# Drug-Resistance and Protective Factors in NSCLC

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# Contents

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# Aims of the study

The aims of the thesis were to understand the different mechanisms that can lead to relapse of targeted therapy and activating mutations that may be beneficial for patient survival. The first part of this thesis focused on squamous cell carcinoma and *PIK3CA* mutations to understand how these mutations may lead to different clinical outcomes in early stage lung cancer. Secondly, to investigate novel mechanisms of drug-resistance in advanced non-small cell lung carcinoma and determine if these are potential therapeutic targets. Therefore the aims were:

- Understand how *PIK3CA* mutations affect patient survival in early stage squamous cell carcinoma in the Norwegian cohort population.
- Understand mechanisms in advanced stage drug-resistant lung adenocarcinoma sub-lines with focus on the Yes-associated protein.
- Evaluate the Yes-associated protein molecular function differences between drug-resistant sub-lines.
- Determine if the Yes-associated protein can be used as a prognostic biomarker of survival in early stage lung cancer patients.

# Abbreviations

AC - Adenocarcinoma

Akt – Protein kinase B

ALK – Anaplastic lymphoma kinase

AREG – Amphiregulin

Axl – Tyrosine-protein kinase receptor UFO

BCA – Bradford coomassie assay

BSA – Bovine serum albumin

CAM – Cell adhesion molecules

CTGF – Connective tissue growth factor

DMSO – Dimethyl sulfoxide

D-PBS – Dulbecco's phosphate buffered saline

ECM – Extra cellular matrix

EGF – Epidermal growth factor

EGFR – Epidermal growth factor receptor

EMA – European Medicines Agency

EML4 – Echinoderm microtubule-associated protein-like 4

FBS – Foetal bovine serum

FDA – Food and Drug Administration

GPCR – G protein-coupled receptor

GTPase - Guanosine triphosphate hydrolizer

KRAS – Kirsten rat sarcoma

LATS – Large tumour suppressor

LATS1/2 – Large tumour suppressor 1/2

MAPK – Mitogen-activated protein kinase

MET – Mesenchymal-epithelial transition factor

MST1/2 – Mammalian Ste20-like kinases 1/2

mTOR – Mechanistic target of rapamycin

NF2 – Merlin

NSCLC – Non-small cell lung cancer

PD-L1 – Programmed death-ligand 1

PDP1 – Phosphoinositide dependant protein 1

P-GP – P-glycoprotein

PI3K – Phosphoinositol kinase 3

PIK3CA/B/D/G – Phosphatidylinositol-4-5 biphosphate 3-kinase catalytic subunit A/B/D/G

PTEN – Phosphatase and tensin homolog

ROS1 – Proto-oncogene tyrosine-protein kinase ROS 1

RTK – Receptor tyrosine kinase

SCC – Squamous cell carcinoma

SCLC – Small cell lung cancer

TAM – Tyro-AXL-Mer

TEAD – Transcriptional enhancer factor TEF

TGF-β – Transforming growth factor receptor beta

TKI – Tyrosine kinase inhibitor

TMA – Tissue microarray

YAP – Yes-associated protein

# Papers

**Paper I**

**PIK3CA Mutations as Prognostic Factor in Squamous Cell Lung Carcinoma.**

McGowan M, Hoven AS, Lund-Iversen M, Solberg S, Helland Å, Hirsch FR, and Brustugun OT.

*Lung Cancer, 2017 January. doi: 10.1016/j.lungcan.2016.11.018*

## Paper II

**NSCLC Depend Upon YAP Expression and Nuclear Localization After Acquiring Resistance to EGFR Inhibitors.**

Marc McGowan, Lilach Kleinberg, Ann Rita Halvorsen, Åslaug Helland, and Odd Terje Brustugun

*Genes & Cancer, 2017 May. doi: 10.18632/genesandcancer.136*

## Paper III

**Yes-Associated Protein Regulates *PIK3CD* in Tyrosine Kinase Inhibitor-Resistant Mutated Lung Adenocarcinoma Cell Lines.**

Marc McGowan, Lise Berven, Anne Hansen Ree, Daniel Nebdal, Åslaug Helland, and Odd Terje Brustugun

*Under review in BMC Cancer*

## Paper IV

**Early Stage NSCLC Prognosis is Independent of YAP Expression**

Marc McGowan, Marius Lund-Iversen, Åslaug Helland, and Odd Terje Brustugun

*Manuscript*

# Introduction

## Lung cancer statistics and aetiology

Global statistics estimates lung cancer to be the most commonly diagnosed cancer type accounting for 12.9% of all cancers diagnosed. When the sexes are segregated male populations show a higher frequency (16.7%) than female populations (8.7%) (Ferlay et al., 2015). Cancers of the lung in the Norwegian population are the second most commonly diagnosed cancer type in the male population (9%) after prostate, and shared second in the female population with colon cancer (10%) (figure 1) after breast cancer (Norway, 2016).

Global figures shows 19.4% of all cancer-related deaths are from cancers of the lung (figure 2). In the male population, deaths by lung cancer account for 23.6% of all cancer-related deaths. In females, 13,8% of cancer-related deaths are from lung cancers (Ferlay et al., 2015). Globally, only 8.9% of lung cancer patients reach five years survival (Dela Cruz et al., 2011). While in Norway lung cancer statistics show 16% reach five years survival, and those with advanced lung cancers show just 1.8% reach five years (Norway, 2016).

Lung cancer is commonly associated with lifestyle, in particular of that from smoking. Smokers are at 96% higher risk of developing lung cancer than those who do not, with smoking-related lung cancers accounting for 71% of lung cancer deaths worldwide (Wu et al., 2015, Novello et al., 2016). Carcinogens from smoking can form free radicals that cause oxidative stress on the DNA. This stress can lead to DNA damage, causing adductions and signalling for repair or apoptosis mechanisms (Pfeifer et al., 2002). Occupational exposure to carcinogens from coal mining, radon gas and burning petrol or diesel can all contribute to the development of lung cancer (Pfeifer et al., 2002). Since the lungs are constantly exposed to environmental factors it is of little surprise that lung cancer is a leading cause of cancer-related deaths world-wide.

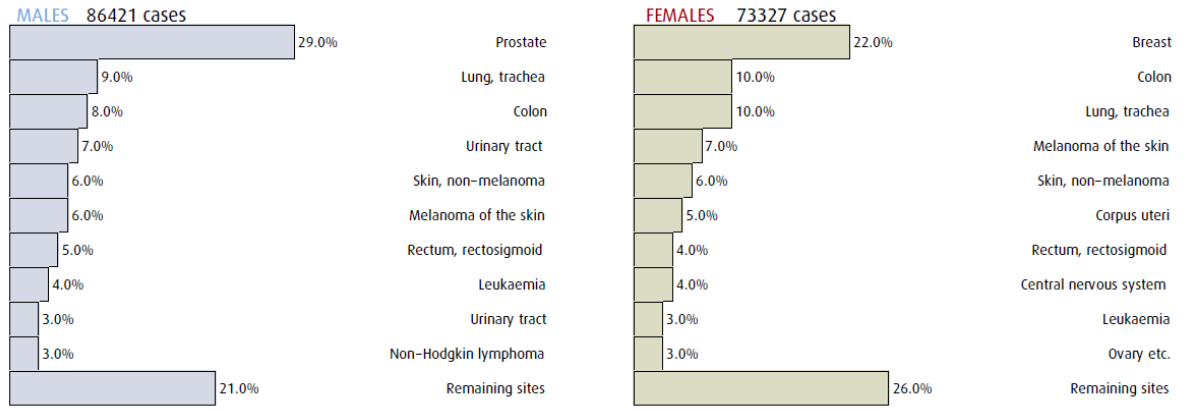


**Figure 1. National statistics of cancer for the Norwegian population.** Prostate and breast cancers remain the most commonly diagnosed cancer types at 29% and 22% for male and females of all ages. Lung cancer is the second most commonly diagnosed cancer type, at 9% for males and 10% in females (Norway, 2016).

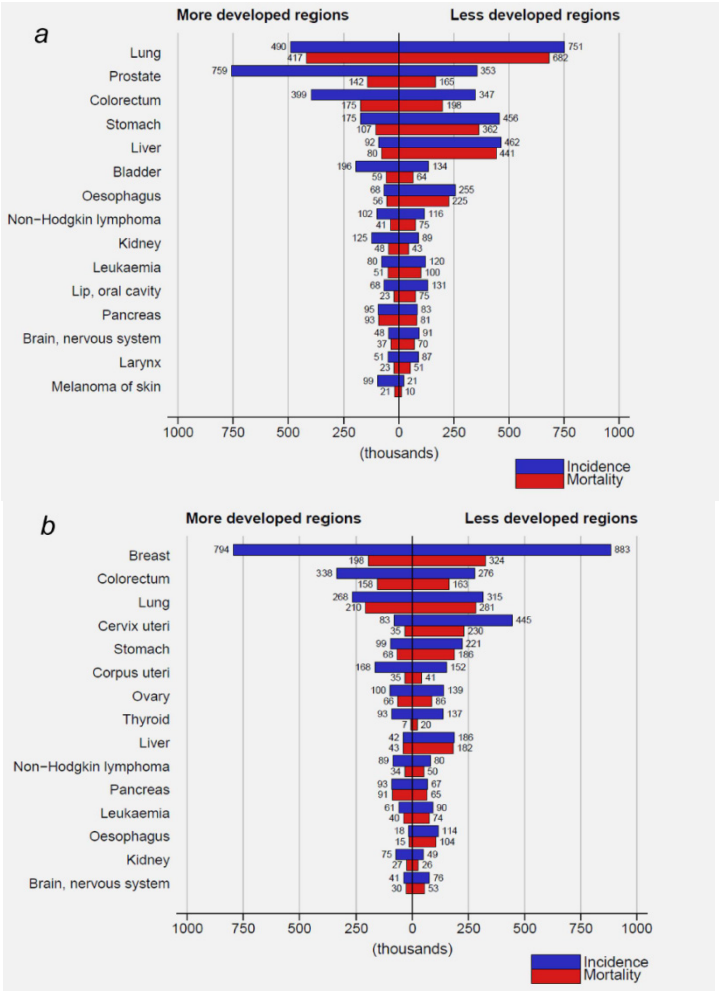


**Figure 2. Global estimates of cancer incidence in 2012.** A) Global cancer estimates for males show lung cancer the second highest diagnosed cancer type after prostrate. Lung cancers are responsible for more cancer-related deaths in males. B) The female population show higher incidence for breast and colorectal cancers than for lung cancer. However, more deaths are observed in the Western female population attributed to lung cancer than for breast or colorectal cancer (Ferlay et al., 2015).

## Lung cancer histology sub-types.

There are two types of lung cancer: small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). NSCLC are the more commonly diagnosed lung cancer type with a frequency of 85% of all lung cancer diagnosis; the remaining 15% of lung cancer diagnosis are SCLC (Gridelli et al., 2015). NSCLC is further divided into squamous and non-squamous. The squamous cell carcinoma (SCC) accounts for 30% of all NSCLC diagnosis while the remaining 70% are non-squamous. Non-squamous NSCLC is sub-divided based

upon their histology into adenocarcinoma (AC) and large cell carcinomas (LCC). AC are more frequently diagnosed accounting for 90% of the non-squamous, and the LCC accounting for the remaining 10% (Gridelli et al., 2015). All histological lung cancer sub-types are diagnosed in former and current smokers, but AC are the most frequent sub-type in never smokers (Dela Cruz et al., 2011, Pesch et al., 2012).

AC and SCC both develop in the bronchi of the lung (figure 3). Histological differences between AC and SCC aid in correctly identifying and diagnosing the NSCLC type. AC is often mucous-producing and can be identified by its glandular growth pattern and by its biomarkers thyroid transcription factor 1 and napsin A. SCC is characterised by its keratin structures and by its biomarker p63 or p40 (Shoshan-Barmatz et al., 2017).

