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Investigating the role of p65BTK as an emerging therapeutic target in NSCLC

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CHAPTER 1

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INTRODUCTION

THE BRUTON'S TYROSINE KINASE (BTK)

BTK GENE

Bruton's tyrosine kinase (BTK) is a TEC family kinase (originally called Tyrosine kinase of hepatocellular carcinoma), which includes other five members: TEC, BTK, IL-2-inducible T-cell kinase (ITK), BMX non-receptor tyrosine kinase (BMX) and TXK tyrosine kinase (TXK) (Mohamed et al. 2009).BTK gene is localized on the X chromosome (Xq21.33-q22) (Fig.1). The coding region of BTK gene is subdivided into 19 exons covering a total length of 37.5 Kb in humans and 43.5 Kb in the mouse and 3 different messengers are present in the NCBI Gene database (https://www.ncbi.nlm.nih.gov/gene/695). Two transcripts differ only for the presence of an alternative first exon (exon 1A and 1B) whereas the third transcript lacks exons 14-16. All transcripts contain a 5' untranslated region (5'UTR) of variable length and a 3' untranslated region (3'UTR) located in the last exon. The promoter of BTK contains no characteristic elements such as TATA-box or CAAT-like except for the transcriptional start site that is similar to the consensus sequence (Smale et al. 1989). Furthermore, bioinformatic analysis of the sequence upstream of the transcription start site demonstrated the presence of binding sites for specific transcription factors, such as NF-kB, Sp1/3, BOB.1/OBF.1, Oct1/Oct2 and Spi-B/PU1 (Mohamed et al. 2009).

Figure 1. BTK localization on X chromosome.

BTK PROTEIN

BTK encodes for a protein of 659 amino acids (referred here as p77BTK, due to its molecular weight) derived from the translation of an ORF whose start codon is in the second exon. This protein is predominantly cytosolic, but upon activation translocate to the plasma membrane; it has also been reported that the kinase is able to shuttle between the cytoplasm and the nucleus, where it can interact with chromatin–bound multimolecolar complexes (Hirano et al. 2004). p77BTK contains five different protein interaction domains: a N-terminal pleckstrin homology (PH) domain, a TEC homology (TH) domain, a SRC homology (SH) SH3 domain, a SH2 domain and a C-terminal region with kinase activity (Fig.2)(Mohamed et al. 2009). Each domain is involved in the interaction with a plethora of proteins critical for intracellular signaling. Moreover, functional association of BTK with many interactors is crucial for its activation and regulation.

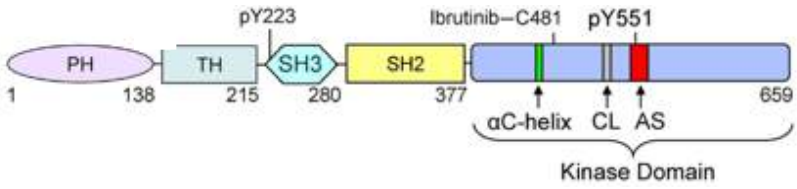

Figure 2: Schematic representation of BTK domains. AS, activation segment; CL, catalytic loop, pY, phosphotyrosine (Roskoski 2016).

The PH domain, located in the N-terminal region, is composed of seven β-sheets and one α-helix (Fig.3). Moreover, between the sheets β-5 and β-6, there is a long aminoacid stretch that ends with a turn and a half α-helix (α1). This domain has a high affinity for the lipidic products derived from the action of phosphatidyl-inositol-3-kinase (PI3K), such as phosphatidylinositol-3,4,5-triphosphate (PIP3). However, BTK has also affinity for other phosphatidylinositols such as phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP2) (Rameh et al. 1997). Following the interaction with these lipidic molecules, BTK translocate to the plasma membrane, where is activated in response to different extracellular stimuli. The PH domain region responsible for the interaction with these lipid molecules can also be recognized and bound by the kinases of the protein kinase C (PKC) family. The latter is a group of serine/threonine-kinases involved in cellular functions such as proliferation, differentiation and cell death. Experimental evidences indicate that several PKC isoforms are able to interact with BTK resulting in the modulation of its kinase activity. It

is known that PKC kinases and the various phosphatidylinositols compete for binding BTK. BTK and PKC kinases are involved in a reciprocal negative feedback regulation. Following activation, BTK can phosphorylate and activate PKC-βI. At the same time, activated PKC-βI can downregulate the activity of BTK itself preventing its binding with inositolphosphate (Qiu et al. 2000). Another feature of the PH domain is the ability to bind actin, which plays a fundamental role in various cellular processes such as chemotaxis, motility and cell division. It has been shown that BTK colocalizes with actin fibers following stimulation of the high affinity IgE receptors (FcεRI) in mast cells. In particular, BTK interacts with F-actin but not with G-actin; the binding site has been mapped into a stretch of 10 basic amino acids of the PH domain. In vitro, the interaction of BTK and F-actin promotes the polymerization of F-actin, suggesting that BTK regulates the reorganization of the cell shape and motility in response to certain stimuli (Qiu et al. 2000). Furthermore, the PH domain enables the interaction of BTK with the “death receptor” FAS, whose stimulation activates the extrinsic apoptosis pathway. The BTK-FAS interaction prevents FAS from binding FAS-associated protein with death domain (FADD) adapter protein, resulting in the inhibition of the death-inducing signaling complex (DISC) and consequently apoptosis (Vassilev et al. 1999).

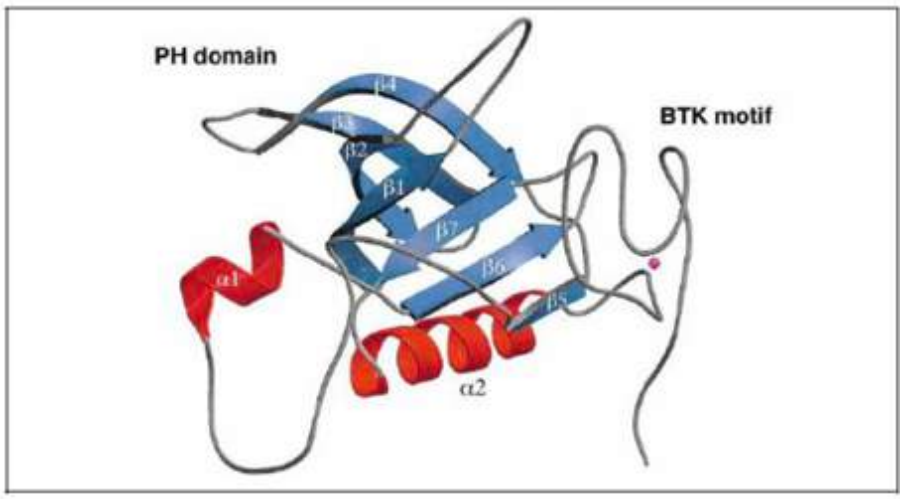

Figure 3: PH domain representation. BTK protein motives. β sheets in blue and α-helixes in red.

Downstream the PH domain there is a TH domain, which is constituted of a BTK motif and a proline-rich region. The BTK motif coordinates a zinc ion (Zn ++) through the extremely conserved residues Histidine 143 (H143), Cysteine 154 and 155 (C154, C155); mutations in these amino acids compromise the ability of binding the ion and greatly destabilize the protein. On the other hand, the proline-rich region is essential for the regulation of the kinase activity. In fact, this region can be recognized by the SH3 domain, which induces the closed conformation preventing the binding from other substrates (Lowry et al. 2001). In addition, mutant forms of BTK lacking the proline-rich region are no longer able to phosphorylate the majority of targets, highlighting the role of the TH domain along with the PH domain in determining the specificity for the targets (Lowry et al. 2001). It is possible that these two domains are also

implicated in binding the substrates given their stabilizing and allosteric properties.

In addition to the PH and TH domains, BTK contains two SRC-Homology domains, SH2 and SH3. SH2 interacts with proteins containing phosphorylated tyrosine residues, while SH3 has affinity for proline-rich regions. As mentioned above, the SH3 domain is able to interact with the proline stretch of the TH domain inducing the inactive "closed" conformation of the protein (Fig.4). Despite the structural homology with the SRC domains, SH2 and SH3 exhibit a different functional role. Differently from SRC, the in vitro interaction of SH2 and SH3 of BTK with an activating peptide (containing residues of phospho-tyrosine and a proline stretch, respectively) does not stimulate its catalytic activity. This evidence implies a different intradomain regulation of BTK by the SH domains. In fact, the activation of BTK kinase is stimulated by the phosphorylation of two tyrosine residues respectively located in the kinase and the SH3 domain (Lowry et al. 2001). Furthermore, the SH3 domain contains the binding site for SAB, a c-Jun N-terminal kinase (JNK)-interacting protein that functions as negative regulator of BTK. SAB inactivates the signals triggered by BCR stimulation in B cells, including the release of second messengers such as calcium and IP3 (Yamadori et al. 1999).

The kinase domain of BTK (referred as SH1 or activation loop (A-loop) consists of an N- and a C-terminal lobe. The N-terminal lobe (residues 397-475) contains 5 antiparallel β sheets and an α-helix (αC). The C-

terminal lobe (residues 479-659) has four α-helices flanked by a short antiparallel β-sheet followed by four additional α-helices. These two regions are connected by a linker region (residues 475-479), which forms a pocket for the ATP binding. In most of the kinases the A-loop negatively regulates the catalytic activity by physically occupying the binding site for the substrate or ATP. However, the crystallographic analysis of the SH1 domain of BTK revealed that the catalytic pocket of this domain is not occupied in an inhibitory manner by its A-loop (Mao et al. 2001). Therefore, the regulation of the BTK catalytic domain differs from the other kinases. In its inactive form, BTK is dephosphorylated and the residues glutamic acid 445 (E445) and arginine 544 (R544) co-interact through a hydrogen bond. The activation of BTK occurs in two steps following its recruitment to the cell membrane. First, BTK is phosphorylated in tyrosine 551 (Y551) in the kinase domain by spleen tyrosine kinase (SYK) or the SRC family kinases. Then, Y551 phosphorylation promotes the catalytic activity of BTK that results in autophosphorylation of Y223 in the SH3 domain. The Y223 phosphorylation is thought to stabilize the active conformation and fully activate BTK kinase activity (Fig.4) (Singh et al. 2018). Finally, it has been shown that the BTK kinase domain interacts with the SH2 domain of PI3K (subunit p85), which activates BTK (Takahashi-Tezuka et al. 1997). Then, the active form of BTK activates PI3K establishing a positive loop.

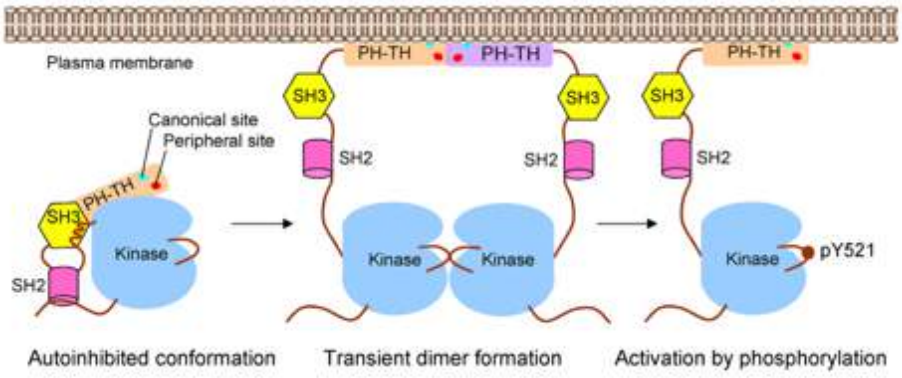

Figure 4: Inactivated and activated BTK conformation (Roskoski 2016).

ROLE OF BTK IN B CELLS

p77BTK is normally expressed in all the cell lineages of the hematopoietic compartment, except for T cells: lymphocytes B, mastocytes, myeloid cells (monocytes/macrophages, granulocytes and dendritic cells) erythroid precursors, thrombocytes/platelets (Mohamed et al. 2009). BTK has been widely characterized as a critical mediator of B cell maturation via its role in the B cell receptor (BCR) signaling (Wang et al. 2016) (Fig.5). BTK is expressed continuously from the late pre-B stage (Hardy et al. 2007) to mature plasmacells (Satterthwaite et al. 1998). However, BTK protein levels are higher and constitutively activated in pre-B cells as compared to mature cells. Upon BCR stimulation, BTK is recruited to the plasma

membrane, where is activated by SRC kinases-mediated phosphorylation in Y551 and auto-phosphorylation in Y223. Phosphorylated BTK modulates Ca^{2+} influx, facilitates phosphatidylinositides metabolism and regulates BCR-mediated antigen processing and presentation (Fig.5). (Sharma et al. 2009).

Loss-of-function mutations in the BTK genes cause the X-linked agammaglobulinemia (XLA), also called Bruton's agammaglobulinemia (Tsukada et al. 1993). To note, XLA is the first identified Mendelian inheritance pathology caused by the loss of functionality of a tyrosine kinase. In XLA, B cell differentiation is impaired at pro- and pre-B cell stages, with a consequent severe decrease of mature B lymphocytes both in the bloodstream and in the lymph node sites. Accordingly, XLA patients showed a complete absence of circulating B cells, resulting incapable of producing antibodies. On the contrary, the lack of BTK does not cause any alteration to the other cellular lineages (Sideras et al. 1995). Due to the absence of the humoral immune response, XLA patients are extremely sensitive to any type of bacterial infection, for which they require both antibiotics and antibody infusion therapies. XLA frequency is estimated around 1 male person for every 200,000 births with a complete penetrance. In women, mutations in the BTK gene induce only the state of healthy carriers (in heterozygosity) for the preferential inactivation of the mutated X chromosome.

The different mutations causing XLA have been described in all the structural domains of BTK, including the non-coding sequences

of the gene. Statistically, missense mutations account for 40% of the total mutations, followed by nonsense (17%), deletions (20%), insertions (7%) and mutations at splicing sites (16%) (Väliaho et al. 2006). All these mutations impair the protein altering either the kinase activity or the structural stability or the interactions with other proteins. The majority of missense mutations are located in the α-helices or at the junction site of the two β-antiparallel sheets, affecting the correct folding of the protein itself. In the SH2 domain, many mutations have been shown to occur in the binding sites for phosphotyrosine residues. In the kinase domain, only few mutations affect the minor lobe, whereas the majority of them are in critical residues such as the binding site for ATP or other proteins. Other mutations affect the self-recognition and interaction sites of BTK domains.

In mice, a single amino acid substitution (R28C) deriving from a point mutation in the PH domain has been associated with X-linked immunodeficiency (Xid) (Rawlings et al. 1993). This mutation impairs the selective binding of BTK to PIP3. Xid mice are characterized by a reduced number of mature B cells (50% reduction compared to wild-type mice), absence of peritoneal B cells, low levels of IgM and altered immune response to T-independent type II antigens. Mechanistically, this phenotype is caused by fact that in response to BCR stimulation, affected B cells instead of proliferating undergo apoptosis (Fruman et al. 2000).

Overall, BTK is crucial for B cells maturation, survival and the proliferative response to BCR stimulation. In particular, BTK mainly acts as a regulator of the signal threshold of this receptor, rather than as an essential component of the pathway (Fig.5).

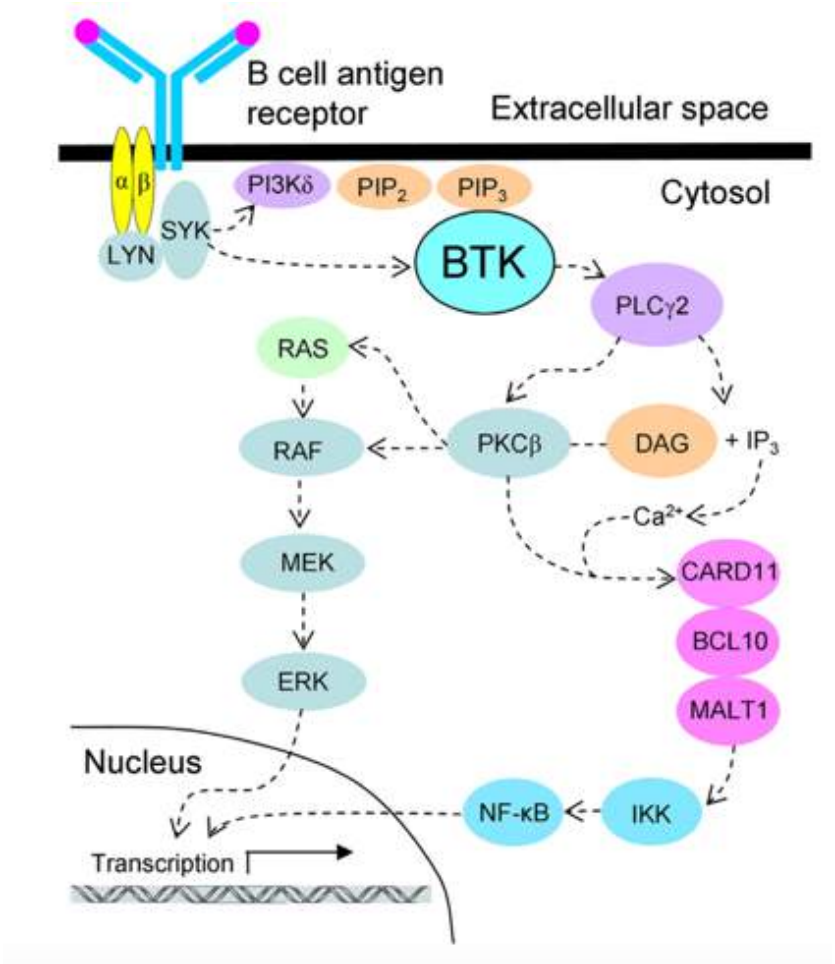

Figure 5: B cell receptor and B cell signaling pathway (Roskoski 2016).

BTK ROLE IN CELL SIGNALING

BTK exerts multiple functions beyond those ones already mentioned above, namely the modulation of the signals transmitted by the immune receptors such as the BCR, the cytoskeletal reorganization mediated by interaction with F-actin and the blockade of apoptosis due to the inhibition of the death receptor FAS (Fig.6).

BTK can act as an activator of NF-kB, a transcription factor indispensable for the stimulation of B cells proliferation. In fact, many experimental evidences indicate that active BTK is able to induce the phosphorylation and therefore the inactivation of IkB, the main inhibitor of NF-kB (Bajpai et al. 2000), resulting in the activation of NF-kB. Following the BCR stimulation, NF-kB induce the transcription of both anti-apoptotic (Bcl-XL, XIAP) and proliferative (cyclin D, MYC) genes (Fig.6).

BTK is also involved in the signaling pathways activated by different cytokines including IL2, IL3, IL6, and erythropoietin. For instance, in B cells IL6 signaling leads to the activation of JAK1, which consequently phosphorylates and activates BTK (Takahashi-Tezuka et al. 1997). In mast cells, IL3 signaling cascade activates BTK through PI3K (Qiu et al. 2000). BTK plays also a crucial role is in the activation pathway of phospholipase C (PLC) and of PKC, which are involved in calcium release and in the mitogen-activated protein (MAP) kinases activation (Fluckiger et al. 1998).

Several evidences show a role for BTK in the apoptotic pathway. However, BTK can have either pro- or anti-apoptotic roles depending on the experimental models and conditions (Uckun et al. 1996, Uckun 1998). BTK pro-apoptotic activity has been initially demonstrated in DT-40 avian cells, a BTK-deficient B lymphocytes cell line. These lymphocytes are normally resistant to radiation-induced ROS generation and apoptosis. However, the reconstitution of BTK restores the apoptotic response. It has been shown that only the kinase domain of BTK is responsible for its pro-apoptotic function (Uckun et al. 1996). In fact, BTK directly phosphorylates STAT3, preventing its translocation into the nucleus and the transcription of anti-apoptotic genes, such as Survivin and BCL-XL (Fig.6) (Uckun et al. 2007). In several models of Pre-B Acute Lymphoblastic Leukemia (LLA), BTK mutants (kinase-deficient) exerted a dominant-negative function by altering the production of second messengers (Ca2 +) in response to BCR stimulation (Fig.6). As a consequence, these cells lines resulted resistant to apoptosis following exposure to ionizing radiation. On the contrary, the phenotype was rescued by adenoviral delivery of the wild-type form of BTK (Feldhahn et al. 2005).

In other models, BTK exerts anti-apoptotic activities. As previously mentioned, BCR stimulation in B cells causes the activation of BTK along with the consequent degradation of IkB. As a result, NF-kB translocate into the nucleus and induces the expression anti-apoptotic genes such as BCL-XL (Bajpai et al. 2000). AKT is considered another possible effector involved in the anti-apoptotic role of BTK.

In fact, it has been reported that BTK can activate AKT, which exerts a strong anti-apoptotic action through phosphorylation and inactivation of several pro-apoptotic mediators such as BAD, FOXO1 FHKRL, Caspase-9 (Amaravadi et al. 2005). As previously mentioned, BTK protects against FAS-induced apoptosis by preventing the formation of the DISC and therefore the transmission of the apoptotic signal (Fig.6) (Vassilev et al. 1999). In erythroid precursors BTK mediates the resistance to TRAIL-induced apoptosis through direct interaction with TRAIL receptor, similarly to what happens with FAS (Schmidt et al. 2004).

Overall, BTK exerts important roles in cellular pathways crucial for cell survival and apoptosis.

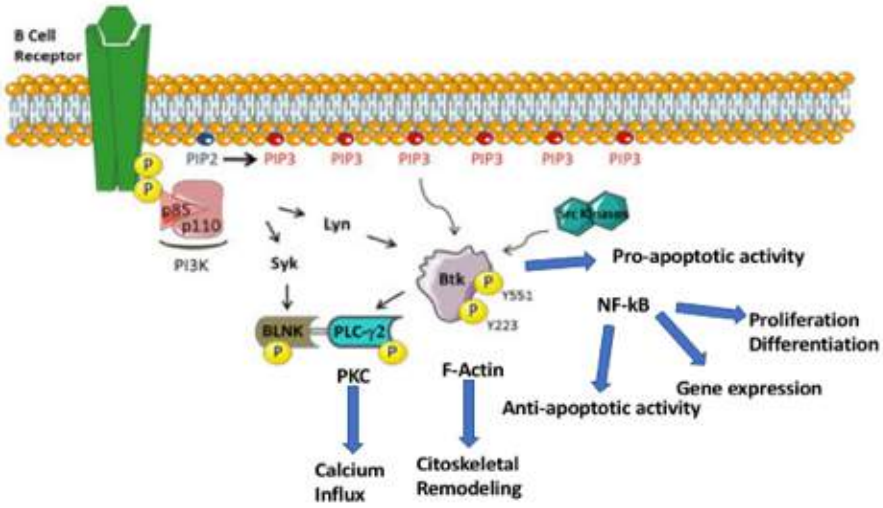

Figure 6: BTK role in B-cells. Following B-cell receptor activation, BTK is involved in different signaling such as cytoskeletal remodeling, cell

proliferation and differentiation, gene expression and apoptosis regulation.

BTK IN B CELL MALIGNANCIES

As explained above, BTK is an important signaling kinase in the BCR pathway. Most of the B cell malignances show impairment of the BCR signaling and, in particular, direct involvement of BTK activity. BTK has been associated with several B cell malignancies such as Chronic Lymphocytic Leukemia (CLL), Mantle Cell Lymphoma (MCL), Waldenström Macroglobulinemia (WM), Diffuse Large B-cell Lymphoma (DLBCL), Follicular Lymphoma (FL) and Multiple Myeloma (MM) (Singh et al. 2018).

CLL is a common adult leukemia characterized by monomorphic round B lymphocytes in peripheral blood (PB), bone marrow (BM) and lymphoid organs (Scarfò et al. 2016). It has been reported that all the BCR signaling kinases, among which BTK, SYK, phosphatidylinositol 3-kinase-δ (PI3Kδ), participates in the survival of CLL cells by eventually activating MAPK, PI3K/AKT and NF-kB signaling pathways. The amplification of these signaling has been associated with the over-expression or constitute activations of these kinases, both in patient's cells and mouse models. BTK has been shown overexpressed and constitutively phosphorylated in CLL patients (Buchner et al. 2009). Therefore, BTK targeting resulted an efficient

therapeutic option for CLL. Ibrutinib, the first orally bio- available irreversible inhibitor of BTK, has shown outstanding clinical activity in CLL patients with extended durable remissions in both untreated and relapsed disease (Singh et al. 2018), which led to the fast-track FDA approval in 2014.

MCL is a rare subtype of non-Hodgkin's B-cell lymphoma caused by the translocation t(11:14)(q13;32) and characterized by malignant transformation of B lymphocytes. MCL often follows an aggressive clinical course with an overall poor prognosis. From the biological point of view, chronic BCR signaling has been implicated in the pathogenesis of MCL. BTK resulted overexpressed in MCL cells and its inhibition by Ibrutinib decreased MCL cell proliferation and survival inducing the inhibition of anti-apoptotic proteins. The Food and Drug Administration (FDA) has approved ibrutinib for treatment of MCL (Wang et al. 2013) in 2015.

