, 2004 Thomas *et al.*, 2007
P332S	U-1240	WT	WT	Not available	---
I646L	HeLa S3	WT	WT	BRAF status not available KRAS WT	Steegamier *et al.*, 2007
T678M	MDA-MB-361	G12V (HE)	WT	BRAF and KRAS WT	Davies *et al.*, 2002
G719S	SW-48	WT	WT	BRAF and KRAS WT	Davies *et al.*, 2002
P753S	SK-MEL-28	WT	V600E (HO)	BRAF V600E KRAS WT	Davies *et al.*, 2002 Sasaki *et al.*, 2004
E922K	818-4	G12R (HO)	WT	Not available	---
A1118T	FTC-133	WT	WT	BRAF and KRAS WT	Ricarte-Filho *et al.*, 2009 Schweppe *et al.*, 2008
A1118T	FTC-238	WT	WT	BRAF and KRAS WT	Ricarte-Filho *et al.*, 2009 Schweppe *et al.*, 2008

Table 4. Genetic alterations identified at previously established mutational "hot-spots" of *kras* and *braf* genes in human cancer cell lines carrying somatic mutations of the epidermal growth factor receptor (*egfr*) gene. Sequences at exons 1 and exon 2 of the *kras* gene and exons 11 and 15 of the *braf* gene were determined for 10 cancer cell lines using genomic DNA as template. Alterations that lead to non-synonymous alterations are indicated here. Sequencing reactions that did not detect any non-synonymous alteration is indicated as wild-type (WT). Whenever available, previously published data (with references) are included for comparison. "HE" and "HO" denote hetero- and homozygous respectively.

4.2.3 Gefitinib resistance of SW-48 cells may be due to a low EGFR protein expression level

Besides activating *kras* / *braf* mutations, another dominant event / property of a cell that may counteract EGFR TKI sensitivity is the expression level of the mutant EGFR protein, simply because an EGFR TKI-sensitizing mutation would not be able to effect a phenotype change if the mutant protein is not expressed in the very first place. To determine if the 10 cell lines in which somatic EGFR mutations were identified express EGFR protein at all, EGFR protein level was determined by immuno-blotting using whole cell lysates obtained from exponentially growing cells.

As seen in Figure 13, EGFR protein can be detected in 8 cell lines. Since 1 out of these 8 cell lines is sensitive to gefitinib, it appears that the EGFR protein levels alone do not correlate with gefitinib sensitivity. For MDA-MB-361 and SK-MEL-28 cells, the low expression level of EGFR protein may be an additional factor contributing to their gefitinib resistance, as there is a lack of primary molecular target for the drug to act on. Similarly, EGFR protein expression level in SW-48 cells appears to be the lowest among the 8 cell lines where the protein could be detected. Therefore it is possible that gefitinib resistance of SW-48 cells may be a result of the low number of EGFR receptors in these cells.

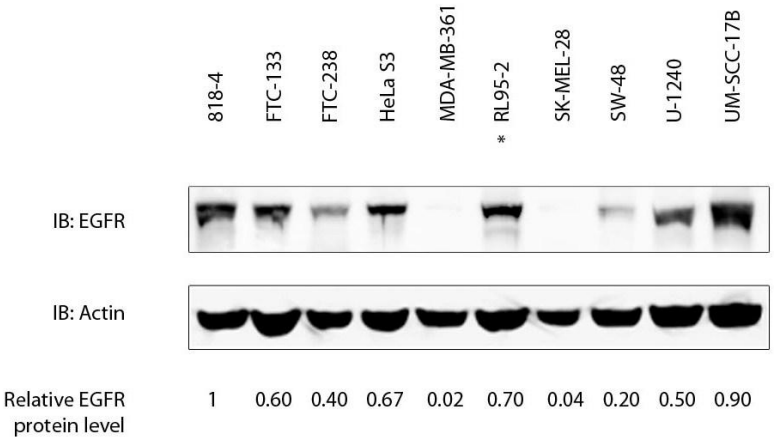

Figure 13. Epidermal growth factor receptor (EGFR) protein is detectable in most cancer cell lines carrying EGFR somatic mutations identified by the Singapore OncoGenome (SOG) Group. EGFR protein level of 10 human cancer cell lines carrying various somatic point mutations was determined using whole cell lysates prepared from non-starved cells. The above immuno-blot images are representative of lysates obtained from 2 independent batches of cells. * denotes a gefitinib-sensitive cell line.

4.2.4 The dynamics of proliferation of RL95-2 cells in the presence of gefitinib

In the earlier end-point viability assays, sensitivity towards EGFR TKIs gefitinib and erlotinib was established in RL95-2 cells (Figures 12d and 12e). To establish a more complete time-dependent profile of the anti-proliferative effect of the inhibitors, cellular viability was determined at multiple time points throughout the duration of the experiment. RL95-2 cells, together with control cell lines A-427 and A-431, were cultured in 6-well plates with either vehicle or 1 μM gefitinib. The number of attached cells in each test well was counted 1, 2, 3, 5 and/or 7 days after drug addition. Expectedly, the proliferation rates of both vehicle and gefitinib-treated A-427 cells (gefitinib-insensitive) are almost identical throughout the duration of the experiment (Figure 14a). On the contrary, massive cell death could be detected in A-431 cells 2 days after treatment with gefitinib but not with DMSO (Figure 14b). By 72 h after drug addition, approximately 95 % of A-431 cells had detached from the surface of the test wells.

The EGFR TKI-sensitive cell line RL95-2 was tested at 2 initial seeding densities because previously cells seeded at different densities displayed dramatically distinct responses towards 1 μM gefitinib (Figure 12d). To determine if a similar behaviour could be seen in the time course assays, the cell line was seeded at 1.5 x 10^5 and 9 x 10^5 cells/well in this set of experiments. These seeding numbers resulted in test wells with cell densities identical to those in end-point assays seeded with 5000 and 30000 cells respectively. Indeed, 1 μM gefitinib caused a transient halt in proliferation of RL95-2 cells, which lasted for 2 days in wells initially seeded with 9 x 10^5 cells (Figure 14d) and 3 days in wells seeded with 1.5 x 10^5 cells (Figure 14c). Subsequently, instead of progressing to cell death, RL95-2 cells resumed proliferation after the halt. In wells seeded with 9 x 10^5 cells, the rate of the subsequent proliferation actually matched that of the vehicle-treated cells, which is indicative of total resistance of the surviving cells. In wells seeded with 1.5 x 10^5 cells, resumption of proliferation is also observed but at a slower rate. Thus, densely populated RL95-2 cells appeared to recover from EGFR inhibition faster and better than sparsely populated ones. In all, these experiments revealed interesting responses of RL95-2 cells to the EGFR TKI gefitinib that were otherwise not seen in end-point assays.

The resumption of proliferation of RL95-2 cells after gefitinib exposure may also be due to events such as drug depletion by metabolism and/or degradation during the course of the

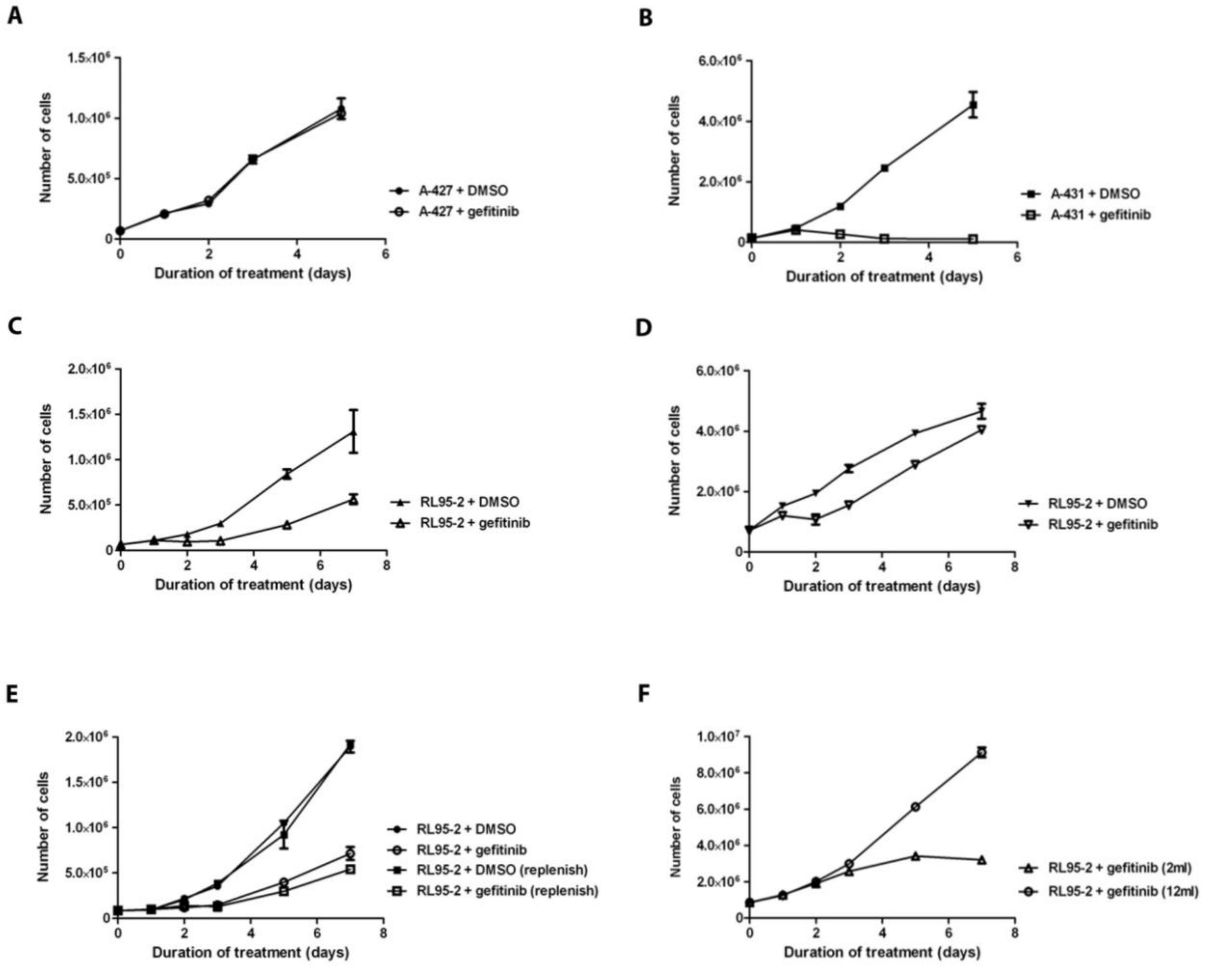

Figure 14. Growth kinetics of RL95-2 cells in the presence of 1 μM gefitinib. (a). A-427 cells, (b). A-431 cells, (c) RL95-2 cells (1.5 x 10^5 cells/well) and (d). RL95-2 cells (9 x 10^5 cells/well) were seeded in 6-well plates and incubated for 24 h before added with 1 μM gefitinib. (e). RL95-2 cells (1.5 x 10^5 cells/well) were cultured in the presence of 1 μM gefitinib, with (squares, indicated by "replenish") or without (circles) change of medium and drug once everyday for 6 days after the start of treatment. (f). RL95-2 cells (9 x 10^5 cells/well) were treated with 1 μM gefitinib in either 2 ml or 12 ml of culture medium for up to 7 days. The number of attached cells in test wells was determined by counting and plotted against time. Graphs shown here are representative of 2 independent experiments, and in each experiment each data point was tested in triplicate. Error bars denote SEM.

experiment. To rule out this possibility, the growth curve experiment was repeated but the culture medium (and therefore the drug) was replaced with fresh medium (and drug) everyday for 6 days. As observed in Figure 14e, the supply of fresh medium and drug did not significantly alter the growth curves. This indicates that the growth resumption observed in gefitinib-treated RL95-2 cells is unlikely to be due to drug depletion during the experiment.

From the above experiments, gefitinib sensitivity of RL95-2 cells appears to be dependent on initial seeding density to the test wells, regardless of the assay format (Figure 12d and compare Figures 14c and 14d). This phenomenon is suggestive of an involvement of autocrine or paracrine factors in the response of these cells against perturbations of EGFR activity. To further explore this idea, RL95-2 cells were treated with 1 μM gefitinib in the presence of different volumes of medium in test wells, namely the usual 2 ml and the much higher 12 ml. The rationale behind the use of different culture volumes is that if RL95-2 cells require secreted factors to counteract the growth-inhibitory effect of gefitinib, the effect of these factors may be diminished in the presence of a larger culture volume due to dilution. If this conjecture turns out to be true, one would expect RL95-2 cells to become more sensitive to gefitinib when cultured in a larger volume of medium. Quite the opposite, in the first 2 days after drug addition, gefitinib-treated RL95-2 cells displayed an identical rate of proliferation regardless of culture volume (Figure 14f). Subsequently the proliferation rate of cells cultured in 2 ml medium gradually decreased due to nutrient depletion and/or building up of metabolic wastes (colour change in the culture medium was observed). Therefore these results suggest an involvement of entities other than autocrine / paracrine factors in the response of RL95-2 cells towards growth inhibition by EGFR TKIs.

4.2.5 The P753S substitution increases auto-phosphorylation of the EGFR protein

Another promising EGFR mutation is the P753S substitution. This is primarily because of its proximity to the highly conserved LREA motif (codons 747 to 750) within the kinase domain of the protein (Pao *et al.*, 2004). This motif is functionally important, as its deletion confers constitutive activity and transformation capability to the protein (Greulich *et al.*, 2005). Moreover the deletion is also clinically relevant, as it is frequently found in EGFR TKI-responsive NSCLC patients. One of the activating LREA motif deletions observed in patients results in the removal of codons L747 to S752 and at the same time replaced the hydrophobic proline residue at codon 753 with the polar serine (Shimegatsu *et al.*, 2005) (this “L747-S752 del S ins” deletion is termed simply the “deletion” for the rest of this section). On the other hand, SK-MEL-28 cells carry the same P753S substitution but without the deletion. By comparing the EGFR-P753S with the deletion mutant proteins, an attempt

was made to determine if the substitution alone would suffice in conferring constitutive activity to the EGFR protein.

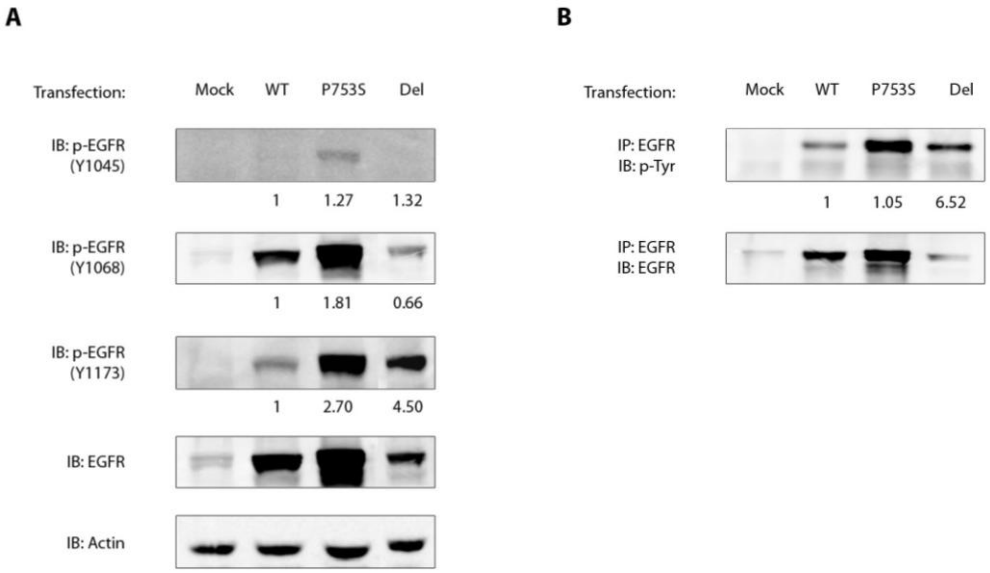

Figure 15. The P753S substitution increases basal phosphorylation at Y1068 and Y1173 of epidermal growth factor receptor (EGFR). HEK-293 cells stably expressing various mutant EGFR proteins were serum-starved for 24 h before lysis to obtain whole cell lysates. Basal phosphorylation levels of these mutant EGFR proteins were determined from these lysates using (a). site-specific antibodies, and (b). immuno-precipitation of EGFR followed by detection of phospho-tyrosine. The numbers below phospho-EGFR immuno-blot images represent relative site-specific basal phosphorylation level with respect to that of the wild-type protein (arbitrarily set at 1). All immuno-blot images are representative of lysates obtained from 2 independent immuno-blotting experiments. "Del" denotes the "L747-S752 del S ins" deletion mutant reported by Shigematsu and colleagues (2005).