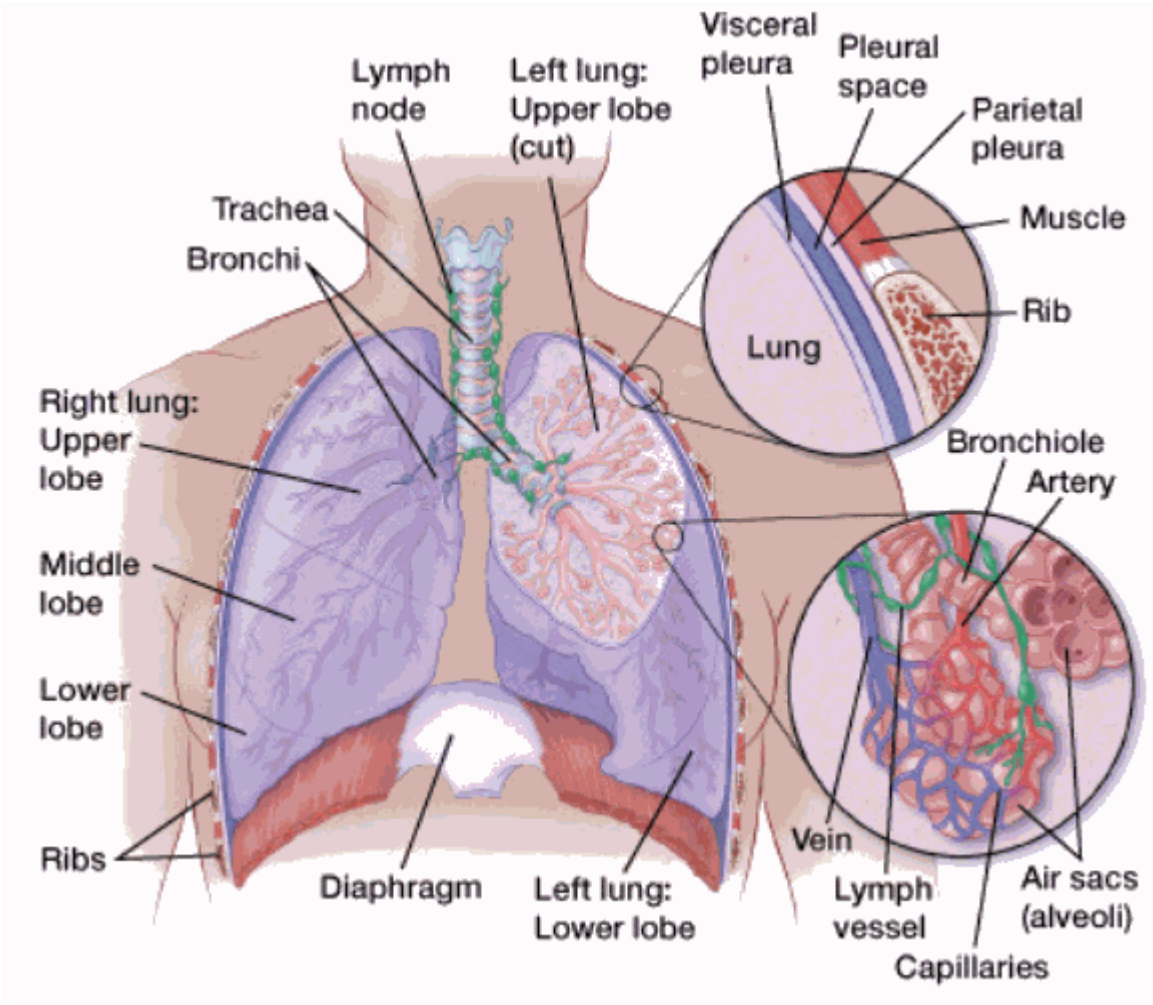


**Figure 3. The upper thorax and lung anatomy.** AC and SCC develop in the bronchi airspace of the lung (Cancer.org, 2015).

## Treatment options for lung cancer

NSCLCs are assigned a stage at diagnosis to determine which course of treatment the patient will take. These are ranked from stages I-IV, with I being localized and potentially curable, to IV which has spread and provides a challenge to treat with few treatment options.

## Part I – Potential curative treatments (stages I-III)

Management and treatment strategies depend upon the stage of the cancer at time of diagnosis. Treatments available to lung cancers diagnosed at stages I-II are surgery, radiotherapy, and adjuvant chemotherapy. Surgery is offered as a first line therapy if the tumour is operable and the patient is healthy to undergo the treatment. Should the tumour

have locally metastasised to inoperable regions then the use of radiation may be used in combination with chemotherapy (chemoradiation) as a first line treatment. Stage I cancers are usually treated with surgery and/or stereotactic radiotherapy, stage II as standard care are treated with chemotherapy following surgery or chemoradiation. Stage III lung cancers are rarely operated. It is more likely that the patient will be treated with chemoradiation as a first line option (Cancer.org, 2018).

## Part II – Treatment of advanced (stage IV) lung cancers

Generally patients that are diagnosed with advanced stage lung cancers have a poor prognosis with a 17% survival at 1 year and just 6% at 5 years (Goldstraw et al., 2016). Surgery is unlikely with these advanced stages and instead chemotherapy, immunotherapy and targeted therapies can be used. It is often seen in the clinic that patients treated with drugs will have a relatively short-term response then relapse. Mechanisms of relapse and resistance will be covered in more detail later in the introduction.

In addition to conventional DNA-damaging chemotherapy, drugs targeting the anaplastic lymphoma kinase (Alk), epidermal growth factor receptor (EGFR) and the v-Raf murine sarcoma viral oncogene homolog B (BRAF) are all now used to treat stage IV lung cancers (see below). Recently, immunotherapy with emphasis on the programmed cell death ligand 1 (PD-L1) has also become a treatment option for some patients. When expressed on the cell surface, PD-L1 can interact with T-cell PD-1 effectively suppressing T-cell activity and allowing the cancer cell to proliferate (Alsaab et al., 2017). Research has focused on inhibiting the PD1/PD-L1 interaction, allowing the host T-cells to recognise and degrade the cancer. Three PD1/PD-L1 inhibitors are approved for use in NSCLC, namely nivolumab and atezolizumab. These are monoclonal antibodies that inhibit the ligand domain of the PD-L1 receptor, prohibiting PD-1/PD-L1 interaction with good overall survival in patients (Rittmeyer et al., 2017, Brahmer et al., 2015).

## Part III – Treatment of EGFR-mutated tumours

The first generation EGFR TKIs were gefitinib (Iressa from AstraZeneca), which first entered the clinic in 2003, and erlotinib (Tarceva from Genentech) in 2004 (figure 4 A and B). These two drugs reversibly bind to phosphorylation sites on the tyrosine kinase domain of the

receptor effectively preventing activation of downstream signalling and halting cell proliferation. An early clinical study showed patients diagnosed with stages III and IV NSCLC treated with erlotinib had a favourable clinical outcome (Pérez-Soler et al., 2004). However, it was not until 2009 that the importance of EGFR mutations became clear in selecting patients for EGFR TKI therapy. Mok, *et al* performed a clinical study comparing gefitinib to standard chemotherapy in pulmonary AC. This study found that mutations in the EGFR sensitized the tumours to gefitinib that resulted in a significant progression free survival compared with standard chemotherapy, but no change in overall survival (Mok et al., 2009). Before this study, patients selected for clinical studies with EGFR TKI were not assessed for mutations. These results highlighted two important finds: careful selection of patients based upon their EGFR mutation status for further clinical studies, and that the first generation EGFR inhibitors may have more specificity to mutant forms of EGFR than with wild-type. Once identified, the mutational status of the EGFR revealed how patients should be selected for first generation inhibitors treatment. In a later study comparing erlotinib and chemotherapy, erlotinib had a greater effectiveness but overall survival resulted in little difference. Patients whom had relapsed from first line therapy, chemotherapy or EGFR TKI, were changed to receive the opposite treatment (crossover effect), which may explain the overall survival similarities (Gao et al., 2012). However, no differences were found between erlotinib and gefitinib when compared together in a phase II clinical trial of advanced stage NSCLC (Yang et al., 2017). Patients treated with EGFR TKIs typically relapse due to resistance between 9 and 14 months after commencing treatment (Sequist et al., 2011a, Kosaka et al., 2011, Oxnard et al., 2011).

Afatinib (Giotrif) is a second generation EGFR-TKI that was introduced into the clinic for advanced stage NSCLC (figure 4 C). Unlike the first generation EGFR-TKI, afatinib is an irreversible EGFR-TKI, which means it covalently binds to its target. A clinical trial comparing afatinib to chemotherapy resulted in no difference of survival in advanced NSCLC. However, those patients enrolled in the study that had the exon 19 ΔE746-A750 deletion responded better to afatinib treatment than those with the L858R point mutation (Yang et al., 2015). Relapse following first and second EGFR TKI therapies led to further research into drug resistance. This revealed a secondary mutation spot T790M on the EGFR exon 20 (explained in more detail later in the introduction).

Osimertinib (Tagrisso, AstraZeneca) (figure 4 D) is a third generation irreversible EGFR TKI inhibitor that binds to the C797 region of exon 20 (Zhang, 2016), which has a high affinity for the EGFR T790M gatekeeper mutation. This drug was approved in 2017 by

the Federal Drug Administration (FDA) and European Medicine Agency (EMA) for clinical use. This third generation EGFR TKI has been shown to improve patients' outcome following failure of first and second line EGFR TKI. A clinical study comparing osimertinib to platinum-based chemotherapy revealed a greater progression-free survival (10.1 months) than platinum therapy (4.4 months), and also as a first-line treatment (Mok et al., 2017). It is too early to assess the long term benefits of osimertinib and survival, but relapse and failure of treatment inevitably occur of which there are few alternative treatment options.

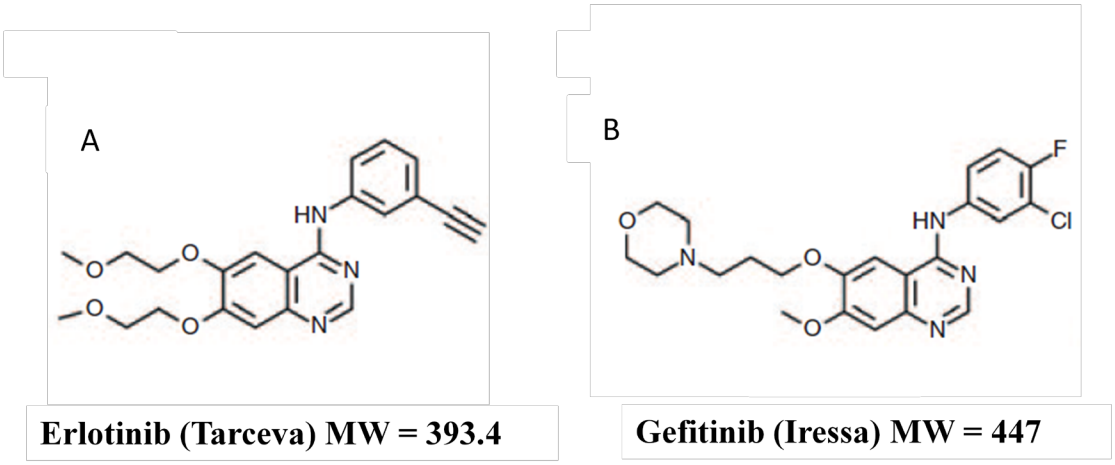


**Figure 4. EGFR TKI molecular structures of erlotinib, gefitinib, afatinib and osimertinib.** A and B show the first generation EGFR TKIs erlotinib and gefitinib with C showing the second generation EGFR TKI afatinib (Gonzalez de Castro et al., 2013). D. The third generation EGFR TKI osimertinib (Cross et al., 2014).