BTK has been involved also in WM, that is a type of non-Hodgkin lymphoma (NHL). WM (also referred as lymphoplasmacytic lymphoma) is a heterogenous disease characterized by the accumulation of lymphoplasmacytic cells in the bone marrow and the secretion of IgM monoclonal paraprotein. MYD88 gene (MYD88 L265P) mutation is considered the hallmark of this disease, commonly identified in more than 90% of the patients. The current findings show BTK as a downstream target of the MYD88 L265P signaling. BTK inhibition by Ibrutinib induced apoptosis of WM cells blocking the NF-κB signaling (Gavriatopoulou et al. 2018).

An increased activity of BTK has been reported in diffuse large B-cell lymphoma (DLBCL), which is divided into two distinct molecular subtypes, activated B cell-like (ABC) and germinal center B cell–like (GCB). In particular, the ABC subtype displays chronic active BCR signaling, which constitutively stimulates NF-κB via BTK. The involvement of BTK in these pathways suggests the use of BTK inhibitors to restore the physiological signaling (Li et al. 2018).

The most common subtype of indolent non-Hodgkin lymphoma is FL. FL is a complex disease that remains largely incurable due to its variable clinical course. The hallmarks of FL are the translocation (14;18) resulting in BCL2 over-expression and the alterations within the tumor microenvironment. Recently, other recurrent mutations of the BCR signaling have been identified, several of them in BTK gene. Some BTK mutations have been found in the tyrosine kinase domain, such as the L528W mutation, previously associated with Ibrutinib resistance in CLL, and an in-frame deletion that also alters this amino acid and the adjacent C527. Moreover, T117P and R562W mutations (previously associated with XLA), have been reported in FL patients. All these mutations affect BTK and consequently B cell functions. The use of Ibrutinib in FL is currently under phase III clinical trial (Krysiak et al. 2016).

MM is considered a malignancy of plasmacells, characterized by heterogeneous plasma cells clonal proliferation in the bone marrow microenvironment, monoclonal protein secretion in the blood or urine, anemia, bone lesion, hypercalcemia and renal

damage. Despite advancement in therapy development, MM remains an incurable disease. BTK was shown to be overexpressed in MM and associated with poor prognosis. It has been reported that BTK activates the AKT signaling resulting in down-regulation of the cell cycle inhibitor cyclin inhibitory protein p27Kip1 (p27) and up-regulation of key stemness genes. These evidences suggest the inhibition of BTK as new therapeutic strategy for suppressing the AKT/p27 signaling (Rajkumar et al. 2016).

Overall, these findings highlight the central and recurrent role of BTK in pro-survival pathways in B cells and provide a rationale for the use of BTK inhibitory agents as novel therapeutic approach in B cells malignancies.

BTK IN SOLID CANCERS

Although BTK has for long time been considered as being expressed and playing important roles only in B lymphocytes and other bone marrow-derived cells, new evidences in recent years demonstrated its expression also outside of the hematopoietic compartment, and in particular in different types of solid cancers (Molina-Cerrillo et al. 2017).

Zucha et al. have shown for the first time the central role of BTK in chemo-resistance and metastasis of ovarian cancer. In this

study ovarian spheroids, enriched of ovarian cancer stem cells (CSCs) exhibited high BTK levels. The authors observed that BTK plays a crucial role in the maintenance and self-renewal of CSCs through JAK2/STAT3 activation. Moreover, they showed that the spheroids derived from chemo-resistant ovarian cells were more resistant when BTK signaling is active. Then, the authors demonstrated the beneficial effect of BTK inhibition by Ibrutinib alone and in combination with cisplatin in re-sensitizing ovarian cancer cells to the treatments (Zucha et al. 2015).

Yue et al. reported high levels of BTK in gliomas, being its expression associated with poor prognosis in glioma patients. The authors demonstrated that BTK inhibition by Ibrutinib suppressed glioma cells proliferation, both in vitro and in vivo, by arresting the cell cycle in G1 and blocking the EGFR-induced NF-κB activation. Moreover, the inhibitory effects of Ibrutinib augmented when used in combination with Temozolomide, a drug commonly used for glioma treatment. Another study on the role of BTK in glioblastoma was reported by Wang et al., who demonstrated that BTK inhibition by Ibrutinib inhibited cellular proliferation and migration, by blocking BTK downstream effectors such as AKT/mTOR signaling and contributed to the initiation of autophagy process (Wang et al. 2017).

Supra-physiologic levels of BTK were reported also in gastric carcinoma tissues and cells. Wang et al. demonstrated that the knockdown of BTK selectively inhibited the growth of gastric cancer cells, but did not affect the normal mucosal epithelium (Wang et al.

2016). These authors showed that inhibiting BTK by Ibrutinib blocked its downstream signaling via PLCγ2, STAT3 and AKT resulting in the apoptosis of gastric cancer cells. In addition, they showed that Ibrutinib had a synergistic effect with docetaxel (DTX) in chemo-sensitizing those cells (Wang et al. 2016).

Similarly, Chong et al. successfully used Ibrutinib for inhibiting BTK in esophageal tumor lines. BTK inhibition resulted in cell cycle arrest and induction of apoptosis in both MYC and HER2 amplified esophageal tumors. Given these encouraging results, a phase II trial of Ibrutinib in patients with MYC and/or HER2 amplified esophageal cancer is currently ongoing (NCT02884453).

A BTK isoform other than p77BTK, BTK-C, has been found by Eifert et al., expressed in human breast cancer cells, where it was responsible for protecting these cells from apoptosis (Eifert et al. 2013). BTK-C differs from the canonical p77BTK isoform because it contains an amino-terminal 34 amino acid extension thus resulting in an 80kDa protein. The extended PH domain derives from the translation of a mRNA containing the alternative exon 1B instead of the canonical exon 1A found in the transcript encoding p77BTK. The same group subsequently reported that treating with Ibrutinib HER2-positive breast cancer cell had a cytotoxic effect due to both BTK and EGFR/HER2 inhibition. In fact, even though at the beginning Ibrutinib was believed to specifically inhibit only BTK it has been subsequently demonstrated that it powerfully inhibits also EGFR family members. Ibrutinib efficiently reduced the phosphorylation of AKT, ERK and

histone H3, increasing cleaved caspase-3 levels. Mechanistically, this treatment inhibited the BTK-dependent reactivation of the PI3K/AKT or MAPK signaling (Wang et al. 2016). Those evidences support the therapeutic utility of BTK inhibition in in HER2-positive breast cancer. The BTK-C isoform has then been described by the same authors also in prostate cancer, where it is highly expressed and its inhibition by BTK inhibitors or down-regulation by RNA interference resulted in decrease of cancer cell survival and induction of apoptosis (Kokabee et al. 2015). Based on these findings a phase I/IIstudy is evaluating Ibrutinib as neoadjuvant therapy in men with localized prostate cancer undergoing radical prostatectomy (NCT02643667).

P65BTK

In the last decade our lab focused on the identification of actionable targets to overcome drug resistance in colon cancer. To this end, a kinase-directed shRNA-mediated phenotype screening was performed that led to the identification of a number of kinases whose inhibition re-sensitized drug-resistant colon cancer cells to chemotherapy (Grassilli et al. 2013, Grassilli et al. 2014). Among those genes, a novel isoform of BTK was identified which was named p65BTK because of its apparent molecular weight. We demonstrated that p65BTK-encoding mRNA comprises a different first exon compared to the first exon of p77BTK-encoding mRNA. Curiously,

p65BTK-encoding transcript is the same as that encoding the 80kDa isoform found in breast and prostate cancer. However, in colon cancer cells and tissues BTK protein is around 65kDa. We demonstrated that this shorter isoform is due to the fact that the translation of the mRNA instead of starting from the ATG present in the 1st alternative exon or the one present in the 2nd exon (from which the translation of the 77kDa isoform starts) initiates from an in-frame ATG present in the 4th exon (thus extending the 5'UTR portion of the mRNA) (Fig.7). As a consequence, the p65BTK isoform lacks the first N-terminal 86 amino acids, resulting in a protein devoid of most of the PH domain, thus unable to bind classical activators, such as PIPs, and to interact with several negative regulators. In fact, it has been recently demonstrated that the loss of most of the PH domain that occurs in p65BTK leads to an increased level of a constitutively active kinase, in absence of external stimuli (Joseph et al. 2017).

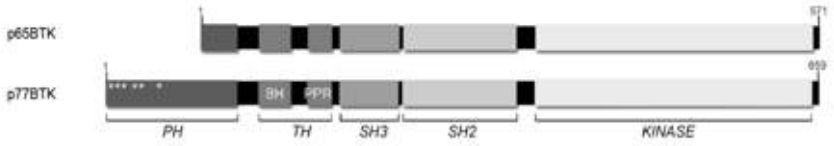

Figure 7: The two isoforms of BTK p77 vs p65. The two isoforms differ only in N-term part (Grassilli et al. 2016).

Analyzing p65BTK 5'UTR we found four putative hnRNPK binding sites. hnRNPK is a heterogeneous nuclear ribonucleoprotein involved in transcription regulation, mRNA transport from the nucleus to the cytoplasm, RNA editing and translation regulation which is also overexpressed in colon cancer where it has an aberrant cellular localization (Carpenter et al. 2006). While in normal colon tissue hnRNPK is exclusively present in the nucleus, in tumoral tissues the protein is exported also in the cytoplasm, subsequently to ERK-mediated phosphorylation on serine 284 and 353 (Habelhah et al. 2001). Accordingly, we demonstrated a crucial role for hnRNPK and ERK1/2 in regulating the translation of p65BTK in colon cancer cells.

It is known that hnRNPK induces the translation of c-myc by assembling the ribosome through an Internal Ribosome Entry Site (IRES) (Notari et al. 2006). IRES-mediated pathway compensates the protein synthesis in conditions of decreased cap-dependent translation, such as during mitosis, cell differentiation or pathological endoplasmic reticulum stress, hypoxia, lack of nutrients and apoptosis (Spriggs et al. 2008). It has been estimated that at least 10% of the human transcriptome contains IRES elements, with high prevalence in mRNAs encoding for oncogenes and regulators of cell stress and apoptosis (Mokrejš et al. 2009). We identified an IRES element in the 5'UTR of p65BTK mRNA and demonstrated that hnRNPK-dependent translation of endogenous p65BTK was IRES-mediated, suggesting for p65BTK a role as a possible oncogenic protein

Due to the over-expression of p65BTK in colon carcinomas and its IRES-mediated translation, as explained above, we investigated the possible oncogenic properties of p65BTK and showed that p65BTK, but not p77BTK, over-expression transformed NIH3T3 fibroblasts. We found that p65BTK oncogenic activity is dependent on its kinase activity and that over-expression of p65BTK in NIH3T3 cells increases also the expression levels of endogenous RAS. We demonstrated that RAS activity was necessary for p65BTK-mediated transformation, which was abolished by a specific RAS inhibitor, as well as by the co-transfection with a RAS-DN. Conversely, also H-RASV12 over-expression increased endogenous p65BTK, whereas the knockdown of endogenous RAS rapidly inhibited p65BTK expression, confirming a role for RAS in regulating p65BTK expression, likely via MAPK/hnRNPK. Moreover, inhibiting endogenous p65BTK by Ibrutinib blocked H-RASV12-mediated transformation indicating p65BTK as an obligate effector of the transforming activity of RAS and suggesting the existence of a reciprocal regulation between p65BTK and H-RAS. Also blocking the RAS/MAPK pathway downstream of RAS, by using a MEK1/2 inhibitor, suppressed p65BTK-mediated transformation activity confirming the crucial role of MAPK/hnRNPK in regulating p65BTK. Finally, we showed a correlation between p65BTK expression, ERK1/2 activation and abnormal hnRNPK cytoplasmic in tumoral, but not normal, colonic tissues from RC patients.

On the whole, these previous results from the lab indicated that p65BTK is an oncogene whose expression and transforming activity are tightly controlled, via hnRNPK, by the RAS/ERK pathway and that p65BTK over-expression in colon carcinomas reflects hyperactivation of the RAS/ERK pathway (Fig.8).

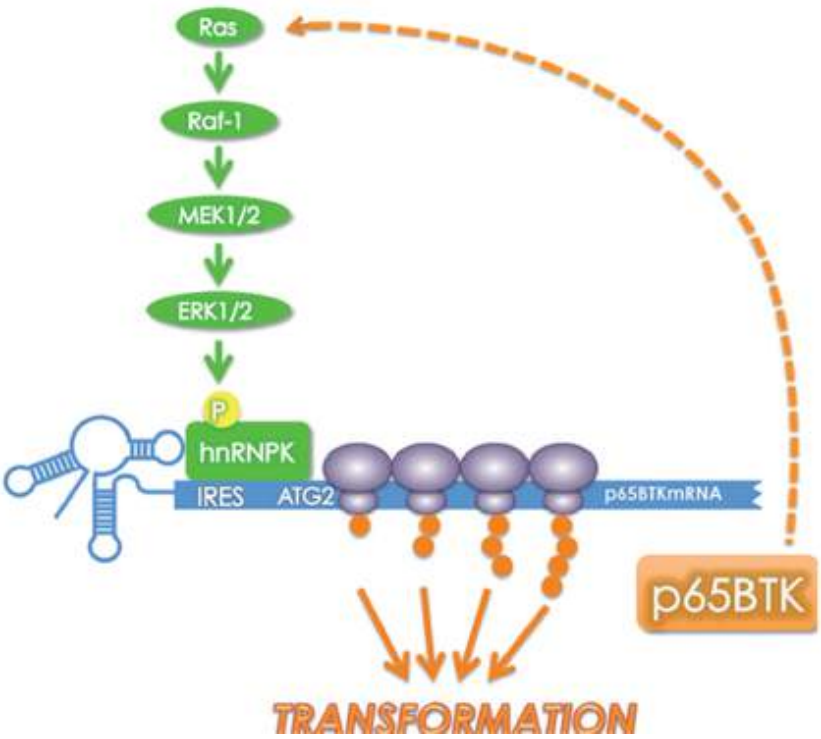

Figure 8: Model of p65BTK regulation (Grassilli et al. 2016).

Recent data from the lab showed that p65BTK expression increases with the stage and the grade of the tumor and it is expressed also in colon cancer stem cells purified from patients'

tumors and colon cancer stem cell-derived organoids. Moreover, we demonstrated that its targeting re-sensitizes, in vitro, ex-vivo and in vivo, drug-resistant colon cancer cell to chemotherapy (Lavitrano et al, submitted).

BTK INHIBITORS

FIRST GENERATION BTK INHIBITORS

In the recent years numerous BTK inhibitors have been synthesized and tested in all the different disorders in which BTK is involved. Among the different inhibitors, Ibrutinib has been the first BTK inhibitor approved in clinic by FDA for the treatment of mantle lymphoma and chronic lymphocytic leukemia (Wang et al. 2013).

Ibrutinib (formerly known as PCI-32765 and on the market as Imbruvica) is a small molecule inhibitor discovered in 2007 by Pharmacyclics. This compound binds covalently to a cysteine residue (Cys-481) in the ATP binding site of BTK, irreversibly abrogates the full activation of BTK by inhibiting its autophosphorylation at Y223, eventually blocking BCR signal transduction (Honigberg et al. 2010). Ibrutinib was shown to specifically and efficiently inhibit BTK in various preclinical models of B-cell lymphoma and was then selected for clinical trials (Honigberg et al. 2010, Byrd et al. 2013, Wang et al.

2013). In 2014, Ibrutinib has been approved by FDA for the treatment of several lymphoproliferative malignancies (Cameron et al. 2014, Novero et al. 2014). In 2017, Ibrutinib became the first non-chemotherapeutic drug ever to receive FDA approval specifically for the treatment of patients with relapsed or refractory marginal zone lymphoma who require systemic therapy and who have received at least 1 previous anti-CD20 based therapy. FDA successively approved Ibrutinib as a first treatment specifically indicated for relapsed/refractory marginal zone lymphoma (MZL)-a rare type of non-Hodgkin's lymphoma (Press release. January 19, 2017. https://news.abbvie.com/news/us-fda-approves-imbruvica-ibrutinib-as-first-treatment-specifically-indicated-for-relapsedrefractory-marginal-zone-lymphoma-mzl--rare-type-non-hodgkins lymphoma.htm. Accessed March 1, 2017). In addition to lymphomas, promising preclinical data showed the efficacy of Ibrutinib also for the treatment of inflammatory and autoimmune disorders (Weber et al. 2017). It is orally administered, potent, selective and has a half maximal inhibitory concentration (IC50) of about 0.5 nmol/L (Hutchinson et al. 2014) (Spaargaren et al. 2015). Regarding the pharmakinetics, Ibrutinib is rapidly absorbed and eliminated after oral administration with maximum serum concentration in 1-2 hours. Ibrutinib has short half-life (2-3 h), being rapidly metabolized by CYP3A4, and it can only be administered once daily. At a dose of 420 mg, Ibrutinib remains covalently bound to BTK for 24 h (Advani et al. 2013). Ibrutinib is generally well tolerated, and usually associated with grade 1-2 side effects, such as diarrhea, nausea, dyspnea,

bleeding, fatigue and infections, that are self-limited and not required therapeutic intervention (Burger 2014). Nevertheless, some toxic effects such as anemia, neutropenia and thrombocytopenia have been reported in a small population of patients (Byrd et al. 2015). Those side effects can be also attributed to the off-target effects of Ibrutinib, which is able to bind and inhibit other kinases with similar cysteine residue in the ATP binding site, including, HER2, HER4, EGFR, BLK and JAK3 (Singh et al. 2010, Brown 2013, Berglöf et al. 2015). Moreover, it has been reported that Ibrutinib can also affects other proteins without the specific residue, such as HCK, LCK, BRK, CSK and FRG. The effects of Ibrutinib on these proteins might also contribute to the efficacy or toxicity of the drug (Berglöf et al. 2015) (Honigberg et al. 2010).

MECHANISMS OF RESISTANCE TO IBRUTINIB

Despite the clinical effectiveness of Ibrutinib in multiple B-cell malignancies many patients develop resistance to the treatment (Zhang et al. 2015). The most common mechanism of primary or acquired resistance to Ibrutinib is due to a mutation of the Cys residue responsible for the binding of the drug (Wu et al. 2016). In CLL or MCL resistant patients, the replacement of Cys481 with a serine residue is the recurrent identified mutation. (Wu et al. 2016). As a consequence, the binding between BTK and Ibrutinib is altered, resulting in decreased affinity for the binding site and only a

reversible inhibition of BTK (Furman et al. 2014). In addition to the BTK C481S mutation, a variety of acquired activating mutations in PLCγ2 have also been detected in patients with CLL. These gain-of function mutations of PLCγ2 bypass BTK inhibition by Ibrutinib inducing the downstream activation of BCR signaling (Maddocks et al. 2015) (Liu et al. 2015). Moreover, other BTK mutations (like C481F, C481R, C481Y, T474S, T474I and L528W) that are associated with BTK C481S or PLCγ2 variations have also been implicated in rare cases (Maddocks et al. 2015) (Albitar et al. 2017). Recently, a study has reported a novel mutation (T316A) in the SH2 domain of BTK in Ibrutinib-resistant CLL patient, which is the first reported BTK mutation outside the kinase domain. However, how this mutation alters the drug binding is not well understood (Sharma et al. 2016).

SECOND GENERATION BTK INHIBITORS

Due to the emerging of resistance and off-target side effects of Ibrutinib, a second generation of BTK inhibitors has been developed showing higher selectivity and efficacy than Ibrutinib.

ACALABRUTINIB

Acalabrutinib (ACP-196) is a second generation BTK inhibitor developed by Acerta Pharma. Similarly to Ibrutinb, Acalabrutinib irreversibly and covalently binds Cys481 in the kinase region, with

higher selectivity and specificity towards BTK and much less affinity for other kinases with similar cysteine residue in the ATP binding site (Wu et al. 2016). In particular, Acalabrutinib showed specific targeting in CLL and no side effects on thrombus formation compared to Ibrutinib. Pharmacodynamic studies have shown a dose-dependent response to Acalabrutinib resulting in cytotoxicity and anti-proliferative effects in a canine lymphoma cell line (Zou et al. 2016). In vivo, the compound was found to be generally safe and well-tolerated in a dosage range of 2.5–20 mg/kg administered every 12 or 24 hours along with clinical benefits observed in 30% of canine patients. However, Acalabrutinib exhibited adverse effects in the gastrointestinal tract such as anorexia, weight loss, vomiting, diarrhea and lethargy (Patel et al. 2016).Currently, Acalabrutinib is under evaluation in more than 20 clinical trials (alone and in combination with other treatments) for various blood cancers and solid tumors and for rheumatoid arthritis (RA) (https://clinicaltrials.gov/ct2/results?term=Acalabrutinib).

TIRABRUTINIB

Tirabrutinib (ONO/GS-4059) is another irreversible and highly selective BTK inhibitor, initially developed by Ono Biomedical for the treatment of B-cell lymphoma and CLL. This compound is able to inhibit BTK by blocking the auto-phosphorylation at the Tyr223 position. The IC50 of Tirabrutinib is in the sub-nanomolar concentration range. This suggests that Tirabrutinib is more specific

for BTK compared to Ibrutinib. Tirabrutinib has been reported effective in a BC-DLBCL TMD-8 xenograft model and DLBCL, FL, MCL and CLL cell lines when used alone or in combination with other targeted agents. A phase I study of Tirabrutinib in relapsed/refractory B-cell malignancies is ongoing (NCT01659255). Notably, in 2017, Tirabrutinib was designated as an orphan drug in the U.S. for the treatment of CLL (Wu et al. 2017).

SPEBRUTINIB

Spebrutinib (AVL-292/CC-292) is an orally available, potent, selective and covalent BTK inhibitor with an IC50 below 0.5 nM. AVL-292 was originally developed by Avila Therapeutics, designed to covalently bind Cys481 with strong affinity, thus blocking the ATP-binding site (Evans et al. 2013). AVL-292 efficiently blocked the BCR signaling in the Ramos human Burkitt's lymphoma cell line by covalent modification of the Cys481 residue of BTK. Moreover, AVL-292 was also able to inhibit BTK autophosphorylation and its downstream effectors, including PLCγ2 and ERK (Arnason et al. 2014). In addition, AVL-292 showed a selective targeting of JAK3 and TEC, which contain a homologous cysteine residue. Therefore, due to the specific inhibition of BTK and the targeting effect on other molecules of the BCR signaling, AVL-292 has been tested for the treatment of B cell malignancies and autoimmune disease. In 2014, Spebrutinib was approved in the treatment of CLL and small lymphocytic lymphoma. Furthermore, this compound has been tested in clinical trial for the

treatment of autoimmune diseases such as RA (phase II clinical study) (Norman 2016). Several clinical studies of Spebrutinib alone or in combination with other agents are under evaluation.

CGI-1746

CGI-1746 is another reversible BTK inhibitor, developed by CGI Pharmaceuticals to target BTK. It has been shown that CGI-1746 binds the SH3 binding pocket, blocks the phosphorylation at Y551 and Y223 and thereby stabilizes BTK in its inactive conformation (Di Paolo et al. 2011). In B lymphomas, CGI-1746 was able to block the BCR signaling resulting in the inhibition of B cells proliferation. In addition, it was shown that CGI-1746 suppresses cytokines production in macrophages blocking FCγRIII signaling. Moreover, CGI-1746 strongly suppresses cytokines and antibodies productions leading to a decrease in the erosion area of the joints in a mouse model of RA (Di Paolo et al. 2011).

RN486

RN486 is a novel BTK inhibitor with a different mechanism of inhibition compared to previous BTK inhibitors, such as Ibrutinib and CGI-1746. RN486 strongly interacts with K430, a residue critical for BTK kinase activity. Differently from the others inhibitors, RN486 inhibits the phosphorylation of both BTK and PLCγ2 in B cells, blocking the BCR signaling pathway (Akinleye et al. 2013). It has been

reported that RN486 inhibits the TNF production following FcεR-mediated mast cell degranulation in a dose-dependent manner with an IC50 of 7.0 nM. Moreover, different studies have reported the role of RN486 in multiple cell types including B cells, monocytes, and mast cells, regulating their activation, proliferation, and degranulation, respectively (Hill et al. 2015). RN486 has also shown robust anti-inflammatory and bone-protective effects in mouse collagen-induced arthritis (CIA) and rat adjuvant-induced arthritis (AIA) models. Additionally, RN486 can effectively abrogate type I and type III hypersensitivity responses in rodent models. Thus, due to the potential of RN486 for the treatment of RA, the drug is currently in preclinical trials at Roche for the treatment of autoimmune and inflammatory diseases.