Due to a low EGFR protein expression level in SK-MEL-28 cells (Figure 13), the EGFR-P753S protein was studied under a stable over-expression system over a HEK-293 background. In particular, site-specific basal phosphorylation levels of wild-type, P753S and deletion mutant proteins in serum-starved cells were determined by immuno-blotting using antibodies that recognise phospho-tyrosines at codons 1045, 1068 and 1173. These phospho-tyrosines were studied because of their association with different downstream activities, including signal transduction via the MAP kinase (Okabayashi *et al.*, 1994; Rojas *et al.*, 1996), STAT3 (Shao *et al.*, 2003) and PI3K-AKT pathways (Mattoon *et al.*, 2004), as well as ubiquitin-mediated degradation (Levkowitz *et al.*, 1999). By comparing the phosphorylation status at specific tyrosine residues, qualitative differences in the activities between these 3 proteins may be detected (Sordella *et al.*, 2004). Moreover, basal phosphorylation at Y1068 has been often

used as an indicator of EGFR auto-phosphorylation and activity (Arao *et al.*, 2004; Nielsen *et al.*, 2003; Uchida *et al.*, 2007). Therefore, it is essential to investigate the phosphorylation of individual tyrosines of the EGFR protein.

Ectopically expressed wild-type EGFR protein demonstrated substantial basal phosphorylation at Y1068, and to a much lesser extent, at Y1173 (Figure 15a). The deletion mutant, on the contrary, is hyper-phosphorylated at Y1173 and hypo-phosphorylated at Y1068. The P753S protein demonstrated hyper-phosphorylation at both Y1068 and Y1173 with respect to the wild-type protein. When compared to the deletion mutant, the P753S protein has a higher basal phosphorylation level at Y1068 but a lower basal phosphorylation level at Y1173. Finally, basal phosphorylation at Y1045 could be detected in the P753S protein. However, after taking into consideration of the total EGFR protein level in these cells, the basal phosphorylation level at Y1045 of both the P753S and the deletion mutant proteins are actually highly similar, and is about 30 % above that of the wild-type protein. Taken together, it appears that the wild-type, P753S and the deletion mutant EGFR proteins each displays a distinct pattern of basal phosphorylation across the 3 auto-phosphorylation sites investigated here. More importantly, the P753S mutation confers increased basal phosphorylation to the EGFR protein at both Y1068 and Y1173, and should therefore be considered as constitutively active.

To obtain an initial idea on the status of the other auto-phosphorylation sites of the EGFR-P753S protein, ectopically expressed EGFR was immuno-precipitated from whole cell lysates obtained from starved cell lines and detected for phospho-tyrosine. This allowed the determination of the sum of basal phosphorylations contributed by all auto-phosphorylation sites. In contrast to the deletion mutant protein, which displayed an increased total basal phosphorylation level when compared to the wild-type protein, the total basal phosphorylation level of the EGFR-P753S protein is similar to that of the wild-type protein (Figure 15b). Since the EGFR-P753S protein displayed hyper-phosphorylation at both Y1068 and Y1173 but not when total phosphorylation was measured, it is likely that significant differences in the basal phosphorylation levels at one or more of the other auto-phosphorylation sites (Y845, Y992, Y1086 and Y1148) exist between the 3 EGFR proteins.

To summarize, P753S is characterized as a novel EGFR mutation that increases auto-phosphorylation. Interestingly, while it elevates auto-phosphorylation to the same extent as the highly homologous LREA deletion mutant, the two mutants exhibit striking differences in

their action on specific tyrosine residues. *In silico* modelling will be helpful to determine if these subtle changes are due to conformational variations at the molecular level.

4.3 Biochemical and functional analyses of genetic alterations of the *tyk2* gene

As described earlier, 10 non-synonymous single amino acid substitutions and 2 truncating deletions were identified from Tyk2 transcripts. Now, the functional relevance of these genetic alterations is being determined by studying the properties of variant proteins carrying these alterations in both over-expression and endogenous settings.

4.3.1 Basal phosphorylation levels of variant Tyk2 proteins

It has emerged recently that Tyk2 activity contributes to the invasiveness of breast, kidney and prostate cancer cell lines (Caldas-Lopes *et al.*, 2009; Ide *et al*., 2008; Wu *et al*., 2007), suggesting that Tyk2 may operate like a classical oncogene in certain pathological scenarios. To date, no one has reported on a naturally-occurring oncogenic mutation in the human *tyk2* gene. It would therefore be interesting to investigate if any of the *tyk2* genetic alterations identified here may confer oncogenic property to the protein by changing its catalytic activity.

To investigate this, basal phosphorylation level of the variant proteins was determined. Oncogenic mutations in proto-oncogenes often confer an enhanced basal phosphorylation level to the protein, which in turn generates elevated signals responsible for driving many oncogenic phenotypes. Such signalling aberrations have already been reported for other members of the JAK family, for example, the gain-of-function JAK2 V617F mutation found in a majority of patients suffering from polycythemia vera (PV) (Baxter *et al*., 2005; James *et al*., 2005; Kralovics *et al.*, 2005; Levine *et al*., 2005). Here, a panel of expression vectors based on the pcDNA-DEST40 backbone housing various Tyk2 alterations was created by site-directed mutagenesis. The plasmids were transiently transfected into HEK-293 cells, and from whole cell lysates harvested from serum-starved transfected cells, basal phosphorylation levels of the ectopically expressed variant Tyk2 proteins were determined.

4.3.1.1 Basal phosphorylation levels at tyrosine residues Y1054 and Y1055

Of the 6 reported tyrosine phosphorylation sites in the Tyk2 protein (Y292, Y433, Y827, Y1054, Y1055 and Y1145) (Daub *et al.*, 2008; Gauzzi *et al*., 1996; Rikova *et al*., 2007; Zheng *et al*., 2005) listed on the PhosphoSitePlus™ website (www.phosphosite.org) (Hornbeck *et al*., 2004), Y1054 and Y1055 were first investigated as they comprise the major phosphorylation sites of the protein: phosphorylation of these 2 residues is essential for ligand-induced Tyk2 activation (Gauzzi *et al*., 1996). In addition, phosphorylations of Y1054 / Y1055 could be easily studied as site-specific antibodies are commercially available.

As seen in Figures 16a and 16b, ectopically expressed Tyk2 proteins carrying the identified single amino acid substitutions exhibited variable basal phosphorylation levels. Using the Multi Gauge software (version 3.0) provided along with the Fujifilm imaging system, basal phosphorylation levels (in terms of intensities of phospho-Tyk2 bands) were quantified, which were then normalized against β-actin and compared against wild-type Tyk2 protein (Figure 16c). From these arbitrary units of densitometric measurements, the variant proteins could be broadly divided into 3 categories, namely (i). hypo-phosphorylated proteins (those with A53T, R701T, A928V, K930R or P1104A substitutions), (ii). proteins with basal phosphorylation levels similar to that of the wild-type protein (G363S, I684S or D883N), and (iii). variants with increased phosphorylation of the tyrosine residues Y1054 / Y1055 (V362F or R901Q).

One of the basally hypo-phosphorylated proteins, the previously described kinase-dead Tyk2-K930R variant protein (Gauzzi *et al.*, 1996), did not demonstrate any detectable basal phosphorylation when ectopically expressed in HEK-293 cells. Similarly, the other 4 basally hypo-phosphorylated Tyk2 variant proteins are likely to possess comparable activities to the kinase-dead Tyk2-K930R protein. Thus, these 4 variations are likely to be loss-of-function in nature and are not pursued further. Similarly, further studies on the 3 variant Tyk2 proteins with basal phosphorylation levels comparable to that of the wild-type protein were not performed. Instead, attention was placed on the Tyk2-V362F and Tyk2-R901Q proteins that display an increased basal phosphorylation. The basal phosphorylation level of the Tyk2-V362F protein is 40 % more than that of the wild-type protein. This effect is mediocre as compared with the Tyk2-R901Q protein, which displays a much higher basal phosphorylation level at approximately 4 times that of the wild-type protein. Therefore the

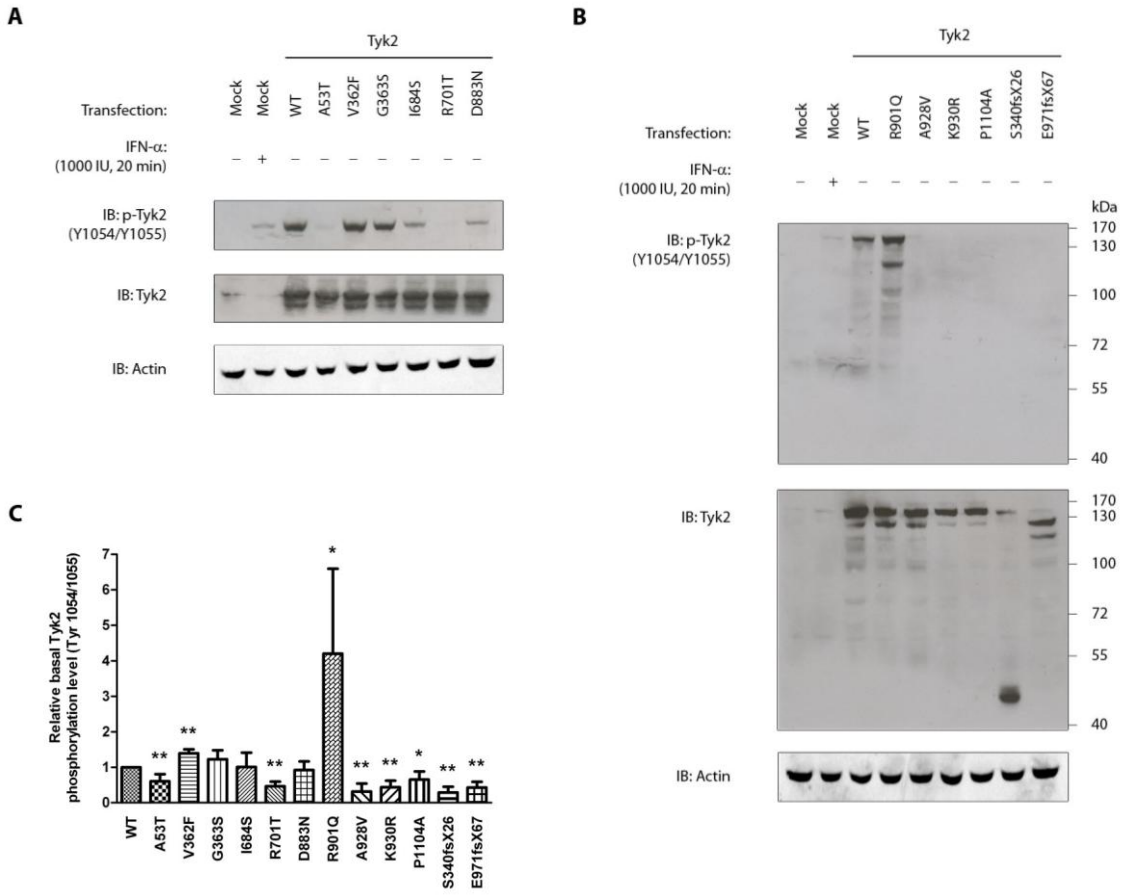

Figure 16. Site-specific basal phosphorylation level of ectopically expressed variant tyrosine kinase 2 (Tyk2) proteins. Whole cell lysates harvested from serum-starved HEK-293 cells transiently over-expressing variant Tyk2 proteins were separated by electrophoresis and detected for Tyk2 phosphorylation at tyrosine residues Y1054 and Y1055. (a). Basal phosphorylation of variant Tyk2 proteins carrying the first series of 6 substitutions. The A53T and R701T substitutions result in variant proteins with decreased basal phosphorylation at Y1054 / Y1055 compared to the wild-type protein. (b). Basal phosphorylation of variant Tyk2 proteins carrying the remaining 4 substitutions and the 2 deletions. The R901Q mutation confers constitutive basal phosphorylation to Tyk2. In contrast, the other 5 variant proteins do not have detectable basal phosphorylation. (c). Relative basal phosphorylation level of variant Tyk2 proteins at Y1054 / Y1055, compared to that of the wild-type protein, which is set at an arbitrary value of 1. Error bars represent standard deviation (SD). * denotes $0.01 < p \leq 0.05$, while ** denotes $p \leq 0.01$ (Student's *t* test). The immnuo-blots shown here are representative of experiments using at least 3 individual batches of transfected cells.

Tyk2-R901Q protein is highly constitutively active and will be further investigated. Lastly, basal phosphorylation at tyrosines Y1054 / Y1055 could not be detected from the 2 truncated proteins (Figure 16b).

4.3.1.2 Basal phosphorylation levels of other tyrosine residues

In addition to Y1054 / Y1055, there are 4 other phosphorylation sites in the Tyk2 protein. To study the effect of Tyk2 mutations on the status of these potential phosphorylation sites, total basal tyrosine phosphorylation levels of these proteins were determined by immuno-precipitation followed by detection of phospho-tyrosine by immuno-blotting. This approach was adopted because site-specific antibodies against these phospho-tyrosines were unavailable.

Similar to the results from Figures 16a and 16b, hypo-phosphorylated variants (those with A53T, G363S, R701T, D883N, A928V, K930R, P1104A substitutions or S340fsX26 deletion), proteins with basal phosphorylation levels similar to that of the wild-type protein (V362F, I684S or E971fsX67) and hyper-phosphorylated proteins (R901Q) were identified (Figures 17a and 17b). By comparing the data between Figures 16c and 17c, it was observed that the relative (compared to wild-type protein) total and site-specific (Y1054 / Y1055) basal phosphorylation levels of 9 out of the 12 variant Tyk2 proteins studied here are similar. Of these 9 variant proteins, the relative total phosphorylation level of the Tyk2-D883N protein seems to be lower than its site-specific phosphorylation level, but the difference is marginally significant (p = 0.077). On the other hand, the truncated protein with the E971fsX67 deletion appears to have a higher relative total phosphorylation level compared to the relative site-specific phosphorylation level, suggesting the presence of basal phosphorylation of one or more of the other tyrosine residues in the protein. However the difference is also not statistically significant (p = 0.34). Finally, 3 out of 12 variant Tyk2 proteins displayed different relative total and site-specific basal phosphorylation status. Tyk2 proteins with V362F, G363S and R701T substitutions appear to have a significantly lower relative total phosphorylation level than the site-specific phosphorylation level ($p \leq$ 0.05).