## NSCLC oncogenes

Somatic alterations and dysregulation of cellular signalling pathways may lead to uncontrolled cell proliferation. Somatic cells that have gained the ability to proliferate uncontrollably and without cell cycle arrest can arise as tumours or cancers. Gene mutations are common in cancers, which may translate into proteins that serve as signal transducers, or orchestrate a range of gene transcriptions aiding proliferation and invasion. These mutations and proteins are referred to as *oncogenes*, which is the term for cancer-causing genes. NSCLC harbour various expressions and mutations that have revealed new therapeutic targets (Vogelstein et al., 2013). Such mutations have been found in *EGFR* and Kirsten rat sarcoma viral oncogene (*KRAS*). Mutations in the *EGFR* are more common in never smokers while *KRAS* mutations are dominant in current and former smokers (figure 5) (Couraud et al., 2012). As illustrated in figure 5, 50% and 52% of American former and current smokers' NSCLCs, no known mutations were found. A similar trend is also observed in American never smokers with 44% of NSCLCs having no known mutations found in biopsies. As research continues it is likely new oncogenes will reduce the unknowns and perhaps produce novel therapeutic targets.

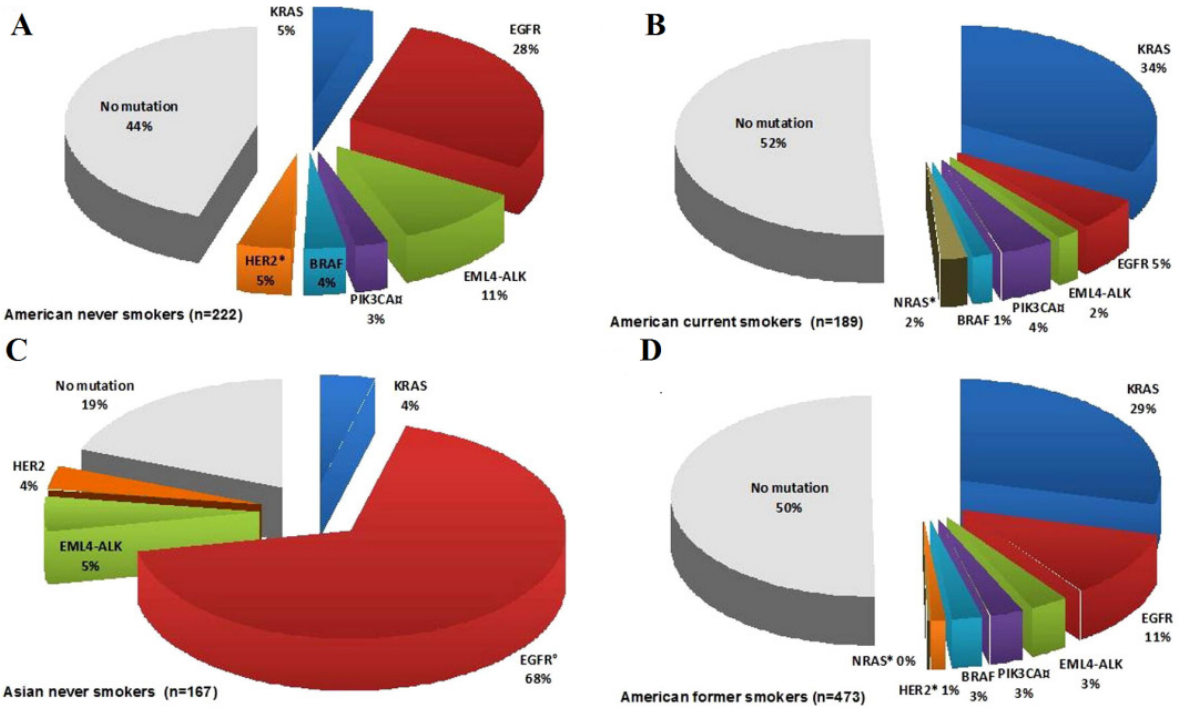


**Figure 5. Common mutations found in NSCLC patient biopsies.** A, B and D show the different mutations between those that have or continue to smoke compared to never smokers in the USA. KRAS is more prevalent in current and former smokers (34% and 29%) with few EGFR mutations (5% and 11%). A higher frequency of EGFR mutations are observed in never smokers (28%) with few patients harbouring KRAS mutations (5%). C. Similar trends are seen in the Asian never smokers population with a higher frequency of EGFR mutations (68%) and fewer KRAS mutations (4%) (Couraud et al., 2012).

The ErbB is a family of receptor tyrosine kinases (RTKs) that consists of ErbB-1 to ErbB-4. ErbB-1 is more commonly known as EGFR, which is over-expressed in most lung ACs (Bethune et al., 2010). The EGFR gene amplification and protein over-expression has been found in 62% of NSCLC biopsies, and is correlated with poor prognosis (Hirsch et al., 2003). The EGFR is a transmembrane RTK (figure 6) which comprises of an extracellular antigen binding domain and an intracellular kinase domain. The extracellular ligand binding domain can interact with several mitogens that can activate the RTK by dimerization – a process in which two EGFR proteins each bind to the ligand allowing sites on the kinase domain to reveal regions of phosphorylation sites. There are eleven ligands with an affinity for EGFR binding: EGF; transforming growth factor-α (TGF-α); heparin-binding EGF; betacellulin; epiregulin and the neuregulins (Singh et al., 2016).

As shown in figure 5, there are 28% and 68% of American and Asian never-smokers with mutations in the EGFR kinase domain (Couraud et al., 2012). The most frequent mutations are exon 19 ΔE746-A750 deletion and the L858R point mutation. Exon 19 ΔE746-A750 account for 40% of mutations in the EGFR and can cause hyperactivation of the kinase domain leading to increased activity of phosphoinositide 3-kinase-protein B- mechanistic target of rapamycin (PI3K-Akt-mTOR) proliferation and survival pathways (Sharma et al., 2007). The point mutation L858R is located on exon 21 and found in 40-45% of patient biopsies (Mitsudomi et al., 2005).

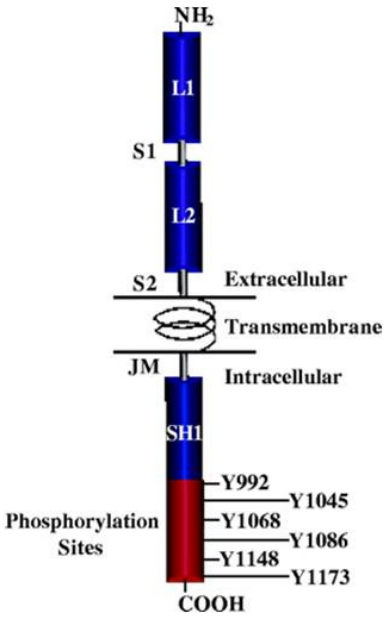


**Figure 6**. **The structure of the EGFR receptor.** This RTK has two ligand-binding sub-domains (L1 and L2) and two cysteine domains (S1 and S2 respectively). JM is the juxtamembrane domain which anchors the cytoplasmic domains to the inner cell membrane (Bazley and Gullick, 2005).

Other mechanisms of oncogenesis involve *KRAS* mutations, loss of PTEN, ALK and ROS1 translocations, and BRAF mutations. *KRAS* is the gene which codes for the Kirsten Rat sarcoma viral oncogene (K-Ras) of which belongs to the Ras family of guanosine triphosphate hydrolizers (GTPases). Wild-type forms of this Ras function as downstream signal transducers, activating cell proliferation pathways via the mitogen activation protein kinase (MAPK) before hydrolysis deactivates the enzyme into its non-active form. Mutations on codons 12 and 13 in *KRAS* are common in ACs, which are found in 29%-34% of current

and former smokers' lung cancers (Riely et al., 2009). These mutations deregulate K-Ras that results in continuous activation leading to increased downstream signalling (figure 7). Additionally, *KRAS* mutations can also lead to drug resistance (Pao et al., 2005b). So far there are no drugs that specifically target K-Ras.

Another rat sarcoma viral oncogene is the v-Raf murine sarcoma viral oncogene homolog B (BRAF). BRAF mutations are found in fewer NSCLC patients, accounting for around 4% of clinical biopsies (Couraud et al., 2012), of which 42% harbour the V600E mutation (Tissot et al., 2016). This oncogene is downstream of KRAS in the RAS-RAF-MEK-ERK signalling pathway promoting cell proliferation gene expressions (Garnett and Marais, 2004). Dabrafenib is a selective V600E BRAF inhibitor that is used in the clinic to treat NSCLC with this mutation (Nguyen-Ngoc et al., 2015).

The tumour suppressor phosphate and tensin homolog (PTEN) is the opposite of a GTPase where it is responsible for inactivating target proteins and impairing their function. A well-documented downstream target of PTEN is the phosphoinositide 3-kinase (PI3K) (Carracedo and Pandolfi, 2008). Inhibition of PI3K results in reduced cell proliferation by inhibiting the PI3K-Akt-mTOR pathway. Loss of PTEN is common amongst NSCLC which results in continuous PI3K signalling and increased cell proliferation. This process has also been identified with reduced EGFR TKI drug resistance (Mellinghoff et al., 2007).

Anaplastic lymphoma kinase (ALK) is an RTK with an extracellular ligand binding domain. This RTK functions in a similar manner to the EGFR following stimulation it phosphorylates a cascade of events targeting the PI3K-Akt-mTOR pathway (Hallberg and Palmer, 2013). ALK was first described in a subset of anaplastic large-cell lymphomas. This discovery observed a translocation of chromosome 2p (*ALK* gene) and chromosome 5q (*nucleophosmin*) (Morris et al., 1994). It was not until 2007 that *ALK* gene rearrangements were discovered in NSCLC (Soda et al., 2007) which led the way to developing the drug crizotinib. ALK is not only conserved to translocate with nucleophosmin, in NSCLC *ALK* has been found to translocate and rearrange with a variety of genes, typically echinoderm microtubule-associated protein-like 4 (*EML4*). Like most targeted therapies, resistance to crizotinib generally occurs 1-2 years following treatment. Analogous to the EGFR T790M mutation following first line TKI resistance, ALK may acquire mutations at sites L1196M (Lovly and Pao, 2012), G1202R and S1206Y (Katayama et al., 2012), which affect the binding ability of crizotinib. In NSCLC failure of EGFR TKI treatment in patients can be linked to the emergence of the ALK-EML4 translocation (Sweis et al., 2016).

The proto-oncogene tyrosine-protein kinase ROS1 is the protein product of the gene *ROS1*. ROS1 is a receptor tyrosine kinase that is able to fuse with other proteins allowing activation of the PIK3-Akt-mTOR pathway (Davies and Doebele, 2013). This RTK is commonly found fused with cluster of differentiation 74 (CD74) forming the *CD74-ROS1* fusion (Bubendorf et al., 2016). ROS1 has been identified in 1-2% of biopsies from NSCLC patients and is targeted using crizotinib (Rimkunas et al., 2012).