LUNG CANCER

Lung cancer (LC) is the leading cause of cancer-related death worldwide both in men and women (Oncology 2014). The American Cancer Society reported the following LC rates in the United States for 2018: about 234,030 new cases of LC (121,680 in men and 112,350 in women); about 154,050 deaths from LC (83,550 in men and 70,500 in women). Moreover, LC occurs mainly starting from age 65 years, while a very small number of people is diagnosed younger than 45. LC is a heterogeneous disease with a multiple complex combination of morphological, molecular and genetic alterations

(Hanahan et al. 2000). Generally speaking, the accumulation of several molecular and genetic changes causing an imbalance between tumor suppressor genes (TSG) and tumor promoting oncogenes, has to occur for cells to become malignant (Fearon et al. 1990). Many critical oncogenes, activated by somatic mutations and resulting in an 'oncogene addiction' effect (Davidson et al. 2013) have been considered as potential 'driver' events in lung carcinogenesis. The identification and characterization of such 'driver' events have provided a rationale for developing a personalized molecular targeted therapy specific to given lung cancer subtypes.

Generally, LC is classified into two main sub-types based on histological features: small cell lung cancer (SCLC, previously known as oat cell carcinoma) and non-small cell lung cancer (NSCLC). SCLCs is a neuroendocrine carcinoma that exhibits aggressive behavior, rapid growth, early spread to distant sites (Jackman et al. 2005). NSCLC is any type of epithelial lung cancer other than SCLC. There are three main subtypes of NSCLC, including adenocarcinomas, squamous cell carcinomas and large cell carcinomas. NSCLC is more common in smokers and accounts for 85-90 % of lung cancer cases (Molina et al. 2008, Jemal et al. 2011). In LC patients the survival depends on the type, stage at diagnosis and treatment available. At the time of diagnosis ~70% of NSCLC patients have locally advanced or metastatic disease and poor survival prognosis (Yang et al. 2005).

RISK FACTORS

The main risk factors for lung cancer are genetics, tobacco smoking, age, radon, asbestos, diet, radiation therapy, air pollution (Fig.9).

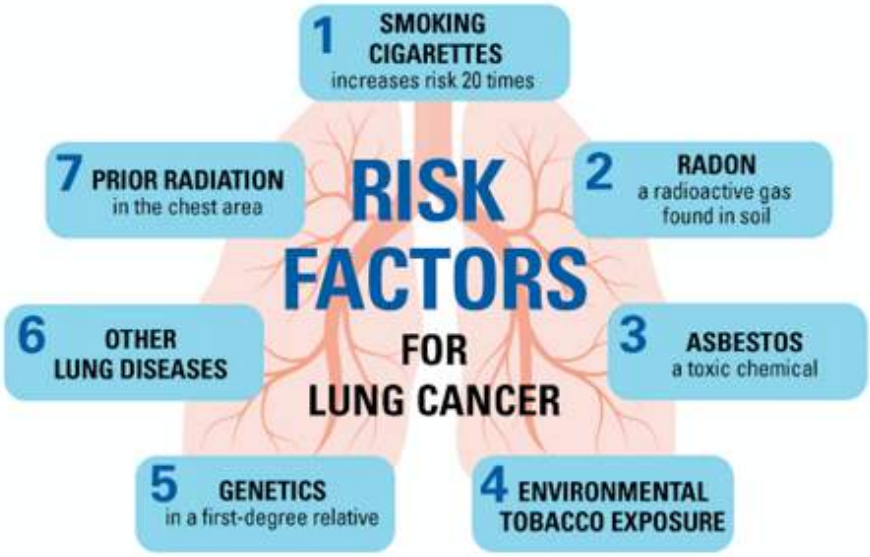

Figure 9: Lung cancer risk factors (Adapted from: https://www.nationaljewish.org/health-insights/health-infographics/lung-cancer-risk-factors).

FAMILY HISTORY

A genetic component involved in the development of LC has been identified as risk factor in several studies. LC incidence is higher in people with a family history of the disease with or without

smoking or exposure to environmental carcinogens (Bermejo et al. 2005). It has also been reported an increased LC risk associated with the Li–Fraumeni syndrome, characterized by germline mutation in the tumor-suppressor gene p53 (Malkin et al. 1990).

TOBACCO SMOKING

Tobacco smoking is the major cause of both SCLC and NSCLC. Starting from the 1950s epidemiological studies have identified a carcinogenic effect of tobacco smoke in the lung, which has then been recognized by public health and regulatory authorities in the mid-1960s (Wynder 1997). The relative risk is tightly related to the number of smoked cigarettes per die, the duration of smoking (years) and the content of tobacco residues (tar). The risk in continuous smokers is 20- to 50-fold higher than in non-smokers. For smokers who stopped, such risk decreases in the following 10-15 years. Regarding passive smokers, it has been estimated a risk increase of 20-50% compared to non-smokers. Non-smokers are 20-30% more likely to develop LC if they are exposed to secondhand smoke at home or at work (Yano et al. 2008). Secondhand smoke, also known as environmental tobacco smoke, includes both the smoke exhaled from a smoker and the smoke from a lighted cigarette, pipe, cigar or hookah. It has also been indicated that secondhand smoke may be involved in other cancer progression including leukemia and brain, bladder, breast cancers (Subramanian et al. 2007) (American Cancer Society. Accessed online on 8/2/16 at http://www.cancer.org/). In

addition, tobacco smoke can potentiate the effect of substances recognized as lung carcinogens (asbestos, chrome, arsenic, beryllium, vinyl cloride, radon, etc).

AGE

Age is an important risk factor for LC, as in many other cancers. Most lung cancers are diagnosed among people 65-74 years of age (Cassidy et al. 2006).

EXPOSURE TO RADON

Radon is a naturally occurring, colorless, tasteless, and odorless radioactive gas that is produced by decaying uranium. It is commonly released by construction material in buildings and is hardly detectable without specialized equipment. It occurs naturally in soil and rocks, thus underground miners have a high risk of exposure to radon (Zappa et al. 2016). Exposure to radon increases a person's risk of developing LC, and according to the US Environmental Protection Agency (EPA) radon is the second-leading cause of lung cancer, especially in never smokers. The outdoor amount of radon is very low and is not considered dangerous. On the contrary, the indoor amount is more concentrated resulting in exposure to small radiation amounts (US EPA. Health Risk of Radon. Available online: https://www.epa.gov/ radon/health-risk-radon).

EXPOSURE TO ASBESTOS AND OTHER WORKPLACE CARCINOGENS

The mortality of people who works with asbestos (such as in mines, mills, textile plants, places where insulation is used, and shipyards) is tightly related with LC development. In addition, the risk of developing LC is even greater for smokers who are workers exposed to occupational carcinogens. People exposed to large amounts of asbestos have also a greater risk of developing mesothelioma, a type of cancer that starts in the pleura (the lining surrounding the lungs) (Berman et al. 2008). Other carcinogens in the workplace that increases LC risk are uranium, arsenic, beryllium, cadmium, silica, vinyl chloride, nickel compounds, chromium compounds, coal products, mustard gas, and chloromethyl ethers from Diesel exhaust. Only recently, industries are starting to protect workers from many of these exposures (Spyratos et al. 2013).

DIET

It is known that the diet is responsible for approximately 30% of all cancers. Several studies pointed out the role of diet as risk factor of LC development. In particular, low concentrations of antioxidants, including vitamins A, C, and E, have been associated with LC development. Other studies have indicated that also beta carotene supplements increase the risk of LC. On the contrary, fruits and vegetables are associated with a decreased risk of cancer (Malhotra et al. 2016)

RADIATION THERAPY FOR BREAST CANCER

Radiation therapy is one of the first treatment for many cancers. Among its side effects, there is an increased risk of developing secondary cancers. It has been reported that breast cancer patients treated with radiation therapy show an increased risk of developing LC. In addition, this risk is further exacerbated when patients are smokers (Neugut et al. 1994).

AIR POLLUTION

Air pollution has long been considered a risk factor for LC. The effect of low levels of air pollution exposure over time are difficult to measure, but some studies have shown that people who live in urban areas or cities have a 10-40% increase in LC deaths than those in rural areas (Vineis et al. 2005).

SCLC

Small-cell lung cancer (SCLC) accounts for around the 15% of all cases of LC worldwide, however geographic differences in smoking habits, combined with gender and ethnic differences, prevent accurate worldwide estimates. SCLC is a particularly aggressive form of cancer widely metastatic and rapidly lethal that kills around 250,000 people worldwide per year (Abidin et al. 2010). The main hallmarks of SCLC are rapid proliferation, high vascularity, apoptotic imbalance, genetic instability, early onset dissemination, inactivation

of TP53/RB1 and frequent deregulation of multiple signaling pathways. Moreover, reports showed a higher risk of developing SCLC in patients with paraneoplastic syndromes compared to the other cancer types (Gazdar et al. 2017). In addition, current or former heavy smoke habits have been linked to the development of SCLC (Alexandrov et al. 2016). Patients who survive more than two years after diagnosis have a significantly increased risk of developing a second primary tumor (Kawahara et al. 1998). Over the past 30 years, only a few advances have been reported in SCLC detection, therapy or survival, classifying SCLC as a refractory cancer (US Congress. H.R.733 — Recalcitrant Cancer Research Act of 2012. Congress.gov https://www.congress.gov/ bill/112th-congress/house-bill/733 (2012).

A significant change in the knowledge of SCLC arose in 1973, when Matthews et al. showed that, differing from NSCLC, SCLC was found already metastatic at the time of diagnosis in patients who underwent resections with curative intent (Matthews et al. 1973). Still, little is understood on the biology of SCLC due to the lack of resected SCLC tissues for research studies, which underline the need for the development of preclinical models to help translational research (including cell lines, genetically engineered mouse models (GEMMs) and patient-derived xenografts (PDXs).

Several alterations were found in SCLC starting from 1982, when Whang-Peng and colleagues described a frequent, nonrandom, acquired chromosomal abnormality (deletion 3p) in SCLC tumors and

cell lines (Whang-Peng et al. 1982). More recently, Notch has been found inactivated in the majority of SCLC tumors, especially in those expressing the full set of pulmonary neuroendocrine (NE) cell marker genes. In SCLC Notch acts as a tumor-suppressor that negatively regulates NE differentiation (Meder et al. 2017). On the contrary, Notch pathway acts as an oncogene in NSCLC. Moreover, alterations have been identified in several MYC family members such as MYC, MYCL and MYCN, which are frequently amplified or overexpressed in SCLC tumors and cell lines (Little et al. 1983). Generally, a modest number of mutated kinase genes has been described in SCLC compared to NSCLC. Also, PIK3CA (which encodes the catalytic subunit of PI3K) and PTEN (inhibitor of the PI3K–AKT–mTOR pathway) have been found mutated in SCLC (Shibata et al. 2009). In particular PTEN inactivation, causes faster tumor growth and increased number of metastases in a Trp53 and Rb1-inactivated GEMM (Cui et al. 2014). A subset of SCLCs is instead characterized by activation of the fibroblast growth factor (FGF)–FGF receptor 1 (FGFR1) pathway. Differently from NSCLC, EGFR or KRAS mutations or alteration of their signaling, including the downstream RAF–MEK–ERK pathway, are extremely rare in SCLC. KRAS mutations are rare, and reduced pathway activation may be advantageous to SCLC tumors (Bunn et al. 2016).

NSCLC

NSCLC is defined as any type of epithelial LC other than SCLC. The World Health Organization (WHO)/International Association for the Study of Lung Cancer (IASLC) has classified three main types of NSCLC: squamous cell carcinoma (25-33% of lung cancers), large cell carcinoma (3-10% of lung cancers) and adenocarcinoma (40% of lung cancers); however, there are several other less frequent types and all the types can occur in unusual histologic variants (Fig.10). NSCLC arises from the lung epithelial cells of the central bronchi to the terminal part of alveoli. The histological type of NSCLC correlates with its origin, reflecting the variation in the respiratory epithelium tract of bronchi and alveoli. Although NSCLC is generally associated with cigarette smoke, adenocarcinoma is more frequent in non-smoker patients. NSCLC is relatively more insensitive to chemotherapy and radiation therapy compared to SCLC (Zappa et al. 2016).

From a diagnostic point of view NSCLC is classified in four main stages, which are determined based on the size and extent of the main tumor (T), the spread to nearby lymph nodes (N) and to distant sites (metastasis; M). The most recent TNM classification is the 8th Edition of TNM in Lung Cancer, which is the standard of NSCLC staging since January 1st, 2017 and has been issued by the IASLC.

Stage I: the cancer is located only in the lungs and has not spread to any lymph nodes.

Stage II: the cancer is in the lung and nearby the lymph nodes.

Stage III: the cancer is found in the lung and in the lymph nodes in the middle of the chest, also described as locally advanced disease. Stage III has two subtypes:

Stage IIIA: the cancer has spread only to lymph nodes on the same side of the chest where the cancer started.

Stage IIIB: the cancer has spread to the lymph nodes on the opposite side of the chest, or above the collar bone.

Stage IV: This is the most advanced stage of lung cancer and is also described as advanced disease. The cancer has spread to both lungs, to fluid in the area around the lungs, or to another part of the body, such as liver or other organs. (https://www.cancer.org/cancer/non-small-cell-lung cancer/detection-diagnosis-staging/staging.html).

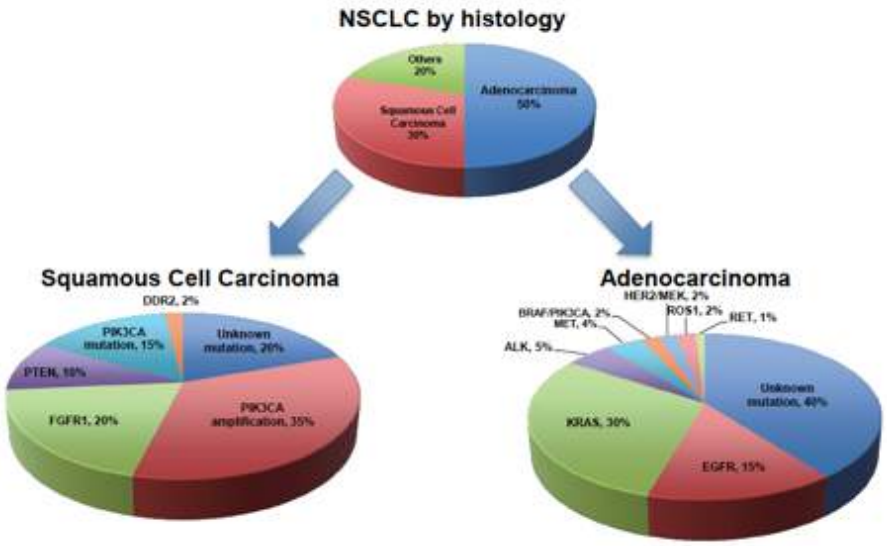

Figure 10: NSCLC histology subtypes and frequent mutations (Chan et al. 2015).

SQUAMOUS CELL CARCINOMA (SCC)

SCC accounts for approximately 25-33% of LC worldwide (Davidson et al. 2013). However, its incidence is decreasing in the last years. This trend is thought to be partly attributable to changes in smoking behaviors. Generally, SCC is considered more strongly linked with smoking habits than the other forms of NSCLC. SCC is centrally localized close to the bronchus, but it has been also identified in the periphery of the lung. It is morphologically characterized by intercellular bridges, individual cell keratinization and squamous pearl formation. The other SCC subtypes identified by the current WHO classification are papillary, clear cell, small cell and basaloid. These subtypes can be only distinguished by descriptive features and are not prognostic. Future sub-classification of SCC will require the establishment of crucial predictors of treatment response and prognosis (Davidson et al. 2013).

ADENOCARCINOMA

Adenocarcinoma represents the most common histologic lung cancer subtype in many Countries, particularly among non-smokers. One of the main problems of adenocarcinomas is its histologic heterogeneity. Indeed, mixtures of adenocarcinoma histologic subtypes are more common than pure tumors originated from a

single pattern of acinar, papillary, bronchioloalveolar, and solid adenocarcinoma with mucin formation (Davidson et al. 2013). Adenocarcinoma and bronchioloalveolar carcinoma predominantly arise peripherally in lung tissue (Woodring et al. 1983). Recently, the diagnostic criteria of bronchioloalveolar carcinoma have been changed. The acronym BAC for bronchioloalveolar carcinoma was discarded and the current WHO/IASLC classification comprises adenocarcinoma in situ (AIS) (previously BAC) and minimally invasive adenocarcinoma (MIA); if completely resected both malignancies show 5-year survival rates approaching 100% (Davidson et al. 2013). However, the future of bronchioloalveolar carcinoma as a distinct clinical entity is unclear; a newer International Association for the Study of Lung Cancer (IASLC)/ the American Thoracic Society (ATS)/ the European Respiratory Society (ERS) classification has been proposed compared to the one of 2011, based on the heterogeneity of this disease.

The actual IASLC/ATS/ERS classification recognizes five adenocarcinomas:

· Well-differentiated fetal adenocarcinoma.

· Mucinous (colloid) adenocarcinoma.

· Mucinous cystadenocarcinoma.

· Signet ring adenocarcinoma.

· Clear cell adenocarcinoma.

A positive prognosis has been associated with AIS and MIA. On the contrary, poor prognosis has been associated with invasive mucinous, colloid variants and predominant solid or micropapillary growth (Davidson et al. 2013).

LARGE CELL CARCINOMA

LCC accounts approximately for 3-10% of all lung cancers (Sawabata et al. 2010). It is essentially diagnosed when no morphological features of adenocarcinoma, SCC or SCLC are verified in the tumor (Travis 2011). LCC is poorly differentiated and is considered the most aggressive NSCLC subtype (Muscat et al. 1997). It occurs more frequently in smokers. In 2004 WHO classification recognized basaloid carcinoma, lymphoepithelioma-like carcinoma, clear cell carcinoma and LCC with rhabdoid phenotype as LCC subtypes. In particular, basaloid carcinoma is also considered as a squamous cell carcinoma variant and, rarely, adenocarcinomas can have a basaloid pattern; however, tumors without either of these features are treated as LCC variant (Travis et al. 2015).

MOLECULAR DEFECTS IN NSCLC

Multiple signaling pathways have been now identified as well as specific oncogenic driver mutations that can be specifically targeted in NSCLC (Fig.11).

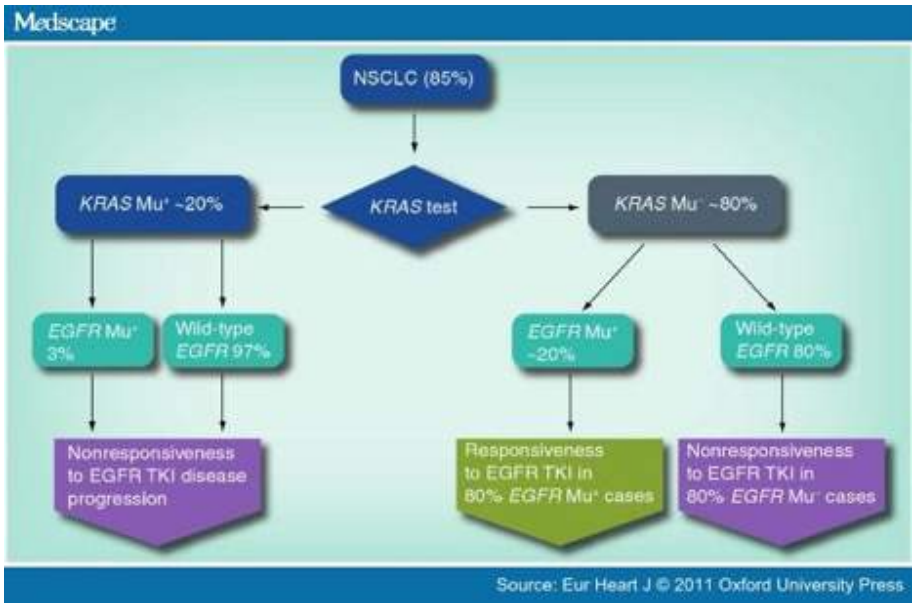

Figure 11: Algorithm of various mutations frequencies for NSCLC patients. EGFR: EGF receptor; Mu: Mutation; NSCLC: Non-small-cell lung cancer; SCC: Small-cell carcinoma; TKI: Tyrosine kinase inhibitor (Cheng et al. 2011).

EGFR

EGFR (ERBB1) is a transmembrane glycoprotein that belongs to the tyrosine kinase family, which also includes ERBB2-4. EGFR is a growth factor receptor upstream of the of RAS-RAF-MEK and PI3K-AKT-mTOR signaling pathways and play key role in carcinogenesis (Fig.12) (Pinho et al. 2015). In NSCLC, EGFR dysregulation may be due to three main mechanisms:

- EGFR over-expression in malignant cells;

- Increased ligand production by malignant cells;
- EGFR activating mutations in malignant cells.

EGFR has been found overexpressed or mutated in 40-80% of NSCLC, becoming the first molecular target to be therapeutically exploited. The most common EGFR mutations are in exons 18-21, which encode part of the EGFR kinase domain which can be targeted by specific tyrosine kinase inhibitors (TKIs). 65% of these mutations are deletions in exon 19 whereas the remaining 35% are L858R missense substitutions that result in constitutive activation of the receptor (Chan et al. 2015). The treatment with EGFR TKIs usually drives the selection of additional mutation that renders the tumors resistant to the treatment: the most common additional mutation is the substitution of threonine to methionine at codon 790 (T790M) in exon 20 (Oxnard et al. 2011) (Maemondo et al. 2010) (Kobayashi et al. 2005). It has been demonstrated that the insertion of the bulky methionine reduces the ability of certain TKIs to bind to the ATP binding pocket, causing an increased ATP binding and therefore kinase activation (Yun et al. 2008). Moreover, other additional mutations have been involved in the resistance to TKI inhibitors, such as T854A in exon 21 (Bean et al. 2008), L747S and D761Y in exon 19 (Costa et al. 2008) (Balak et al. 2006).

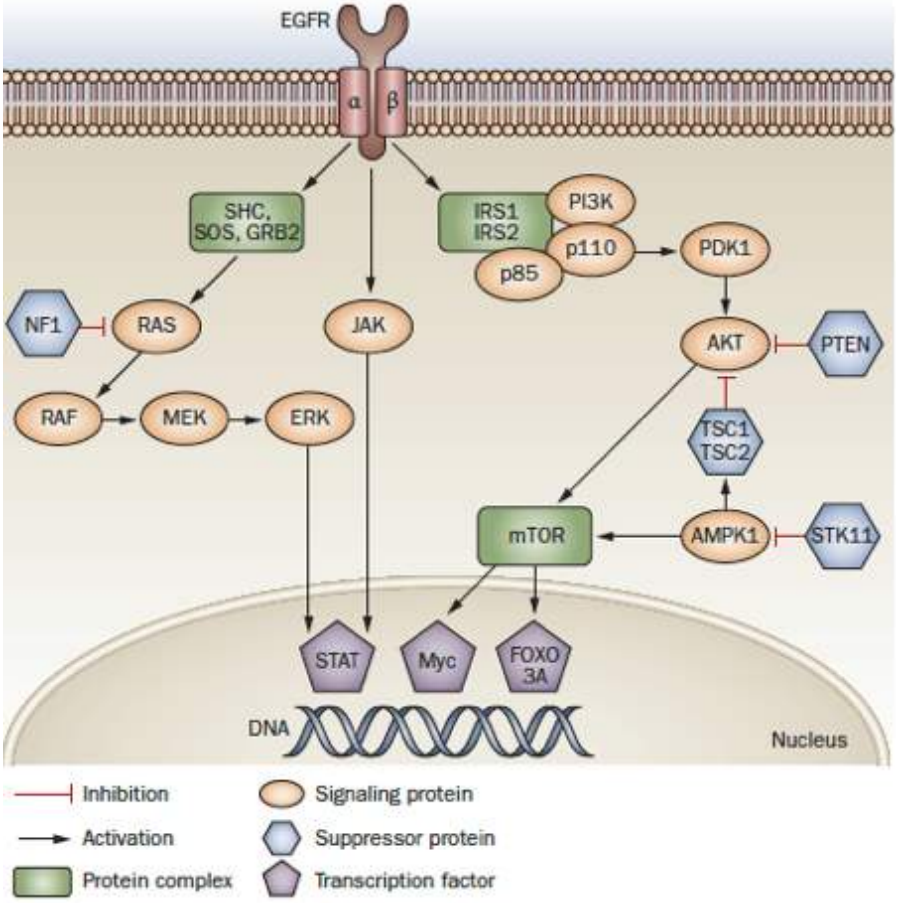

Figure 12: EGFR signaling and related pathway (Harris et al. 2010).

ALK

ALK is a tyrosine kinase receptor, whose intracellular kinase domain has been found fused to the amino terminal portion of different proteins. Approximately ALK is expressed in form of a fusion protein in 3–7% of all lung tumors, especially in young patients. In particular, the EML4–ALK fusion is a rare abnormality detected in 3–13% of patients with adenocarcinomas (Zappa et al. 2016). The fusion

protein is encoded by a rearranged gene on chromosome 2p23 ensuing from the joining the 5' of the EML-4 gene and the 3' end of the ALK gene; nine different fusion variants have been reported (Zappa et al. 2016). Generally, ALK rearrangement is mutually exclusive with other crucial oncogenic mutations like EGFR or RAS. It has been reported that ALK-fusion positive lung cancers are resistant to the EGFR TKIs, but on the contrary they are sensitive to small molecule TKIs against ALK.