In summary, basal tyrosine phosphorylation of Tyk2 variant proteins has been determined, and both loss-of-function and constitutively active alterations were identified. Notably, the Tyk2-R901Q protein consistently demonstrated an approximately 3-fold increase in basal phosphorylation levels compared to that of the wild-type protein. Thus this activating somatic mutation will be subjected to in-depth investigation.

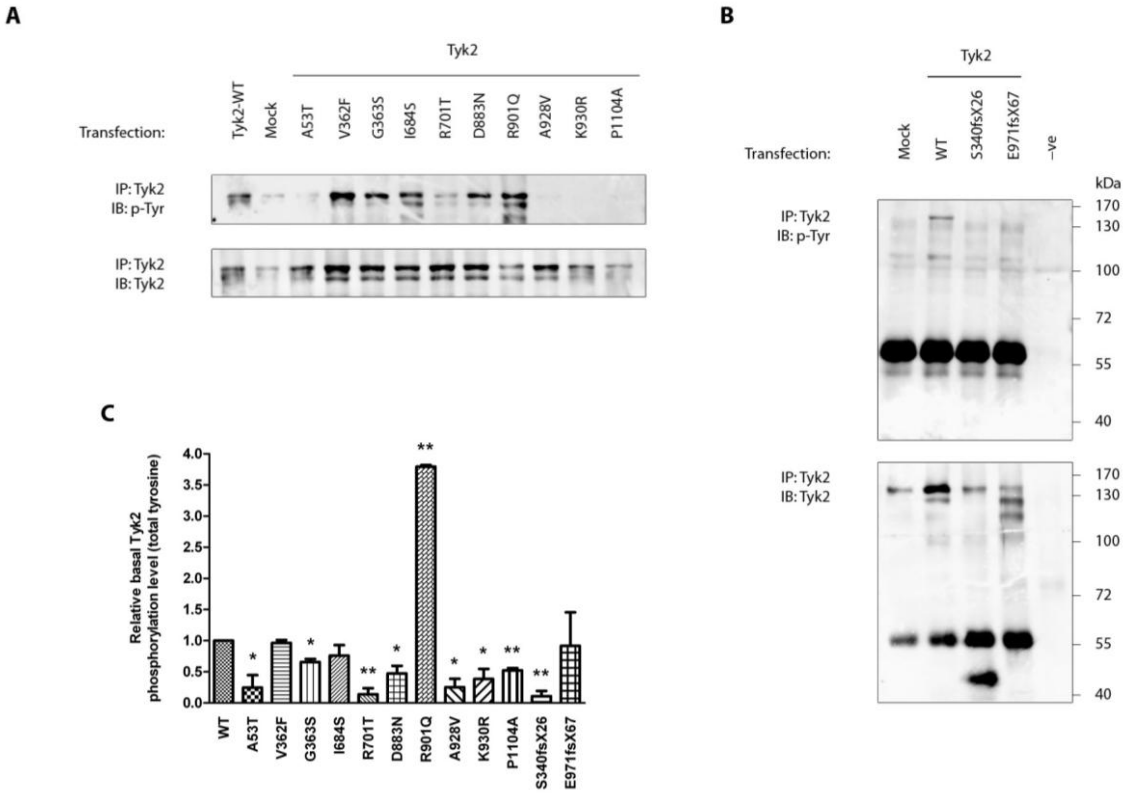

Figure 17. Total basal phosphorylation level of ectopically expressed variant tyrosine kinase 2 (Tyk2) proteins. Tyk2 protein was immuno-precipitated from whole cell lysates harvested from serum-starved HEK-293 cells over-expressing variant Tyk2 proteins and detected for protein phosphorylation using an antibody against phospho-tyrosine. (a). Total basal phosphorylation levels of Tyk2 proteins carrying the substitutions. Tyk2 protein with R901Q mutation demonstrates increased basal phosphorylation, whereas the A53T, G363S, R701T, D883N, A928V, K930R and P1104A substitutions result in proteins with decreased basal phosphorylation level. (b). Total basal phosphorylation levels of Tyk2 proteins carrying the 2 deletions. Decreased basal phosphorylation is detected in the S340fsX26 protein. (c). Relative total basal phosphorylation level of variant Tyk2 proteins, compared to that of the wild-type protein. Error bars represent SD. * denotes $0.01 < p \leq 0.05$, while ** denotes $p \leq 0.01$ (Student's *t* test). "-ve" denotes a negative control immuno-precipitation experiment, in which the lysate was replaced by an equal volume of 1 X HNTG buffer. The immnuo-blots shown here are representative of experiments using 2 or more individual batches of transfected cells.

4.3.2 Effects of over-expression of variant Tyk2 proteins on selected signalling pathways

Tyrosine kinases relay stimulatory signals towards the nucleus via activation of a cascade of messenger proteins (Schlessinger, 2000). In a pathological scenario, deregulated signalling by either mutated tyrosine kinases or excessive ligand engagement often results in constitutive activation of these signalling molecules (Blume-Jensen and Hunter, 2001). To

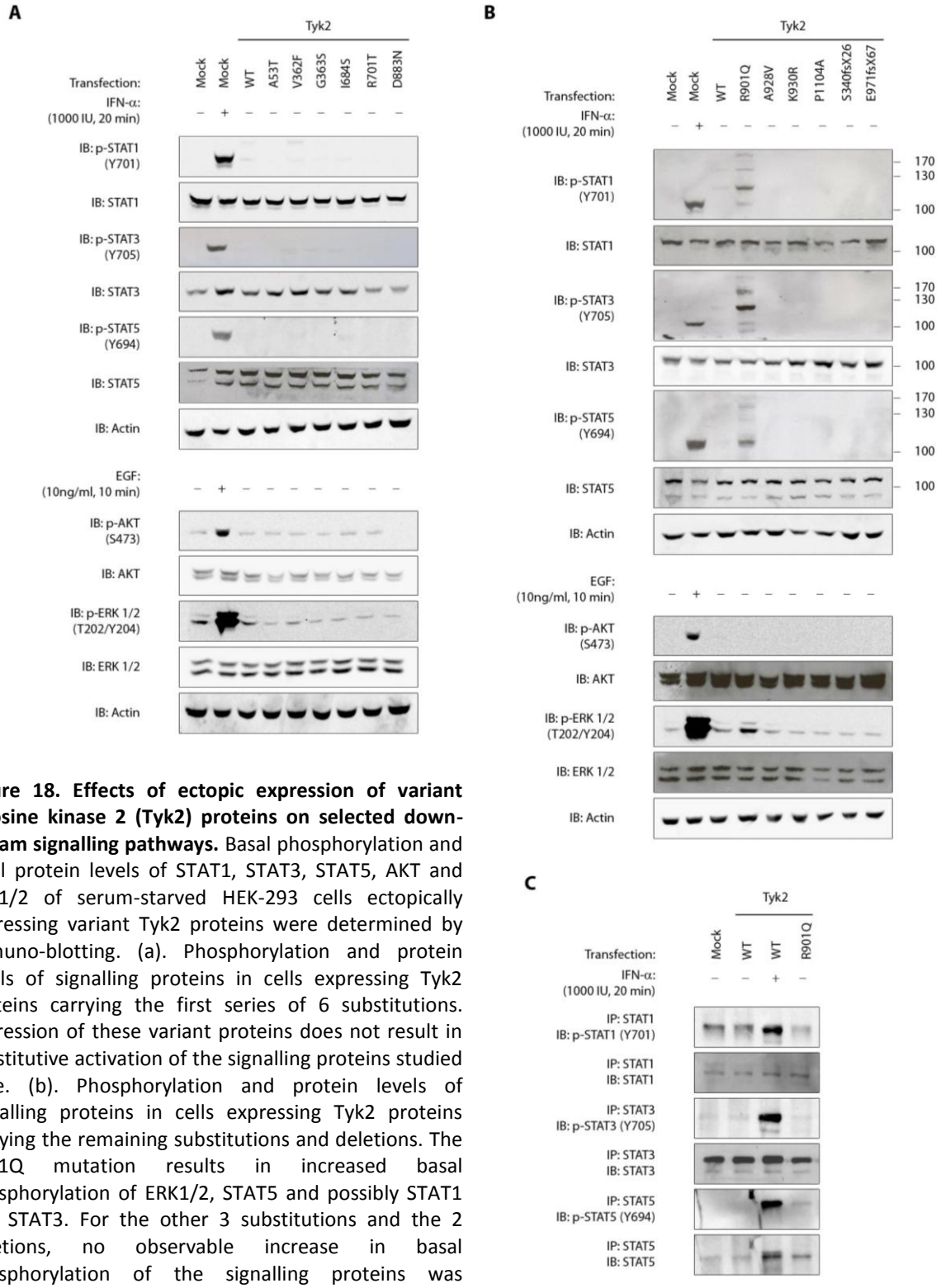

Figure 18. Effects of ectopic expression of variant tyrosine kinase 2 (Tyk2) proteins on selected down-stream signalling pathways. Basal phosphorylation and total protein levels of STAT1, STAT3, STAT5, AKT and ERK1/2 of serum-starved HEK-293 cells ectopically expressing variant Tyk2 proteins were determined by immuno-blotting. (a). Phosphorylation and protein levels of signalling proteins in cells expressing Tyk2 proteins carrying the first series of 6 substitutions. Expression of these variant proteins does not result in constitutive activation of the signalling proteins studied here. (b). Phosphorylation and protein levels of signalling proteins in cells expressing Tyk2 proteins carrying the remaining substitutions and deletions. The R901Q mutation results in increased basal phosphorylation of ERK1/2, STAT5 and possibly STAT1 and STAT3. For the other 3 substitutions and the 2 deletions, no observable increase in basal phosphorylation of the signalling proteins was detected. (c). Various STAT proteins were immuno-precipitated from whole cell lysates prepared from cells expressing wild type or R901Q mutant Tyk2 protein, and detected for site-specific STAT phosphorylations. Tyk2-R901Q protein results in increased phosphorylation of STAT5 but not STAT1 and STAT3. In all experiments lysates of serum-starved HEK-293 cells stimulated with either 1000 IU of IFN-α for 20 min or 10 ng/ml of EGF for 10 min were loaded onto PAGE gels to confirm the proper functioning of the antibodies. This is especially useful when the levels of phospho-proteins were low or un-detectable in test lysates. The immnuo-blots shown here are representative of experiments using 2 or more individual batches of transfected cells.

determine if any of the variant Tyk2 proteins identified here is able to modify downstream signalling events, basal phosphorylation levels of ERK1/2, AKT, STAT1, STAT3 and STAT5 were determined in cells ectopically expressing the variant proteins. Data presented here showed that expression of the Tyk2-R901Q protein results in increased basal phosphorylations of ERK1/2 and STAT5 (Figure 18b), again suggesting functional importance of this mutation. On the other hand, over-expression of all variant Tyk2 proteins does not appear to alter the basal phosphorylation levels of STAT1, STAT3 and AKT, as well as the total protein levels of all signalling proteins studied here.

According to the immuno-blotting results, STAT1 and STAT3 may be phosphorylated by the Tyk2-R901Q protein (Figure 18b). Intriguingly, a distinct band of approximately 120kDa in size was detected when site-specific antibodies against phospho-STAT1 or phospho-STAT3 were used. These bands are significantly larger in size than the expected sizes of STAT1 (84 and 91kDa) and STAT3 (79 and 86kDa) proteins, and could represent either protein complexes containing the STAT proteins or could be a result of non-specific binding of the antibodies. To determine which of these 2 possibilities is likely the actual reason behind the observations, STAT1 and STAT3 were separately immuno-precipitated from whole cell lysates and detected for site-specific phosphorylations. The motivation behind performing this experiment is that if the approximately 120 kDa bands are really STAT-containing complexes, they are expected to be pulled down in the immuno-precipitation reactions and subsequently to appear on the immuno-blots as approximately 120 kDa bands when the immuno-precipitated proteins were separated on a PAGE gel and probed with phospho-STAT antibodies. If these bands are non-specific in nature, they should otherwise not appear on these immuno-blots.

To demonstrate the efficacy of the immuno-precipitation reactions, STAT5 was similarly immuno-precipitated and detected for tyrosine phosphorylation at codon 694 of the protein. Indeed, an increased basal STAT5 phosphorylation could be detected again from cells expressing the Tyk2-R901Q protein (Figure 18c), thereby confirming the earlier immuno-blotting results (Figure 18b). In contrast, cells expressing both wild-type and Tyk2-R901Q proteins demonstrated similar basal phosphorylation levels of STAT1 and STAT3. Importantly, no band of approximately 120 kDa in size could be seen on these immuno-blots. These observations indicate that firstly basal phosphorylations of Y701 of STAT1 and Y705 of STAT3 are not up-regulated in cells over-expressing the Tyk2-R901Q protein, and

secondly, the appearance of the approximately 120 kDa bands seen in Figure 18b are likely a result of non-specific binding by the antibodies.

4.3.3 Tyk2-R901Q mutant protein is likely to be expressed in the ovarian cancer cell line IGROV-1

Next, the properties of the mutant protein Tyk2-R901Q were studied in the cell line IGROV-1 where it was first identified. Proving of the activity of this mutant protein in an endogenous system would further support the claim that the Tyk2-R901Q substitution is indeed an activating mutation.

The presence of the Tyk2 R901Q mutation in IGROV-1 cells is confirmed by repeat sequencing of both genomic DNA and cDNA prepared from these cells (Figure 19a). In addition, Tyk2 protein is abundantly expressed in IGROV-1 cells at a level much higher compared to the 4 other colon carcinoma cell lines CaOv-3, CaOv-4, OVCAR-3 and Sk-OV-3 as indicated in Figure 19b. Taken together, it is therefore likely that the Tyk2-R901Q protein is expressed in IGROV-1 cells. Hence, IGROV-1 is a suitable system that endogenously

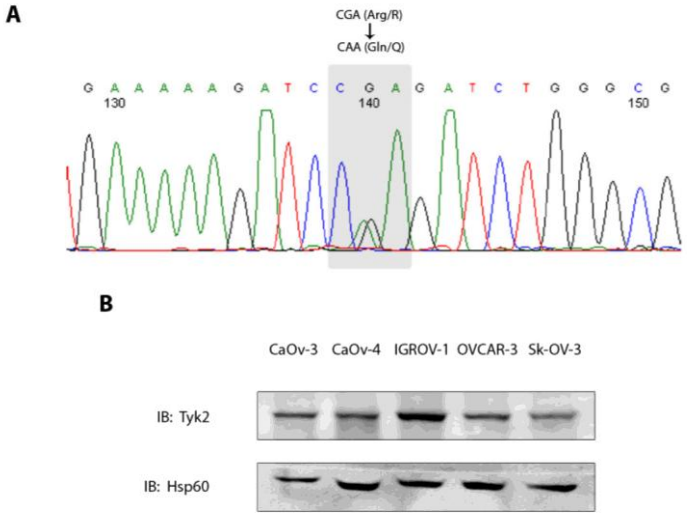

Figure 19. The tyrosine kinase 2 (Tyk2)-R901Q protein is likely to be expressed in IGROV-1 cells. (a). Representative chromatogram showing the presence of the heterozygous G to A substitution responsible for the R901Q substitution of Tyk2 protein in IGROV-1 cells at both genomic and transcript levels. The mutation was confirmed in sequencing reactions in both directions. (b). Tyk2 protein level of 5 ovarian cancer cell lines. Non-serum-starved whole cell lysates extracted from these cell lines were separated and detected for Tyk2 protein using a specific antibody. The level of heat shock protein 60 (Hsp60) was also determined as a loading control. Among the cell lines, IGROV-1 appears to have the highest level of Tyk2 protein.

expresses this mutant Tyk2 protein to support the investigation of its *in vitro* oncogenic phenotypes.