Another RTK proto-oncogene (a gene that can function in both non-cancerous and cancerous cells) is the mesenchymal-epithelial transition factor (*MET*). There have been disputes regarding *MET* amplification and *MET* overexpression in NSCLC. *MET* gene over-expression has been identified in 37-67% of NSCLC biopsies. While *MET* amplification has been identified in 3-4% of AC (Salgia, 2017). Interestingly, this RTK was first identified in the AC cell line HCC827 (Engelman et al., 2007). Following interaction with its ligand hepatocyte growth factor, MET can recruit the PI3K-Akt-mTOR pathway (Janku et al., 2010)

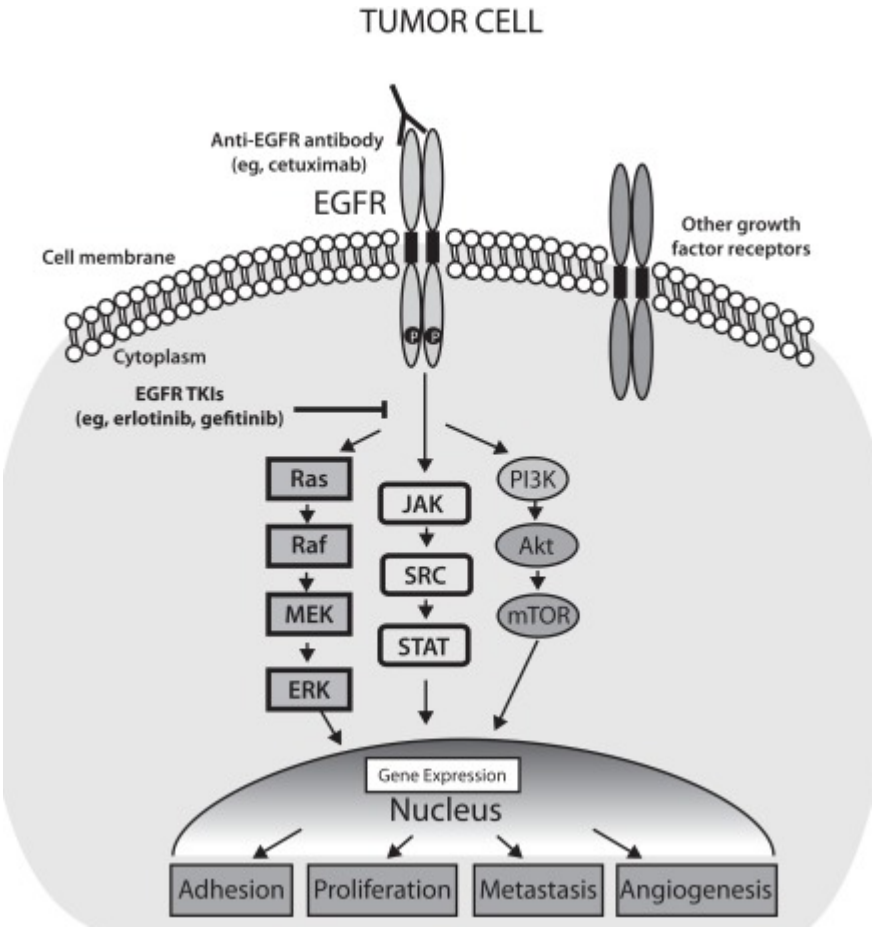


**Figure 7. Various pathways activated through EGFR RTK signalling with EGFR inhibitors.** EGFR TKIs erlotinib and gefitinib have been shown to inhibit downstream signalling pathways of PI3K-Akt-mTOR, along with RAS-RAF-MEK-ERK (Mehta, 2012).

## Mechanisms of drug-resistance

There is still an ongoing debate whether cells have the ability to acquire drug resistance or if this process is more of a selection of a heterogeneous cluster of cells. However, this section will cover some of the main concepts of drug resistance in NSCLC.

Two of the more classical mechanisms of drug resistance are mutations in the p53 and the ability to remove cytotoxic drugs via the p-glycoprotein (p-gp) efflux pump (Ahrendt et al., 2003, Miyatake et al., 2003, Skaug et al., 2000). P53 is a tumour-suppressor protein responsible for repair mechanisms following damage to the cell's DNA. This process of cell repair orchestrate by p53 either holds the cell in senescence activates apoptosis mechanisms. Mutations in the p53 may result in loss of apoptosis or senescence mechanisms allowing DNA damaged cells to proliferate uncontrolled.

Patients whom relapse to first and second generation EGFR TKIs are frequently found to harbour the EGFR gatekeeper mutation T790M on exon 20 in biopsies following treatment (Pao et al., 2005a). This mutation in the EGFR is responsible for resistance to erlotinib and gefitinib in over 50% of patients (figure 8). However, research has shown that time off EGFR TKI for these patients harbouring the T790M mutation can lead to a positive second round of treatment using the same drug (Watanabe et al., 2011, Sequist et al., 2011b). In addition, exon 20 of the EGFR can also harbour the C797S mutation which has been responsible for the failure of all EGFR TKI, including the newly approved osimertinib in 40% of treated patients (Thress et al., 2015). Figure 8 illustrates the most common mutations found on the EGFR kinase domain and also highlights some of the mutations found following EGFR-TKI drug resistance.

AXL is a RTK and a member of the TAM receptor family (tyro-AXL-Mer) which share a conserved kinase domain. This receptor was identified as a mechanism of EGFR TKI resistance in the cell line HCC827 following erlotinib resistance *in vitro* and *in vivo* (Zhang et al., 2012). This RTK has also been found in patient biopsy material from cancers of the breast (Meric et al., 2002), colorectal (Martinelli et al., 2015) and lung (Shieh et al., 2005). Like EGFR, AXL can utilize and recruit the PI3K-Akt-mTOR pathway.

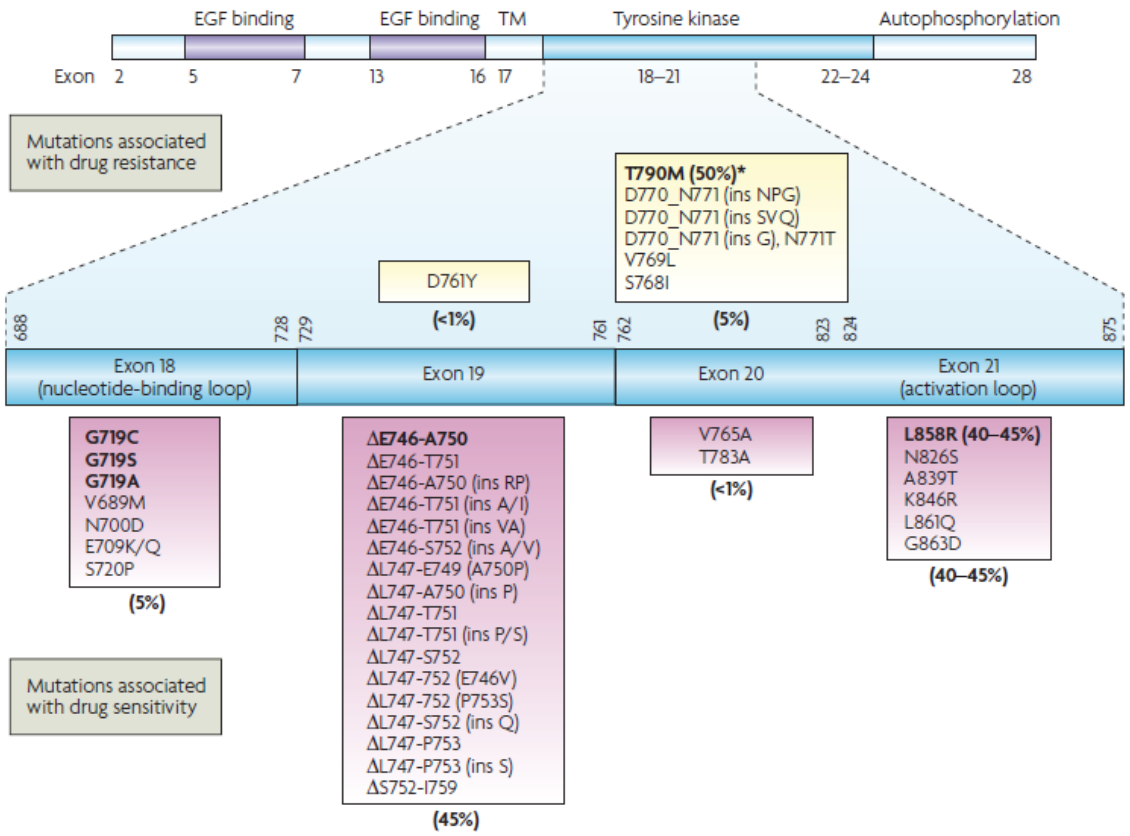


**Figure 8. EGFR kinase domain exon map of mutations.** The lower part of the figure shows known mutations associated with drug sensitivity (not following drug-resistance). Patients harbour mutations on the exon 19 with ΔE746-A750 deletion being the most common (45%). The upper yellow boxes highlight known mutations following drug resistance. The exon 20 T790M point mutation accounts for 50% of patient relapse to EGFR-TKI therapy (the asterisk shows clinical-relevance) (Sharma et al., 2007).

## Yes-associated protein

Yes-associated protein (YAP) is a co-transcription factor that has been associated with poor prognosis in lung cancer (Wang et al., 2010), breast cancer (Kim et al., 2015a), and ovarian cancer (Xia et al., 2014). It has been identified as an oncogene orchestrating a multitude of gene transcriptions by binding to a nuclear transcription factor such as the transcriptional enhancer associate protein domain (TEAD) family (Mo et al., 2014) and p73 (Downward and Basu, 2008), which results in the gene expression of the connective tissue growth factor (CTGF) (Zhao et al., 2008) and amphiregulin (AREG) (Zhang et al., 2009). This interaction promotes the epithelial to mesenchymal transformation (EMT) (Overholtzer et al., 2006) , cell migration and many other cellular activities (Ling et al., 2017).

The structure of YAP mainly consists of WW-domain-containing transcription regulators, proline/x amino acid/x amino acid/hydrophobic region/ proline (PxxφP) TEAD binding domain, and a transcription activation domain (TAD). In 2012, Gaffney *et al* successfully cloned 8 splice variations of the *YAP* gene revealing size differences (Gaffney et al., 2012). The YAP protein can be divided into two main types based upon how many WW domains are present. The WW domain (figure 9) is a protein-protein interacting domain which is responsible for binding YAP to a nuclear receptor. The WW domain interacts with proline/proline/x amino acid/tyrosine (PPxY) motif (Webb et al., 2011). YAP 1-1 has only one WW domain, while YAP1-2 has two. Both variations have four splice isoforms each; these are named α, β, γ, and δ (Gaffney et al., 2012); so far the biological affects if each YAP isoform has yet to be understood. The difference of having two WW domains instead of one is that the second has been shown to bind to p73 (a homologue to p53) while YAP1-1 isoforms are unable to bind and promote apoptosis pathways (Oka et al., 2008). The TEAD binding interacts with TEAD 1-4 which can promote cell survival mechanisms. The TAD domain consists of a PDZ-binding motif which has been suggested to interact with cytoskeleton or transmembrane proteins that direct the localization of YAP (Varelas, 2014).

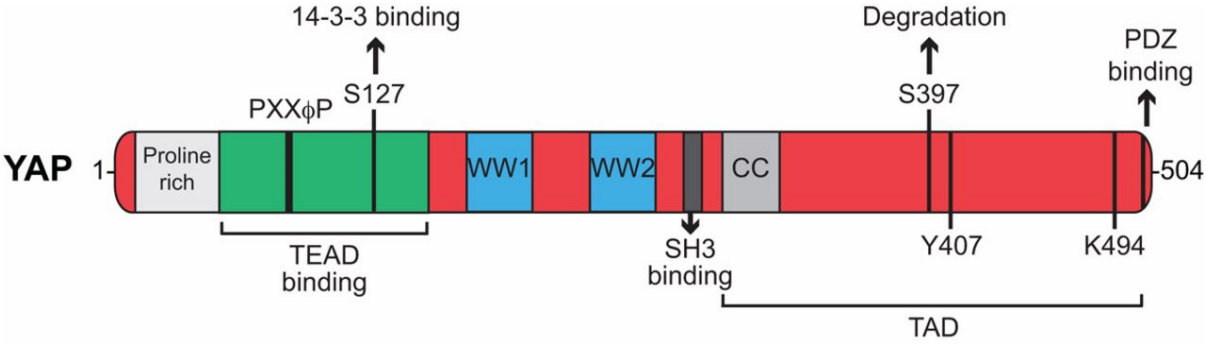


**Figure 9. A schematic of YAP protein arrangement.** YAP has several phosphorylation binding sites that inhibit migration to the nucleus by Lats1/2. More importantly the binding of YAP to TEAD is inhibited by Lats1/2 on the Ser127 phosphorylation site. Degradation of the protein by ubiquitin is on the Ser397 phosphorylation domain. The schematic shows the YAP1-2 isoform due to having two WW domains; while YAP 1-1 has one (Varelas, 2014).