K-RAS

K-RAS (Kirsten rat sarcoma 2 viral oncogene homolog) is one of the most mutated oncogenes across all cancers. It belongs to the GTPases family and transduces growth signals from multiple tyrosine kinases, including EGFR and MET (Fig. 12). KRAS mutation is another common alteration in NSCLC where a substitution of amino acids at position 12 or 13 or 61 usually occurs, being G12 and G13 the most frequently mutated residues. These mutations lead to an unrestricted and constitutive KRAS activation which results in an EGFR-independent activation of the intracellular signal cascade downstream of EGFR. KRAS mutations have been frequently identified in adenocarcinomas, in Caucasians and in tobacco smokers and are mutually exclusive from EGFR or ALK mutations (Riely et al. 2008). It has been reported that KRAS is mutated in 10-25% of patients with an adenocarcinoma, with a poor prognosis accompanied by non-responsiveness to chemotherapy and EGFR TKI

therapy (Brose et al. 2002). Despite KRAS is one of the first identified oncogenic drivers in NSCLC, its targeting remains a therapeutic challenge. New approaches are currently attempting to inhibit downstream molecules in the RAS/RAF/MEK/ERK and PI3K/AKT/mTOR pathways. One recent STUDY reported that the specific targeting of KRAS mutations with small molecule inhibitors directed towards G12C (common in smokers and non-smokers) did not affect the wild type KRAS (Zappa et al. 2016).

MET

Mesenchymal epithelial transition factor (MET) is a proto-oncogene located on chromosome 7q21 that encodes the hepatocyte growth factor receptor (HGFR), a transmembrane receptor tyrosine kinase involved in cell proliferation, migration, invasion and metastasis (Pinho et al. 2015). MET/HGFR pathway can be activated in LC through different alterations: protein over-expression; increased expression of its ligand, the hepatocyte growth factor; single aminoacidic mutations; gene amplification; and alternative splicing or exon 14 skipping. In particular, the over-expression of MET occurs in 25–75% of NSCLC tumor cells compared to adjacent normal tissues and is associated with poor prognosis (Benedettini et al. 2010). Each of these mutagenic mechanisms have been investigated as a potential target for the treatment of cancer patients. Among those, MET exon 14 alterations, which result in increased MET protein levels due to disrupted ubiquitin-mediated

degradation, occur at a prevalence of around 3% in adenocarcinomas and around 2% in other lung neoplasms, making them attractive targets for the treatment of lung cancer. At least five MET-targeted TKIs, including Crizotinib, Cabozantinib, Capmatinib, Tepotinib, and Glesatinib are being clinically investigated in patients with MET exon 14 altered-NSCLC (Reungwetwattana et al. 2017).

BRAF

BRAF is a proto-oncogene belonging to the RAF kinase family along with B-RAF and RAF-1. It is known that BRAF has a central role in the KRAS signaling pathway, inducing cell proliferation and survival. In addition, BRAF and KRAS are found involved in the signaling cascade of the EGFR family proteins (Fig. 12) (Zappa et al. 2016). Mutations in BRAF have been reported in about 1-4% in all NSCLC cases, mainly in adenocarcinoma and commonly associated with smokers. These mutations are mutually exclusive with EGFR and KRAS and confer poor responsiveness to EGFR TKI treatment (Cheng et al. 2012).

ROS1

ROS1 gene (located at chromosome 6q22) encodes for a receptor tyrosine kinase, which belongs to the insulin receptor family. Similarly to ALK aberrant kinase activity, rearrangements involving the ROS1 gene lead to a constitutively activated downstream

signaling of several oncogenic pathways. The homology to ALK has been particularly relevant in the development of ROS1-directed therapies. ROS1 rearrangements occur in 1% to 2% of NSCLC. In 2016, Crizotinib, an anaplastic lymphoma kinase (ALK)/ROS1/MET proto-oncogene, receptor tyrosine kinase (MET) inhibitor, became the first targeted agent approved by the FDA for the treatment of advanced ROS1-rearranged NSCLC (Clavé et al. 2016).

CURRENT TREATMENT OPTIONS IN LC

The first line of NSCLC treatment generally involves surgery, chemotherapy and/or radiotherapy. Surgery has been shown to be the most effective treatment for NSCLC and offers the best prognosis, achieving long-term cure (Myrdal et al. 2001). However, surgery is only an option for patients with early stage disease (Stage I, II, IIIA). Since the majority of NSCLC patients are diagnosed at later stages they are not eligible for surgery. On the other hand, SCLC is already metastatic from the outset and so rarely tractable with surgery. Therefore, chemotherapeutics such as cisplatin or carboplatin combined with etoposide with or without additional radiotherapy are the current standard of care (SOC) for SCLC patients (Available:http://emedicine.medscape.com/article/2007031-overview).

NSCLC patients in advanced non-operable stage require radiotherapy and/or combination chemotherapy. Patients with locally advanced inoperable disease are often treated initially with radical radiotherapy with or without additional chemotherapy, while those with metastatic disease receive combination of chemotherapy with or without additional radiotherapy. The type of chemotherapy is different also depending on LC histology. Adenocarcinoma patients are treated with Pemetrexed in combination with Cisplatin or Carboplatin, whereas SSC patients with Gemcitabine and Platinum-derived agents. Despite the advances in treatment, the overall prognosis of NSCLC is still poor, with a 5-year survival rate (percentage of patient's survival at least 5 years after diagnosis) of around 16% (Siegel et al. 2012).

Currently, the goal of NSCLC management directions is to identify and validate a range of predictive and prognostic biomarkers defining a subpopulation of patients eligible for targeted therapy and estimate prognosis respectively (Thunnissen et al. 2014). In the last decades, up to 60% of lung adenocarcinoma and up to 50-80% of SCC oncogenic driver mutations have been identified (see above) which allowed the development of targeted therapies, i.e., molecules specifically designed to block/inhibit the mutated protein from which the cancerous cell usually depends, due to the phenomenon of oncogenic addiction: as a result, the cell stop to proliferate or die. The epidermal growth factor receptor (EGFR) and the abnormal fusion of the anaplastic lymphoma kinase (ALK) are the best studied

targets so far in NSCLC (Fig.13). Both proteins are often mutated in patients and have been found to be efficiently inhibited by EGFR TKIs and Crizotinib respectively (Blais et al. 2014)(http://emedicine.medscape.com/article/2007153-overview).

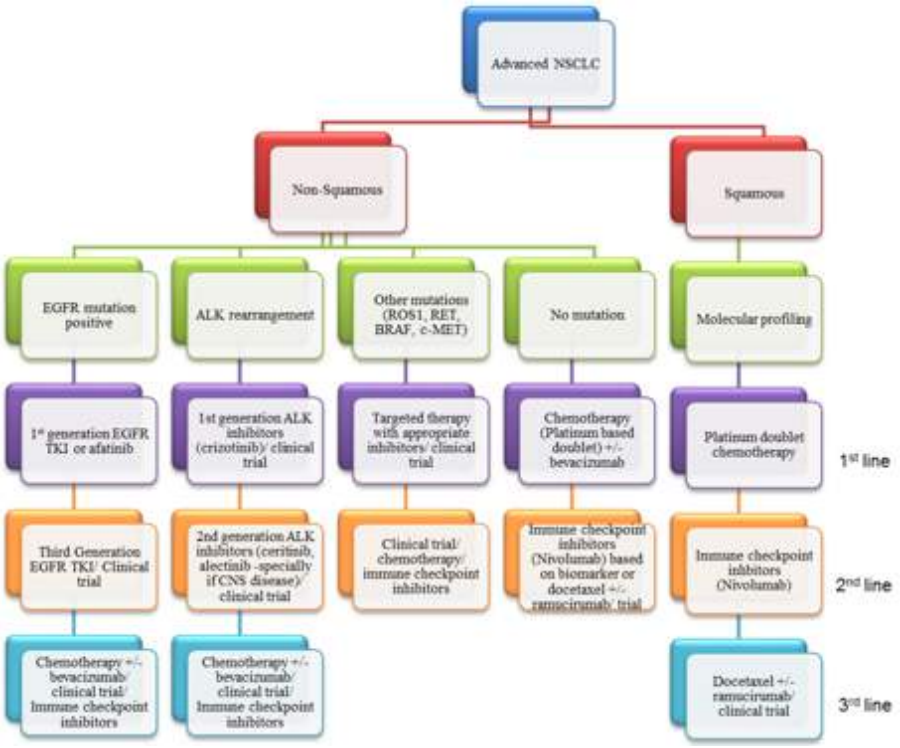

Figure 13: Treatments in advanced NSCLC (Boolell et al. 2015).

CHEMOTHERAPY IN NSCLC

Several classes of chemotherapeutic drugs can be identified based on the different mechanism of action:

- alkylating agents which damage DNA;

- anti-metabolites that replace the normal blocks of RNA and DNA;
- antibiotics that interfere with the enzymes involved in DNA replication;
- topoisomerase inhibitors that block either topoisomerase I or II (enzymes involved in unwinding DNA during replication and transcription);
- mitotic inhibitors (inhibit mitosis and cell division) (Huang et al. 2017).

Currently the guidelines identified platinum-based doublet (Cisplatin or Carboplatin) in combination with Gemcitabine, a taxane (Paclitaxel, Docetaxel), or Vinorelbine as the first-line of chemotherapeutic treatment. From a meta-analyses study, it has been reported that the clinical outcome of Cisplatin doublets is slightly superior compared to Carboplatin-based chemotherapy, with acceptable toxic effects (Hotta et al. 2004) (Ardizzoni et al. 2007).

CISPLATIN

The current chemotherapeutic treatment of solid tumors found to be most effective is Cisplatin (cis-diamminedichloroplatinum, DDP). DDP and its second-generation derivative, Carboplatin, are alkylating agents that, by forming intra- and inter-strand adducts with DNA, induce DNA damage and interfere with DNA repair in most

cancer cell types (Cohen et al. 2001). DDP was first approved by FDA in 1978 for the treatment of testicular and bladder cancer (Galluzzi et al. 2012). Moreover, high-dose DDP– based combination chemotherapy regimens are used as front-line treatments of SCLC and NSCLC. Unfortunately, many patients relapse due to an intrinsic resistance to platinum-based agents or to the development of an acquired resistance, following few cycles of DDP-based chemotherapy (Kartalou et al. 2001) (Rabik et al. 2007). According to Chih-Yang Huang et al. DDP resistance is developed in part by: (1) an enhanced drug detoxification system due to elevated levels of intracellular scavengers such as glutathione (GSH) and/or metallothioneins; for example, GSH may be involved in DDP resistance by inactivating DDP itself or preventing DDP-adducts formation. Intrinsic resistance to DDP in lung cancer cell lines has also been linked to enhanced GST-π isoenzyme expression (Seve et al. 2005); (2) changes in the expression/activity of the components of the DNA repair machinery such as an increased activity of the nucleotide excision repair system or the inter-strand crosslink repair system or the loss of activity of the mismatch repair system; (3) changes in DNA damage tolerance mechanisms; (4) alterations of the apoptotic cell death pathways (loss of pro-apoptotic functions; enhancement of anti-apoptotic functions) (Huang et al. 2017). For current regimens, DDP is usually administered in combination with third-generation cytotoxic agents (Rajeswaran et al. 2008). Currently, platinum-based chemotherapy has shown promising results in patients with advanced NSCLC and without a drug-targetable driver

(approximately 85–90%). Platinum-based therapy remains also the principal clinical therapy following the development of resistance to targeted therapy (Fennell et al. 2016).

GEMCITABINE

Gemcitabine (2′ , 2′ -difluoro-2′ -deoxycytidine) is a deoxycytidine analogue, which is wrongly inserted (instead of dCTP) into duplicating DNA, thus inhibiting DNA synthesis itself. This process, known as 'masked chain- termination', also inhibits removal of Gemcitabine by DNA repair enzymes (Huang et al. 1991). As a consequence, Gemcitabine blocks cell cycle progression at the G1/S phase and kills the cells in S phase. Previously employed as an antiviral drug, now it is widely used as an anti-cancer agent, representing the first-line chemotherapy as a single agent for patients with advanced pancreatic cancer and being included in current therapeutic protocols for patients with NSCLC, breast cancer, bladder cancer and ovarian cancer (Jia et al. 2015). It has been shown that Gemcitabine is more effective in NSCLC, but good results were also observed in the above mentioned tumors, especially when used in combination with other drugs (Huang et al. 2017) (de Sousa Cavalcante et al. 2014). Indeed, a better efficacy has been also been reported in the case of the combination of Gemcitabine with other drugs for the treatment of locally advanced or metastatic NSCLC. However, the survival rate of many patients is affected by the low

therapeutic index and the intrinsic or acquired resistance to this drug (Siegel et al. 2012).

PEMETREXED

Pemetrexed is an antifolate chemotherapeutic drug. It inhibits multiple enzymes of the pyrimidine and purine synthesis pathway. Among these, thymidylate synthase (TS) is a folate-dependent enzyme that catalyzes the transformation of deoxyuridine monophosphate to deoxythymidine monophosphate. The inhibition of TS by Pemetrexed drastically reduces the amount of thymidine necessary for DNA synthesis. Moreover, Pemetrexed is also able to inhibit dihydrofolate reductase (DHFR) and glycinamide ribonucleotide formyltansferase (GARFT) (Adjei 2004). The cytotoxicity of Pemetrexed has been associated with these targets due to the role of thymidine and hypoxanthine in bypassing cellular death caused by high doses of Pemetrexed. Pemetrexed is recognized as an efficient treatment for advanced NSCLC. Several trials have shown its activity in both first- and second-line settings alone or in combination with other compounds (platinum-based) with an acceptable overall toxicity profile (Fuld et al. 2010). Additionally, it has been shown by West et al. that Pemetrexed shows improved outcomes in non-squamous patients compared with their counterparts with squamous cell cancer (West et al. 2009).

TARGETED THERAPY IN NSCLC

EGFR INHIBITORS

To date, over 20 well-characterized EGFR-TKIs have undergone clinical development and many of them have been or are being tested for the treatment of NSCLC. Among the different molecules, the so-called first-generation inhibitors are currently used in the clinic.

The majority of first-generation EGFR-TKIs (for instance, Gefitinib and Erlotinib) are able to selectively but reversibly bind the active site of the tyrosine kinase domain (TKD) (Yun et al. 2007). Two factors are mainly involved in this binding: 1) the recognition of the active state of the kinase by EGFR-TKIs (Stamos et al. 2002) (Wood et al. 2004); and 2) the higher ATP affinity of mutant enzyme compared to the wild type EGFR (Yun et al. 2008). Despite the increased responsiveness of mutant EGFR patients to TKIs, resistance usually arises toward these molecules after some cycle of therapy commonly because of additional mutations occurring within the receptor (Sharma et al. 2007).

To overcome resistance to first-generation inhibitors second-generation EGFR-TKIs (Afatinib and recently Dacomitinib and Neratinib) were designed to irreversibly bind the TKD in attempts to overcome acquired resistance. The beneficial effects of these compounds have been reported in LC models with T790M-mediated

EGFR-TKI resistance suggesting the second-generation as more effective than the first-generation EGFR TKIs (Kwak et al. 2005) (Engelman et al. 2007). Nevertheless, these novel inhibitors have not been approved for clinical trials in lung adenocarcinoma T790M-positive EGFR-mutant patients, due to their limited therapeutic window (Girard 2018).

The third-generation TKIs (Osimertinib, EGF816, Olmutinib, PF-06747775, YH5448, Avitinib and Rociletinib) were designed to irreversibly bind the TKD active site in the presence of T790M mutation (Tan et al. 2018). Indeed, the third-generation agents have shown higher efficiency in EGFR-mutant compared to EGFR-wild type cells. Right now, Osimertinib is the only third-generation TKI approved by the FDA for the first-line treatment of metastatic NSCLC patients with EGFR mutations. However, it has been reported that patients developed resistance also to Osimertinib suggesting that alternative strategies and/or EGFR-TKIs are needed.

The resistance to the third-generation inhibitors can be attributed to the additional mutation C797S, which blocks the covalent binding of these drugs. This has led to the development of fourth-generation inhibitors (EAI001 and EAI045). These compounds bind EGFR allosterically (non-ATP competitive), with higher specificity for EGFR mutant compared to the wild type (Tan et al. 2018). EAI001 was efficient against the double L858R/T790M mutant EGFR, but less against individual L858R or T790M mutant EGFR. EAI045 was effective in overcoming EGFR C797S-mediated resistance in cell lines

with either L858R or T790M mutation, or with both mutations. Furthermore, EAI045 decreases cell proliferation in cell lines bearing L858R/T790 M/C797S mutations only when used in combination with Cetuximab (a monoclonal antibody blocking from the outside of the cell the EGFR). These results were validated in both L858R/T790M/C797S and L858R/T790M mutant-driven LC mouse models, where the combined treatment of EAI045 and Cetuximab resulted in reduction of tumor growth (Jia et al. 2016). These data support the use of the last generation TKIs in clinic. However, the potential development of new form of resistance remains still a barrier that needs to be fully overcome in LC treatment.

GEFITINIB AND ERLOTINIB FOR THE TREATMENT OF ADVANCED NSCLC

Gefitinib is a substituted anilinoquinazolin that reversibly and competitively inhibits EGFR, blocking the binding of ATP to the tyrosine kinase catalytic domain (Tiseo et al. 2010). This process results in the impairment of the receptor autophosphorylation and the inhibition of the downstream signal transduction (Tiseo et al. 2010). As a consequence, inhibition of cancer cells proliferation occurs due to an arrest of cell cycle in G1 that in turn ensues from the up-regulation of cell cycle inhibitors such as p27 and down-regulation of transcription factors such as c-FOS (Di Gennaro et al. 2003). Gefitinib has also been shown to induce autophagy and apoptosis, blocking the PI3K/AKT/mTOR pathway (Zhao et al. 2016). Gefitinib

has been approved by the FDA as monotherapy treatment for patients with locally advanced or metastatic NSCLC, which are resistant to Platinum or Docetaxel chemotherapies (Cohen et al. 2003). However, the approval was later retracted due to the absence of long-term clinical benefits and to several adverse effects associated with the treatment (Available:http://www.fda.gov/downloads/drugs/drugsafety/postmarketdrugsafetyinformationforpatientsandproviders/ucm126182.pdf). No exhaustive explanation was found to explain this toxicity. However, it has been suggested an association of Gefitinib with glutathione (GSH) by Li and colleagues. The authors showed that Gefitinib can be converted in hepatic, intestinal, and pulmonary microsomes, to a reactive metabolite that reacts with GSH to form Gefitinib-GSH adducts (Paez et al. 2004, Li et al. 2009) . Due to the limited efficiency and toxicity, Gefitinib was subsequently indicated only for patients who had previously shown a benefit from its use.

Erlotinib has been approved by the FDA for the treatment of NSCLC, in patients whose tumors have specific EGFR mutations. However, it is also being used in NSCLC patients with wt EGFR, previously treated with chemotherapy. Erlotinib presents similar structure, pharmacokinetic and toxicity profiles of Gefitinib. However, up to 49 differences have been identified in their clinical effectiveness (Wu et al. 2011). These evidences may be attributed to different binding to the catalytic site of the mutant active EGFR.

Indeed, it has been shown that mutations around the catalytic domain affect the sensitivity of EGFR to the different inhibitors (Yun et al. 2007). When compared to Gefitinib, Erlotinib treatment showed increased progression-free survival (PFS) and reduced adverse hepatic and pulmonary effects (Urata et al. 2016). However, patients inevitably developed acquired resistance to these drugs, mainly because of a secondary T790M mutation (Godin-Heymann et al. 2008).

RESISTANCE TO EGFR TARGETED THERAPY

EGFR TKIs treatments have shown promising results in EGFR-mutant NSCLC patients. However, the beneficial effects last only for a short period and many patients have reported relapses after one year. Therefore, resistance remains the main problem of the EGFR TKIs therapy. Resistance can be distinguished in “primary” (de novo) or “acquired” following the exposure to targeted agents. The majority of patients develops acquired resistance, either through secondary EGFR mutations (see above) or activation of EGFR-independent pathways (for example MET up-regulation). Moreover, the acquired resistance can remain in form of resistant clones within a tumor or in different tumors of the same patients. Therefore, re-biopsies are needed during the treatment to verify the tumor progression (Chan et al. 2015). In 5-20% of cases, the amplification of MET causes the activation of PI3K-AKT-mTOR signaling despite the

inhibition of EGFR (Cheng et al. 2014). Increased MET levels therefore result in the second cause of EGFR TKI acquired resistance. Moreover, mutations in PIK3CA, HER2, BRAF, STAT3, AXL kinase, CRKL amplification have been associated with the development of resistance to the therapy (Chan et al. 2015). Unfortunately, the origin is unknown in more than 30% of resistance cases and chemotherapy remains the only treatment option (Sequist et al. 2011).

Differently from the resistance to chemotherapy, the resistance to targeted therapy can be bypassed identifying the altered pathway. It has been reported that the second-generation TKI Afatinib can overcome the T790M mutation (Miller et al. 2012). Moreover, successful results have been obtained with dual inhibition of EGFR by EGFR TKIs and Cetuximab in murine models. However, this combination showed no significant results when tested in clinical trial in 2011. The combined inhibition of MET and T790M has also shown efficacy in murine models (Xu et al. 2012). However, this combination showed toxicity in patients (ClinicalTrials.gov identifier: NCT01121575). Third-generation EGFR TKIs (such as CO-1868, AP26113, AZD9291) that specifically target T790M have shown higher selectivity and efficacy in acquired resistance along with limited toxicity. (Sequist et al. 2011) (Camidge et al. 2013). In a phase I trial, AZD9291 showed an overall response rate of 51% (91 of 177 patients with EGFR-mutant NSCLC). Moreover, the response rate of the patients with tumors harboring the EGFR T790M mutation was around 64% (89 of 132 patients). Currently AZD9291 is under

investigation in a phase III trial (NCT02151981), which includes patients with NSCLC tumors previously treated with an EGFR TKI and with the EGFR T790M resistance mutation (Janne et al. 2014).

The optimal strategy for overcoming drug resistance is to rationally choose the best drug combinations and predict if such combination can be more effective than the first-line single-agents whilst balancing toxicity and costs.

OTHER INHIBITORS

Recently, Crizotinib has been approved by FDA as the first ALK TKI, because of its ability to target the constitutively active receptor tyrosine kinases resulting from EML4-ALK and other ALK fusions (Solomon et al. 2014). Overall, Crizotinib showed significant improvements in progression-free survival (PFS) compared to the standard chemotherapy along with an adequate safety profile (Solomon et al. 2014). Currently the gold standard for ALK positive patients is Alecitinb (Vavalà et al. 2018).

Several strategies have been developed to inhibit MET/HGFR mediated growth such as HGF antagonists, anti-HGFR mAb, anti-MET mAb and MET-TKIs such as Tivantinib (ARQ197), abozantinib (XL184) and Crizotinib (Gelsomino et al. 2014). Interestingly, it has been shown that MET and EGFR have a synergic effect in blocking tumor cell proliferation.

Finally, the BRAF inhibitors Vemurafenib and Dabrafenib have been approved by the FDA as effective for the specific mutation BRAF-V600E, which is present in 40 to 50% of patients with BRAF mutations (Okimoto et al. 2014) (Forde et al. 2013). BRAF mutation is more frequently associated with melanomas or colon cancers but given that it can be present also in a small percentage of NSCLC patients, Vemurafenib is currently under phase 2 clinical trial investigation in NSCLC patients with BRAFV600 mutations, HER2 amplification and ALK/RET gene rearrangements (NCT02314481).

SCOPE OF THE THESIS

To date, resistance to the current treatments in NSCLC remains one of the main problems. To this end many combined treatments among chemotherapy and targeted therapy have been used. Until now many therapeutic targets have been discovered and associated to the development of NSCLC and the research of new targets is still ongoing. Previous data from our lab demonstrated that a novel isoform of BTK, p65BTK, is a novel oncogene highly expressed in colon carcinoma and a pivotal downstream effector of RAS. Moreover, we have shown that its inhibition affects growth and survival of colon cancer cells and sensitize drug-resistant cells to chemotherapy, thus suggesting that its targeting may be a promising

therapeutic approach. In order to establish which solid tumors, other than colon carcinoma possibly express p65BTK, we analyzed cell lines derived from different types of tumors and found a high expression of p65BTK in NSCLC. Therefore, the aim of this project is to investigate whether p65BTK could be a novel theranostic target in NSCLC and to study its role in NSCLC cell biology.