4.3.4 Effect of Tyk2 knock-down on proliferation of IGROV-1 cells

To address the question whether the expression of Tyk2-R901Q variant is indeed enhancing proliferating signal as shown with ERK1/2 phosphorylation, RNA interfering technique was used to silence Tyk2 in IGROV-1 cells. To look at the effect on proliferation, IGROV-1 cells were first transfected with Tyk2 siRNAs for 2 days before siRNA removal and transfer to new wells to allow further growth. The number of cells was determined daily for 3 days by counting with a Coulter particle counter and plotted against time to generate a growth curve.

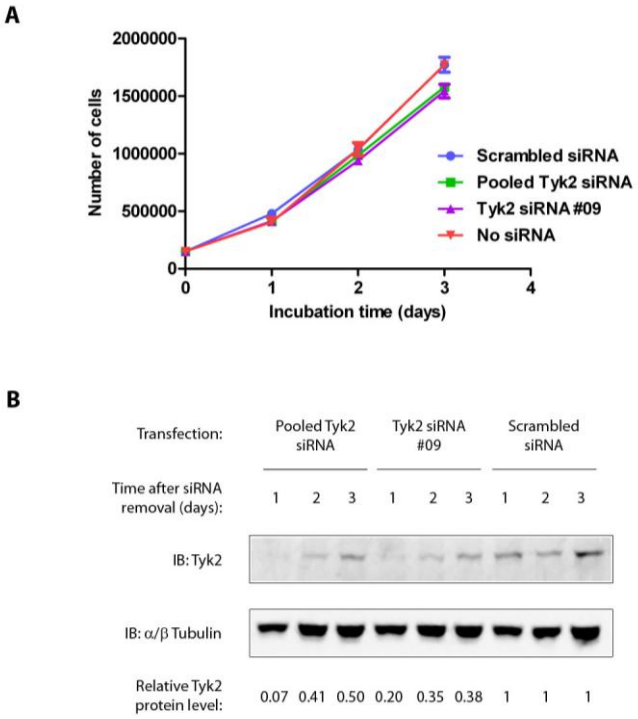

Figure 20. Transient knock-down of tyrosine kinase 2 (Tyk2) protein by siRNA does not result in changes in proliferation rate of IGROV-1 cells. (a). IGROV-1 cells transfected with either scrambled or Tyk2 siRNA were seeded at 1.5 x 10^5 cells per well of a 6-well plate in triplicates and allowed to grow for 3 days. The average number of cells in each set of the triplicate wells was plotted against time. Insignificant difference in growth rate was observed between cells transfected with scrambled or Tyk2 siRNA. Error bars represents SD. (b). Cells used to determine the growth curves in (a) were harvested and detected for Tyk2 protein. Tyk2 knock-down is observed 3 days after siRNA removal. After correction with loading control, relative Tyk2 protein levels of cells transfected with Tyk2 siRNAs were expressed as a fraction of Tyk2 protein level in cells transfected with scrambled siRNA (arbitrarily set at 1) on the same day of harvest. Growth curves and immnuo-blots shown here are representative of experiments using 2 separate batches of transfected cells.

From Figure 20a, it was observed that the growth rates of Tyk2 siRNA-treated and scrambled siRNA-treated IGROV-1 cells are comparable throughout the duration of the experiment. The same result was observed regardless of whether pooled or deconvoluted Tyk2 siRNA was used to transfect the cells. A growth curve of un-transfected cells was included to show that the transfection reagents and/or process have no significant effect on the growth rate of IGROV-1 cells.

To rule out the possibility that the lack of changes in the growth rates of IGROV-1 cells after Tyk2 siRNA knock-down seen here could be due to unsuccessful knock-down and/or a quick wearing off of the knock-down effect after transfer of the transfected cells into new wells, cells were lysed and immuno-blotted for Tyk2 protein. As seen in Figure 20b, there is at least 50 % decrease in Tyk2 protein level in cells transfected with Tyk2 siRNA as opposed to controls throughout the duration of the experiment. Therefore siRNA-mediated knockdown of Tyk2 in IGROV-1 cells does not appear to affect proliferation of this cell line.

4.3.5 Tyk2 knock-down decreases migratory capability of IGROV-1 cells

IGROV-1 cells were first isolated and cultured from the primary tumour of a 47 year-old patient suffering from stage 3 ovarian cancer (Bénard *et al*., 1985). During surgery, spread to cervix and rectum was noted. This observation is indicative of the migratory and invasive capabilities of these cells. To investigate if Tyk2 protein may play a role in its ability to spread, migration of IGROV-1 cells upon Tyk2 siRNA treatment was investigated.

Both untreated, scrambled siRNA-treated and Tyk2 siRNA-treated cells were subjected to modified Boyden chamber assay with 10 % serum as a chemo-attractant. As shown in Figure 21, Tyk2-silenced cells migrate 20 % less compared to scrambled siRNA-treated cells ($p < 0.05$) (Figure 21). These data support a role of Tyk2 in the migration of IGROV-1 cells.

4.3.6 Effects on STAT3 phosphorylation level in IGROV-1 cells upon Tyk2 knock-down and STAT3 inhibitor treatment

Next, the signalling pathways in IGROV-1, which endogenously expresses the Tyk2-R901Q protein, were looked at. A siRNA-mediated decrease in Tyk2 protein in these cells (Figures 20b, 22a and 22c) was found to be accompanied by the abolishment of site-specific STAT3

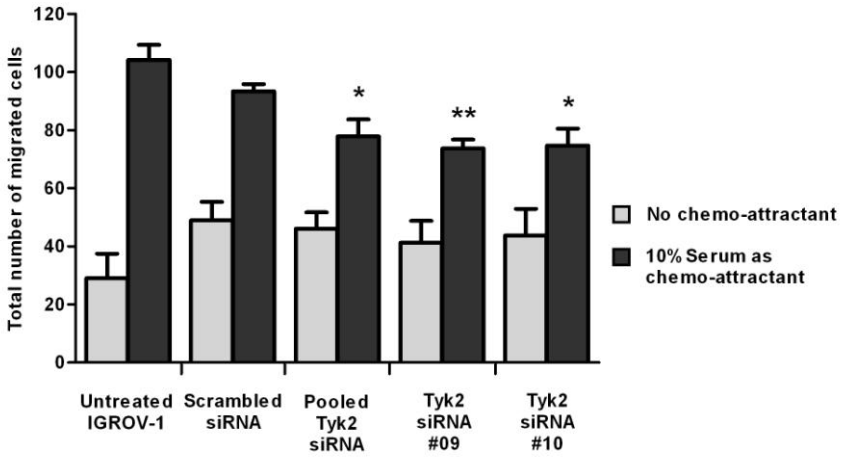

Figure 21. Tyrosine kinase 2 (Tyk2) knock-down by siRNA decreases migration in IGROV-1 cells. Serum-starved IGROV-1 cells transfected with either scrambled or Tyk2 siRNA were seeded at 4 x 10^4 cells per Transwell chamber in 6-replicates and allowed to move towards the outer chamber in the presence or absence of 10 % serum as a chemo-attractant for 24 h at 37 °C in the presence of 5 % CO_2. The number of migrated cells from 3 separate batches of transfected cells were counted and shown here as an average. The "total number of migrated cells" refers to the sum of cells observed in each Transwell within 5 set fields, with the help of an eyepiece grid, under 100 X magnification. Error bars represent SEM. Statistical significance of the difference in migratory capabilities was determined between scrambled and Tyk2 siRNA-transfected cells in the presence of chemo-attraction. * denotes $0.01 \leq p \leq 0.05$, while ** denotes $p \leq 0.01$.

phosphorylation, while STAT3 protein level remains unchanged (Figure 22a). In contrast, Tyk2 knock-down does not alter the phosphorylation and total protein levels of both AKT and ERK1/2 (Figure 22a). In addition, site-specific STAT1, STAT5 and Tyk2 phosphorylations were not detected in IGROV-1 cells (Figure 22d). Identical observations can be made in IGROV-1 cells transfected with 2 out of the 4 deconvoluted siRNAs that constitutes the pooled siRNA (Figure 22b). The siRNAs #09 and #10 are effective in suppressing Tyk2 protein levels. To summarize, these data indicate that in IGROV-1 cells, at least some intracellular signals generated by Tyk2 is mediated via STAT3.

The decreased migration (Figure 21) and abrogation of STAT3 phosphorylation (Figures 22a and 22b) of IGROV-1 cells after Tyk2 silencing suggested that Tyk2 may modulate on the migration of these cells via STAT3 activity. To investigate if Tyk2-mediated basal STAT3 phosphorylation contributes to migration in IGROV-1, cells were treated with the STAT3 inhibitor S3I-201 (also known as NSC 74859), which prevents dimerization of STAT3 monomers (Siddiquee *et al.*, 2007). IGROV-1 cells were incubated with S3I-201 for up to 24 hours at a concentration of 100 μM, a concentration previously shown to cause a 50 % reduction in proliferation rate in the hepatocarcinoma cell line Huh7 (Lin *et al.*, 2009) and an

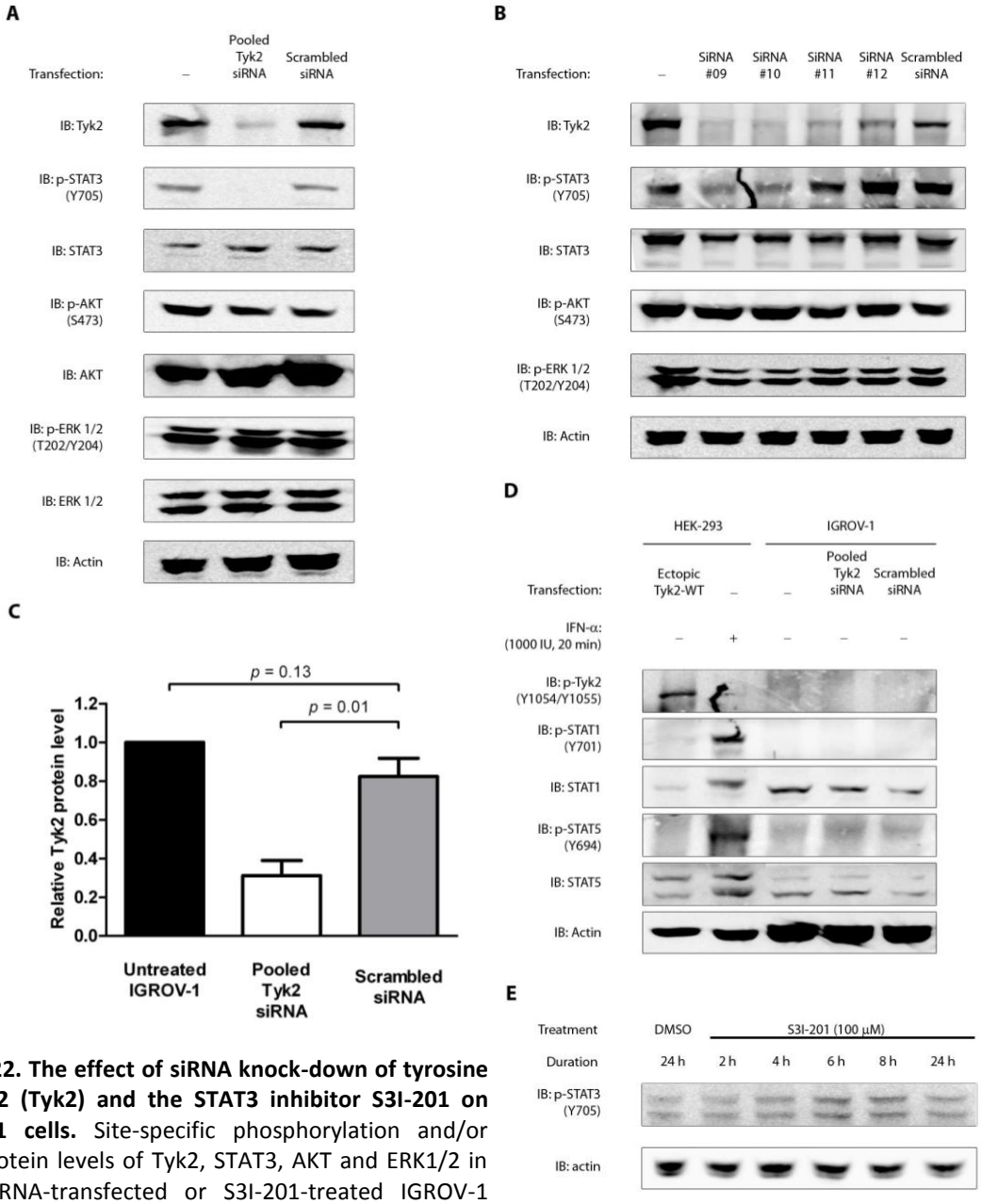

Figure 22. The effect of siRNA knock-down of tyrosine kinase 2 (Tyk2) and the STAT3 inhibitor S3I-201 on IGROV-1 cells. Site-specific phosphorylation and/or total protein levels of Tyk2, STAT3, AKT and ERK1/2 in Tyk2 siRNA-transfected or S3I-201-treated IGROV-1 cells were determined by immuno-blotting. (a). Phosphorylation and/or protein levels of Tyk2 and signalling proteins in cells transfected with pooled siRNA. Tyk2 knock-down abrogates STAT3 phosphorylation but has no effect on phosphorylation and protein levels of AKT and ERK1/2. (b). Phosphorylation and/or protein levels of various proteins in IGROV-1 cells transfected with deconvoluted Tyk2 siRNAs. Significant decrease in STAT3 phosphorylation in pooled Tyk2 siRNA-transfected IGROV-1 cells seen in (a) was also observed when 2 deconvoluted Tyk2 siRNAs (#09 and #10) were used for transfection. (c). Relative Tyk2 protein level in IGROV-1 cells after Tyk2 siRNA transfection, compared to that of scrambled siRNA-transfected and un-transfected cells. A statistically significant drop in Tyk2 protein level upon siRNA knock-down was observed. (d). Site-specific phosphorylation levels of Tyk2, STAT1 and STAT5 in Tyk2 siRNA-transfected and un-transfected IGROV-1 cells. Phosphorylations of these 3 proteins are un-detectable. (e). Phosphorylation level of STAT3 in IGROV-1 cells after incubation with 100 μM S3I-201 for various periods of time. No observable change in STAT3 phosphorylation level was observed in drug-treated cells. Serum-starved lysates of HEK-293 cells stimulated with 1000 IU of IFN-α for 20 min were included in this set of PAGE gels to confirm the proper functioning of the antibodies. The immnuo-blots shown here are representative of experiments using 2 or more individual batches of transfected cells, and the graph in (c) shows average Tyk2 protein level from 3 independent batches of transfected cells.

almost 80 % reduction in phospho-STAT3 level in a rat renal interstitial fibroblast cell line (Pang *et al.*, 2010). As seen in Figure 22e, no observable change in the phosphorylation level of STAT3 was observed in IGROV-1 cells treated with this concentration of S3I-201 for all the time points tested. The underlying cause(s) for this apparent resistance require further investigation.