YAP is a downstream target of the Hippo signalling pathway (figure 10) Merlin (NF2) phosphorylates and activates the serine/threonine protein kinases mammalian Ste20-like kinases 1/2 (MST1/2) which in turn activates the large tumour suppressor 1/2 (LATS1/2)

that phosphorylates YAP on the Ser127 domain. This phosphorylation cascade withholds YAP in the cytoplasm, which is targeted by degradation proteins (Serrano et al., 2013). When active, YAP is able to interact with chaperone proteins, sequestering the co-transcription factor to the nucleus where it binds to a transcription factor. An important protein in both EMT and YAP regulation is the CAM E-cadherin. Reports have shown the loss of E-cadherin and the expression of vimentin is required for cell invasion (Lamouille et al., 2014). A report showed E-cadherin to be part of the Hippo-signalling pathway by inactivating YAP through phosphorylation of the Ser127 domain. This action required E-cadherin to be bound to B-cantanin or Lats 1/2 (Kim et al., 2011) suggesting E-cadherin regulates the Hippo pathway. The important factor is the loss of E-cadherin during EMT which may account for the activation of YAP.

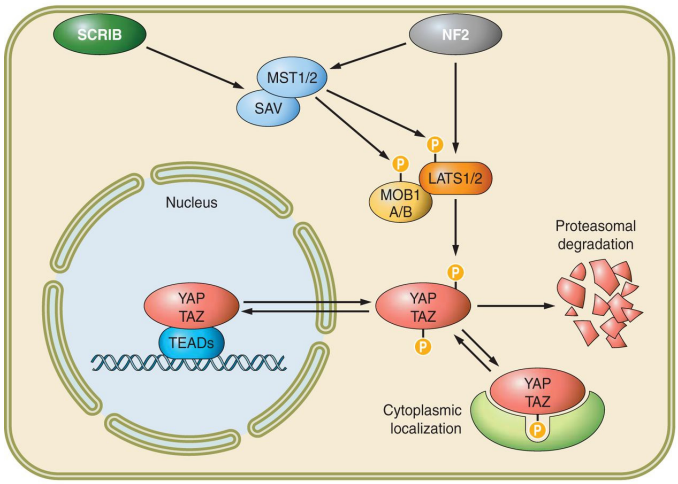


**Figure 10. A cartoon showing the regulation of YAP**. The Hippo signalling pathway inhibits YAP by phosphorylation after the activation of Merlin (NF2) (Piccolo et al., 2014).

YAP has recently become an interesting area for oncology research since this co-transcription factor is seen as a mechanism of drug resistance in a variety of cell types including breast and lung cancers (Kim et al., 2015a, Steinhardt et al., 2008). Survival studies have shown a correlation between YAP nuclear localization and poor prognosis in patients diagnosed with lung ACs (Kim et al., 2015b) along with a meta analysis studying various cancers highlighting furthering YAP's involvement in prognosis (Sun et al., 2015). Molecular studies have also shown YAP to regulate AXL and PD-L1 expression (Boin et al., 2014, Lee et al., 2017).

## PI3K variants

Phosphoinositol 3 kinase (PI3K) is a two sub-unit enzyme with a catalyst (p110) and a regulator (p85) domain. The catalytic domain is responsible for all downstream phosphorylation and activation of various targets, mainly Akt. PI3K is recruited to the cell membrane by p85 where it is phosphorylated by an RTK or G-protein coupled receptor (GPCR) into an active conformation. This enzyme then adds a phosphate group to phospatidylinositol (4,5) biphosphate (PIP2) producing phosphatidylinositol (3,4,5) trisphophate (PIP3), which continues the signal transduction to phosphoinositide-dependent kinase-1 (PDK1)-Akt pathway (Hemmings and Restuccia, 2012) (figure 11). This process is abbreviated to the PIK3-Akt-mTOR pathway. This pathway has been associated with oncogenesis and has been found to be responsible for cell proliferation and survival.

There are four classes of the catalyst domain found in mammalian cells; these are the p110α, β, δ and γ. The p110α is coded by the gene *PIK3CA*, and p110δ is in turn coded by the *PIK3CD* gene. Mutations are common in the *PIK3CA* on exon 9, which are E542K, E545K and less common on the exon 20 (Scheffler et al., 2015). Mutations in the p110α are have been found to be associated with poor prognosis (Mangone et al., 2012), whereas in other studies, mutations have been associated with improved survival (Dupont Jensen et al., 2011, Maruyama et al., 2007, Kalinsky et al., 2009).

The p110δ is a common PI3K subtype found in T-cells (Soond et al., 2010). Although mainly associated with cells of the blood, this catalytic subtype has also been described in various solid tumours. Thyroid cancers (Bu et al., 2017) and some lung carcinomas (Nakamura et al., 2007) have all described *PIK3CD* expressed and activation; it has also been associated with poor prognosis in glioblastoma (Suda et al., 2014). *PIK3CD* gain of function mutations have been described in cells of the immune system that lead to reduced immune defence, increased infections and autoimmune disorders (Lucas et al., 2016).

Co-expression of *PIK3CA* and *YAP* can lead to the development of hepatocellular carcinoma in mouse and human livers (Li et al., 2015b). Furthermore, YAP has been identified to regulate the expression of p110β in cardiomyocytes (Lin et al., 2015). Therefore, the PI3K variants were of interest in this thesis.

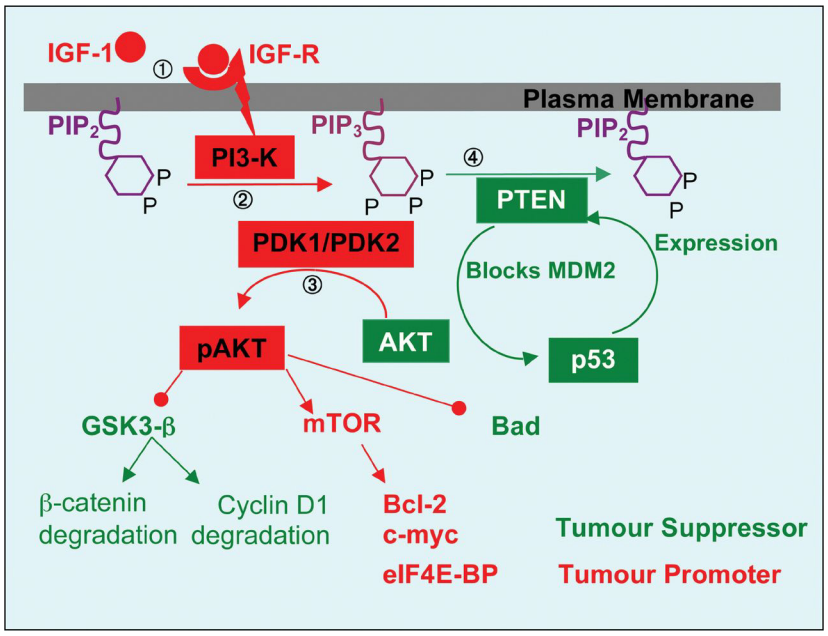


**Figure 11. A cartoon of the PIP2 to PIP3-mediated by PI3K.** In this schematic PI3K is stimulated by the insulin growth factor receptor (IGF-R). PI3K phosphorylates PIP2 to its active form of PIP3 which in turn donates a phosphate group to PDK1 activating the Akt pathway (Assinder et al., 2009).

## Cell invasion and EMT

Cascades of events are required to enable cancer to become metastatic. The epithelial to mesenchymal transformation (EMT) is a mechanism that grants a cell the ability to break away from the tumour mass and invade other areas of the body. The expression of the cell surface receptors, such as the transforming growth factor receptor beta (TGF-β), induces the cascade of events leading to EMT (Moustakas and Heldin, 2007). The TGF-β orchestrates the gene expression of Slug, Snail, Twist and Zeb1/2 which in turn down regulate the CAM E-cadherin, which relinquishes the cell's reliance on cell contact for growth. Down-regulation of cytoskeleton cytokeratin and up-regulation of vimentin (Kim et al., 2013, Sakuma et al., 2013) depolarises the cell and improves focal adhesion for migration (Liu et al., 2015). Cell invasion (illustrated in figure 12) fundamentally consists of:

1) Development and signalling from tumours to recruit new blood supply vessels – **angiogenesis**.

2) Cells begin to regulate surface proteins and down-regulate CAM to escape the tumour mass.
3) Extra cellular matrix (ECM) remodelling and digesting through the basement membrane through the secretion of metalloproteases
4) Cells migrate and invade the blood stream – **intravasation**.
5) Cells alter their morphology again to enable adhesion to the endothelial cells of the blood vessels – **cancer cell arrest**.
6) Cells exits the blood stream – **extravasation**.
7) Proliferation of secondary tumour – **secondary colonisation site**.

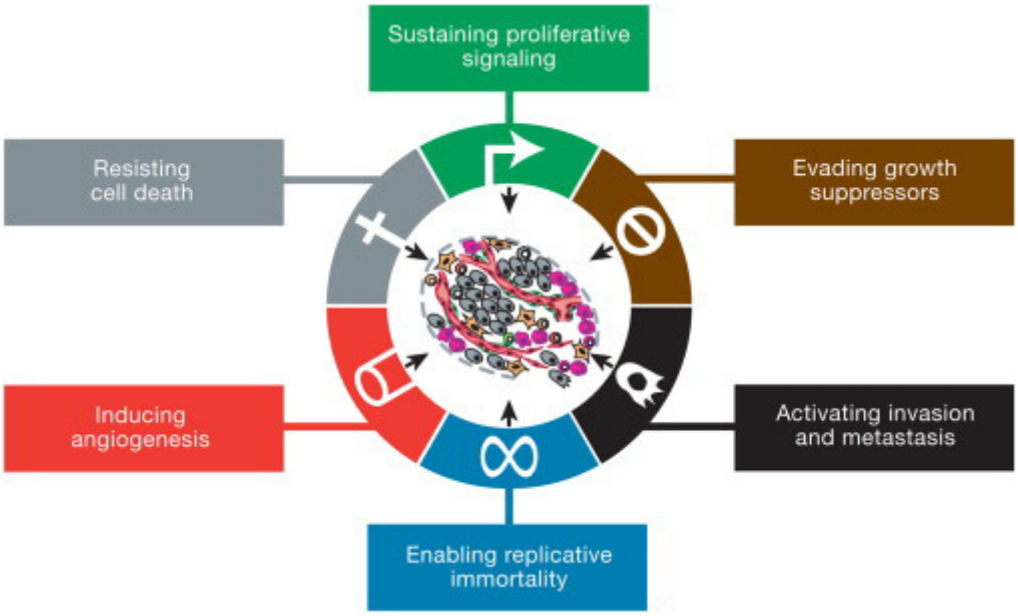


**Figure 12. The fundamentals of cancer cell invasion and metastasis.** For a tumour cell to invade it must first secure signalling activities that promote self-renewal and proliferation. (Hanahan and Weinberg, 2011).

## Patient selection and cell line models

NSCLCs are fraught with treatment issues with many first and second line therapies improving survival but ultimately lead to relapse. However, in some cases treatments result in complete remission. Therefore the research question of this thesis was to determine why some NSCLC respond better to treatments while others fail. To answer this both cell lines

and patient material were employed. Lung cancer patient biopsies were used to evaluate the role of *PIK3CA* mutations, and also YAP expression in early stage diagnosis as prognostic factors following surgery.

The human NSCLC cell lines HCC827 and H1975 were chosen for advanced stage NSCLC experiments. The HCC827 are characterised with the over-expression of the EGFR harbouring the exon 19 ΔE746-A750 deletion. The HCC827 cell line is responsive to all EGFR TKI therapies. The H1975 cell line harbours the EGFR T790M along with the L858R point mutation. This cell line is resistant to all first and second generation EGFR TKI but sensitive to osimertinib.