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CHAPTER 2

P65BTK IS AN EMERGING ACTIONABLE TARGET IN NON-SMALL CELL LUNG CANCER

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ABSTRACT

Background: Lung cancer is the leading cause of cancer-related death worldwide, being the most common (~80%) histotype the Non-Small Cell Lung Cancer (NSCLC). Despite significant improvements in understanding the biology of this tumor, lung cancer mortality remains elevated because of intrinsic and acquired resistance. Therefore, new strategies are urgently needed to overcome these limitations.

Methods: We studied a cohort of 382 NSCLC patients and we reported clinicopathological records smoking habits, mutational and overall survival status. In this cohort by immunohistochemistry we evaluated p65BTK expression. By MTT we evaluated the effect of p65BTK inhibition by BTK and/or EGFR inhibitors on the viability of drug-resistant NSCLC cell lines and tumor-derived primary cell lines with hyper-activation of the RAS/MAPK pathway. The effect of BTK inhibitors on the proliferation and clonogenicity of the cell lines was assessed by crystal violet and colony assay, respectively. Cell toxicity assays were performed to study the effect of the combination of non-toxic concentrations of BTK inhibitors (Ibrutinib, AVL-292, RN486) with EGFR inhibitors (Gefitinib, Erlotinib) and standard-of-care (SOC) drugs (Cisplatin, Gemcitabine, Pemetrexed).

Results: In NSCLC patients p65BTK was significantly overexpressed in Adenocarcinoma (AdC) from non-smoker patients with a wild type EGFR. Notably, these patients are not eligible for treatments with

EGFR inhibitors due to a lack of EGFR mutation. We confirmed this observation both *in vitro*, in NSCLC human cell lines mutated for KRAS or for one of the components of the RAS/MAPK pathway, and *ex vivo,* in the tumor-derived primary cell lines from *Kras/Trp53* null mice, suggesting that in NSCLC p65BTK overexpression correlates with a hyper-activated RAS/MAPK pathway. Moreover, we showed that BTK inhibitors affected viability and strongly impaired proliferation and clonogenicity of NSCLC cell lines and that the addition of non-toxic concentrations of BTK inhibitors re-sensitized drug-resistant NSCLC cell lines to both target and chemotherapy independently of the mutational status.

Conclusions: Our data indicate that p65BTK is an emerging actionable target in NSCLC and suggest that the combined treatment of BTK inhibitors and SOC chemotherapy or targeted therapy could represent a novel approach to overcome drug resistance in NSCLC.

KEYWORDS:

NSCLC; drug resistance; p65BTK; BTK Inhibitors; EFGR; EFGR inhibitors; targeted therapy; chemotherapy.

BACKGROUND

Lung cancer is the leading cause of cancer-related deaths worldwide with the highest mortality rate of 47.6 % in men (Central and Eastern Europe) and of 23.5% in women (Northern America) (Wong et al. 2017). Histologically, lung cancer can be divided into two major forms: NSCLC (85%) and Small Cell Lung Cancer (SCLC) (15%) (Jemal et al. 2008). The most prevalent histotypes of NSCLC are Adenocarcinoma (AdC), Squamous Cell Carcinoma (SCC), and Large Cell Carcinomas (LCC). Unfortunately, NSCLC is often diagnosed late, when the disease is in an advanced phase and the prognosis is poor (Herbst et al. 2008, Jones et al. 2018). In addition, despite considerable advances in its treatment - e.g. combined SOC chemotherapy, molecular targeted therapy, immunotherapy (Aldarouish et al. 2016)- lung cancer mortality continues to remain high, largely due to intrinsic and acquired resistance to therapy (Chan et al. 2015). In the evolution toward a more personalized and efficient therapeutic approach, particular emphasis is on the understanding of lung cancer biology and on the consequent identification of new actionable targets. As far as biology is concerned, NSCLC is a heterogeneous disease, where different sub-populations of cells with distinct molecular features, coexist within the same tumor (Chen et al. 2014) . The most frequent driver mutations identified in AdC and SCC are in receptors or protein kinases related to RAS/MAPK, PI3K/AKT/mTOR and JAK/STAT

pathways (Chan et al. 2015). In particular, the most commonly mutated genes are epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK), mesenchymal epithelial transition factor (MET) and KRAS. The resulting dysregulation of all of these proteins leads to hyper-activation of the MAPK pathway (Chan et al. 2015). However, while for the first three targets several inhibitors have been developed and introduced into the clinic, for the latter no clinical inhibitors exist yet. Indeed, despite KRAS mutations are the most frequent in NSCLC, KRAS represent a difficult target. It has been previously shown that these gene alterations are also implicated in mechanisms of drug sensitivity and primary/acquired resistance to kinase inhibitors (Galvani et al. 2013, Maione et al. 2015). Notably, mutations in KRAS are associated with worst prognosis and resistance to chemotherapy or EGFR inhibitors (Eberhard et al. 2005). Hence, the identification of novel actionable targets downstream of KRAS is particularly urgent in NSCLC.

Bruton tyrosine kinase (BTK) is a 77 kDa non-receptor tyrosine kinase that plays a crucial role in B-cell activation, proliferation, maturation, differentiation and survival (Mohamed et al. 2009). Notably, in the last years BTK emerged as a novel molecular target in some B-cell leukemias and lymphomas where it is found commonly overexpressed (Singh et al. 2018). Ibrutinib, the first irreversible BTK inhibitor, has been recently approved by the FDA for the treatment of certain B-cell malignancies (Roskoski 2016). This has led to a rapid

development in the field and several other BTK inhibitors, among which AVL-292, are currently in advanced phase of clinical trial for several types of leukemia.

Recently, our lab identified and characterized p65BTK, a new isoform of BTK, overexpressed in colon cancers. Notably, in this tissue only the messenger encoding p65BTK and not that for p77 is expressed. Compared to the hematopoietic-specific p77KDa isoform, p65BTK lacks the first 86 N-terminal amino acids and results from the translation of a transcript containing an alternative first exon in the 5'UTR. The translation of the p65BTK protein is hnRNPK-dependent, IRES-driven and post-transcriptionally regulated by the MAPK pathway. In addition, p65BTK is a novel and powerful oncogene acting downstream the MAPK pathway and an obligate effector of RAS-mediated transformation (Grassilli et al. 2016). Finally, p65BTK inhibition reverted drug resistance to 5-Fluorouracil in colon cancers lacking p53 (Lavitrano et al. submitted). Given the role of p65BTK in colon cancer cells, we aimed to investigate p65BTK in other solid tumors. Here, we reported that p65BTK is expressed in more than 50% of NSCLC, is significantly overexpressed in AdC (vs SCC) from non-smoker patients with a wild type EGFR, where high p65BTK expression correlate with worst survival. Using NSCLC commercial cell lines and primary tumor cells from mutated *Kras* and mutated *Kras*/*Trp53* null mice, we demonstrated that p65BTK inhibition reduced cell proliferation, survival and clonogenicity in a dose-dependent manner. Finally, we showed that the combination of BTK

inhibitors with chemotherapeutic agents (Cisplatin, Pemetrexed, Gemcitabine) or EGFR inhibitors (Gefinitib, Erlotinib) induced cytotoxicity of NSCLC cells poorly responsive to chemotherapy and targeted therapy.

MATERIALS AND METHODS

Lung Cancer Patients

A previously described series of 383 chemo- and/or radio-naïve NSCLC patients who underwent surgery for therapeutic purposes at Fondazione IRCCS Ca' Granda-Ospedale Maggiore Policlinico Hospital (Milan, Italy) between 2004 and 2010 were enrolled for this study (Del Gobbo et al. 2016). Patients' informed consent was obtained and the study was approved by the Fondazione IRCCS Ca' Granda Institutional Review Board (Institutional Review Board 179/2013; approval date: 19 March 2013). All clinical investigation has been conducted according to the principles expressed in the Declaration of Helsinki and data were analyzed anonymously. Clinicopathological records and overall survival status were available for the entire cohort. Smoking habits was available for 348 patients. Moreover, the presence of ALK rearrangements or EGFR mutations was analyzed for all cases as described (Del Gobbo et al. 2016). Patients' features are described in Additional File 1: Table S1.

Tissue Microarray (TMA) construction and immunohistochemical (IHC) staining

Representative tissue blocks of tumor and non-neoplastic lung tissue derived from each patient were used to construct TMAs, as previously described (Vaira et al. 2011). Briefly, for all lung cancer samples five

representative cores were selected by a pathologist whereas for non-neoplastic parenchyma one core was chosen. Then, 4-microns-thick sections were cut from each TMA and subjected to IHC staining for p65BTK using BN30 antibody, in a BenchMark Ultra automatic system (Ventana Medical Systems). Reactions were revealed using the UltraView Universal DAB, according to the manufacturer's instructions (Ventana Medical Systems) and all slides were counterstained with hematoxylin. As positive control we used a colon carcinoma specimen, whereas negative controls were prepared in the absence of primary antibody and included in each reaction. p65BTK cytoplasmic staining was evaluated and scored in all cases, by two pathologists independently, as percentage of positive neoplastic cells.

Anti-p65BTK antibody production and characterization

BN30 polyclonal antibodies were obtained in rabbits by immunization with the N-terminal decapeptide of p65BTK, conjugated to keyhole limpet hemocyanin via an additional C-terminal cysteine residue. Specificity of BN30 polyclonal antiserum (IgG fraction), used for IHC, was assessed by western blot analysis on lysates HCT116p53KO cells transfected with control (luc) or p65BTK-specific siRNA (Additional file 1: Figure S1a) and by immunocytochemistry, on sections from cell blocks of HCT116p53KO p65BTK197 expressing and p65BTK-silenced cells (Additional file 1: Figure S1b). Data not shown indicated the absence of cross reactivity of BN30 with commercial purified p77BTK confirming the specificity of the antibody towards p65BTK.

Cell lines, culture, and treatments

All commercial human NSCLC cell lines used were from ATCC. The mutational background of the four cell lines used for the *in vitro* experiments is reported in table 2. Mouse lung cancer primary cell lines were originally derived in Silve Vicents' lab (Vallejo et al. 2017). Upon reception, cells were expanded and frozen as seed stocks of first or second passage. All cells were passaged for a maximum of 3 or 4 weeks, after which new seed stocks were thawed for experimental use. All cells were grown at 37°C in 5% CO_2 and were maintained as a sub confluent monolayer using the following media: Dulbecco's modified eagle's medium (DMEM) for SK-LU-1 and Calu-6 supplemented with non-essential amino acids (NEAA) and 1% sodium

pyruvate; Roswell Park Memorial Institute 1640 (RPMI 1640) supplemented with 1% sodium pyruvate for NCI-H1975 and NCI-H2228. Mouse lung cancer primary cell lines were grown in DMEM. In addition, all media were also supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Media, serum, and supplements were all from Invitrogen. BTK inhibitors Ibrutinib, AVL-292, RN486 and EGFR inhibitors Erlotinib and Gefitinib (all inhibitors were purchased from Selleckchem), were dissolved in DMSO and stored in aliquots at -80°C. Chemotherapeutic drugs Cisplatin, Pemetrexed and Gemcitabine were kindly provided by S. Gerardo Hospital (Monza).

Western blot analysis

Protein extracts were prepared using high-salt lysis buffer (50 mM Hepes (pH 7.5), 500 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.1% NP-40) supplemented with 1% protease inhibitor cocktail (Sigma-Aldrich). 20 µg of cell lysates were separated on 10% tris-glycine Wedge-wells gels (Invitrogen), transferred onto a nitrocellulose membrane (Invitrogen) and incubated with the following antibodies: anti-p65BTK BN49 (Grassilli et al. 2016); anti-Actin (A1978, Sigma-Aldrich). Images were acquired using G:BOX XT4 Chemiluminescence and Fluorescence Imaging System (Syngene) and processed with Adobe Photoshop.

Cell proliferation/viability assay

Cells were seeded in 96-well plate at 70% confluency in octuplicates for overnight attachment. For survival curves, cells were treated with the different concentrations of inhibitors (day 0) and cell number was evaluated after 72 hours using an MTT-based assay (Sigma-Aldrich) according to the manufacturer's instructions. For growth curves, 3,000 cells were seeded and their number was evaluated at 0, 24, 48 and 72 hours by crystal violet staining. Briefly, after washing with PBS, cells were fixed with Formalin 10% (Bio-Optica) for 1 hour on the shaker and then stained with a crystal violet solution (Sigma-Aldrich) in 35% ethanol (Sigma-Aldrich) for 20 minutes at room temperature. After washing extensively with tap water, color was extracted by adding 0.1 M acetic acid and quantified by spectrophotometer at 595 nm. Graphs represent the average of 3 to 5 independent experiments. Average ± s.e.m. are plotted in the graphs.

Cell toxicity assay

Cells were seeded in octuplicates at 70% confluency and the next morning treated or not with drugs and inhibitors and their combination as indicated in the figures. Cell viability was evaluated by CellTiter-Glo® Luminescent Cell Viability Assay (Promega)

following manufacturer's instructions. Graphs represent the average of 3 to 5 independent experiments. Average ± s.e.m. are plotted in the graphs.

Colony forming assay

Cells were seeded at low density (1000 cells/well in 6-well plate) in triplicate and left untreated or treated with different concentrations of Ibrutinib, AVL-292 and RN486. Medium (alone or supplemented with the inhibitors) was replaced every 3 days. After 10 days, colonies were fixed and stained with 1% crystal violet in 35% ethanol. Images were acquired using G:BOX XT4 Chemiluminescence and Fluorescence Imaging System (Syngene, Cambridge, UK) and processed with Adobe Photoshop. Colony assays were repeated 3 times.

Immunofluorescence staining

NSCLC cell lines were seeded at a density of 10×10^5 cells/well on glass slides pretreated with Polylysine (Sigma) and grown for 2 days. After treatments cells were washed with PBS and fixed for 10 minutes in 1.6% Paraformaldehyde (Sigma-Aldrich) and washed again with PBS. The slides were then permeabilized with ice-methanol at -

80°C overnight. The day after, slides were incubated with anti-pBTK tyr551 (1:100 in 3% BSA in PBS; Bioss) for 1 hour at room temperature and then washed 3 times with PBS. Then, the slides were incubated with secondary antibodies diluted 1:2000 in 3% BSA in PBS for 40 minutes at room temperature, washed 3 times with PBS and evaluated using a fluorescence microscope (Zeiss, Germany). Nuclei were counterstained with DAPI (Sigma-Aldrich).

Statistical analysis

The experimental data were analyzed using Microsoft Excel and expressed as the mean ± s.e.m. of 3 to 5 experiments. The statistical significance of differences was determined by Student's T-test with a p-value of 0.05 or less considered as significant.

RESULTS

p65BTK is overexpressed in wild type EGFR NSCLC patients and in KRAS-mutated NSCLC cell lines and tumors

Using the isoform-specific polyclonal antibody that we previously developed and characterized (Grassilli et al. 2016) we examined p65BTK expression in cancer tissues derived from a cohort of 383 NSCLC patients. 382 NSCLC cases out of 383 (99.7%) were

available for immunohistochemical analysis (Additional file 2: Table S1).

We found that p65BTK was expressed in 51 % of cases, being significantly ($p<0.0001$) more expressed in AdC vs SCC (Fig. 1, panels a and b, Table 1).

p65BTK expression	***All (382)***	***AdC (293)***	***SCC (89)***	***Smokers (283)***	***Non-smokers (65)***
Negative	***189 (49.3%)***	***129 (43.9%)***	***60 (67.4%)***	***151 (53.3%)***	***18 (27.7%)***
1-10% positive cells	*131 (34.2%)*	*106 (36%)*	*25 (28.1%)*	*101 (35.7%)*	*21 (32.3%)*
11-20% positive cells	*12 (3.1%)*	*10 (3.4%)*	*2 (2.2%)*	*5 (1.8%)*	*5 (7.7%)*
21-50% positive cells	*24 (6.3%)*	*23 (7.8%)*	*1 (1.1%)*	*15 (5.3%)*	*8 (12.1%)*
51-100% positive cells	*26 (6.8%)*	*25 (8.5%)*	*1 (1.1%)*	*11 (3.9%)*	*13 (20%)*
Positive	***193 (50.4%)***	***164 (55.8%)***	***29 (32.6%)***	***132 (46.7%)***	***47 (72.3%)***

Table 1. p65BTK score of the NSCLC patients' tissue analyzed by IHC. The analysis was performed on TMA using the antibody BN30, produced and characterized in the lab (Additional file 1: Figure S1). AdC: adenocarcinoma, SCC: squamous cell carcinoma.

We also observed that p65BTK expression correlated with smoke habits. In fact, we found that p65BTK was significantly more expressed in non-smoker than in smoker AdC patients ($p<0.0001$; Fig.

1c). In details, the difference was significant only in patients bearing wt EGFR ($p<0.0001$), whereas in patients with mutated EGFR the expression level of p65BTK was very low in both groups, with no difference between smoker and non-smoker patients (Fig. 1d). Finally, survival analysis indicated that p65BTK expression correlated with a poor prognosis for non-smoker patients (Fig. 1e).

Then, using an isoform-specific polyclonal antibody previously developed and characterized (Grassilli et al. 2016) we confirmed that p65BTK was abundantly expressed in several commercial human NSCLC cell lines bearing a mutation in KRAS or in the RAS/MAPK pathway (Fig. 2a).

As already shown for colon cancer, also in lung cancer cell lines only p65BTK-encoding transcript, but not that coding for p77BTK, was expressed (Additional file 3: Table S2). Moreover, we found also that p65BTK was overexpressed in primary cell lines derived from tumors spontaneously arising in *KrasLSL-G12D* mice, a genetically engineered mouse model of *Kras*-driven lung cancer (Fig. 2b). These data suggest that p65BTK levels are dependent on RAS/MAPK hyper-activation, in line with what we already reported for colon cancer (Grassilli et al. 2016). Interestingly, p65BTK levels were even higher in cell lines derived from tumors from *Kras LSL-G12D;Trp53* null mice, a model of *Trp53*-null *Kras*-mutated aggressive lung AdC (Kim et al. 2005) (Fig. 2 panels b and c).

Overall, these data indicate that p65BTK is widely expressed in a sub-population of AdC non-smoker patients with wild type EGFR, and that its expression correlates with a worst prognosis, likely depend on RAS/MAPK hyper-activation.

p65BTK targeting affects cell viability of NSCLC cell lines and tumor-derived primary cells scarcely responsive to EGFR inhibition

To investigate whether p65BTK could be a novel target in NSCLC, we first evaluated whether p65BTK was constitutively activated in NSCLC cell lines with different mutations along the EGFR/RAS/MAPK pathway (Table 2).

Cell line	***Hystotype***	***Mutational status***			
		TP53	***EGFR***	***KRAS***	***other mutations/ epigenetic alterations***
Calu-6	*anaplastic carcinoma*	*mut*	*wt*	*Q61K*	*JAK1, BRCA1, p16INK4A methylation*
SK-Lu-1	*adenocarcinoma*	*mut*	*wt*	*G12D*	*KIT, CSF3R, FLT4*
NCI-H1975	*adenocarcinoma*	*mut*	*L858R+ T790M*	*wt*	*PDGFRA, PIK3CA*
NCI-H2228	*adenocarcinoma*	*mut*	*wt*	*wt*	*ALK transl, PDGFRA, RB*

Table 2. Known genetic alterations characterizing the different NSCLC lines used in the paper. Information about genetic defects

were retrieved from the database of the Wellcome Trust Sanger Institute Catalogue Of Somatic Mutations In Cancer COSMIC, http://www.sanger.ac.uk/genetics/CGP/cosmic).

To this end, we checked the phosphorylation of Y465, the tyrosine residue in p65BTK corresponding to Y551 in p77BTK, whose phosphorylation reflects its activation (Nisitani et al. 1999). Results indicated that in all the cell lines expressing high levels of p65BTK the kinase was constitutively active (Additional file 4: Figure S2, panel a) and its activation was turned off by BTK inhibitors (Additional file 4: Figure S2, panel b). We also tested the effects of EGFR inhibition in the same cell lines by treating them with increasing concentrations of the two specific EGFR inhibitors currently used in therapy, Erlotinib and Gefitinib (Fig. 3a). After 72 hours, only a dose-dependent reduction of cell number but no cytotoxic effects were observed. The same results were confirmed in tumor-derived primary cell lines from *KrasLSL-G12D* and *KrasLSL-G12D;Trp53* null mice (Fig. 3b). Next, we tested the effect of BTK inhibitors in the same cell lines and we found that p65BTK inhibition resulted in a stronger anti-proliferative effect than the treatment with EGFR inhibitors: significant reduction in cell number was observed even at the lower doses, and in the case of the highest concentration of RN486 the effect was cytotoxic in all cell lines (Fig. 4 panels a and b).

These results indicate that cell lines bearing mutations along the EGFR/RAS/MAPK pathway are very sensitive to p65BTK inhibition.

The inhibition of p65BTK strongly impairs proliferation and clonogenicity of NSCLC cell lines

Given the significant reduction in cell number obtained with BTK inhibitors, we then investigated the effects of p65BTK inhibition on cell proliferation and clonogenicity of NSCLC cell lines. First, we performed growth curves of NSCLC cell lines in the presence of increasing concentrations of p65BTK inhibitors and observed that p65BTK inhibition strongly impaired proliferation of all NSCLC cell lines. As shown in Fig. 5a, in all the cell lines analyzed 10 μM Ibrutinib caused a slight to moderate decrease of proliferation whereas a strong proliferation decrease was observed only at the highest dose of Ibrutinib 20 μM. AVL-292 and RN486 instead strongly decreased cell proliferation when used at concentrations as low as 5 μM (Fig. 5a).

Moreover, we investigated whether p65BTK inhibition could affect the clonogenicity of NSCLC cell lines by evaluating clonogenic growth in the presence of increasing concentrations of p65BTK inhibitors. Our results indicated that Ibrutinib was able to reduce colony formation only at the highest dose of Ibrutinib (20 μM) whereas AVL-292 and RN486 were efficient already at lower concentration (5 μM) (Fig. 5b). In particular, NCI-H1975 and NCI-H2228 cell lines, bearing a double mutation in the EGFR and an ALK translocation, respectively, resulted more sensitive to all the inhibitors tested (Fig. 5b).

All together these data show that p65BTK inhibition impairs proliferation and clonogenicity of NSCLC cell lines, being AVL-292 and RN486 effective at low concentration.

p65BTK inhibition sensitizes NSCLC cell lines scarcely responsive to target therapy and chemotherapy independently of the EGFR and KRAS status

Having studied the effect of BTK inhibitors on NSCLC cell proliferation, next we tested whether the addition of Ibrutinib, AVL-292 and RN486 could sensitize cells to EGFR inhibitors (Fig. 6). We showed that the combination of 20 μM Ibrutinib and 20 μM Gefitinib was highly cytotoxic for all the NSCLC cell lines scarcely responsive to EGFR inhibition. At variance, the combination of 20 μM Ibrutinib with 20 μM Erlotinib was cytotoxic only for NCI-H1975 cells (which bear a double mutation in the EGFR). The combination of 10 μM AVL-292 with 20 μM Gefitinib was cytotoxic in Sk-Lu-1 and Calu-6 cells, cytostatic in NCI-H1975 cells and ineffective in NCI-H2228 cell. On the contrary, the combination of 10 μM AVL-292 and 20 μM Erlotinib was mildly cytotoxic only in the NCI-H1975 cells. 10 μM RN486 either in combination with 20 μM Gefitinib or combined with 20 μM Erlotinib was cytotoxic in all the cell lines.

We then studied the effect of combining p65BTK inhibitors with SOC drugs currently used in the clinic such Cisplatin, Pemetrexed and Gemcitabine. As shown in Fig. 7, the combination of

RN486 with any of the SOC drugs dramatically reverted the chemo-resistance in all the cell lines analyzed. The co-treatment Ibrutinib/Cisplatin was cytotoxic only in Sk-Lu-1 and NCI-H1975 cells whereas had a cytostatic effect in Calu-6 and NCI-H2228 cells. The combinations of Ibrutinib with Pemetrexed or Gemcitabine were ineffective. Finally, the combination of AVL-292 with SOC drugs had a stronger anti-proliferative effect than each of the drugs administered alone in all cell lines.

Overall, the inhibition of p65BTK in combination with the EGFR target therapy or chemotherapeutic drugs is effective in sensitizing NSCLC cells scarcely responsive to the current treatments. However, different inhibitors show or not synergy depending on which EGFR inhibitor or chemotherapeutic drug they are combined with.

DISCUSSION

In the last decade, significant advances at the molecular level have afforded an improved understanding of the underlying pathology and significant heterogeneity of NSCLC. Multiple signaling pathways have now been identified as well as specific oncogenic driver mutations that lead to malignant transformations. Indeed, a number of clinical series has been profiled for the identification of key actionable alterations (Chan et al. 2015). Despite the continuous discoveries in cancer treatment, the problem of the intrinsic or

acquired resistance is still unsolved. Therefore, it is of uttermost importance to find new molecular targets involved in drug resistance.