4.3.7 Changes in the expression of selected downstream target proteins in IGROV-1 cells upon Tyk2 siRNA-mediated abrogation of STAT3 signalling

It has emerged in the recent years that STAT3 is a protein "at a crossroad" of multiple intracellular signalling cascades, and is thus responsible for many cellular processes, of which proliferation and cell survival are closely associated with cancer (Yu and Jove, 2004).

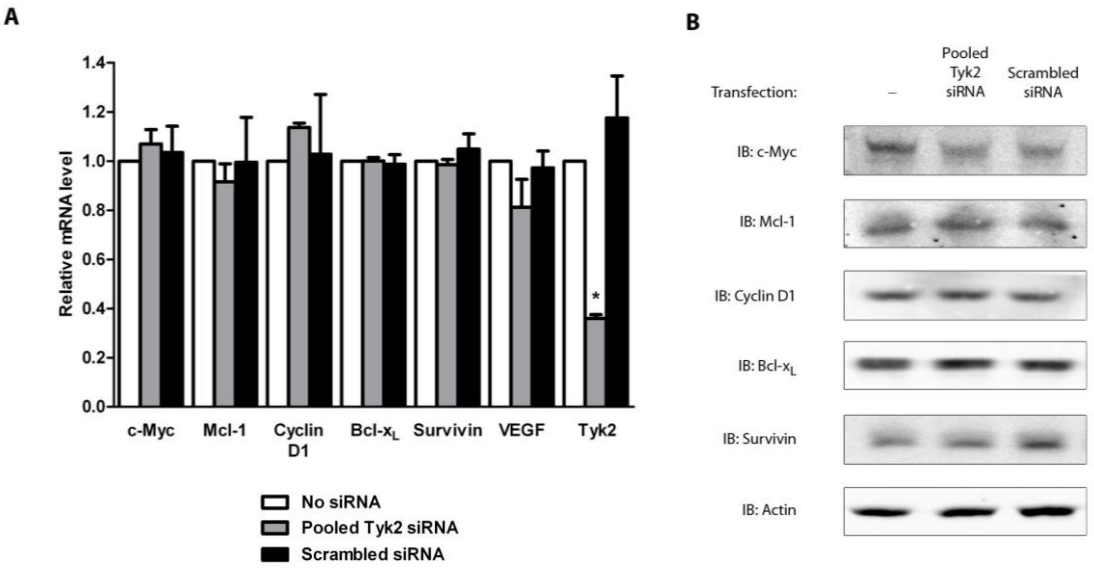

Figure 23. siRNA-mediated tyrosine kinase 2 (Tyk2) knock-down in IGROV-1 cells does not alter the expression levels of previously identified downstream target genes of STAT3 signalling. Transcript and protein levels of Bcl-x_L, cyclin D1, Mcl-1, c-Myc, survivin and transcript level of vascular endothelial growth factor (VEGF) were determined in IGROV-1 cells transfected with scrambled or Tyk2 siRNAs by real-time PCR and immuno-blotting. (a). Relative transcript levels of the 6 genes of interest. Transcript levels were first normalized against that of the house-keeping gene *gapdh*, and then against that of the un-transfected IGROV-1 cells. Transcript levels were also normalized against another house-keeping gene *rn18s1* (18S rRNA), and similar results were obtained. Tyk2 knock-down removes approximately 60-70 % of Tyk2 transcripts but has no effect on the transcript levels of the 6 genes. * denotes $p \leq 0.05$. (b). Level of the proteins of interest, except VEGF. Similar to the results in (a), Tyk2 knock-down does not alter the levels of these 6 proteins. The immnuo-blots shown here are representative of experiments using 2 individual batches of transfected cells. The full names of the other proteins included in this Figure are already mentioned in the main text.

In many cancer types, intervention of constitutive STAT3 signalling changes the expression levels of numerous proteins including the anti-apoptotic, mitochondrial membrane proteins Bcl-x_L and Mcl-1 (Catlett-Falcone *et al.*, 1999; Epling-Burnette *et al.*, 2001; Grandis *et al.*, 2000; Zamo *et al.*, 2002), caspase inhibitor survivin (Aoki *et al.*, 2003), G1/S checkpoint protein cyclin D1 (Ccnd1) (Masuda *et al.*, 2002), transcription factor c-Myc (Bowman *et al.*, 2001) and angiogenesis factor vascular endothelial growth factor (VEGF) (Niu *et al.*, 2002, Wei *et al.*, 2003). Based on the earlier observation that in IGROV-1 cells Tyk2 knock-down by siRNA suppresses STAT3 phosphorylation, it was tempting to speculate that in this cell line the expression of one or more of the above-mentioned genes may be regulated by Tyk2 via modulation of STAT3 activity. Here, expression levels of these 6 genes were investigated by determining their mRNA and protein levels in Tyk2 knock-down cells by real-time PCR and Western blotting respectively.

In Figure 22a, Tyk2 knock-down by siRNA was shown to reduce Tyk2 protein level in IGROV-1 cells by approximately 70 %. Appropriately, Tyk2 transcript level in these cells is also about 60-70 % lower than that of the scrambled siRNA-transfected cells (Figure 23a). However, transcript levels of the 6 genes of interest remain unchanged upon Tyk2 knock-down. This is mirrored by a lack of changes in the levels of 5 of those proteins (Figure 23b). Therefore, abrogation of STAT3 phosphorylation by Tyk2 siRNA does not affect the steady-state transcript and protein levels of the 6 genes investigated here.

V. Discussion

5.1 PTKs are valid targets for anti-cancer therapeutic intervention

Based on decades of research work it is generally accepted that cancer arises from a sequential accumulation of genetic alterations, which confer a cell with the ability to proliferate independently and to resist controlling measures from the host (Hanahan and Weinberg, 2000). Since PTKs are involved in a myriad of essential physiological processes, most notably growth and survival, anomalies of these proteins are very likely to result in changes in cellular activities, summarized as the "hallmarks of cancer" in the landmark paper by Hanahan and Weinberg (2000), which are in turn responsible for the development of pathological states such as cancer. Since these changes in cellular activities are a result of altered signal transduction and many of such signalling is initiated from receptor PTKs, receptor PTKs represent a starting as well as a focal point in the deregulation of cellular activities leading to cancer. Despite substantial effort, cancer-causing anomalies of PTK genes have been successfully established in only a handful of cancer types, such as NSCLC (*egfr* gene mutations), gastro-intestinal stromal tumour (GIST) (*c-kit* gene mutations), breast cancer (ErbB2 protein over-expression) and CML [t(9:22) chromosome translocation] (de Klein *et al.*, 1982; Hirota *et al.*, 1998; Lynch *et al.*, 2004; Paez *et al.*, 2004; Slamon *et al.*, 1987). The aetiologies of many other cancers, such as ovarian carcinomas and gliomas, still await elucidation. Given the importance of PTKs in cellular function, yet unknown anomalies of PTK genes is likely to underlie at least some of these other cancers. On the other hand, some anomalies of non-PTK genes, such as those of *kras*, *braf*, *tp53* and phosphatase and tensin homolog (*pten*), also contribute to cancer. In spite of this, a large proportion of anti-cancer drugs approved for clinical use nowadays are PTK inhibitors probably because (i). PTKs are regarded as highly "druggable" targets for drug development (Hopkins and Groom, 2002), (ii). a role of an anomaly of PTK in some cancers is well established (both validate the choice of target) and (iii). detailed 3-dimensional structures and mechanism of actions of the kinase domains of many PTKs are available (which assist in the design of high affinity drug-like compounds). Furthermore, many currently available PTK inhibitors display dramatic efficacy in targeted subsets of cancer patients (for example, imatinib in BCR-ABL fusion protein-positive CML patients and trastuzumab [Herceptin®, Genentech, South San

Francisco, USA] in breast cancer patients with ErbB2 over-expression). In all, PTKs are and will remain as important targets for the development of anti-cancer therapeutics in the future.

5.2 Large-scale screening is a feasible approach to identify cancer-related genetic alterations

To identify these unknown cancer-related anomalies of PTKs, both cancer and (preferably matching) control material should be screened across all known PTK genes, and increasing the number of samples would be necessary to increase the chance of finding these anomalies. Performing a large-scale mutational screen of cancers has only become both technically and financially feasible at around the turn of the century, as a result of advances in the chemistry and the throughput of both PCR and DNA sequencing technologies, coupled with increasing computing power (Chan, 2005). These developments allowed the SOG Group (quoted as "SOG" in the remaining text) and others (Bardelli *et al.*, 2003; Davies *et al.*, 2005; Greenman *et al.*, 2007; Sjöblom *et al.*, 2006; Stephens *et al.*, 2005; Thomas *et al.*, 2007) to embark on screening projects to catalogue the mutational profiles of cancer material. The recent demonstrations of the oncogenic properties of mutant proteins of fibroblast growth factor receptor 4 (FGFR4) (Roidl *et al.*, 2010) and ACK1 (Chua *et al.*, 2010), which were first identified by SOG, exemplify the feasibility of the employment of large-scale mutational screens for the identification of novel and functionally important alterations of PTK genes.

5.3 A catalogue of genetic alterations of PTKs in human cancer cell lines is urgently needed

Despite the widespread usage of human cancer cell lines for a long period of time, prior to the effort by SOG no one has ever systematically profiled the mutational status of PTK genes in them. This missing information is important because genetic alterations that affect the activity of PTKs is also likely to affect how the cell lines carrying them will respond to experimental manipulations, and subsequently how results should be interpreted (Wellcome Trust Sanger Institute Cancer Cell Line Project website). Therefore prior

knowledge of PTK-related genetic anomalies in commonly used cancer cell lines would be of great aid to both experimental design and understanding of data, and thus could only be beneficial to the cancer research community. For example, in this study the choice of a negative control cell line for gefitinib sensitivity tests was assisted by the trend that NSCLC cell lines with wild-type *egfr* alleles and a normal EGFR transcript level, in which both are previously observed in A-427 (McDermott *et al.*, 2007; Ruhe *et al.*, 2007), tend to be resistant to the drug (Noro *et al.*, 2006; Tracy *et al.*, 2004). Fittingly, this rationale used for choosing A-427 was later validated experimentally in this study (Figures 12c, 12e and 12f). With this in mind SOG has taken the task to establish the mutational profiles of all PTK genes in 254 commonly used human cancer cell lines of various cellular origins. By using cell lines of diverse origins, the information obtained would benefit more researchers working on different cancer types. Moreover, the inclusion of multiple cancer subtypes in the screen may facilitate the discovery of trends and/or signatures universal to cancers in general.

Unlike similar mutational screening efforts by other groups, the effort here involves exclusively cancer cell lines. There are several issues associated with cancer cell lines that may limit their usefulness in scientific research, including (i). unresolved issues on authenticity / contamination (which can be, and need to be tackled urgently by increasing awareness among researchers to the importance of performing routine checks) (Masters, 2002; Capes-Davis *et al.*, 2010), (ii). the possible acquisition of additional alterations during *in vitro* growth and (iii). the possibility that cell lines do not represent all tumour types found at a specific site or organ. On the other hand, there are numerous advantages and conveniences brought about by the use of cell lines, which cannot be provided by other experimental systems. First, the cell lines represent an almost unlimited supply of material. This removes the need to repeatedly obtain specimen from patients, which can be difficult to achieve as a result of, for example, the unavailability of patients (they may have succumbed to the disease before completion of the study) and the need to obtain approval from research ethics committee. Moreover, since cell lines generally closely resemble the tumours they originate from even after multiple (but not infinite) passages, observations made from them are usually both clinically relevant and reproducible. Other advantages include the relative ease in the culture and manipulation of cell lines, and an absence of contamination from normal tissues, which is an issue often associated with tumour biopsies

(Borrell, 2010; Masters. 2000; Paz *et al.*, 2003). Therefore cancer cell lines remain a highly valuable research tool when carefully used.

5.4 The advantages and disadvantages of screening for genetic alterations using cDNA

Another characteristic that distinguish the cancer mutational screen carried out here from similar efforts by others is the usage of cDNA as the template for screening, in contrast to the usage of genomic DNA by others. For the purpose of identifying single amino acid substitutions, both genomic DNA and cDNA are equally competent as a source material. This is exemplified by the confirmations of the N882K mutation of the c-KIT protein and the A289V mutation of the EGFR protein previously identified from the genomic DNA of the AML cell line KASUMI-1 (Beghini *et al.*, 2002) and the endometrial cell line RL95-2 (Thomas *et al.*, 2007) respectively. However, for the identification of other types of genetic alterations, genomic DNA and cDNA each have its advantages and disadvantages: the major difference lies in the types of genetic alterations that can be detected from each type of source material. Screens using cDNA as template allow the detection of splice variants and the acquisition of information on whether a genetic alteration is being transcribed. The latter advantage is especially important because in order for a genetic anomaly to exert its effect, it has to be present in the corresponding transcript and subsequently the variant protein. Screens using genomic DNA, on the other hand, enable the identification of genetic alterations at the introns and intron-exon junctions, as well as gene duplications. Interestingly, some mutations located in exons do not alter the activities of the proteins they encode for. Instead, they are associated with the steady-state levels of the corresponding transcripts. Examples of these mutations include the R661X truncation of the catalytic A-subunit of the fibrin cross-linking enzyme factor XIII (Mikkola *et al.*, 1994), the R897X truncation of the insulin receptor (Kadowaki *et al.*, 1990) and various mutations of an enzyme involved in heme synthesis called porphobilinogen deaminase (Mustajoki *et al.*, 1997). These mutations are likely to be detectable in genomic DNA but not cDNA. Detecting mutations from genomic DNA, though, would require a much larger sequencing effort (and thus the time spent and cost incurred) simply because many more primer pairs are needed to sequence the numerous exons of each gene.

5.5 Categorization of genetic alterations depends on the usage of non-disease "control" material

From cDNAs generated from 254 cancer cell lines, 389 non-synonymous genetic alterations were identified. To provide an insight to the origin of these alterations, each of them was marked as either a somatic mutation or a polymorphism. This categorization should ideally be achieved using data generated from the sequencing of genetic material extracted from non-cancerous tissues of the same patient where each cancer cell line was derived from. Since this material is unavailable, alternative sources of non-cancer "control" material were used. To this end, a representative collection of "controls" containing a large number of samples of different nature (genomic DNA and cDNA) acquired from multiple sources (purchased from different companies) was put together. To further enhance the accuracy of the categorization, available data from the literature as well as from major public cancer mutation databases were also reviewed.

To illustrate the efficacy of the various measures employed to ensure the accurate categorization of the 389 genetic alterations identified, pertaining to this study the assignment of the EGFR R521K and the Tyk2 V362F, G363S, I684S, A928V and P1104A substitutions as polymorphisms conforms to that reported in 2 other large-scale mutational screens of cancers (Greenman *et al.*, 2007; Tomasson *et al.*, 2008). The one exception is the Tyk2 A53T substitution, which was assigned as a somatic mutation here, but as a polymorphism by Tomasson and colleagues (2008) [this alteration was not found by Greenman and colleagues (2007)]. In their mutational screen of genomic DNAs of 94 AML patients, Tyk2 A53T was found in both blood (tumour material) and skin biopsies (matching, non-cancer material) obtained from 4 individuals. The discrepancy between results obtained from different screens could be attributed to the usage of different sources of "controls" in each screen, and is more likely to surface when a genetic alteration is rare in occurrence or when the number of "control" samples is small.