# Materials and methods

## Cell lines and reagents

The cell lines HCC827 and H1975 were purchased from ATCC (CRL-2868 and CRL-5908). RPMI1640 (Sigma, R8758) was supplemented with 0.1% v/v penicillin-streptomycin (Gibco, 15140-122), 0.1% v/v L-glutamine (Sigma, G7513) and heat-inactivated 10% v/v foetal bovine serum (FBS) (VWR, S1810-500) – referred to as supplements from now on. Cells were incubated at 37$^{o}$C in a 95 % humidified 5% $CO_2$ atmosphere. Hela cells were a kind gift from Dr. Inga Hansine Rye and were maintained in DMEM/F12 with supplements (Thermo Fisher, 21331020). Additional culture chemicals and reagents were 0.25% trypsin (Sigma, T4049), sterile Dulbecco's phosphate buffered saline (D-PBS) (Gibco, 14190-094) and dimethyl sulfoxide (DMSO) (Sigma, D2650). The EGFR inhibitors erlotinib (LC Labs, E-4007), gefitinib (LC Labs, G-4408), osimertinib (MedChem Express, HY-15772), and the AXL inhibitor BGB324 (MedChem Express, HY-15150) were all dissolved in DMSO and stored at -20$^{o}$C. The recombinant human epidermal growth factor (EGF) (R&D Systems, 236-EG-200) was dissolved in D-PBS, and recombinant human growth arrest specific 6 (Gas6) (R&D Systems, 885-GSB-050) was dissolved in distilled $H_2O$ ($dH_2O$); both at the manufacturer's specifications and stored at -20$^{o}$C.

Tris/glycine/SDS buffer (Bio-Rad, 161-0732) and Tris-buffered saline (Bio-Rad, 170-6435) was diluted to 1x in $dH_2O$. Tween 20 TBS (TTBS) was made by diluting Tween 20 (Bio-Rad, 161-0781) to 0.1% v/v in TBS.

For the cell viability tests resazurin sodium salt (Sigma, R7017) was diluted to 2.3 mg/ml in sterile D-PBS, sterile filtered through a 0.2 µm syringe and stored at 4$^{o}$C. This was further diluted to 1/20 in sterile D-PBS, warmed to 37$^{o}$C and added to the cells at a final dilution of 1/10.

Lysis buffer for western blot was made by diluting sodium orthovanadate (Sigma, S6508) Pefabloc (Merck, 1.24839.0500), and sodium fluoride (Sigma, 30105) in Ripa lyses buffer (Thermo Scientific, 89901), all to a final concentration of 50 mM. In addition to the lysis buffer, both PhosStop and Protease inhibitor cocktail (Roche, 04906845001 and

05892970001) were added at the manufacturer's specifications. The buffer was aliquot and stored at -20$^{o}$C and thawed on ice before use. This will be defined as "WB lysis buffer".

Lysis buffer for PamChip tyrosine phosphorylation assay was made by diluting PhosStop and Protease inhibitor cocktail (at the manufacturer's specifications) in Mammalian protein extraction reagent (M-PER) (Thermo Scientific, 78503) and stored at -20$^{o}$C.The buffer was thawed on ice before use. This will be defined as "M-PER lysis buffer".

## Cell culture and the generation of drug resistant sub-lines

The HCC827 and H1975 parental cells were maintained in RPMI1640 + supplements in accordance with ATCC recommendations and incubated in a 37$^{o}$C, 95% humidified 5% $CO_2$ atmosphere. Cells were maintained in constant exponential growth phase until 80% confluence was observed. Cells were then washed once with warm sterile D-PBS and incubated for 5 minutes in 0.25% trypsin to disassociate from the culture flask bottom. Flasks were washed with D-PBS and cells collected for centrifugation at 300 x g for 5 minutes. D-PBS was decanted and cell pellets were re-suspended in media + supplements and seeded at 1/15 into new culture flasks. Media were changed twice weekly.

For EGFR TKI resistant sub-line generation the HCC827 parental cells were seeded into large culture dishes and subjected to increasing doses of erlotinib, gefitinib or osimertinib from their $EC_{50}$ value until cell proliferation was observed. Cells were deemed resistant once the drug concentrations surpassed clinical Cmax (concentration maximum) values of 0.3 μM gefitinib (Swaisland et al., 2005), 2.2-2.5 μM erlotinib (Hamilton et al., 2006), and 2-3 μM osimertinib (Jänne et al., 2015). Cloning cylinders were used to reduce heterogeneity by isolating small colonies of cells and expanding in RPMI1640 +supplements and drug. Twenty-five sub-colonies of each erlotinib-resistant HCC827 (HCC827/ER) and gefitinib-resistant HCC827 (HCC827/GR) sub-lines were expanded and checked for EMT and YAP expression using western blot. Only ten osimertinib-resistant HCC827 (HCC827/OR) sub-lines were successfully expanded and checked for differences in heterogeneity. All sub-lines' EGFR exons 18-21 were sequenced using PCR-based analysis with Cobas® EGFR Mutation Test v2 (Roche, 07248563190).

A cell viability assay was used to determine if the H1975 cell line was resistant to both erlotinib and gefitinib due to the T790M mutation. As with the HCC827, H1975 cells were treated with low concentrations of osimertinib until proliferation was observed at 2.5

μM. The cells were confirmed resistant following a cell viability assay. Three sub-lines were expanded from cloning cylinders and maintained in drug. Cells were frozen and stored long term using 20% v/v FBS and 5% v/v DMSO in drug-free RPMI1640 with supplements and left for 24 hours at -80$^{o}$C before transferring to liquid nitrogen. Thawing of cells was achieved by rapidly warming to 37$^{o}$C and diluting in media with supplements without drug. Cells were then centrifuged at 300 x g for 5 minutes, re-suspended in warm media and left for 24 hours before changing the media.

The HCC827/ER were maintained in 3.5 μM erlotinib; HCC827/GR were maintained in 2.5 μM gefitinib; HCC827/OR and H1975/OR were maintained in 2.5 μM osimertinib (all with 0.1% v/v DMSO final concentrations); and parental cell lines in RPMI1640 + supplements defined hereon as "relevant media". Routine media changes occurred twice weekly. All drug-resistant cells were kept in exponential phase until 80% confluent before passaging. All cell lines were periodically checked for mycoplasma using MycoAlert ™ detection kit (Lonza, LT07-218) at their specifications.

## Cell viability assay

Parental HCC827 and H1975 were initially used to determine how sensitive they were to EGFR inhibitors. For this 5,000 cells were seeded into a 96-well plate and left overnight in media + supplements with 8 replicas. The following day the media was removed and replaced with half-log dilutions of erlotinib, gefitinib or osimertinib. Dilutions ranged from 10 μM to 0.001 μM for the treatment groups, and DMSO only for the control; keeping the final DMSO concentrations consistent at 0.1% v/v. A column of empty wells served as blanks with media + supplements and 0.1% v/v DMSO. Cells were incubated for 5 days and on the fifth day resazurin was added to each well and incubated for 4 hours. The effective concentration at 50% ($EC_{50}$) was measured using fluorescence with the excitation at 544 nm and emission at 590 nm using the Synergy 2 with software Gen5 v1.11.5. This was repeated once more and the data pooled. $EC_{50}$ were presented as % of viable cells by calculating the mean for each drug concentration and deducting the blank. These values were then normalized to the control wells and presented as percentages using GraphPad v7 using the Non-linear regression curve and dose dependant calculation. The same procedure was used to test drug-resistant sub-lines to their respective inhibitor and also for cross-resistance to other EGFR inhibitors.

## Western Blot

Parental cell lines and drug-resistant sub-lines were seeded at 2.5 x $10^5$ cells per well of a six-well plate and left in relevant media overnight. The following day media was removed and replaced with RRPMI1640 + supplements containing 0.1% v/v DMSO for the HCC827 and H1975 parental cells. HCC827/ER were cultured in 3.5µM erlotinib, HCC827/GR in 2.5µM gefitinib, HCC827/OR and H1975/OR both in 2.5 µM osimertinib all with a final concentration of DMSO at 0.1% v/v. Cells were left for 3 days and washed once with ice-cold TBS to prevent degradation of proteins before harvesting with WB lysis buffer and cell scrapers on ice. Lysate samples were incubated on ice for 30 minutes and centrifuged at 13,000 x g for 10 minutes. The supernatant was collected and samples stored at -20°C until use.

The total protein concentration was measured using the Pierce BCA Protein Kit (Thermo Scientific, 23227) at 562 nm absorbance using the Synergy 2 from BioTek® with Gen5 1.11.5 software. All western blots used 15 µg of protein lysate which was heated to 95°C with Laemmli sample buffer (Bio-Rad, 1610747) and 100 mM dithiothreitol (AppliChem, A3668) for 5 minutes. Lysates were briefly centrifuged and loaded into 12% SDS mini PROTEAN® TGX™ gel (Bio-Rad, 456-1046) along with Precision Plus Protein™ WesternC™ ladder (Bio-Rad, 161-0376) and ran for 60 minutes at 200V in 1x TGS running buffer. Once complete, gels were transferred to a Midi Format nitrocellulose membrane (Bio-Rad, 170-4159) using the Turbo Blot transfer module from Bio-Rad for 30 minutes. Membranes were incubated at room temperature in blocking buffer (Table 1) for 60 minutes. The membranes were washed three times for 5 minutes in TTBS before sectioning with a clean scalpel. Each section was incubated in 50 ml tubes with antibodies listed in Table 1. Sectioned membranes were incubated overnight rotating at 4°C. The following day membranes were washed three times for 5 minutes using TTBS before transferring the membranes to new 50 ml tubes containing secondary antibodies (listed in Table 2) with 1:10000 Precision Plus streptactin-HRP conjugate (Bio-Rad, 161-0381). Membrane sections were incubated at room temperature for 60 minutes before repeating the previous washing steps and developed using SuperSignal® (Thermo Scientific, 34096). Membranes were imaged using the Bio-Rad Chemi Doc™ MP imager and observed using the Image Lab v4.1 software.

**Table 1**. **Dilutions and concentrations for each primary antibody used for Western Blot.**

| Primary antibody | Block in | Dilute in | Dilution |
|---|---|---|---|
| **Anti-AXL (Cell Signalling, 8661)** | 5% w/v fat-free milk in TTBS | 5% w/v BSA in TTBS | 1:1000 |
| **Anti-EGFR (Cell Signalling, 2085)** | 5% w/v fat-free milk in TTBS | 5% w/v BSA in TTBS | 1:1000 |
| **Anti-Vimentin (abcam, ab92547)** | 5% w/v fat-free milk in TTBS | 5% w/v fat-free milk in TTBS | 1:1000 |
| **Anti-E-cadherin (abcam, ab1416)** | 5% w/v fat-free milk in TTBS | 5% w/v fat-free milk in TTBS | 1:1000 |
| **Anti-YAP (Thermo Scientific, PA5-13504)** | 5% w/v fat-free milk in TTBS | 5% w/v fat-free milk in TTBS | 1:1000 |
| **Anti-Merlin (Thermo Scientific, PA5-35316)** | 5% w/v fat-free milk in TTBS | 2% w/v fat-free milk in TTBS | 1:200 |
| **Anti-Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH) (Millipore, MAB374)** | 5% w/v fat-free milk in TTBS | 5% w/v fat-free milk in TTBS | 1:3000 |

**Table 2. Secondary antibodies used against each primary antibody for Western Blot**.

| Primary antibody | Secondary antibody | Diluted in | Dilution |
|---|---|---|---|
| **Anti-AXL** | Goat anti-rabbit IgG HRP (Invitrogen, 65-612) | 3% fat-free milk w/v in TTBS | 1:5000 |
| **Anti-EGFR** | Goat anti-rabbit IgG HRP | 3% fat-free milk w/v in TTBS | 1:5000 |
| **Anti-Vimentin** | Goat anti-rabbit IgG HRP | 3% fat-free milk w/v in TTBS | 1:5000 |
| **Anti-E-cadherin** | Goat anti-Mouse IgG HRP | 3% fat-free milk w/v in TTBS | 1:5000 |
| **Anti-YAP** | Goat anti-rabbit IgG HRP | 3% fat-free milk w/v in TTBS | 1:5000 |
| **Anti-Merlin** | Goat anti-Mouse IgG HRP (Invitrogen, G2140) | 3% fat-free milk w/v in TTBS | 1:5000 |
| **GAPDH** | Goat anti-Mouse IgG HRP (Invitrogen, G2140) | 3% fat-free milk w/v in TTBS | 1:5000 |

## Immunocytochemistry staining

The immunocytochemistry staining was used to identify YAP localization. Parental HCC827 and H1975 along with HCC827/ER and GR as well as H1975/OR were seeded directly onto Superfrost Plus® glass slides (Thermo Scientific, I6172PLUS) and left overnight in a petri dish with sterile D-PBS to prevent media evaporation. The following day the slides were washed twice with D-PBS before being fixed with 4% v/v paraformaldehyde in D-PBS for 20 minutes. Slides were washed again 3 times for 5 minutes with D-PBS then permeabilized in 0.3% v/v Triton-X100 (Sigma, T8787) in D-PBS for 5 minutes at room temperature. Slides were washed again 3 times and blocked with 5% v/v goat serum (Thermo Fisher, PA1-46189) in D-PBS for 60 minutes. Slides were gently blotted on tissue paper to remove goat

serum then incubated in primary antibody anti-YAP 1:500 (Thermo Scientific, PA1-46189) in 5% v/v goat serum in D-PBS or blocking solution for secondary antibody controls for 60 minutes. Slides were washed three times again and incubated in goat anti-rabbit IgG Alexa Fluor® 594-conjugated secondary antibody (Thermo Scientific, A-11037) 5 µg/µl in blocking solution and left for 60 minutes in the dark. Slides were washed again as before and excess D-PBS removed before staining slides with ProlLong® Gold Antifade Mountant with DAPI nucleus dye (Thermo Scientific, P36931) for 10 minutes. Slides were imaged using the Zeiss AxioImager M1 with the software AxioVision 4.8.2.