In a screening to identify actionable targets supporting drug resistance in colon cancer, we previously demonstrated a functional role of non-receptor tyrosine kinase BTK. BTK has been for long time considered only expressed in cells derived from the hematopoietic cell lineage, where it is crucial for B cell maturation and proliferation (Mohamed et al. 2009). In the last decade BTK has also been reported as new molecular target for the treatment of lymphoproliferative disorders and Ibrutinib, the first specific BTK inhibitor, has been approved in 2014 for the treatment of mantle cell lymphoma and chronic lymphocytic leukemia. Moreover, other BTK inhibitors, such as AVL-292, are in clinical trials for hematological malignancies and autoimmune diseases (Novero et al. 2014). Lately, the application of BTK inhibitors has been explored also in solid tumors, given their established effect on multiple kinases involved in cancer growth such as the EGFR family members (Campbell et al. 2018). Among the solid tumors in which BTK inhibitors have shown promising results, there are lung cancer(Young et al. 2009, Singh et al. 2010), breast cancer, gastro-esophageal cancer, pancreatic cancer, ovarian cancer, prostate cancer, renal cell carcinomas and glioma (Molina-Cerrillo et al. 2017, Wang et al. 2017, Yue et al. 2017). Recently we demonstrated that a new isoform of BTK, p65BTK, but not the already known 77kDa isoform, is highly expressed in colon cancer cells and tissues. We also demonstrated that BTK is an

oncogene, being its transforming activity carried out by the p65, but not the p77 isoform. p65BTK acts downstream of the RAS/MAPK pathway. In fact, p65BTK transforming activity depends on active signal-regulated protein kinases-1/2 (ERK1/2) and on RAS transforming activity. Accordingly, p65BTK overexpression in colon cancer tissues correlates with ERK1/2 activation. Finally, we showed that p65BTK inhibition affects cell growth and survival of colon cancer cells (Grassilli et al. 2016). Here, we reported that p65BTK, but not p77BTK, is also highly expressed in NSCLC cells and tumors similarly to what reported in colon cancers. Our data indicate p65BTK as: i) a prognostic marker in a sub-population of AdC non-smoker patients with wt EGFR, where its expression correlates with a worst prognosis (Fig.1, panel e); and ii) an emerging actionable target in NSCLC cells resistant to chemotherapy and scarcely responsive to target therapy. First, we found p65BTK expression in more than half of the NSCLC patients analyzed (n=382) (Table 1). More interestingly, p65BTK is significantly over-expressed in AdC from non-smoker patients with a wt EGFR (Fig. 1, panels b-d). Notably, these patients are not eligible for treatments with EGFR inhibitors due to a lack of EGFR mutation (Chan et al. 2015, Tan et al. 2018). Then, we confirmed this observation both *in vitro*, finding that p65BTK is overexpressed in NSCLC human cell lines mutated for RAS or for one of the components of the RAS/MAPK pathway, and *ex vivo,* in primary cell lines from spontaneously occurring tumors of mutant *Kras/Trp53* null mice (Fig. 2). These finding suggest that p65BTK over-expression correlates with a hyper-activated RAS/MAPK pathway also

in NSCLC. This is particular important given that activating KRAS mutations are found in 15–30% of all patients with NSCLC, predicting poor outcome in response to conventional treatment regimens (Mascaux et al. 2005, Douillard et al. 2010) and that there are still no effective targeted therapies, despite the fact that RAS signaling has been studied for over 25 years (Young et al. 2009). Furthermore, at least one BTK inhibitor is already FDA-approved and several other are in advanced phases of clinical trials (Roskoski 2016).

Based on these observations, we investigated the effect of p65BTK inhibition on a subset of NSCLC cell lines with a mutation in the RAS/MAPK pathway other than in the EGFR (SK-Lu-1, Calu-6, NCI-H2228). As a control, we selected a cell line scarcely sensitive to EGFR inhibition due to a double mutation in the receptor (NCI-H1975). We confirmed that all these cell lines are scarcely sensitive to EGFR-inhibition: in fact, we showed a dose-dependent anti-proliferative but not a cytotoxic effect, when blocking EGFR phosphorylation with Erlotinib and Gefitinib (Fig. 3). Interestingly, a stronger dose-dependent anti-proliferative effect was observed when we tested different p65BTK inhibitors. Ibrutinib and AVL-292 are two irreversible inhibitors that act by targeting the same critical Cys481 residue in the kinase domain (Singh et al. 2010), a residue conserved also in the EGFR family members. On the contrary, RN486 is a reversible allosteric inhibitor, whose mechanism of action is via interaction with K430 (Liang et al. 2018), a residue critical for kinase activity (Liang et al. 2018). It has been reported that Ibrutinib is able

to arrest the cell cycle and induce apoptosis in EGFR-mutated NSCLC cells (Gao et al. 2014, Wu et al. 2015, Wang et al. 2016). In these cells, no detectable expression of BTK was shown, attributing the therapeutic effect only to the inhibition of mutant, but not wild type, EGFR. These Authors could not detect BTK expression in NSCLC cells since they used a commercial antibody raised against the 77kDa isoform. Using our homemade antibody, specifically reacting with p65 but not p77BTK (Grassilli et al. 2016) we demonstrated high levels of expression of p65BTK in both, mutated and non-mutated, EGFR NSCLC cell lines and we excluded p77BTK expression due to the lack of expression of the mRNA encoding for it (Additional file 3: Table S2). Moreover, cell number was significantly reduced in both mutated and wt EGFR NSCLC cell lines when treated with the irreversible inhibitors Ibrutinib and AVL-292, whereas cytotoxicity was triggered by high doses of the reversible inhibitor RN486 (Fig. 4). Therefore, we showed that the biological effect of p65BTK inhibition is independent of the EGFR mutational status in NSCLC cells. In addition, all the BTK inhibitors have an anti-proliferative activity on all the cell lines bearing a defect on the EGFR/RAS/MAPK pathway, regardless of other molecular characteristics (Fig. 4) and of the intrinsic variability of growth rates. Data from proliferation and clonogenic assays (Fig. 5) clearly indicate that Ibrutinib significantly decreased proliferation in all the NSCLC cell lines examined and that lower concentrations of AVL-292 and RN486 showed even a more powerful effect. Notably, the most effective drug is RN486 that was cytotoxic at the highest concentration. A possible explanation might

involve differences in the ADME of the different inhibitors. For example, it is known that Ibrutinib is metabolized and inactivated by the two isoforms of the CYP3 detoxifying enzyme 3A4 and 3A5 (Scheers et al. 2015), both of them overexpressed in NSCLC (Lolodi et al. 2017): this might reduce the quantity of Ibrutinib molecules effectively inhibiting p65BTK.

Having demonstrated the strong anti-proliferative effect of BTK inhibitors in NSCLC cell lines, we then tested the potential benefit of these compounds in combination with targeted therapy and chemotherapy. First, we showed that in most cases the combined treatment of EGFR and BTK inhibitors sensitized wt EGFR-bearing NSCLC cell lines to EGFR-targeted therapy (Fig. 6). Importantly, in all cell lines where the treatment with Gefitinib only reduced cell proliferation, the addition of Ibrutinib was very effective in inducing high levels of cytotoxicity, thus excluding that the action of Ibrutinib might be due to its binding to EGFR. Instead, the synergistic effect of such combination may be explained by the fact that the inhibitors act at different levels on the pathway downstream the EGFR, given that p65BTK acts downstream of the RAS/MAPK cascade (Grassilli et al. 2016). Hence, the effect of one inhibitor is likely enhanced by the other drug.

Cisplatin, Pemetrexed and Gemcitabine are the first-line chemotherapeutic agents used in lung cancer therapy but unfortunately resistance to their action usually arise very quickly. Given the sensitizing effect of BTK inhibitors to target therapy we

aimed to verify if the addition of BTK inhibitors could be useful also in the case of SOC chemotherapy. In particular, we showed a limited effect of Ibrutinib and AVL-292 in re-sensitizing NSCLC cell lines to chemotherapy whereas the addition of RN486 to all of the three chemotherapeutics always induced high levels of cytotoxicity (Fig. 7) confirming it as a very powerful inhibitor.

In summary, we showed that p65BTK is a prognostic marker; in fact, its expression is significantly higher in NSCLC AdC tissue from non-smoker patients' bearing a wt EGFR and correlate with a worst prognosis. We also demonstrated in an *in vitro* system the beneficial effect of BTK inhibitors in NSCLC treatment suggesting a potential role for p65BTK as theranostic marker. In fact, the addition of BTK inhibitors to EGFR-targeted therapy or SOC chemotherapy is effective in re-sensitizing NSCLC cells with defects in the RAS/MAPK pathway and scarcely responsive to the current treatments. Due to the different efficacy of the diverse inhibitors further studies are needed to identify criteria useful to select the right inhibitor/combination of drugs effective in a given setting.

CONCLUSIONS

Our results indicate that p65BTK inhibition might be an effective strategy for overcoming the resistance of NSCLC to chemotherapy and targeted therapy. Moreover, p65BTK resulted a

prognostic marker for AdC NSCLC patients EGFR wt and might be a novel theranostic marker in NSCLC.

ABBREVIATIONS

NSCLC: Non–Small Cell lung Cancer; **SCLC:** Small Cell Lung Cancer; **AdC:** Adenocarcinoma; **SCC:** Squamous Cell Carcinoma, **LCC:** Large Cell Carcinomas; **EGFR:** epidermal growth factor receptor; **ALK:** anaplastic lymphoma kinase; **MET:** mesenchymal epithelial transition factor; **BTK:** Bruton tyrosine kinase; **WT:** wild type.

DECLARATIONS:

Funding

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Author's contributions

FG wrote the manuscript and with VV, designed, performed experiments and analyzed data; SaB, JG, FB, AC, LI, IF performed experiments and analyzed data; MGC and RG analyzed data; GF production and characterization of BN49 and BN30 antibodies; SV provided tumor-derived primary cell lines from *Kras/Trp53* null mice; ET provided critical revision of the manuscript; SiB provided clinical samples, analyzed data and provided critical revision of the manuscript; EG conceived the research, supervised the research and provided critical revision of the manuscript. ML conceived the research, coordinated the research activity, and provided critical revision of the manuscript.

Ethics approval and consent to participate

All the samples were collected according to the national and international legislation. Patients' informed consent was obtained and the study was approved by the Fondazione IRCCS Ca' Granda Institutional Review Board (Institutional Review Board 179/2013; approval date: 19 March 2013). All clinical investigation has been conducted according to the principles expressed in the Declaration of Helsinki and data were analyzed anonymously according to the regulations.

Consent for publication

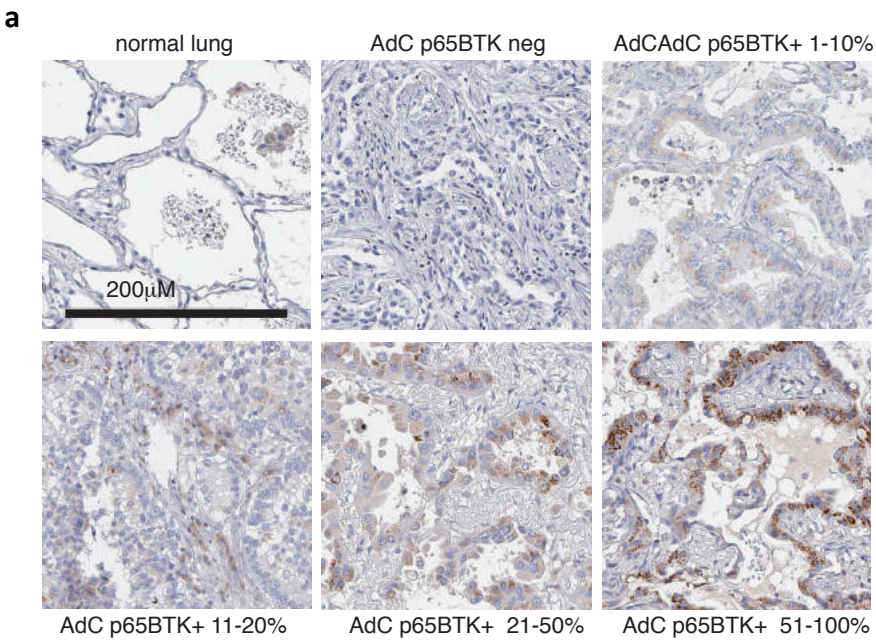
The manuscript has not been published previously and is not being considered concurrently by another publication. Moreover, we declare under our responsibility that all the authors and acknowledged contributors have read and approved the manuscript.

Competing interests

The authors declare that they have no financial and non-financial competing interests.

FIGURES AND FIGURE LEGENDS

Figure 1



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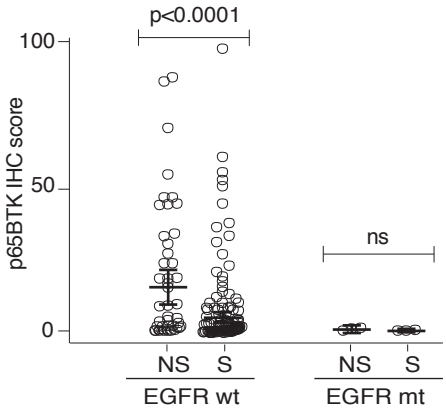

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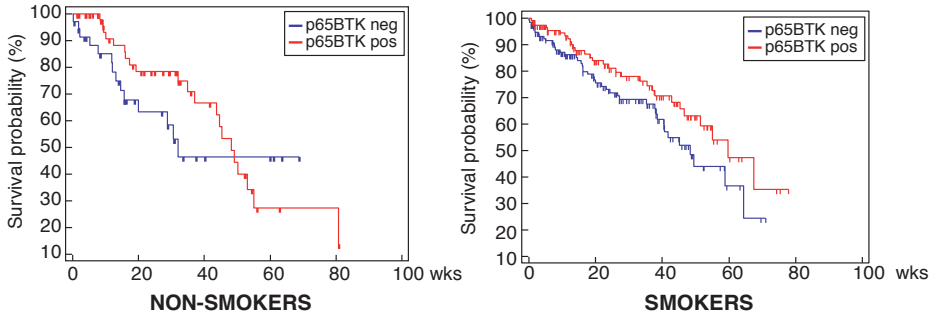
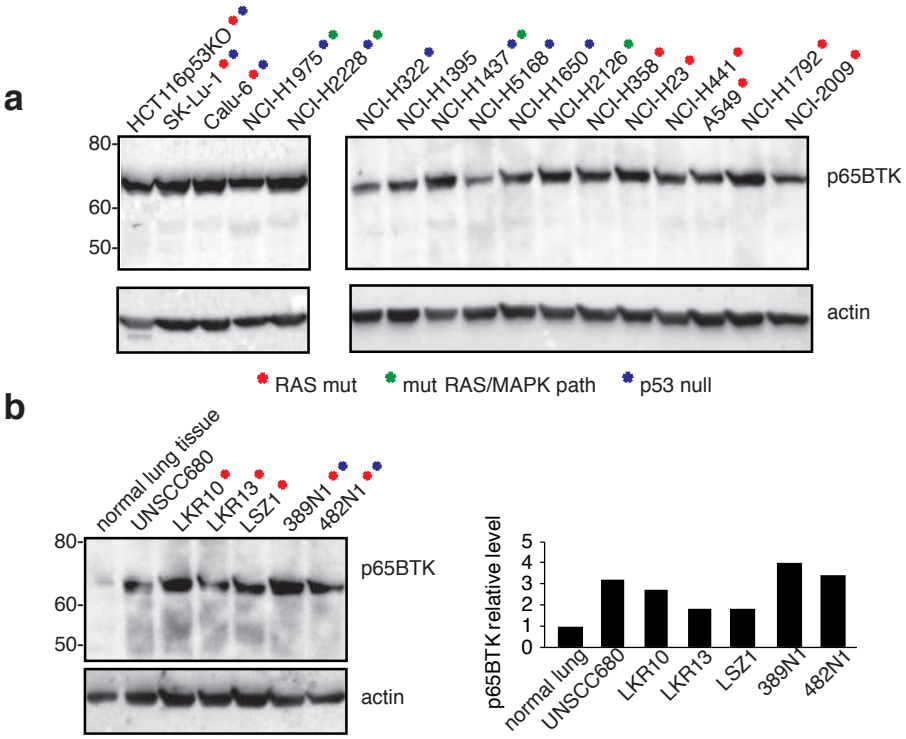


Figure 1. p65BTK expression correlates with histotype, smoke habit and wtEGFR in NSCLC.

a IHC analysis of p65BTK in lung cancer tissue samples from a cohort of NSCLC patients using the BN 30 antibody. The percentage of positive cells is indicated in the panel. Representative images are shown. **b** Quantitative analysis of p65BTK expression in SCC and AdC patients. **c** Quantitative analysis of p65BTK expression in smoker and non-smoker patients. **d** Quantitative analysis of p65BTK expression in smoker and non-smoker patients with either wt or mutated (mt)

EGFR. ns = not significant**. e** Kaplan Meier curve for the duration of survival in non-smoker p65BTK negative group (blue line) and p65BTK positive group (red line) versus smoker patients. Although not statistically significant, there is a strong correlation between p65BTK positivity and worse prognosis in non-smokers patents.

Figure 2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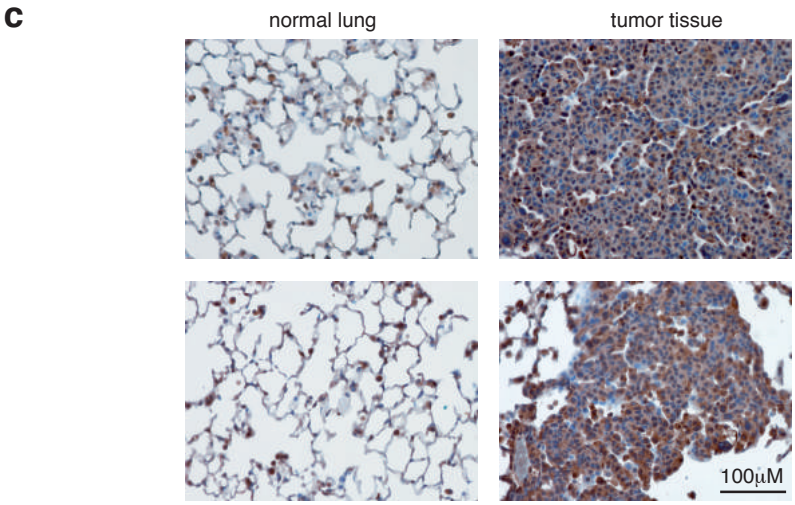
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normal lung
tumor tissue
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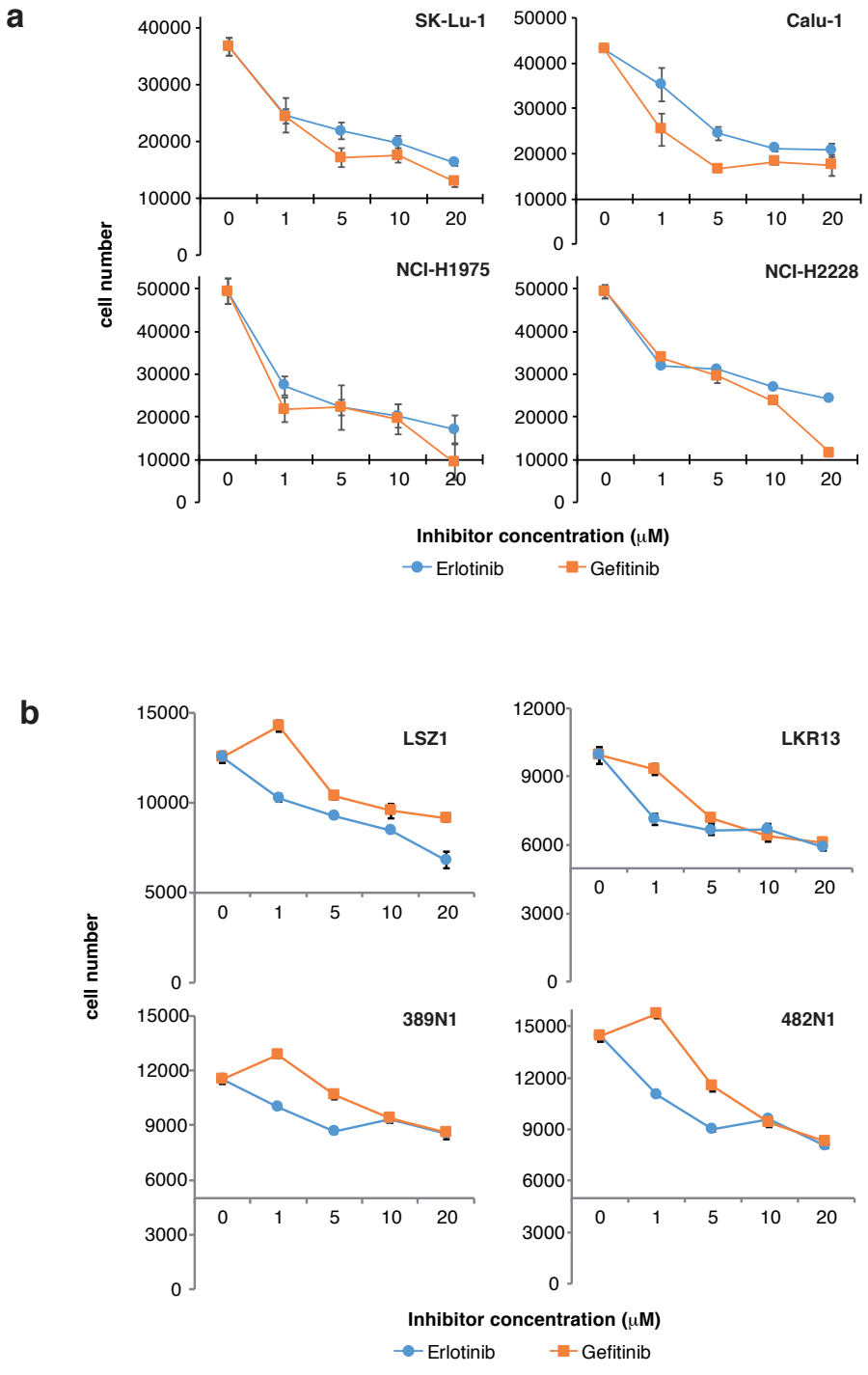
Figure 2. p65BTK is overexpressed in KRAS-mutated NSCLC cell lines and mouse tumors.

a Western Blot analysis of p65BTK expression in NSCLC human cell lines with different mutations along the RAS/MAPK pathway and in p53. **b** Western Blot analysis of p65BTK expression in primary lung cancer cells derived from KrasLSL-G12D and KrasLSL-G12D;Trp53 null mice. On the right: fold change of p65BTK protein expression normalized to beta actin. In **a** and **b** p65BTK was detected by BN49 antibody (Grassilli et al. 2016) and beta actin was used as a loading control. **c** IHC analysis of p65BTK in normal and tumoral lung tissue samples from KrasLSLG12D; Trp53f/f mice using BN30 antibody. Representative images are shown.

Figure 3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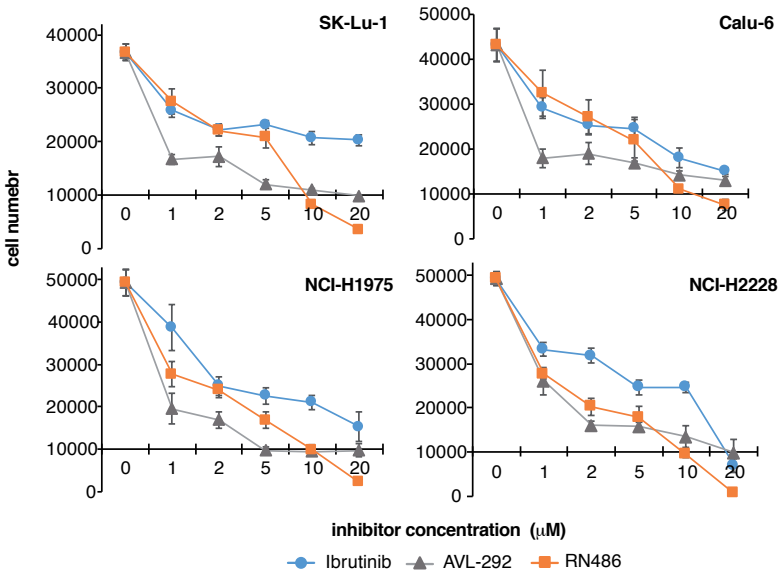
Inhibitor concentration (μM)
Erlotinib
Gefitinib
b
LSZ1
LKR13
389N1
482N1
cell number
Inhibitor concentration (μM)
Erlotinib
Gefitinib

Figure 3. EGFR inhibition does not affect cell viability of NSCLC cell lines and tumor-derived primary cells with mutations along the EGFR/RAS/MAPK pathway.