5.6 Characterization of functionally important genetic alterations of EGFR

Based on the mutational profiles of the PTK transcriptomes of the "control" samples gathered by SOG as well as information from public databases, the 9 EGFR alterations

identified from the cancer cell lines are tagged as somatic mutations (Table 1). To determine the clinical relevance of these EGFR mutations, somatic mutations in primary tumours of breast, kidney and prostate origins, in which the genetic material is already available, were checked. The fact that no genetic alteration of EGFR was detected in any of the clinical samples does not come as a surprise at all, as they were also rarely or not found in the 254 cancer cell lines used in the primary screen. Furthermore, in the published data on large-scale mutational screens reviewed during the preparation of this report, EGFR mutations are rare in occurrence as well. In one study, Greenman and colleagues (2007) identified only 3 non-synonymous "germ-line variants" of EGFR and no mutation from a collection of 210 samples, which consisted of a mixture of tumours, cell lines and normal tissues of various origins. Another much larger study involving a total of 1000 samples containing a mixture of tumours and cell lines, also of various origins, reported single amino acid substitutions at glycine-719 of EGFR in a mere 3 lung tumours, and various LREA motif deletions in 22 samples, of which 21 are lung cancers (Thomas *et al.*, 2007). Among these 1000 samples are 60 breast cancers, 95 prostate cancers and 83 renal cell cancers. None of the 4 EGFR substitutions screened here or any LREA motif deletion was found in these 238 samples. The absence of these 5 alterations in the primary breast, prostate and kidney tumours screened here is agreeable with the data obtained from these 2 studies. Furthermore, from the above data EGFR kinase domain mutations appear to be predominant in lung cancers but not other cancer types. Therefore in these other tissue types, deregulation of EGFR signalling pathway may be manifested in ways other than EGFR mutations, such as over-expression, copy number variation or mutations of various genes downstream of the pathway (of which some are listed in section 5.6.1).

In the current study, the EGFR G719S and P753S mutations were each discovered in 1 colon and skin cancer cell line respectively (Table 1). These 2 mutations were not identified at all in the 12 colorectal carcinomas and 136 melanomas included in the study by Thomas and colleagues (2007). Thus, both sets of data indicate the very low prevalence levels of these 2 mutations in these 2 cancer types. Additionally, the T678M substitution found in the breast adenocarcinoma cell line MDA-MB-361 must be a very rare event as well, as it was not detected in the 55 breast tumours screened in this study as well as in the 60 breast tumours screened by Thomas and colleagues (2007).

Next, an attempt was made to establish the functional significance of the 9 EGFR mutations by determining if any of these mutations may confer EGFR TKI sensitivity to the cell lines carrying it. Drug testing represents a rapid and straightforward method to pinpoint novel mutations worthy of further characterization. Moreover, the usage of EGFR TKIs may be extended to cancer types in which EGFR targeted therapy is not currently indicated, as long as these cancers are found to be carrying these novel EGFR mutations. Therefore, the 10 cell lines carrying the 9 somatic EGFR mutations were assessed for their response to various concentrations of gefitinib. A 1 μM drug concentration was set as the cut-off to distinguish sensitive from resistant cell lines. This concentration was used because it is clinically significant, as a drug concentration of 1 μM in an *in vitro* setting is roughly equivalent to the steady-state drug concentration observed in the plasma of patients treated with standard doses of gefitinib (Baselga *et al.*, 2002; Sharma *et al.*, 2007). This may also be the reason behind the decisions by several other groups to use this "consensus" value to categorize cancer cell lines used in their studies (Bianco *et al.*, 2003; Engelman *et al.*, 2005; Janmaat *et al.*, 2003; Tracy *et al.*, 2004).

Roughly in line with the comment that a substantial portion of mutations are phenotypically silent "passengers" (Davies *et al.*, 2005; Greenman *et al.*, 2007; Sjöblom *et al.*, 2006; Stephens *et al.*, 2005), 9 out of the 10 cell lines tested here were resistant to gefitinib (Figures 12a and 12b). Although 4 cell lines displayed observable inhibition of proliferation when treated with 10 μM of gefitinib for 72 hours, they displayed minimal inhibition at the physiologically relevant cut-off concentration of 1 μM, and were thus labelled as resistant. Furthermore, at such a high concentration of gefitinib any observed inhibition may be due to "off-target" effects against other proteins. At this concentration, gefitinib can inhibit interleukin 1-mediated phosphorylation of the c-Jun protein (Mitsos *et al.*, 2009) and can bind to the cyclin G-associated kinase (GAK) (Fabian *et al.*, 2005). In another report, siRNA knockdown of GAK results in increased expression and activity of EGFR, coupled with altered downstream signalling of AKT and ERK5 as well as EGFR trafficking (Zhang *et al.*, 2004). These observations suggest a possibility that "off-target" inhibition of GAK by a high concentration of gefitinib may actually lead to EGFR activation. Overall, viability assay results obtained from cells treated with 10 μM gefitinib need to be carefully interpreted.

The above viability assays were performed based on the reduction of the tetrazole dye MTT by mitochondrial reductase enzymes (for example, succinate dehydrogenase) present only in viable cells into the purple formazan, which can be subsequently quantified spectrophotometrically (Mosmann, 1983). Despite its low cost, reproducibility and ability to scale-up to a high throughput format, there are some limitations which may need to be taken into consideration. These factors include the amount of glucose in test wells (Vistica *et al.*, 1991), the level of glutathione S transferase in cells (York *et al.*, 1998) and compounds that can spontaneously reduce MTT and/or alter the activities of mitochondrial enzymes (Zhai and Maibach, 2004). In this study, the potential impact of these factors on the assay was removed by the inclusion of control wells containing vehicle-treated cells. Whether gefitinib can spontaneously reduce MTT was not assessed, though.

Nonetheless, to provide further assurance to the MTT assay results, viability of selected cell lines was determined by measuring the number of cells still attached to test wells after gefitinib treatment. Results from these experiments (Figure 12e) are highly similar to those obtained in the MTT assays, indicating that the above-mentioned compounds are unlikely to interfere with the performance of the assays. Based on factors such as the presence of known EGFR TKI-sensitizing mutations (SW-48 and RL95-2), EGFR TKI sensitivity (RL95-2) and location of the mutation (SK-MEL-28), further investigation on the influence of EGFR somatic mutations on the behaviour of the 3 cancer cell lines mentioned above was carried out.

5.6.1 EGFR G719S mutation in SW-48 cells

Prior to the detection of the EGFR G719S substitution in the colon carcinoma cell line SW-48, this mutation was exclusively found in tumours removed from NSCLC patients who had responded to treatment with EGFR TKIs (Lynch *et al.*, 2004; Paez *et al.*, 2004). This discovery raised an interesting prospect that the aetiology of some colorectal carcinomas, albeit probably of a very small proportion (Barber *et al.*, 2004; Fumagalli *et al.*, 2010; Moroni *et al.*, 2005), may be attributed to aberrant signalling generated by constitutively active mutant EGFR proteins, and therefore EGFR TKIs may be useful against these tumours (Ruhe *et al.*, 2007).

The subsequent display of gefitinib resistance by SW-48 cells (Figure 12a) is contrary to that expectation, but is actually analogous to earlier observations made by Johnson and Jänne

(2005). In the 3 clinical studies they have reviewed, a portion of NSCLC patients (ranging from 17 % to 35 %) failed to respond to gefitinib despite their tumours carrying established EGFR TKI-sensitizing mutations. Conversely some EGFR TKI responders (10 % to 14 %) do not carry any EGFR mutations. Therefore, it is apparent that EGFR mutational status is not the sole determining factor for sensitivity to EGFR TKIs. This view is also supported by correlating EGFR mutational status with gefitinib sensitivity of both SW-48 and RL95-2 cells. Both cell lines carry recognized EGFR TKI-sensitizing mutations, but only RL95-2 cells are sensitive to the drug (Figure 12d). Thus, the scenario surrounding SW-48 cells reiterates the importance to consider other elements within the EGFR signalling pathway when evaluating drug response.

Naturally it would be interesting to identify the "other element(s)" responsible for overriding the gefitinib sensitivity brought about by the EGFR G719S substitution in SW-48 cells. Clearly illustrating the heterogeneity of individual tumours, EGFR TKI resistance may be manifested by a myriad of anomalies, including:

(1) a resistance-conferring secondary mutation of EGFR, such as the "gate-keeper" T790M mutation (Pao *et al.*, 2005a),
(2) activating mutations of the *erbb2* (Wang *et al.*, 2006), *kras* (Uchida *et al.*, 2007) and *braf* genes.
(3) an amplification of the *met* proto-oncogene (Engelman *et al.*, 2007),
(4) a loss in the activity of the PTEN protein (Bianco *et al.*, 2003),
(5) an increase in the internalization of ligand-bound EGFR proteins (Kwak *et al.*, 2005),
(6) expression of the cell adhesion molecule epithelial membrane protein-1 (EMP-1) (Jain *et al.*, 2005),
(7) alternative signalling via insulin-like growth factor 1 receptor (IGF1R) (Buck *et al.*, 2008; Chakravarti *et al.*, 2002) and ErbB2 protein (Learn *et al.*, 2006), and
(8) the epithelial-mesenchymal transition (EMT) status of the cell (Frederick *et al.*, 2007; Thomson *et al.*, 2005).

The involvement of some of these anomalies could be checked simply by analyzing data from mutational screens performed here and by others. From such searches, it is ascertained that SW-48 cells carry neither the EGFR T790M "gatekeeper" secondary mutation (Table 1) (anomaly #1 list above) nor activating somatic mutations of the *kras* and *braf* genes (Table 4 and Davies *et al.*, 2002) (anomaly #2). The absence of an activating KRAS

mutation in SW-48 cells provides yet another example for the frequently made observation that activating mutations of the *egfr* and *kras* genes can, but seldom appear together (Eberhard *et al.*, 2005; Han *et al.*, 2006; Kosaka *et al.*, 2004; Pao *et al.*, 2005b).

In addition to KRAS, a somatic mutation of the *erbb2* gene (anomaly #2) was not detected in SW-48 cells. Instead, a heterozygous ERBB2 I655V polymorphism was found, which was also detected in 40 other cancer cell lines and 6 non-cancerous samples SOG has screened (Ruhe *et al.*, 2007). This polymorphism, which affects a residue in the trans-membrane domain, was earlier reported to be associated with an increased risk to breast cancers (Montgomery *et al.*, 2003; Rutter *et al.*, 2003; Xie *et al.*, 2000), especially in younger patients (<40-45 years of age) and in patients with familial histories. However, subsequent studies failed to confirm these findings (Benusiglio *et al.*, 2005; Breyer *et al.*, 2009; Nelson *et al.*, 2005). Very recently, one report also linked this polymorphism to an increased risk to thyroid cancers (Rebaï *et al.*, 2009). Currently, the functional significance of this polymorphism remains unknown. However, it must be noted that some polymorphisms affecting residues within the trans-membrane domain can be functionally important. For example, the FGFR4 G388R substitution is associated with tumour progression, motility and resistance to adjuvant therapy (Bange *et al.*, 2002; Thussbas *et al.*, 2006). Moreover, both ERBB2-G659D and G659Q mutant proteins are constitutively active and transforming (Segatto *et al.*, 1988). Therefore, it is possible that the ERBB2 I655V polymorphism may also confer oncogenic properties to SW-48 cells, similar to those above-mentioned mutations.

Another resistance-conferring “element” that has come to attention is the expression level of the mutant EGFR protein in SW-48 cells. Since the constitutively active mutant protein must be expressed in order to confer drug sensitivity, gefitinib resistance may be caused by a lack of mutant protein expression. Indeed, the expression level of EGFR in SW-48 is the lowest among all the cancer cell lines tested in this study in which EGFR protein level is detectable (Figure 13). Therefore the failure of the drug-sensitizing G719S mutation to confer gefitinib sensitivity to SW-48 is likely to be related to its diminished EGFR protein expression. On the other hand, despite the presence of mutant EGFR proteins in 8 out of the 10 cell lines tested here, 7 of them are resistant to gefitinib (Figure 12d and 12e). Thus, EGFR protein level in these cell lines apparently does not correlate with gefitinib sensitivity. This phenomenon was also observed in other cancer cell lines of various origins (Arao *et al.*, 2004; Janmaat *et al.*, 2003; Tracy *et al.*, 2004).

The low level of EGFR protein expression in SW-48 cells raises an interesting issue as to whether this cell line depends on the EGFR pathway for signalling. Based on currently available data, it is likely that SW-48 cells use other signalling pathway(s) to generate pro-survival and/or proliferative signals, with or without the use of the EGFR pathway. Indeed, in many solid tumours, including colon cancer, the overall oncogenic signal is often a sum of signals generated by multiple genetic determinants. This could explain why SW-48 cells fail to respond to gefitinib, which has a very narrow activity spectrum.

Recently, the basal phosphorylation levels of AKT and ERK1/2 in SW-48 were investigated and an absence of basal AKT phosphorylation was observed in this cell line (W. Wong, SOG Group, unpublished data). Interestingly, it was reported earlier that gefitinib-resistant lung cancer cell lines generally display a low p-AKT level (Noro *et al.*, 2006) and NSCLC patients treated with gefitinib tend to have a poorer clinical outcome if their tumours display low p-AKT levels (Cappuzzo *et al.*, 2005). The observation on the co-existence of (i). EGFR TKI resistance and (ii). absence of AKT phosphorylation in SW-48 cells seems to support the suggestion made in these 2 reports of a correlation between these 2 features in cancer cells. It appears that AKT phosphorylation status may be indicative of a cancer cell's response to EGFR TKIs. On a different note, the absence of AKT phosphorylation in SW-48 appears to suggest that this cell line is unlikely to harbour a loss-of-function mutation of PTEN (anomaly #4), because otherwise this cell line should display constitutive activation of AKT (Vivanco and Sawyers, 2002).

5.6.2 EGFR A289V mutation in RL95-2 cells

Of the 10 human cancer cell lines tested here, the uterine endometrial cell line RL95-2 displays significant sensitivity to the EGFR TKIs gefitinib and erlotinib (Figure 12d to 12f). This cell line carries the heterozygous EGFR A289V mutation, which was also detected in gliomas (Arjona *et al.*, 2005; Lee *et al.*, 2006; Thomas *et al.*, 2007) and the glioma cell line SF-268 (Ikediobi *et al.*, 2006). It is believed that RL95-2 is the first endometrial cell line reported to be carrying this mutation.

The alanine residue at codon 289 is located at the interface between domains I and II (the first ligand-binding and the first cysteine-rich domain, respectively, near the N-terminal of the protein, as illustrated in Figure 9) of the extra-cellular domain of the EGFR protein. It is

believed that the A289V mutation confers constitutive activity (Lee *et al.*, 2006) by preventing auto-inhibitory interactions between domains II and IV (the 2 cysteine-rich domains in Figure 9) of the protein. As a result, the mutant protein permanently assumes an "open" and active conformation (Huang *et al.*, 2009). Fittingly, over-expression of this constitutively active protein also confers anchorage-independent growth, ability to form tumour in mice and sensitivity to erlotinib (Lee *et al.*, 2006). Here, it was demonstrated that this protein also confers EGFR TKI sensitivity in an endogenous setting. Thus, these results provide an example to support the use of data generated from studies using an over-expression system.