## YAP siRNA knockdown for drug-combination treatment

For the siRNA-drug combination treatment of EGFR TKI-resistant sub-lines Lipofectamine RNAiMAX (Thermo Scientific, 13778075) was used. A density of 5,000 cells per well were seeded into a 96 well plate with four replicas per treatment group. The upper four wells of the 96 well plates served as the YAP siRNA-drug combination, while the lower four wells served as the negative siRNA-drug combination controls. Cells were left to adhere overnight in their respective media. RNAiMAX-siRNA complex was made by diluting either Flexitube YAP siRNA (Qiagen, S104438651) or non-targeting negative siRNA (Qiagen, 1022076) in Opti-MEM (Thermo Scientific, 31985070) and mixing with RNAiMAX lipofectamine at the manufacture's specifications. The RNAiMAX-siRNA complex was incubated at room temperature for 5 minutes. The media was removed from the wells and cells washed once with warm sterile D-PBS to remove residual drugs. RNAiMAX-siRNA complex was mixed with fresh media + supplements and loaded into the wells. Cells were incubated for 48 hours. After two days half-log drug dilutions ranging from 10 µM to 0.001 µM were mixed with media + supplements and loaded into the wells and incubated for a further 3 days before assessing cell viability using the resazurin assay previously described. For data interpretation YAP siRNA was normalised to their negative siRNA controls and presented as a bar graph in GraphPad V7. A student's t-test with the parameters of two-tailed and assuming unequal variance was used to evaluate the difference between the treated and control groups.

Western blot analysis was used to confirm siRNA knockdown of YAP. Drug-resistant sub-lines were seeded at a density of 2.5 x $10^5$ per well of a 6 well plate and incubated overnight in their relevant media + supplements. The following day media was removed and

replaced with RNAiMAX-siRNA complex as above but scaled for 6 well plates. Cells were left for 5 days before harvesting using WB lysis buffer as before.

## RT-qPCR

Reverse transcription quantitative PCR (RT-qPCR) was used to quantify and differentiate between mRNA expression levels. A cell density of 2.5 x $10^5$ was seeded into a well of a 6 well plate and left to adhere overnight. HCC827 and H1975 were seeded with media + supplements while all drug-resistant sub-lines were seeded in their respective media. For siRNA knockdown experiments cells were treated the following day as with the western blot. Total RNA was isolated (along with DNA for sequencing the EGFR exons 18-21) using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, 80224) at their specifications, and stored at -80$^o$C until use. Total RNA was quantified using NanoDrop (Thermo Scientific, 3300). For the PCR 500 ng of total RNA was mixed with High Capacity cDNA Reverse Transfection kit (Thermo scientific, 4368814) on ice. The PCR cycle was ran once for:

25$^o$C for 10 minutes
37$^o$C for 3 x 40 minutes
85$^o$C for 50 seconds
4$^o$C hold

All cDNA PCR products were kept at 4$^o$C overnight. For the qPCR procedure, 2 µl of cDNA product was mixed with 1x TaqMan Gene Expression Master Mix (Thermo Scientific, 4369016) and one of the following: *PIK3CA* (HS00180679); *PIK3CB* (HS00178872); *PIK3CD* (HS00192399); *PIK3CG* (HS00176916); *YAP* (HS00902712); *Beta-actin* (HS99999903); RPL32 (HS00851655) all with FAM-conjugated fluorochrome; and *GAPDH* (4326317) conjugated with VIC-BG fluorochrome. All of the previous were purchased from Applied Bioscience. All were diluted to 1x in $dH_2O$ and ran for 40 cycles at:

50$^o$C – 2 minutes - annealing
95$^o$C – 10 minutes - denature
95$^o$C – 15 seconds - denature
60$^o$C – 1 minute - extension

Experiments were ran in triplicates and repeated. Data analysis was performed using the $2^{-\Delta\Delta^{CT}}$ method (Livak and Schmittgen, 2001). Data was pooled and presented as a box-whisker plot using GraphPad with standard error means (SEM±).

## Tyrosine Kinase Phosphorylation array

The PamChip phosphorylation array targets the phosphorylation residues on 144 peptides for phosphorylation pathway activation or suppression. This was used to evaluate downstream phosphorylation of PI3K. For baseline experiments both parental cells and all drug-resistant sub-lines were seeded at a cell density of 2.5 x $10^5$ per well in a 6 x well plate and incubated in media + supplements along with 0.1% v/v DMSO (for parental cells only), or with EGFR-TKI (erlotinib, gefitinib or osimertinib) for the drug-resistant sub-lines overnight. The following day the media was replaced by erlotinib (3.5 μM), gefitinib (2.5 μM) or osimertinib (2.5 μM) at 0.1% v/v DMSO. Parental cells were incubated with 0.1% v/v DMSO and incubated for three days. On the third day media was removed and cells washed once with warm sterile D-PBS. Cells were incubated in media + supplements with reduced 2% v/v FBS and 0.1% v/v DMSO for three hours. Cells were then washed three times with ice-cold D-PBS and harvested using cell scrapers and D-PBS on ice. The centrifuge was cooled to 4$^{o}$C and cells were placed into pre-frozen microcentrifuge tubes and centrifuged at 300 x g for ten minutes. Supernatant was aspirated and replaced with M-PER lysis buffer. The cells were incubated on ice for 20 minutes and centrifuged at 4$^{o}$C for 15 minutes at 13,000 x g. Supernatant was collected and stored at -80$^{o}$C.

For inhibition and stimulation experiments only HCC827/ER, HCC827/GR, HCC827/OR and H1975/OR were used. As with the baseline experiment, 2.5 x $10^5$ cells were seeded per well in a 6 well plate and incubated in media + supplements with erlotinib (3.5 μM), gefitinib (2.5 μM) or osimertinib (2.5 μM) and left to adhere overnight. The following day the media was removed and cells were washed twice with warm sterile D-PBS. Each sub-line was divided into two treatment groups: those that would receive EGFR-TKI and those that would be treated with BGB324. For the EGFR-TKI treatment groups HCC827/ER were treated with 3.5 μM erlotinib, HCC827/GR with 2.5 μM gefitinib; HCC827/OR and H1975/OR were both treated with 2.5 μM osimertinib. Each sub-line counterpart were treated with 1 μM BGB324 – all with a final DMSO concentration of 0.1% v/v. All sub-lines were incubated for three days. On the third day media was removed and cells were washed twice

with warm sterile D-PBS, cells were incubated for 2.5 hours media + supplements with reduced FBS to 2% v/v. After 2.5 hours 60 ng of EGF was given to the EGFR-TKI treatment group, and 100 ng Gas6 was given to the BGB324 treatment groups. Cells were incubated for a further 30 minutes before harvesting with M-PER lysis buffer for as above.

PamChip analysis was performed at Akershus University Hospital EpiGen laboratory by Dr. Lise Berven. The experiment was repeated once more and results were evaluated in a heatmap using Rstudio before selecting phosphoinositide dependant protein kinase (PDK1) $tyr^{9/373/376}$ as this is a downstream target of PI3K signalling. Repeats were pooled and presented as a Box-Whisker plot using Graphpad Prism software v.7. RStudio was used to transform the data into $\log_{10}$ and process in the "*pheatmap*" package to generate a heatmap.

## Wound heal and doubling time assays

A 96-well plate was used for the wound heal assay. All sub-lines were seeded at a density of 15,000 cells per well with three replicas and left overnight in their respective media. The following day the cells were washed twice with D-PBS and incubated with one of the following: two siRNAs against *PIK3CD* (Thermo Scientific, S102223809 and S102223816), *YAP*, or negative non-targeting siRNA. Cells were incubated for three days. The IncuCyte® Cell Migration kit (Essen Bioscience, 4493) was used to make uniform scratches followed by two washes with D-PBS. Cells were incubated with media + supplements (without drug or siRNA) and live microscopy taken automatically every 3 hours for 42 hours in three defined well locations and measured using IncuCyte 2011A software (Essen Bioscience). The experiment was repeated and the data was pooled with a calculated mean, and presented as a linear graph plotting time (hours) against wound distance (microns). Error bars were SEM± and treatment groups were assessed using calculated velocity.

The doubling time assay was conducted similar to the wound healing assay to assess if growth rates were different between treatment groups. A cell density of 5,000 per well with three repeats were set up as with the wound heal assay. The 96-well plate was left for 24 hours to allow cells to adhere with a media change the following day and visual assessment of cell density before adding siRNA as described above and incubated with images taken every 3 hours. Repeats were pooled and the mean was calculated. The data was presented as a growth curve using GraphPad v.7 with doubling times displayed. Error bars were SEM±.

## Patient biopsy staining and gene analysis

For the YAP tissue microarray (TMA) staining, 474 patient biopsies were prepared from paraffin-embedded biopsy material from both normal lung and cancerous tissue of the same patient. Punctures were made using the Beecher Instrument Micro-Arrayer technology. Punches were deparaffinised by heating the samples in Flex Retrival Solution High (need manufacturer info) to 97$^{o}$C for 20 minutes before cooling to 65$^{o}$C. Sections were sliced at 4 µm and placed onto slides for fixing and staining using the EnVision+System-HRP (DAB) (Dako, K4007) at the manufacturer's recommendations. Slides were stained for YAP (Thermo Fisher, PA1-46189) at 1:200, and 5 µg/ml AXL (R&D Systems, AF154) for 30 minutes. Slides were washed and stained with either anti-rabbit or anti-goat HRP-conjugated secondary antibody for 30 minutes before being counter stained with DAB-Chromogen solution for 10 minutes. Finally, slides were stained with Hagens haematoxylin for 15 seconds and mounted with xylene-based glue.

For the *PIK3CA*, 308 early stage SCC patient biopsies were taken at time of surgery between 2003 and 2013 with follow-up. DNA was isolated from fresh-frozen tumour tissue and adjacent non-tumour normal tissue for controls off-site. P110α mutation detection was conducted using the Cobas-platform along with SNaPshot assay to evaluate other oncogenic markers.

## Survival and prognosis statistics

Statistics used for survival curves were time to relapse (TTR) and overall survival (OS). TTR were calculated from date of surgery to first metastasis or recurrence. OS was calculated from date of surgery to time to death. Data was calculated in GraphPad Prism software v6 and v7 using the Log-Rank Kaplan-Meier assessment. The chi-square statistic was calculated in GraphPad to compare YAP expression to patient characteristics, setting the statistical significance $\alpha = 0.05$.

## Ethics approvals

All patients' biomaterial used in this thesis was ethically approved by the Regional Ethics Committee (No. 2009/1904/REK/ Sør-Øst B). Patients signed informed consent forms prior to donating material for research.