Dose-response curves of **a** human NSCLC cell lines (SK-Lu1, Calu-6, NCI-H1975 and NCI-H2228) and **b** primary lung cancer cells derived from KrasLSL-G12D and KrasLSL-G12D;Trp53 null mice (LSZ1, LKR13, 389N1 and 482N1) treated with increasing concentrations of EGFR inhibitors (Erlotinib and Gefitinib). Cell viability was evaluated by MTT assay. X-axis crosses in correspondence of T0 values (before starting the treatment); 72 hours values are then expressed as the variation relative to the initial cell number. Scale on Y-axis is adapted to the different growth rates shown by each cell line. Data are presented as mean ± SEM. n ≥ 3 independent experiments**.**

Figure 4

a


SK-Lu-1
Calu-6
NCI-H1975
NCI-H2228
cell numebr
inhibitor concentration (μM)
Ibrutinib
AVL-292
RN486

b

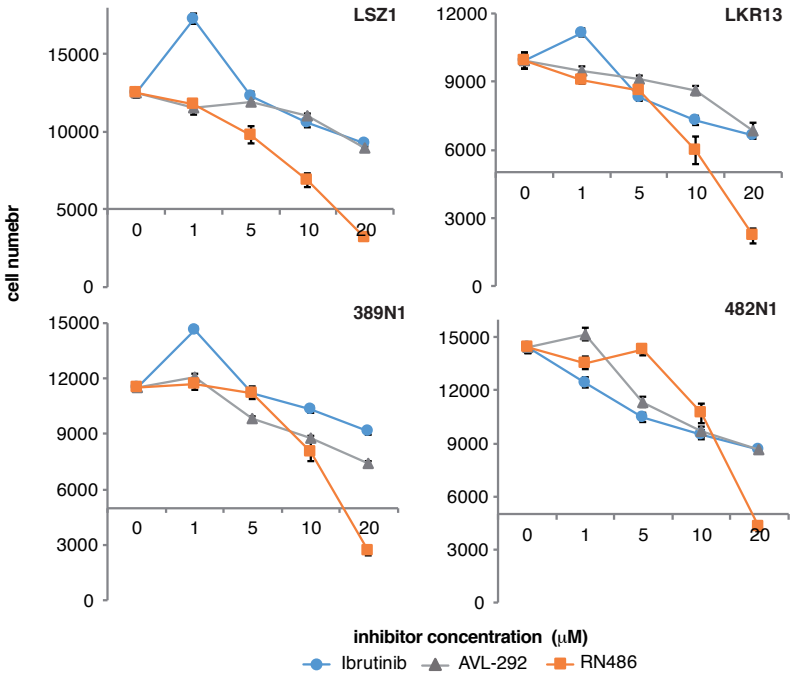
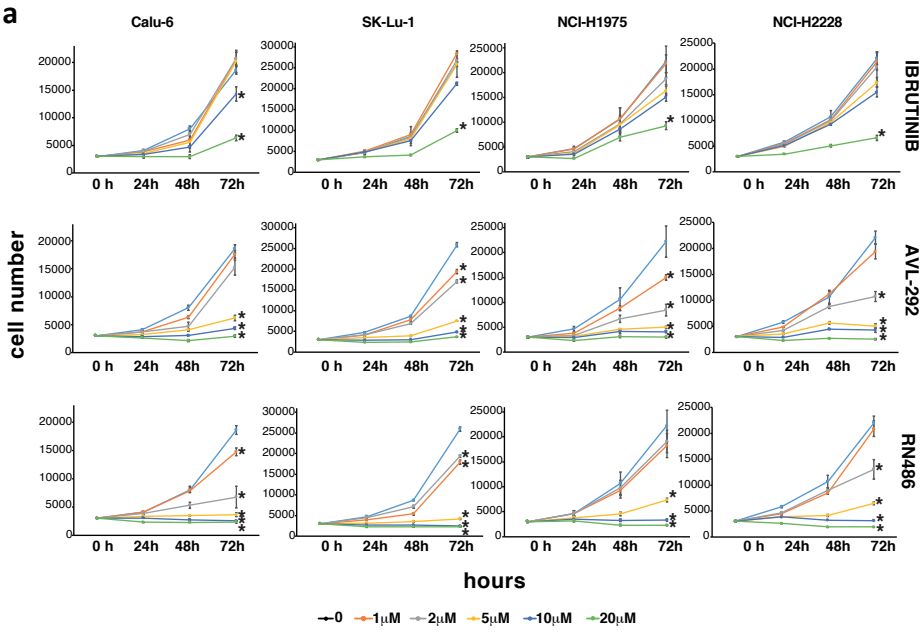

LSZ1
LKR13
389N1
482N1
cell numebr
inhibitor concentration (μM)
Ibrutinib
AVL-292
RN486

Figure 4. p65BTK targeting affects cell viability of NSCLC cell lines and tumor-derived primary cells scarcely responsive to EGFR inhibition.

Dose-response curves of **a)** human NSCLC cell lines (SK-Lu1, Calu-6, NCI-H1975 and NCI-H2228) and **b)** primary lung cancer cells derived from KrasLSL-G12D and KrasLSL-G12D;Trp53 null mice (LSZ1, LKR13, 389N1 and 482N1) treated with increasing concentrations of BTK inhibitors (Ibrutinib, AVL-292, RN486). Cell viability was evaluated by crystal violet staining. X-axis crosses in correspondence of T0 values (before starting the treatment); 72 hours values are then expressed as the variation relative to the initial cell number. Scale on Y-axis is adapted to the different growth rates shown by each cell line. Data are presented as mean ± SEM. n ≥ 3 independent experiments**.**

Figure 5


a
Calu-6
SK-Lu-1
NCI-H1975
NCI-H2228
IBRUTINIB
AVL-292
RN486
cell number
0 h
24h
48h
72h
hours
0
1μM
2μM
5μM
10μM
20μM

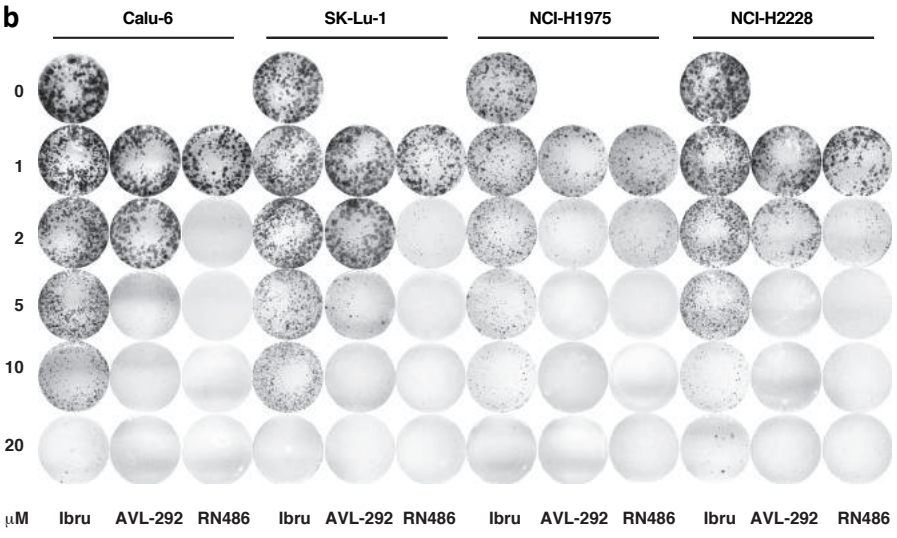

b
Calu-6
SK-Lu-1
NCI-H1975
NCI-H2228
0
1
2
5
10
20
μM
Ibru
AVL-292
RN486

Figure 5. p65BTK inhibition strongly impairs proliferation and clonogenicity of NSCLC cell lines. a Growth curves of human NSCLC cell lines treated with increasing concentrations of BTK inhibitors; cell number was evaluated each 24 hours by MTT assay. Scale on Y-axis is adapted to the different growth rates shown by each cell line. Data are presented as mean ± SEM. n ≥ 3 independent experiments**.** * indicates $p<0.05$ vs untreated**. b** Clonogenicity assay of human NSCLC cell lines treated with increasing concentrations of BTK inhibitors for 10 days. A representative image of one experiment out of 3 is shown.

Figure 6

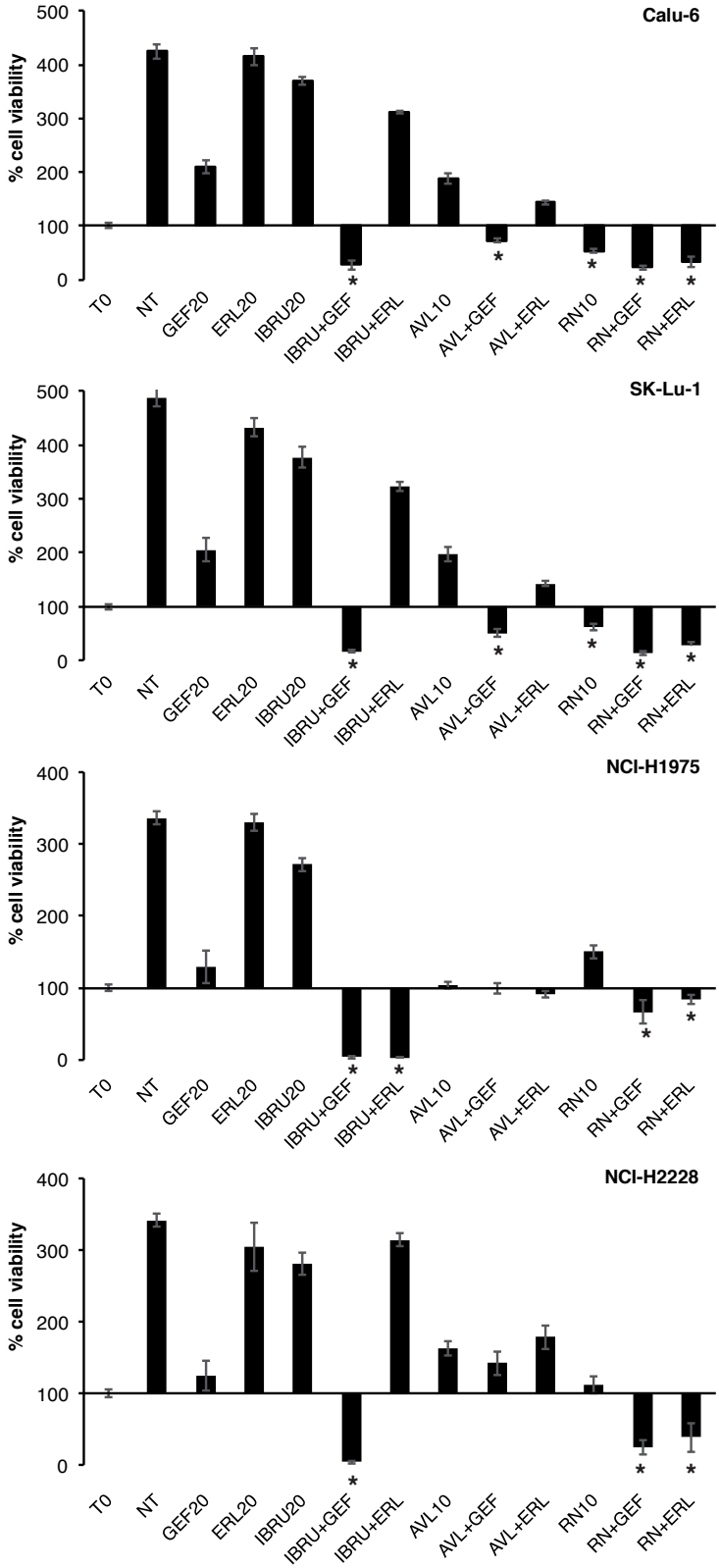

Calu-6
SK-Lu-1
NCI-H1975
NCI-H2228
% cell viability
T0
NT
GEF20
ERL20
IBRU20
IBRU+GEF
IBRU+ERL
AVL10
AVL+GEF
AVL+ERL
RN10
RN+GEF
RN+ERL

Figure 6. p65BTK inhibition sensitizes NSCLC cell lines scarcely responsive to EGFR-targeted therapy.

Cell viability of human NSCLC cell lines in response to different combinations of BTK and EGFR inhibitors (T0= time 0; NT = untreated; GEF20 = Gefitinib 20 μM; ERL20 = Erlotinib 20 μM. IBRU20 = Ibrutinib 20 μM; AVL10 = AVL-292 10 μM; RN10 = RN486 10 μM). X-axis crosses in correspondence of T0 values (before starting the treatment); 72 hours values are then expressed as the percentage variation relative to the initial cell number. Scale on Y-axis is adapted to the different growth rates shown by each cell line. Data are presented as mean ± SEM. n ≥ 3 independent experiments. * indicates $p<0.05$ vs T0 values.

Figure 7

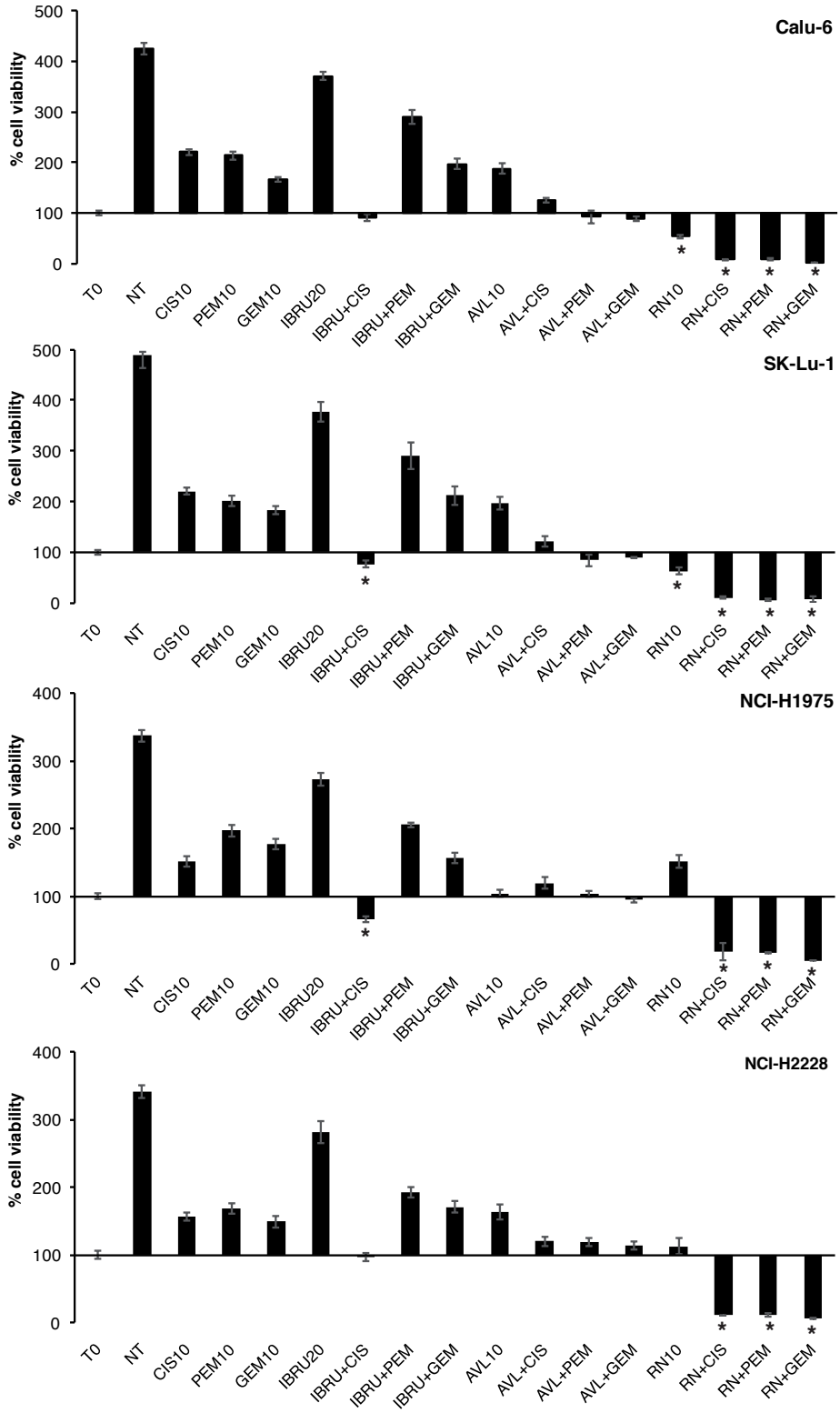
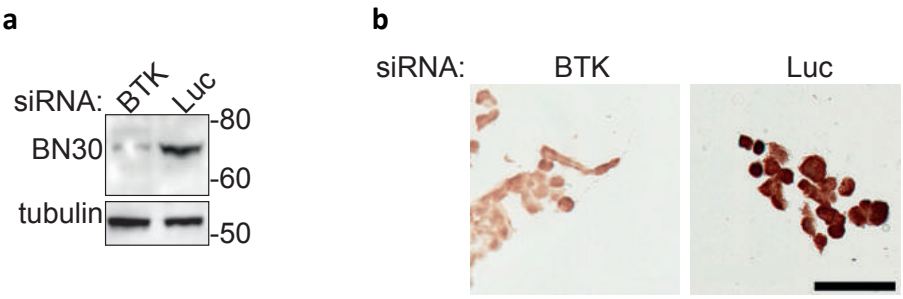

Calu-6
SK-Lu-1
NCI-H1975
NCI-H2228
% cell viability
500
400
300
200
100
0
T0
NT
CIS10
PEM10
GEM10
IBRU20
IBRU+CIS
IBRU+PEM
IBRU+GEM
AVL10
AVL+CIS
AVL+PEM
AVL+GEM
RN10
RN+CIS
RN+PEM
RN+GEM

Figure 7. p65BTK inhibition reverts resistance of NSCLC cell lines to chemotherapy.

Cell viability of human NSCLC cell lines in response to different combinations of BTK and SOC chemotherapeutic agents (T0 = time 0; NT = untreated; CIS10 = Cisplatin 10 μM; PEM10= Pemetrexed 10 μM; GEM10= Gemcitabine 10 μM; IBRU20 = Ibrutinib 20 μM; AVL10 = AVL-292 10 μM; RN10= RN486 10 μM). X-axis crosses in correspondence of T0 values (before starting the treatment); 72 hours values are then expressed as the percentage variation relative to the initial cell number. Scale on Y-axis is adapted to the different growth rates shown by each cell line. Data are presented as mean ± SEM. n ≥ 3 independent experiments. * indicates $p<0.05$ vs T0 values.

ADDITIONAL FILES

Additional file 1



Additional file 1: Figure S1.pdf

Anti-p65BTK BN30 antibody characterization. a Western blot analysis of lysates from SW480 cells harvested 48hs after transfection with control (Luc) or p65BTK-specific (BTK) siRNA and used to produce cells blocks. **b** IHC using BN30 on slides from cells blocks; bar: 50µM.

Additional file 2

	Histotype	n	Smoke (yes)	pT				pN				Grade				ALK	EGFR		OS status Censored
				1	2	3/4	x	0	1	2	x	1	2	3	na	R	L858R	Δex19	
AdC	LGT	7	6	5	1	1	-	7	-	-	-	7	-	-	-	-	-	-	7
	AdC	265	182	114	111	31	9	173	51	41	-	14	140	101	10	8	3	5	158
	LC	22	19	3	11	5	3	9	7	6	-	-	-	19	3	-	-	-	10
SCC	SCC	84	69	24	44	12	4	54	27	2	1	1	44	37	2	3	1	-	55
	AdC/SCC	5	1	2	1	2	-	2	1	2	-	-	3	2	-	-	-	-	2

Additional file 2: Table S1.pdf

Clinicopathological characteristics of NSCLC patients (n=383). LGT: Lepidic growth type; AdC: adenocarcinoma; LC, large cell AdC; SCC: squamous cell carcinoma; AdC/SCC, mixed adeno-squamous carcinoma; R, rearranged. pTx or pNx, this information could not be established.

Additional file 3

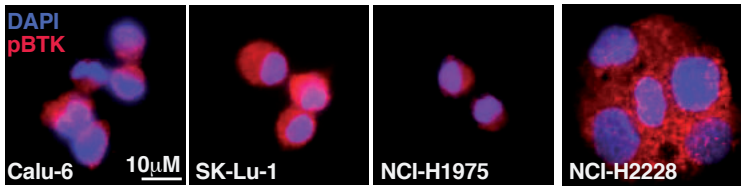
	CYCLE THRESHOLD	
	p65	**p77**
Calu-6	33 ± 0.5	undetermined
SK-Lu-1	34 ± 0.1	undetermined
NCI-H1975	34 ± 0.2	undetermined
NCI-H2228	34 ± 0.3	undetermined
A549	34 ± 0.2	undetermined

Additional file 3: Table S2. pdf

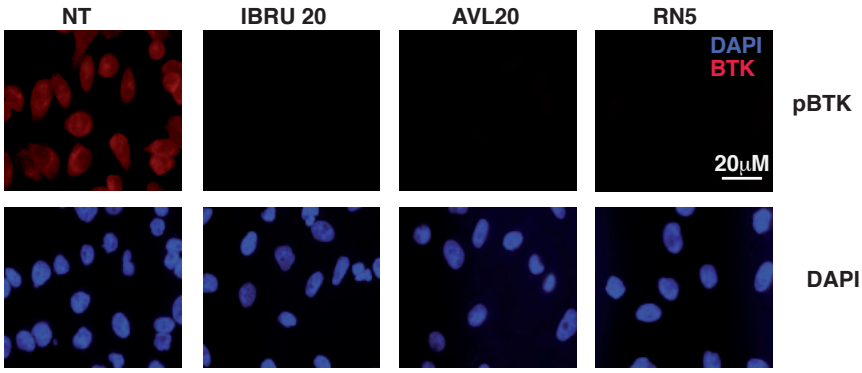
p65BTK mRNA but not p77BTK mRNA is expressed in NSCLC cell lines. mRNA expression was evaluated by RT-PCR using primers specific for each of the two isoforms (Grassilli et al. 2016).

Additional file 4

a



b



Additional file 4: Figure S2. Pdf

p65BTK is overexpressed and active in NSCLC cell lines scarcely responsive to EGFR inhibition. **a** Immunofluorescence staining of phosphorylated p65BTK (pBTK) in untreated human NSCLC cell lines. Nuclei were counterstained with DAPI. **b** Immunofluorescence staining of pBTK after 2 hours treatment of SK-Lu-1 cells with BTK inhibitors (IBRU20 = Ibrutinib 20 μM; AVL10 = AVL-292 10 μM; RN10 = RN486 10 μM).

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CHAPTER 3

CONCLUSIONS AND FUTURE PERSPECTIVES

SUMMARY

LC is one of the top five most diagnosed cancers and can be distinguished in two main types: 1) NSCLC, representing 85-90% of diagnoses and grouped in 3 subtypes: Adenocarcinoma (40%), Squamous Cell Carcinoma (25-33%) and Large Cell Carcinoma (3-10%); 2) SCLC, amounting to 15% of diagnosed LCs. NSCLC is relatively insensitive to chemotherapy and radiation therapy. Moreover, despite the use of inhibitors targeting proteins mutated in lung cancer (EGFR, ALK and MET), NSCLC mortality remains very high because of intrinsic and acquired resistance to the different therapies. Beyond traditional therapies, immunotherapy is considered as a promising approach to treat advanced NSCLC. This strategy aims to boost the own immune system to fight cancer and involves a complex interaction between various components of the innate and adaptive immune systems. Recently, a large number of studies has been conducted to evaluate the relative efficacy and safety of various immunotherapies in NSCLC, however only 20%–25% of patients have shown positive response towards this relatively new approach. Several clinical trials are exploring the use of checkpoint inhibitors for targeting important immune regulators at various stages of SLC and NSCLC, as a part combination or single treatment. As of December 2017, over 250 clinical trials are listed on www.clinicaltrials.gov for treatment of lung cancer with Pembrolizumab, Nivolumab or Atezolizumab. Nevertheless, it is

difficult to predict the beneficial effect and safety of this approach. Despite the development of immune checkpoint inhibitors and neoantigen specific vaccines for lung cancer treatment, there is still a long way ahead for obtaining desirable success in this field (Shine Raju et al. 2018). Therefore, the identification of novel biomarkers and therapeutic approaches in NSCLC is particularly urgent.

In the past years our lab identified new targets to overcome drug resistance in colon cancer; in particular, we discovered and characterized p65BTK, a novel isoform of the BTK, whose abundance correlates with grade and stage of colon cancer. p65BTK is a novel oncogene and a pivotal downstream effector of RAS and its inhibition affects growth and survival of colon cancer cells, suggesting that its targeting may be a promising therapeutic approach (Grassilli et al, 2016). Moreover, the addition of BTK inhibitors re-sensitizes p53-null drug-resistant colon cancer cells, organoids and xenografts to 5FU. The aims of my PhD project are: 1) to study the role of p65BTK in NSCLC cell biology; 2) to verify whether p65BTK might be a novel theranostic target in NSCLC.