Additionally a cell density-dependent effect on drug response was observed in RL95-2 cells (Figure 12d). Based on this observation, it is speculated that the growth inhibitory effect of EGFR TKIs on RL95-2 cells may be offset by pro-survival and/or proliferative signals generated by autocrine / paracrine factors, which are presumably accentuated at a high cell density. Quite the opposite, subsequent experiments suggested that this response is not associated with autocrine / paracrine factors secreted by the cell line (Figure 14f). Interestingly, EGF-mediated proliferation and invasion of RL95-2 cells also display density-dependence. At a high seeding density, 0.83 nM of EGF promotes proliferation and enhances invasiveness of RL95-2 cells. At a low seeding density the same concentration of EGF inhibits proliferation and invasion (Korc *et al.*, 1987; Sundareshan *et al.*, 1991). These results hint at the complex mechanisms regulating the behaviour of this cell line, and Korc and colleagues (1987) speculated that the density-dependent effect of EGF activity on RL95-2 cells may be related to the ratio between the number of ligands and EGFR receptors on the cell surface. As an extension to this view, the ratio between the number of drug molecules and receptors, which changes with seeding concentration, may play a part in determining the overall response of RL95-2 cells towards EGFR TKIs.

Subsequently, the growth characteristics of gefitinib-treated RL95-2 cells were further explored in the form of growth curve assays. These experiments were performed because while end-point assays serve well as an initial screen to pinpoint sensitive cell lines, growth curve assays could provide extra information on the behaviour of these cell lines throughout the duration of drug treatment. Indeed, RL95-2 cells displayed a delayed resistance phenotype towards EGFR TKIs, which was otherwise not indicated in the end-point assays. This phenomenon may be attributed to an emergence of secondary resistance shortly after

drug exposure or to the cells' ability to maintain the generation of pro-survival and/or proliferative signals in the event of a blockade of the EGFR activity by switching to an alternative signalling pathway, akin to the up-regulation of IGF1R activity in cancer cells in response to EGFR inhibition (Buck *et al.*, 2008). In RL95-2 cells, a candidate source of such an alternative signal may be the autocrine activities of the human GH. Recently, it was reported that treatment with the GH receptor antagonist B2036 results in a decrease in proliferation of RL95-2 cells (Pandey *et al.*, 2008). Incubation of RL95-2 cells with 1 μM B2036 for 48 h resulted in an approximately 20 % decrease in the number of viable cells. This is followed by a resumption of growth at a rate similar to vehicle-treated cells upon prolonged drug exposure. The ability of RL95-2 cells to circumvent the inhibitory effects of B2036 to resume proliferation is similarly observed when the cells were challenged with gefitinib. It is possible that activities of the EGF and GH receptors both contribute to the pro-survival and/or proliferative signals seen in this cell line. It would therefore be interesting to investigate how RL95-2 cells would respond to concurrent treatment with both B2036 and gefitinib.

On the other hand, the delayed resistance phenotype displayed by gefitinib-treated RL95-2 cells may also be due to the presence of distinct sub-populations of cells, each with differential sensitivities to gefitinib, within the cell line. Indeed, the group of researchers who first described this cell line detected the presence of a small, morphologically distinct sub-population of cells characteristic of squamous epithelium among a predominant population of glandular epithelial cells (Way *et al.*, 1983). One may speculate that if one of these sub-populations is sensitive to gefitinib, treatment is expected to result in the killing of this group of cells, which is observed as an initial drop in cell number. Subsequently, the resistant sub-population that remains would continue to proliferate. Together, these 2 events may result in a growth curve that resembles what was observed in this study.

Finally, the observation that RL95-2 cells are sensitive to gefitinib suggests that EGFR TKIs may represent a therapeutic option for the treatment of endometrial cancers, at least of those carrying activating EGFR mutations. Therefore, an important follow-up work will be to determine the frequency of occurrence of A289V and other activating mutations in primary endometrial cancers. Currently, endometrial cancers are primarily treated with hysterectomy (surgical removal of the uterus). When necessary, surgery is followed by radio-, chemo- and/or hormone therapy. EGFR over-expression is frequently observed in endometrial cancers, and it is associated with a poor prognosis (Khalifa *et al.*, 1994). Thus

fittingly, multiple anti-EGFR chemotherapeutics (including antibodies and TKIs) are either currently undergoing or have undergone clinical evaluation (summarized in Zagouri *et al.*, 2010). In a completed phase II trial in recurrent or metastatic endometrial cancer, 4 out of 32 patients (12.5 %) displayed a partial response to erlotinib (Oza *et al.*, 2008). These 4 responders do not carry any previously established EGFR TKI-sensitizing mutation. It is possible that pre-selecting endometrial cancer patients with known EGFR TKI-sensitizing mutations, such as A289V, for future clinical trials (akin to what has been done for some NSCLC trials) may yield a better response rate. Having said, the current findings caution the possibility of a compensatory cellular response when EGFR activity is inhibited, a phenomenon also described by several other groups previously (Buck *et al.*, 2008; Chakravarti *et al.*, 2002; Learn *et al.*, 2006). On that note, the need to administer combinatorial or multi-targeted therapy to treat endometrial cancers carrying EGFR mutations cannot be undermined.

5.6.3 EGFR P753S mutation in SK-MEL-28 cells

The last EGFR mutation further investigated in the study, the P753S substitution found in the melanoma cell line SK-MEL-28, comes to attention because this substitution is believed to be potentially oncogenic. This view is based on the observations that (i). the substitution occurs at a “strategic” location near the highly conserved (Pao *et al.*, 2004) LREA motif (codons 746 to 750) and the ATP-binding site (K745) of the protein, and (ii). various LREA motif deletion mutants of EGFR are constitutively active (Shigematsu *et al.*, 2005). It is envisaged that a mutation at proline-753 may alter the conformation of the nearby LREA motif, which may consequently lead to increased protein activity. In addition, according to the published 3-dimensional structure of the kinase domain of EGFR, proline-753 is located at the first turn of the αC-helix (serine-752 to serine-768), which constitutes one side of the ATP-binding pocket (Zhang *et al.*, 2006). This helix maintains the inactive conformation of the pocket via a close association with the A-loop, thus preventing access of peptide substrates (Huse and Kuriyan, 2002). Proline is the only amino acid with the end of its side chain covalently bonded to the nitrogen involved in the formation of peptide bonds with an adjacent amino acid, forming a rigid 5-membered pyrrolidine ring. Due to its unique chemical structure and as a result, ability to distort the spatial arrangement of adjacent

motifs and elements within a protein, prolines are usually found at positions indicative of changes in secondary structure, for example, (i). at positions where α-helices and β-sheets are disrupted, (ii). in turns or loops (for example, at the short loop joining 2 anti-parallel strands of a β-sheet), and (iii). at the first turn of an α-helix (MacArthur and Thornton, 1991). For the same reason, proline residues in proteins tend to be evolutionally conserved. Therefore substitution of such a conserved proline residue is likely to disrupt the αC-helix, and as a result, may destabilize the inactive conformation, locking the protein in a constitutively active state.

In support of the above speculation, results from a recent computer-based modelling exercise predicted that the EGFR-P753S protein is likely to be oncogenic (Liu *et al.*, 2007). In the same study, it was also established that SK-MEL-28 cells over-express EGFR transcripts (2.8 copies), do not express EGFR protein and is resistant to erlotinib. The 2 latter observations are encouragingly identical to findings presented here (Figures 12b and 13), and they correspond to the comment by the same authors that a low EGFR expression level appears to be associated with resistance to EGFR TKIs. Since there is no detectable endogenous EGFR-P753S protein in SK-MEL-28 cells, an ectopic expression system was employed as an alternative to assess the functional properties of this protein. Transient over-expression was not adopted because at least 3 groups have earlier observed that transiently expressed constitutively active mutant EGFR proteins did not demonstrate any increase in basal phosphorylation level as compared to similarly expressed wild-type EGFR proteins, but did so in a stable expression system (Amann *et al.*, 2005; Greulich *et al.*, 2005; Sordella *et al.*, 2004). Therefore, a substantial effort was made to generate a stable cell line over-expressing the mutant protein.

In accordance to the above-mentioned speculation as well as modelling data, the current results have provided biochemical evidence that the EGFR P753S substitution is indeed a novel activating mutation (Figure 15a). It has been shown that similar to the LREA motif deletion, a point mutation at an amino acid residue surrounding the ATP-binding pocket of the EGFR protein, such as the P753S substitution studied here, can also confer constitutive activity. Based on the EGFR TKI-sensitizing properties of many previously identified EGFR kinase domain mutations, it is possible that this mutation also confers sensitivity to these drugs. Further experiments to determine the response of the stable cell line expressing the

EGFR-P753S protein generated in this study towards EGFR TKIs should be able to ascertain if this mutation really confers drug sensitivity.

5.7 Characterization of functionally important genetic alterations of Tyk2

Next, the prevalence of genetic alterations of the *tyk2* gene in clinical samples was determined and their biochemical and functional characteristics were investigated. Based on recently published data suggesting a link between Tyk2 activity and cancer cell invasiveness, the emphasis of the investigation was placed on the identification of oncogenic genetic alterations of the *tyk2* gene. The first set of alterations being looked at are those located within the kinase (JH1) domain, since a majority of oncogenic mutations involving other PTKs affect amino acid residues in this domain.

An attempt was made to establish the clinical prevalence of the Tyk2 R901Q, A928V, K930R and P1104A substitutions as well as the E971fsX67 deletion in the collection of cDNAs generated from primary tumours of breast, kidney and prostate origins already available. In addition, genomic DNA of healthy individuals was screened to aid categorization of these alterations as either mutations or polymorphisms.

The absence of the Tyk2 R901Q alteration in the SOG collection of primary tumours and DNA of non-cancer subjects and the similar absence of the Tyk2 K930R alteration in the SOG collection of cancer cell lines and non-cancer DNA samples affirm their status as unique somatic mutations: now they were each found once among a total of approximately 400 cancer samples (cell lines plus primary tumours) screened, and they are also not detected in the 210 samples used in the cancer mutational screening effort by Greenman and colleagues (2007). On the contrary, prevalence of the A928V and P1104A polymorphisms is quite diverse between different sample sets. The incidence of the A928V substitution among the SOG collection of cancer cell lines (0.4 %) and primary tumours (0.7 %) is comparable to that observed by Greenman and colleagues (2007) (0.95 %), but is lower than the detection rate in the SOG collection of non-cancer genomic DNAs (3.4 %). Incredibly, the NCBI reference single nucleotide polymorphism (dbSNP) website (cluster ID: rs35018800) reported a much higher (61.7 %) prevalence of this polymorphism in a collection of 60 DNA samples of European descent (Data from subjects of other ethnicities are not considered here because all samples used by SOG and in the publications where data are compared are

likely to be of European ancestry). This discrepancy may be attributed to the different samples and technologies used to determine these frequencies. For P1104A, its prevalence in the SOG collections of cancer cell lines (5.3 %) and primary tumours (5.4 %) is fairly similar to that observed in the non-cancer genomic DNAs (6.7 %), on the dbSNP website (cluster ID: rs34536443) (3.3 % of European subjects) and in the report by Greenman and colleagues (2007) (2.4 %). Lastly, the E971fsX67 deletion appears to be under-represented in kidney tumours (2 %, compared to 6 % in the SOG collection of cancer cell lines) but greatly over-represented in both breast (27 %) and prostate (31 %) tumours. This suggests a possible positive selection for this deletion in these 2 tumour types.

Interestingly, the prevalence of the Tyk2 A928V and P1104A polymorphisms in AML patients are significantly higher (5.3 % for A928V, and 9.6 % for P1104A) (Tomasson *et al.*, 2008) than the rates observed in the SOG collection of cancer cell lines. This difference may be explained by the fact that all samples screened were of the same cellular origin. It is possible that these 2 polymorphisms are indeed related to AML, but the association maybe masked by the inclusion of cancer samples of non-AML origins in the analysis. Indeed, of the 13 cancer cell lines where Tyk2 P1104A was found, 5 are of haematopoietic origin (Ruhe *et al.*, 2007). Taken together, these observations suggest that this polymorphism may be positively selected in leukaemias and lymphomas.

Next, a mechanistic study was performed to biochemically characterize all Tyk2 genetic alterations identified here. An over-expression system was used because it enables the removal of interfering factors intrinsic to direct study of cell lines, which is the heterogeneous genetic background of the cell lines. Secondly, to study all Tyk2 alterations in an endogenous setting would mean a study of 138 cell lines, which poses a challenge in terms of both logistics and time.

HEK-293 cells were chosen for the expression of various Tyk2 proteins in this part of the study because of its fast growth rate, ease of maintenance, high efficiencies of transfection and protein expression and ability to fold ectopically expressed proteins into correct conformations (Thomas and Smart. 2005). Indeed, the fact that results obtained here (Figures 16a and 16b) are able to confirm earlier reports on the lack of basal phosphorylation of the kinase-dead Tyk2-K930R protein (Gauzzi *et al.*, 1996) as well as the Tyk2-A53T and Tyk2-P1104A proteins (Tomasson *et al.*, 2008) indicates that the over-expression system employed in this study is suitable for the study of variant Tyk2 proteins.

On a different note, these 3 substitutions are phenotype-changing because they affect residues that are highly conserved among JAK kinases (Kawamura *et al.*, 1994).

5.7.1 Tyk2 R901Q mutation in IGROV-1 cells

Using the over-expression system, the Tyk2 R901Q mutation, identified in the ovarian cancer cell line IGROV-1, was found to confer basal hyper-phosphorylation to the protein (Figure 16b). Prior to this discovery, 3 reports have described on the characterization of activating mutations of the *tyk2* gene, all of which are artificially generated and are located within the JH2 pseudokinase domain. Two of them worked on the V678F substitution, which is homologous to the constitutively active JAK2 V617F mutation implicated in PV (Gakovic *et al.*, 2008; Staerk *et al.*, 2005), while the third studied a panel of Tyk2 proteins carrying PCR-generated point mutations at the JH2 pseudokinase domain (Yeh *et al.*, 2000). The demonstration of the high basal phosphorylation level of the Tyk2-R901Q mutant protein at its major phosphorylation sites Y1054 / Y1055 here therefore represents the first description of a naturally occurring activating genetic alteration of human Tyk2.

Besides Y1054 / Y1055, the Tyk2 protein has 4 other minor tyrosine residues which may be phosphorylated. Here, an attempt was made to investigate the contribution of these 4 sites to the total phosphorylation level of this protein. Due to an absence of commercially available antibodies able to specifically recognize these residues, an indirect method was employed to study them by comparing the relative basal phosphorylation level at Y1054 / Y1055 against that of the whole protein (the sum of phosphorylations at all 6 sites). It is believed that if the R901Q mutation alters the phosphorylation state at the other 4 sites, the relative total basal phosphorylation level of this mutant protein would be significantly different from its relative site-specific basal phosphorylation level at Y1054 / Y1055. Since the relative total and site-specific phosphorylation levels of the Tyk2-R901Q protein are actually similar (compare Figure 16c and 17c), it is likely that in this mutant protein basal phosphorylation occurs mainly at Y1054 and Y1055. On the other hand, an alternative scenario may also result in the same observation: an increase in phosphorylation at one or more residues is being offset by a decrease, to a similar extent, in phosphorylation in other residues, resulting in an undetectable net change in the total phosphorylation level. Therefore, pursuant to the availability of the 4 phospho-specific antibodies, more work is

required to investigate the phosphorylation status of the remaining tyrosine residues. Alternatively, these sites may be studied by determining the change in basal phosphorylation level of the Tyk2-R901Q protein upon the removal of individual tyrosines by site-directed mutagenesis. Nonetheless, the current experiments provide a simple means to catch a glimpse of the status of the 4 minor phosphorylation sites of Tyk2.