The results presented in this thesis show that p65BTK is highly expressed in NSCLC tissues from a cohort of patients and that its expression correlates with histotype, smoke habit and wt EGFR. In particular, p65BTK is significantly more expressed in adenocarcinoma tissue from non-smoker patients bearing wild type vs mutant EGFR.

In order to determine the effect of p65BTK inhibition on viability, proliferation and clonogenicity of NSCLC cells, survival curves, growth curves and colony formation assays in presence of increasing concentrations of different BTK inhibitors (Ibrutinib, AVL-292, RN-486) have been performed. The effect of p65BTK inhibition has been tested on commercial NSCLC human cell lines mutated for RAS or for one of the effectors of the RAS/MAPK pathway and on primary cell lines derived from spontaneously occurring tumors from mutKRAS/p53KO mice. All cell lines analyzed overexpress active p65BTK and when treated with EGFR-targeted therapy underwent a dose-dependent reduction of cell number without cytotoxicity. Instead, treatment with BTK inhibitors had a strongest anti-proliferative effect than EGFR inhibitors, even at lower doses, and in the case of the highest concentration of RN486 the effect was cytotoxic in all cell lines. Moreover, p65BTK inhibition had also a strong anti-clonogenic effect. Finally, cytotoxicity assays have been performed on representative cell lines treated with EGFR-targeted therapy (Gefitinib, Erlotinib) or chemotherapy (Cisplatin, Gemcitabine, Pemetrexed) alone or in combination with non-toxic concentrations of BTK inhibitors. Notably, p65BTK inhibition sensitized NSCLC cell lines to EGFR-targeted therapy and chemotherapy, with different efficiencies depending on the combined treatment and independently of the mutational status. In conclusion, the data presented in this thesis indicate that targeting p65BTK re-sensitizes NSCLC cells scarcely responsive to chemotherapy and targeted therapy and suggest that combining BTK

inhibitors with classic chemotherapy or targeted therapy could represent a novel approach to overcome drug resistance.

CONCLUSIONS

LC is the leading cause of cancer death worldwide and in particular NSCLC is one of the most aggressive and most frequent subtypes. LC treatment has been revolutionized over the years by the identification of actionable oncogenic driver mutations (Chan et al. 2015). The current SOC for patients with NSCLC who harbor genetic driver mutations is the treatment with the corresponding TKI. The clinical use of TKIs substantially improved outcomes for patients with NSCLC replacing conventional chemotherapy and reducing side effects. For example, EGFR inhibitors improved the median survival from approximately 1 year to 3-4 years in NSCLC patients with EGFR mutations (Mayekar et al. 2017). Nevertheless, there are limitations that still need to be fully overcome: off-target activity and development of resistance.

A common approach of targeted therapy is to design inhibitors toward the crucial ATP-binding pocket of oncogenic kinases. This strategy has shown promise for its ability to enhance the drug binding efficiency and the inability of cancer cells to gain mutations in these regions. In fact, the accumulation of resistant mutations in

these sites could affect the function of the oncogenic kinase itself, which would impair the ability of cancer cells to grow (Mayekar et al. 2017). However, the conservative region in the ATP-binding pocket of oncogenic kinases shares similarity to those of other kinases thus making specific targeting of the ATP-binding pocket difficult. In fact, TKIs have often shown an increase in unwanted side effects targeting other pathways and reducing the amount of inhibitor available for therapy, thus resulting in an incomplete inhibition of the target, with a concomitant reduction of the efficacy of the therapy. For this reason, one of the remaining challenges for designing ATP-binding specific TKIs is addressing the limitations regarding off-target activity (Mayekar et al. 2017).

The other limitation is the development of resistance to TKIs. Despite the initial high response rates to the different targeted therapies, tumor patients often develop acquired resistance. Several mechanisms of acquired resistance have been identified including secondary mutations in the target gene, the activation of alternative signaling pathways and phenotypic or histologic transformation. Recently, the double targeting of different effectors of the same pathway has shown promising results in overcoming this limitation. For instance, the dual inhibition of MET and ALK by Crizotinib was effective in overcoming the resistance to Alectinib in an ALK-fusion positive patient (Gouji et al. 2014). Similarly, it has been reported that the concurrent inhibition of BRAF and MEK significantly improved PFS and OS in patients with advanced BRAFV600e mutated

melanoma (Eroglu et al. 2016). This targeted therapy is now the SOC in patients with BRAFv600e mutated advanced melanoma.

Aiming to identify new molecular targets for overcoming drug resistance in solid tumors, our lab performed a shRNA-mediated phenotype screening in drug-resistant colon carcinoma cell lines. Among the genes identified, we found and characterized a novel isoform of BTK, p65BTK, overexpressed in colon cancers. We demonstrated that p65BTK is a novel oncogene acting as a downstream effector of the RAS/MAPK pathway (Grassilli et al. 2016) and that its inhibition by Ibrutinib re-sensitized p53-null drug-resistant colon cancer cells to 5FU (Lavitrano et al, in submission). These promising results led us to investigate the role of p65BTK in other solid tumors. In particular, we found p65BTK overexpressed in NSCLC, ovarian carcinoma and melanoma.

The scope of my PhD project was to investigate whether p65BTK could be a novel actionable target in NSCLC and to study its role in NSCLC cell biology. The first important finding is that in more than 50% of cancer specimens from NSCLC patients there is p65BTK expression, suggesting that p65BTK might be a widely actionable target. Importantly, p65BTK is significantly more expressed in adenocarcinomas from non-smoker patients bearing wt EGFR, a population of patients not eligible for targeted therapy due to a lack of mutations in the EGFR. Proving that p65BTK is an actionable target would therefore open new therapeutic opportunities for this kind of patients. A second important finding is that p65BTK overexpression

has been observed in NSCLC cell lines with mutations in RAS, or one of the effectors of the RAS/MAPK pathway, and in spontaneously occurring tumors of mutKRAS/p53KO mice. This finding is in line with what we previously demonstrated in colon cancer, i.e. that p65BTK overexpression depends on a hyperactivated RAS/MAPK pathway (Grassilli et al. 2016). Notably, despite KRAS mutation is one of the most prevalent oncogenic driver mutations in NSCLC (>30%) its targeting still remains elusive, mainly because of the lack of molecules able to successfully pass the clinical trial step (Garrido et al. 2017). The possibility of targeting a downstream effector of KRAS would therefore represent an alternative strategy for overcoming KRAS "undruggability". Interestingly, mutations in KRAS are also associated with a lack of sensitivity to EGFR-TKIs and primary resistance of lung adenocarcinomas to gefitinib or erlotinib (Pao et al. 2005).

In accordance with this, only a dose-dependent reduction but no cytotoxicity was observed after treatment with EGFR-TKIs of SK-Lu-1 and Calu-6 (both KRAS-mutated) cells and all primary cell lines from Kras-mutant mouse lung tumors. Interestingly, p65BTK targeting in these cells lines more strongly reduced the number of cells than EGFR inhibitors. In particular, Ibrutinib strongly reduced cell number only at the highest concentration, whereas a 2/3 reduction was already evident at the lowest dose of AVL-292 and a stronger cytotoxic effect was observed with the highest dose of RN486. The different potency of the various BTK inhibitors may be very well due

to their diverse mechanisms of action and/or pharmacokinetics profiles. In fact, RN486 reversibly binds to K430, a residue critical for BTK kinase activity, whereas Ibrutinib and AVL-292 irreversibly bind to Cys481, a conserved residue present also in the EGFR family members, which could reduce the efficacy in binding p65BTK. In addition, Ibrutinib is rapidly metabolized – and thus inactivated – by the two isoforms of the CYP3 detoxifying enzyme 3A4 and 3A5 (Lolodi et al. 2017) both of them overexpressed in a large number of LCs (Hohmann et al. 2016).

Particularly remarkable results are those obtained when combining non-toxic concentrations of a BTK inhibitor (Ibrutinib, AVL-292 and RN-486) and an EGFR inhibitor (Gefitinib and Erlotinib): all BTK inhibitors sensitized wt EGFR NSCLC cell lines to Gefitinib, even though with different grades of efficiency depending on the combination, whilst RN486 induced cytotoxicity also when used in combination with Erlotinib. These results are in line with the above reported double targeting approach and suggest that the simultaneous inhibition of two components of the RAS/MAPK pathway (EGFR and p65BTK) sensitizes NSCLC cells bypassing possible feedback regulation and increasing the efficacy of the treatment. Notably, only NSCLC patients with mutations in EGFR are treated with small-molecule TKIs, such as Gefitinib and Erlotinib, whereas patients with wt EGFR are not generally eligible for treatments with EGFR inhibitors. However, one exception is the use of Erlotinib in NSCLC patients with wt EGFR, previously treated with chemotherapy.

Here, we showed that the combination of with BTK inhibitors with EGFR inhibitors is effective independently of the EGFR mutational status. These data suggest the potential beneficial effect of the combined therapy not only in patients with mutations in EGFR but also in patients with wt EGFR.

Chemotherapy is still the first line of NSCLC therapy, but LC patients often display early resistance to the treatments. Currently, platinum-based chemotherapy is the first-line therapy for metastatic squamous NSCLC. Recently, it has been demonstrated that Pembrolizumab (an immunotherapeutic used in patients with programmed death ligand 1 [PD-L1] expression on ≥50% of tumor cells) plus chemotherapy significantly prolong overall survival among patients with non-squamous NSCLC (Paz-Ares et al. 2018). The attempt to combine targeted therapy with chemotherapy has not been successful in NSCLC. The reason of this failure is not always clear and sometimes has been attributed to a possible antagonism between EGFR inhibitors and chemotherapy in tumor cells with mutated EGFR. In theory, mutant tumor cells should exhibit enhanced sensitivity to the concomitant treatment with chemotherapy and EGFR TKIs, due to the massive apoptosis effect showed by the TKIs treatment in NSCLC cell harboring EGFR mutation (Johnson 2006). Similarly to what shown for the combination of a BTK inhibitor and a EGFR inhibitor, we demonstrated that targeting p65BTK in the presence of a chemotherapeutic agent could trigger

cytotoxicity in p53-mutated drug-resistant cells, even though with different efficacy depending on the BTK inhibitor used. In fact, a very modest effect, depending on the cell line and on the chemotherapeutic agent used, was observed using Ibrutinib and AVL-292; at variance, very high levels of cytotoxicity (from 70 to 100%) were observed when RN486 was used in combination with any chemotherapeutic drug (Cisplatin, Pemetrexed and Gemcitabine) indicating that the combination effectively overcome the resistance of p53-mutant NSCLC cells to chemotherapy. Therefore, all these data support p65BTK inhibition as a versatile approach for overcoming the limitations of the current SOC therapy.

BTK has been considered for long time a tyrosine kinase essential for B cell proliferation and differentiation and its role was considered restricted only to bone marrow-derived cells (Mohamed et al 2009). Ibrutinib was used as the first BTK-specific inhibitor for the treatment of MCL and CLL and other BTK inhibitors are currently in clinical trials for hematological malignancies and autoimmune diseases (Novero et al. 2014). In the last years, BTK has emerged as an important player in solid cancers (Molina-Cerrillo et al. 2017), even though in some cases its involvement was not at the level of the tumor cells themselves. For example, the beneficial effect of Ibrutinib in treating pancreatic xenografts has been attributed to the inhibition of the BTK expressed by the mast cells present in the desmoplastic stroma that is abundant in the tumor microenvironment. In this setting Ibrutinib

effectively diminished fibrosis, extended survival, and improved the response to clinical standard-of-care therapy (Massó-Vallés et al. 2015). In another paper it has been reported that pharmacologic inhibition of BTK in pancreas ductal adenocarcinoma can reactivate adaptive immune responses by reprogramming macrophages toward a T(H)1 phenotype that fostered CD8(+) T-cell cytotoxicity, and suppressed tumor growth (Gunderson et al. 2016). In other cases, such as in lung cancer and breast cancer the beneficial effect of Ibrutinib has been attributed to the inhibition of EGFR family members (Gao et al. 2014, Grabinski et al. 2014) (Chen et al. 2016).

However, in recent years a number of reports demonstrated the expression of BTK directly in solid tumors and showed promising results by using Ibrutinib in solid tumors (Campbell et al. 2018). Even tough in many cases such as glioma and glioblastoma, esophageal and gastric cancers, renal cell and ovarian carcinoma the isoform of BTK expressed in those cancers was not investigated and it has been assumed that it was p77, other reports pointed out the existence of different isoforms (Campbell et al. 2018). Unpublished data from our lab demonstrate that in the case of ovarian cancer p77BTK is not expressed at all and it is p65BTK that is overexpressed; interestingly, high p65BTK expression correlates with early relapse and worst progression-free survival of ovarian carcinoma patients and the treatment of patient-derived ovarian cancer cells with Ibrutinib is more effective in decreasing cell survival than SOC treatments (cisplatin, paclitaxel, bevacizumab). In breast and prostate carcinoma

cells Eifert et al. have identified an additional BTK isoform of 80-kDa (referred as BTK-C). These authors showed that BTK-C protects breast and prostate cancer cells from apoptosis. In particular, they showed that BTK-C inhibition by Ibrutinib, AVL-292 and CGI-1746 increases the expression of apoptotic genes. Therefore, BTK-C can be considered a potential biomarker and a therapeutic target for those cancers, being a survival factors for these cells (Eifert et al. 2013, Kokabee et al. 2015). This 80 kDa isoform is transcribed starting from an alternative promoter and differs from the canonical p77 for an extended N-term domain of 34 amino acids. Curiously, this 80kDa isoform is translated from the same messenger as p65BTK but using a different start of translation. Previous work from our lab demonstrated that in colon cancer the translation of p65BTK is dependent on the RAS/MAPK pathway and the data presented in this thesis support this finding also in NSCLC. It would therefore be interesting to investigate what dictates the preference for one or the other isoform in different solid tumors, and whether the preferential translation reflects the activation of different pathways de-regulated in diverse type of tumors. Given an essential pro-survival and anti-apoptotic role of BTK – as pointed out by the above-mentioned papers and from unpublished data of our lab (obtained in the colon cancer model)- it would be important for a translational perspective to understand which up-stream signaling regulates it.

Overall, the results presented in this thesis on p65BTK in NSCLC are in line with the recent findings on the new role of BTK in solid tumors.

In particular, p65BTK resulted a prognostic marker for adenocarcinoma NSCLC patients EGFR wt and might be a novel theranostic marker in NSCLC. Moreover, the demonstration that p65BTK inhibition re-sensitizes NSCLC cells to both chemotherapy and targeted therapy support p65BTK as a potential actionable therapeutic target in NSCLC, to overcome drug resistance to the current therapies. In conclusion, the inhibition of BTK and the new isoforms in solid tumors could be a new precision medicine strategy applicable as a therapy for many types of cancer.

FUTURE PERSPECTIVES AND TRANSLATIONAL RELEVANCE

The heterogeneity of NSCLC indicates that combination regimens that include targeted therapy are a promising strategy to overcome resistance. This approach can target multiple pathways that may be involved in the disease. Based on the in vitro results presented here I would like to test the same combined treatments in a spheroid tumor model. In fact, conventional 2D cell cultures do not mimic the overall complexity and heterogeneity of tumors: cells that grow in 2D plastic support are missing numerous signals that regulate different cellular processes. 3D growth of immortalized or primary established cell lines is regarded as a more stringent and representative model for in vitro drug screening. In particular, tumor spheroids are considered one of the most common and versatile scaffold-free methods for 3D

cell culture. Spheroids are either self-assembling or are forced to grow as cell clusters starting from single cell suspensions (Zanoni et al. 2016). Recently, I started to confirm the effect of BTK inhibitors on a NSCLC cell-derived spheroid model. Spheroids were derived as previously described (Pastò et al. 2014) and characterized by flow cytometry analysis, which showed an increased expression of the stem cell markers CD117 and CD133, thus confirming that the population was more homogenous and enriched in cancer stem cells compared to the 2D cell cultures (Fig.14 and 15). By performing an extreme limiting dilution analysis (ELDA) the ability of spheroid formation in response to increased concentration of Ibrutinib was evaluated: after a 10 days treatment, the ability of NSCLC cells to form spheroids was inhibited by Ibrutinib in a dose-dependent manner, showing a significant effect already at 5μM (Fig.16). Future experiments will be aimed at confirming the hypothesis that Ibrutinib can be used on spheroids alone or in combination with targeted therapy (Gefitinib and Erlotinib) and chemotherapy (Cisplatin, Pemetrexed and Gemcitabine) at lower concentration compared to the combined treatment used in 2D cell cultures. In addition, having demonstrated that the inhibition of p65BTK re-sensitizes NSCLC cells scarcely responsive to chemotherapy and targeted therapy in in vitro models, I plan future experiments to validate the effects of p65BTK inhibition in ex vivo and in vivo model. It has been demonstrated that primary cultures derived from tumor cells isolated from NSCLC patients' malignant pleural effusion (MPE), can be used to screen for sensitivity to chemotherapeutic agents and for genetic profiling

useful to select the most effective therapy regimen (Roscilli et al. 2016). Therefore, to validate p65BTK as an actionable target in NSCLC in a more relevant model, closer to the clinical setting, I intend to repeat the experiments shown in this thesis using spheroids derived from NSCLC patients' MPEs. To assess whether the ex-vivo testing effectively reflect therapy the results obtained using SOC chemotherapy and targeted therapy will be matched with the clinical response observed during therapy and compared to the results obtained using BTK inhibitors alone or in combination. Finally, for the in vivo validation, the therapeutic effect of BTK inhibition either alone or in combination with chemotherapy or targeted therapy will be assessed in mice bearing a Kras mutation (KrasLSL-G12D/+) in the epithelial lung tissue, which spontaneously develop lung adenocarcinomas. In order to increase the efficiency of the treatment and reduce the amount of p65BTK inhibitors, nanoparticles as drug delivery system will also be tested. So far, several kinds of nanoparticles have been designed and developed for the purpose of drug delivery in cancer treatment, among which the Liposomes. This technology has already been tested for NSCLC treatment and for targeting tissue inflammation (Cheng et al. 2014). Hence, p65BTK inhibitors-loaded liposomes will be evaluated as drug delivery system for targeting lung tumors and reducing the drug dosage and degradation.

Overall, these experiments will give the proof-of-concept that p65BTK targeting is a valuable and personalized treatment in NSCLC.

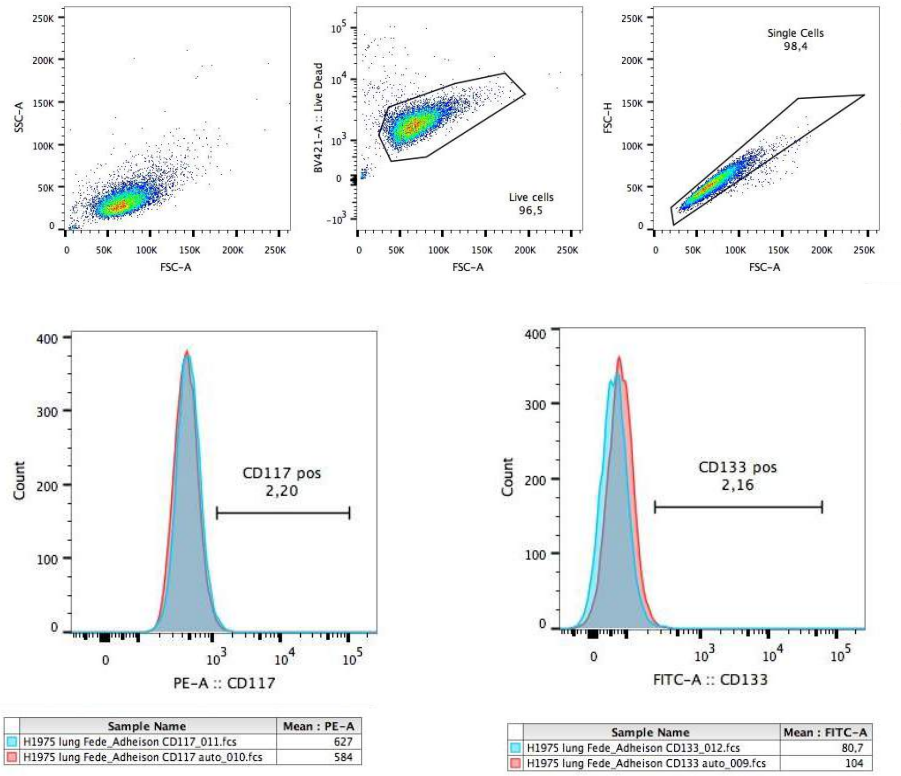
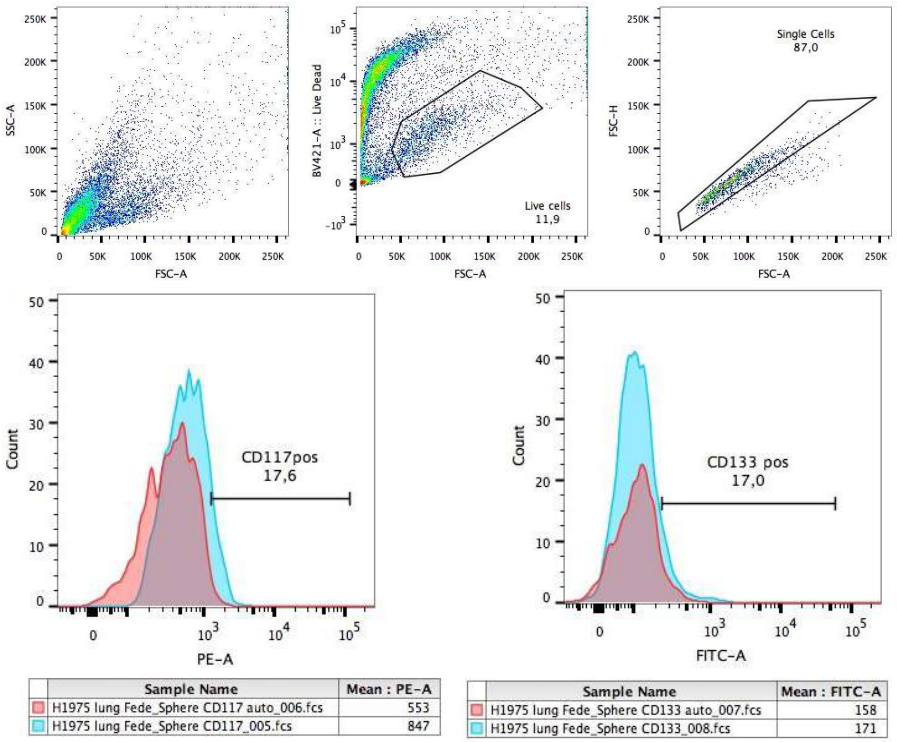


Figure 14: Flow cytometry analysis of the stemness markers CD133 and CD117 on H1975 2D-cultured cells. Only alive cells were analyzed following staining with Live/Dead Fixable Dead Cell Stains (Thermo-Fisher). FITC-conjugated anti human CD133 and PE-conjugated anti human CD117 were used at 1:100 dilution.



Sample Name	Mean : PE-A
H1975 lung Fede_Sphere CD117 auto_006.fcs	553
H1975 lung Fede_Sphere CD117_005.fcs	847

Sample Name	Mean : FITC-A
H1975 lung Fede_Sphere CD133 auto_007.fcs	158
H1975 lung Fede_Sphere CD133_008.fcs	171

Figure 15: Flow cytometry analysis of the stemness markers CD133 and CD117 on H1975 derived spheroids cultured for 10 days. Only alive cells were analyzed following staining with Live/Dead Fixable Dead Cell Stains (Thermo-Fisher). FITC-conjugated anti human CD133 and PE-conjugated anti human CD117 were used at 1:100 dilution.

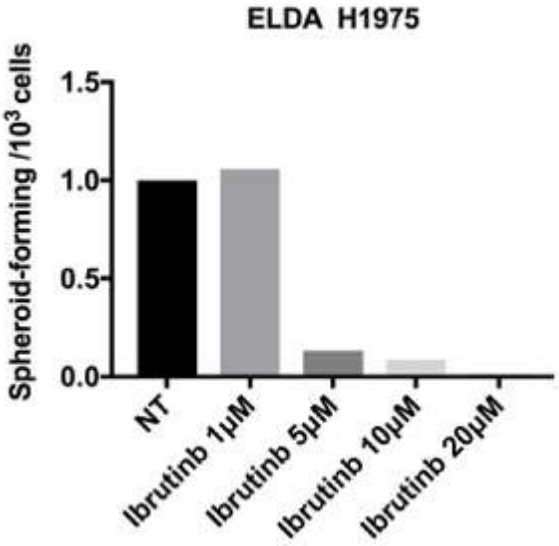


Figure 16: H1975-derived spheroids were cultured in the absence of FBS and with increasing concentrations of Ibrutinib. After 10 days, the ability to generate spheroids was evaluated by extreme limiting dilution analysis (ELDA). The frequency of spheroid-forming precursors was calculated by ELDA web tool (http://bioinf.wehi.edu.au/software/elda) and was expressed as the number of spheroid-forming cells/10^3 cells.

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