Consistent with the high basal phosphorylation level of the Tyk2-R901Q mutant protein at its major phosphorylation sites Y1054 / Y1055, current data confirm that ectopic expression of this protein activates ERK1/2 and STAT5 (Figures 18b and 18c). Accordingly, over-expression of the Tyk2-V678F protein in Ba/F3 cells also results in an increased phosphorylation of the same 2 signalling molecules (Staerk *et al.*, 2005). Therefore it appears that these molecules represent common downstream targets of distinct activating mutations of Tyk2, at least in an over-expression setting.

ERK1/2 is one of the signalling molecules of the MAP kinase pathway, which is traditionally associated with proliferation. Interestingly, emerging data indicate that this pathway regulates additional downstream activities, including both cancer-promoting activities such as anti-apoptosis and anti-cancer activities such as differentiation, cell cycle arrest and senescence (McCubrey *et al.*, 2007). Similarly, aberrant STAT5 signalling is also responsible for both pro-cancer and anti-cancer traits (Nosaka *et al.*, 1999). Since the Tyk2-R901Q protein up-regulates ERK1/2 and STAT5 activities, this protein may modulate a wide range of phenotypes in addition to uncontrolled cell growth.

To validate the pro-survival role of Tyk2 R901Q, siRNA was used to reduce Tyk2 protein expression in IGROV-1 cells. From these experiments, the relationships between (i). Tyk2 and STAT3 activity, as well as (ii). Tyk2 and migration were established. The abolishment of STAT3 phosphorylation in IGROV-1 cells upon siRNA-mediated removal of Tyk2 protein seen here is strikingly similar to what was observed when Tyk2 activity in head and neck squamous cancer and kidney cancer cell lines was disrupted (Sen *et al.*, 2009; Wu *et al.*, 2007). Furthermore, the migratory capability of IGROV-1 cells and invasiveness (contributed in part by the ability of a cell to migrate; and in part by the ability to produce metallo-proteinases to digest the extra-cellular matrix) of several types of cancer cells can be attenuated by inhibition of Tyk2 activity (Figure 21 and Caldas-Lopes *et al.*, 2009; Ide *et al*., 2008; Wu *et al*., 2007). Together, these data further support the view that oncogenic signals in multiple cancer types may be contributed in part by Tyk2.

Further experiments whereby IGROV-1 cells were treated with the STAT3 inhibitor S3I-201 were performed to determine if Tyk2-mediated STAT3 activity and migration may be related events. Results presented in Figure 21e indicate that IGROV-1 is apparently resistant to the drug at 100 μM. It is possible that a higher inhibitor concentration is required to result in a detectable decrease in STAT3 phosphorylation in IGROV-1 cells. Alternatively, some resistance mechanism(s) that maintain constitutive STAT3 phosphorylation may exist in these cells.

Besides migration, proliferation of IGROV-1 cells upon Tyk2 siRNA treatment was also looked at. Similar to the prostate cancer cell line DU-145 (Ide *et al*., 2008), the growth rates of Tyk2 siRNA-treated and scrambled siRNA-treated IGROV-1 cells are comparable throughout the duration of the experiment (Figure 20a). This corresponds well with the lack of change in basal phosphorylation of ERK1/2 in this cell line after Tyk2 knock-down (Figures 22a and 22b). On the contrary, the unchanged basal phosphorylation levels of ERK1/2 and STAT5 in Tyk2 siRNA-treated IGROV-1 cells (Figures 22a, 22b and 22d) is somewhat different from the observations made when the Tyk2-R901Q protein was over-expressed in HEK-293 cells (Figures 18b and 18c). This disparity may be attributed to the genetic background of the cell lines and the methods used in these studies. Alternatively, it may also be a consequence of forced protein-protein interactions brought about by the presence of a large number of proteins in the cytoplasm in an over-expression environment (Jones *et al.*, 2006; Pfleger and Eidne. 2003; Ramsay *et al.*, 2002). This highlights the need to investigate the properties of an oncogenic protein in both endogenous and over-expression settings.

A peculiar observation that further exemplifies such a need is the absence of basal Tyk2 phosphorylation in IGROV-1 cells (Figure 22d), but an increased basal phosphorylation of the same protein when it is over-expressed in HEK-293 cells. The observation that removal of the basally hypo-phosphorylated Tyk2 (Figure 22d) from IGROV-1 cells results in a decrease in STAT3 phosphorylation suggests that the role played by Tyk2 in STAT3 phosphorylation in this cell line may be structural rather than catalytic. Alternatively, phosphorylation of STAT3 in IGROV-1 may require phosphorylation of Tyk2 at tyrosine residue(s) other than Y1054/Y1055.

So far, based on both over-expression and knock-down studies, current data established a link between Tyk2 mutation and both MAP kinases and STAT proteins, with a significant impact on STAT3. Yet, it was subsequently observed that in IGROV-1 cells, Tyk2 siRNA-

mediated abrogation of STAT3 activity does not affect the expression of the 6 proteins which were previously quoted as downstream targets of STAT3 activity in other cancer cells (Figure 23). It is possible that the expression level(s) of one or more of these 6 genes is/are indeed altered upon Tyk2 knockdown, but the effect is either annulled by some yet unidentified compensatory mechanism(s) or the effect is transient and is absent at the time of harvest of the cells. Alternatively, in IGROV-1 cells Tyk2 may modulate expression of genes other than the 6 investigated here via STAT3 phosphorylation. Tyk2 in these cells may also alter the activity, but not expression, of downstream signalling proteins via STAT3. Very recently, a link between STAT3 and cell migration was reported, whereby STAT3 promotes persistent directional migration of mouse embryonic fibroblasts by inhibition of Rac1 via sequestration of its activator βPIX (Teng *et al.*, 2009). Therefore a loss of STAT3 results in cells "wondering around" without making substantial net positional change, which is likely to be observed as a lack of migration if migration is tested using Transwell pores. Importantly, this migration does not require STAT3 activity, as a "kinase-dead" STAT3, in which the major phosphorylation site Y705 is replaced with phenylalanine, is also capable of maintaining directional migration. Therefore migration of IGROV-1 cells attributed by Tyk2 may be mediated via pathways that do not include Rac1, as siRNA treatment of IGROV-1 cells does not result in a decrease in the level of the STAT3 protein (Figures 22a and 22b). Nevertheless, to identify the actual, and most likely novel, target(s) of STAT3 activity in IGROV-1 cells, broad-based approaches such as microarrays or phospho-protein arrays may be adopted. Finally, the current data warrants further work to ascertain the conjecture that Tyk2 may modulate the migratory properties of IGROV-1 cells via the signalling protein STAT3. This may be achieved by studying the migration of IGROV-1 cells treated with either a small molecule STAT3 inhibitor or transfected with a STAT3-specific siRNA. Migration of Tyk2 inhibitor-treated IGROV-1 cells may also be tested to determine if it is the presence of the Tyk2 protein or its activity that is responsible for the decreased STAT3 phosphorylation and/or migration observed in this study.

5.7.2 Other genetic alterations of Tyk2

As stated, the main aim of the current study of genetic alterations of Tyk2 is to identify genetic alterations possibly associated with cancer, which is achieved with the identification

and characterization of the R901Q mutation. Although the other Tyk2 alterations do not display “gain-of-function” properties that would have associated them to cancer, some of them may be linked to other disease states. Sigurdsson and colleagues (2005) reported that the Tyk2 V362F and I684S polymorphisms were less frequently found in Swedish SLE patients than controls, while V362F is also under-represented in Finnish patients. Similarly, the P1104A polymorphism is found to be less often detected in MS patients (Mero *et al.*, 2010). In both studies, the authors employed the programs Polymorphism Phenotyping (PolyPhen) and Sorting Intolerant from Tolerant (SIFT) to predict the effect of these substitutions on the activity of the Tyk2 protein. Results from these modelling efforts suggest that these variant proteins have “reduced” or “disrupted” function, which echoes observations made in similar efforts earlier (Kaminker *et al.*, 2007). Thus, the authors speculated that these polymorphisms may be “loss-of-function” alterations that may confer protection to SLE and/or MS respectively. For the Tyk2-P1104A protein, the “loss-of-function” prediction correlates well with the observation that it is basally hypo-phosphorylated (Figures 16b, 17a and Tomasson *et al.*, 2008). In contrast, more information is needed to reconcile data from the modelling and biochemical studies of the Tyk2-V362F and Tyk2-I684S proteins. Nevertheless, an abrogation of Tyk2 activity in humans appears to be associated with non-cancer diseases of the immunological system, such as MS and HIES (Minegishi *et al.*, 2006). Interestingly, different genetic alterations of Tyk2 seem to be associated with distinct immunological diseases, which suggest that each of these alterations is phenotypically distinct.

The Tyk2 S340fsX26 deletion was detected in 3 melanoma cell lines (Table 2). The fact that all 3 cell lines in which this deletion was detected are of the same cellular origin hints at its potential importance in the aetiology of skin cancers. This truncation is expected to generate a protein of 366 amino acids in size, in which the 2 kinase domains and the SH2-like domain are all absent. Therefore this protein is likely to be catalytically inactive and is unable to recruit downstream signalling molecules. However, it may still be able to bind to the cytoplasmic portion of surface receptors because it contains approximately three quarters of the FERM domain. If this speculation turns out to be true, this truncated protein may serve to block signals generated from ligand-bound receptors from reaching downstream effectors. The same line of reasoning may also apply to the Tyk2 E971fsX67 deletion, which is also expected to be catalytically inactive due to the removal of more than

half of the JH1 kinase domain in this variant protein, including the major phosphorylation sites Y1054 / Y1055. Indeed, the removal of Y1054 / Y1055 in both truncated Tyk2 variant proteins studied here explains why phosphorylation specific to these 2 sites was not detected (Figure 16b). Alternatively, these truncations may result in the creation of novel binding sites or exposure of sites previously hidden in the wild-type protein, thus possibly conferring new functions to the variant proteins.

The potential links of the above-mentioned Tyk2 variants to immunological diseases serve as a reminder to the fact that the role of polymorphisms in cancer should not be undermined. One such polymorphism is the FGFR4 G388R substitution, which is present in almost 50 % of the general population, although mostly in heterozygous form (Bange *et al.*, 2002). This polymorphism itself does not appear to bestow FGFR4 the ability to initiate breast cancer, but instead appears to promote tumour progression, cell motility and resistance to adjuvant therapy (Bange *et al.*, 2002; Thussbas *et al.*, 2006). It is a chilling thought that possibly every other breast cancer and/or head and neck squamous cell carcinoma (HNSCC) (Streit *et al.*, 2004) patient is destined to suffer from a poor prognosis. This certainly would have a wide-ranging effect on the management of these diseases.

5.8 Summary and concluding remarks

The current study represents an effort to identify and characterize novel (i). drug sensitivity-modulating genetic alterations of the *egfr* gene and (ii). oncogenic alterations of the *tyk2* gene. For EGFR, it was first determined that among the 10 cancer cell lines in which a somatic EGFR mutation was identified by SOG, the endometrial cancer cell line RL95-2 displayed sensitivity towards the EGFR TKI gefitinib. The extent of this initial sensitivity, which is subsequently replaced by resistance, is density dependent, and this dependence is unlikely a consequence of activities from autocrine / paracrine factors secreted by the cells. The colon carcinoma cell line SW-48, which is resistant to gefitinib despite carrying the established EGFR TKI-sensitizing mutation G719S, was also studied. Initial effort to characterize the mechanism(s) underlying this resistance confirmed that it is possibly due to a low expression level of the mutant protein but not a secondary resistance-conferring mutation of KRAS or BRAF. Finally, over-expression studies indicate that the EGFR P753S mutation, identified in the melanoma cell line SK-MEL-28, confers increased basal

phosphorylation to EGFR. Therefore this mutant protein is likely to be constitutively active and EGFR TKI-sensitizing.

For Tyk2, screening of ectopically expressed variant Tyk2 proteins enabled the detection of basal hyper-phosphorylation of the R901Q protein, suggestive of a potentially oncogenic mutation. This claim is supported by the observation that over-expression of this mutant protein results in increased basal phosphorylations of ERK1/2 and STAT5, both previously shown to be able to drive proliferation and transformation. The behaviour of this mutant protein was also studied in an endogenous setting. siRNA-mediated removal of Tyk2 protein in IGROV-1, the ovarian cancer cell line where the mutation was identified, results in the attenuation of migration and abrogation of STAT3 phosphorylation without affecting signalling via AKT and ERK1/2. An effort to delineate the relationship between Tyk2, STAT3 phosphorylation and migration in this cell line could be rewarding.

The one common theme that epitomizes the studies of these 3 EGFR and 1 Tyk2 mutations is that the mutation is not the sole factor that determines the phenotype of the cell lines carrying it. In these 4 cell lines, the supposed functions and/or biochemical properties conferred by the mutations appears to be masked by those properties brought about by other cellular events. In particular, the increased basal phosphorylation and possible gefitinib sensitivity of SK-MEL-28 cells are not manifested presumably due to a lack of mutant protein expression, while the expected gefitinib sensitivity of SW-48 cells is confounded by yet unidentified mechanism(s). On the other hand, EGFR TKI-sensitivity of RL95-2 cells is coupled with a possible secondary resistance mechanism. Finally, the Tyk2-R901Q mutant protein displays different basal phosphorylation levels and associates with non-overlapping sets of downstream signalling molecules when it is studied under different experimental systems, which may be attributed to the presence of regulatory elements present in IGROV-1 but not HEK-293 cells. The implication of these observations, which exemplify the heterogeneity of individual cancers, is that one must take into consideration of many factors before a decision can be made on the treatment most beneficial to a cancer patient. This decision may be aided by profiling the status of genes known to be associated with a certain type of cancer. For example, a test kit that determines ErbB2 expression level has been approved by the USFDA in July 2008 to aid clinicians to better decide which breast cancer patients would likely benefit from treatment with the anti-ErbB2 antibody Herceptin. With the maturation of “sequencing by synthesis” technologies and plummeting cost,

profiling of panels of candidate genes can and will soon be scaled up to a whole-genome level. In fact, whole genome sequencing has been very recently demonstrated to be technically achievable, affordable, and able to provide useful information on both risk to diseases and potential drug response (Ashley *et al.*, 2010). Certainly, it is foreseeable that in the near future partial or whole genome profiling of cancer patients will be routinely performed; the information will be analyzed with reference to mammoth cancer genome databases such as those envisioned by the International Cancer Genome Consortium (ICGC), before effective “personalized medicine” is prescribed.

VI. References

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VII. Appendix

7.1 Description of Tyk2 deletions at the protein level

Tyk2 S340fsX26

This deletion results in a frame-shift (the letters “fs” in the nomenclature of the deletion) after the serine residue at codon 340 (“S340”). As a result, a totally different sequence containing 26 amino acids followed by a stop codon (“X26”) replaces the original sequence at the C-terminal end. The single-letter sequences of these 26 amino acids are: HLCRPSCGPRTACTSFTGAPATPTA.

Tyk2 E971fsX67

This deletion results in a frame-shift (“fs”) which replaces Tyk2 amino acid sequence from the glutamic acid residue at codon 971 (“E971”) onwards with an un-related sequence of 67 amino acids in length followed by the stop codon (“X67”). The single-letter sequences of these 67 amino acids are: HGLSALAALHPPRPSRAQRAAGQRQAGQDRGLWPSQGRARRPRVLPR ARGWGQPRVLVCPRVPEGV.