# Summary of individual papers

## Paper I

Three-hundred and eight patients diagnosed with squamous cell carcinoma were examined for *PIK3CA* hotspot mutations on exons 9 and 20. Isolated DNA from biopsy material at time of surgery was sequenced using either the SnapShot, Cobas system, or both. Results showed 11.4% (n = 35) harboured *PIK3CA* mutations, with 80% (n = 28) harboured exon 9 helical domain mutations, while 14.3% (n = 5) were located on exon 20 kinase domain. Further discrimination between exon 9 mutations revealed 60% of patients (n = 21) had the E545K mutation, while 20% (n = 7) had the E542K mutation. The overall survival favoured of patients harbouring *PIK3CA* mutations than those with wild-type (p = 0.04 Kaplan-Meier). Further analysis showed mutations outside of the E545K had improved time to relapse than wild type or the E545K mutation (p = 0.04). It was concluded that mutations in the *PIK3CA* may be a protective factor with improved patient survival than wild-type, which may reduce the need for adjuvant therapy following surgery. Also, PD-L1 expression was less common in patients with *PIK3CD* mutations than in those with wild-type; although no difference in survival was observed with PD-L1 expression.

## Paper II

This paper examined EGFR TKI resistance using the NSCLC AC cell lines HCC827 and H1975. The HCC827 cell line harbours the EGFR ΔE746-A750 deletion and is sensitive to erlotinib and gefitinib, while the H1975 cell line harbours the T790M and L858R mutation and is resistant to first and second generation EGFR inhibitors, but sensitive to osimertinib treatment. YAP expression and nuclear localization was assessed following the successful generation of HCC827/ER and HCC827/GR, and H1975/OR. A difference in phenotype was observed between the HCC827/ER and HCC827/GR. The HCC827/ER was able to undergo full EMT while HCC827/GR resembled the parental cell line and was only in partial EMT state. This was evaluated using western blot and showed HCC827/ER had down-regulated E-cadherin and up-regulated vimentin. However, HCC827/GR maintained E-cadherin expression indicating this sub-line had not fully transformed successfully into a mesenchymal state. A similar pattern was also seen in the H1975/OR sub-line where they resembled their

parental line and yet had a reduced vimentin expression. Immunocytochemistry was used to evaluate if YAP was localized to the nucleus, as this co-transcription factor was also up-regulated as a protein and also in mRNA data. All sub-lines exhibited nuclear localization of YAP and thus was considered active. Using a combination of siRNA targeting YAP and reintroduction of EGFR TKI sub-lines responded to the drugs after normalising to their negative siRNA controls. Drug dosage from 1 μM onwards showed a statistical significance using a student's t-test ($p = <0.05$). These results showed that EGFR TKI treatment can lead to different phenotypes that all appear to utilize YAP as a mechanism of drug resistance. Also, drug-resistant NSCLC AC can be sensitized to EGFR inhibitors following YAP siRNA-mediated knockdown *in vitro*. In conclusion, YAP presented itself as a plausible mechanism of EGFR TKI resistance and a potential therapeutic target for further development in NSCLC ACs.

## Paper III

To further understand the differences between the EGFR TKI resistant sub-lines following YAP nuclear localization, evaluation of PI3K catalytic sub-units were assessed. Using RT-qPCR it was revealed that *PIK3CD* was amplified in the HCC827/ER sub-line but not in the HCC827/GR or H1975/OR sub-lines. Evaluation of the HCC827/OR sub-line revealed *PIK3CD* was also amplified in these sub-lines along with YAP nuclear localization and had undergone full EMT. To further evaluate the protein expression differences between the EGFR TKI-resistant sub-lines, a CyTOF analysis focused on PD-L1 was conducted. Results showed HCC827/ER and HCC827/OR had a greater expression of PD-L1 while HCC827/GR had little expression. No PD-L1 was found in the HCC827 parental lines. Using siRNA-mediated knockdown of YAP it was found that *PIK3CD* responded and was down-regulated in the HCC827/ER and HCC827/OR sub-lines but not in the HCC827/GR or H1975/OR suggesting the full EMT phenotype may require *PIK3CD* expression, which is mediated by YAP. However, interestingly all sub-lines had YAP expression yet only two out of the four sub-lines were able to amplify *PIK3CD*. Due to limitations of p110δ-specific antibodies, *PIK3CD* protein expression could not be assessed and instead direct siRNA targeting *PIK3CD* using two different siRNAs was used. Results showed *PIK3CD* knockdown reduced cell migration of all sub-lines. Silencing *PIK3CD* in the doubling time assay resulted in slower cell division in HCC827/ER and HCC827/OR but not in the HCC827/GR. Silencing

YAP did have a negative effect on but the HCC827/GR. In conclusion, *PIK3CD* may be regulated by YAP in NSCLC AC following drug-resistance, which is used to aid in migration and cell proliferation. The differences in phenotype may also indicate drug effects or micro-RNA expression/down-regulations.

## Paper IV

A lot of research has focused on YAP as a poor prognostic factor on advanced cancers, including lung cancers. It was therefore an interest to know if YAP could be used as a prognostic marker in early stage lung cancers. Using immunohistochemistry staining for YAP in 474 lung cancer patients 371 (78.3%) stained positive for YAP expression, of those patients 56 scored high for YAP (11.8%) and 315 (66.5%) scored for low YAP expression; the remaining 103 (21.7%) patients were negative for YAP expression. No association was found between YAP expression and age, smoking status or gender (chi squared $p = >0.05$). All lung carcinoids and SCLC patients were negative for YAP expression. No difference in overall survival (OS) was found between high, low and negative YAP expression in all cancers. It was concluded that YAP isoforms may differ in early and advanced stage lung cancers which may explain why the results differed in survival. Emphasis on understanding the YAP 1-1 and YAP 1-2 biological functions along with p73 expression and activation may reveal more understanding on how YAP functions as a tumour suppressor and oncogene in patient survival following surgical treatment.

# Discussion

## Methods considerations

### *PIK3CA* Patient material

Out of 308 patients 35 patients (11.4%) were positive for *PIK3CA* mutations. This limited the number of events in the survival analysis. Most of the patients with *PIK3CA* mutations were diagnosed with early stage SCC, which have a better prognosis over later stages. The increase of patients increases the confidence of the results. To increase confidence in the data in Paper I it would have been better to have included more patients with *PIK3CA* mutations to equal the number of wild-type. The PD-L1 expression frequency was mostly in wild-type *PIK3CA* patients and in very few mutated cases. Again, the limited number of patients with *PIK3CA* mutations probably led to the reduced number of PD-L1 positive patients in that cohort. It is possible to request patient biomaterial from other groups and expand the material from Paper I. However, 308 patients biomaterial was used from several local hospitals. If this study was to be repeated equal numbers of patients with *PIK3CA* mutations and wild-type would need to be included.

### The choice of cell lines and generation of drug-resistant sub-lines.

The cell lines chosen were HCC827, which have the exon 19 ΔE746-A750 in frame deletion and are responsive to EGFR-TKI treatments, and H1975 which harbours the T790M gatekeeper mutation rendering them resistant to first generation EGFR-TKIs and thus candidates for osimertinib treatment. Ideally two more cell lines would have been excellent to add, such as the A549 with wild type EGFR. In addition, it was of interest to find YAP following drug treatment. It is unknown which AC cell lines harbour activated YAP since this is not well documented. The use of some SCC cell lines to identify YAP expression along with *PIK3CA* mutations would have also been of benefit.

The EGFR TKI-resistant HCC827 and H1975 sub-lines were generated by increasing concentrations of drug until proliferation was seen. These took place on large culture dishes until small colonies of cells were seen to proliferate. At that point the colonies were isolated

and expanded further. This was to try and reduce heterogeneity within the colonies. Another option of achieving this was to perform single cell cloning. This is achieved by either serial dilution of cells until a single cell is left, or using a cell sorter to isolate single cells. Both of these methods are timely and do not necessarily result in a range of phenotypes. It is also possible to use small hairpin RNA (shRNA) to insert genes known to cause drug resistance. This would be a much quicker but the main issue with that method are the pharmacokinetics of the drug within the cell. As shown between the HCC827/ER and HCC827/GR sub-lines, erlotinib resistance resulted in an EMT phenotype while gefitinib-resistant cells did not.

## Protein identification

Initially, western blot was used to identify EMT biomarkers and also YAP expression. This was fine for simple identification purposes but not ideal for quantification. This would have been especially interesting if an antibody against *PIK3CD* was reliable. Two antibodies against *PIK3CD* were tried and tested but neither worked even on the control 293 T-cells, which expresses *PIK3CD* and utilizes this protein in migration and downstream processes. It was therefore capable to use qPCR to quantify *PIK3CD* both with drug and with siRNA knockdown of *YAP* and *PIK3CD*. Phosphorylation antibodies have been used before to show whether YAP is active or inactive. It was chosen to identify YAP in the nucleus by using immunocytochemistry. By using this method YAP can be seen in the nucleus or withheld in the cytoplasm or both. It was argued that this method alone should be treated with caution since the YAP-TEAD or YAP-p73interaction was not directly evaluated. Using RT-qPCR with probes targeting *CTGF* and *AREG* would confirm activated YAP. However, siRNA-mediated knockdown of YAP significantly increased drug-resistant sub-lines to EGFR inhibitors and *PIK3CD* expression.

## siRNA targeting YAP over experimental drug inhibition.

It was shown that siRNA-mediated *YAP* knockdown re-sensitised drug-resistant sub-lines to EGFR inhibitors. Short-term knockdown was sufficient for the experiments as results showed the reintroduction of EGFR TKI reduced cell viability. Because the cells needed to be under

drug selection during incubation the use of long term gene silencing via short hairpin RNA or CRISPR could mean that drug selection can no longer continue due to toxicity.

Some researchers have proposed repurposing drugs to target and inhibit YAP-TEAD interaction with some interesting results. Therefore, using a range of different drugs and compounds that have been shown to interact with YAP would have been of interest. However, verteporfin was tried and did not inhibit YAP as reported (Wang et al., 2016a).

## Wound healing and doubling time

Wound healing assays are a cheap and quick way of identifying cells which are capable of migration over a surface. Ideally, using matrigel-coated invasion inserts with a low percentage of FBS in the upper compartment and regular 10% v/v FBS in the lower would have greatly increased the confidence of *PIK3CD* knockdown effect on cell invasion. In this thesis a basic migration using a wound heal assay was found to be acceptable and did give some more understanding as to how *PIK3CD* functions in the mesenchymal sub-lines.

## Patient biopsy selection for YAP in early stage lung cancers.

Following the identification of YAP as a plausible mechanism of EGFR-TKI drug-resistance in advanced NSCLC, patient biopsy material was used to determine if YAP could be used a prognostic biomarker in early stage lung cancer. The results were different to the published data where YAP was identified in biopsies and strongly associated with poor survival outcome (Kim et al., 2015b). Biopsy material was taken at time of surgery prior to patients having routine chemotherapy. This is a large factor which may have influenced the data. However, for such a study to harvest biomaterial following drug treatment, research and consent forms would need to be approved. How biopsies will be taken and to what discomfort to the patient over the course of treatment is also a factor, which may result in patients leaving the study. These are challenges facing clinical research.

# Results discussion

Lung cancer is responsible for 19.4% of all cancer deaths worldwide (Ferlay et al., 2015). Early stage NSCLC are easier to treat with good patient survival. Patients with advanced NSCLC provide therapeutic challenges and have poor survival prognosis. Even with the advances of chemotherapy, targeted therapy and careful patient selection, relapse and resistance is common. Therefore the focus of this thesis was to understand why some NSCLC patients with early stage diagnosis have prolonged survival following surgery, while others do not. And for the advanced inoperable cancers, understanding mechanisms by which the cancers become resistant to targeted therapy may provide new therapeutic opportunities for future research.

## Prognostic biomarkers in lung cancer

The data presented in Paper I found patients harbouring mutations in the *PIK3CA* had better survival than their wild-type counterparts following treatment by surgery. These results have also been found in breast cancer with *PIK3CA* mutations (namely on exon 9) having favourable time to recurrence post surgery (Dupont Jensen et al., 2011). The immune-suppressor protein PD-L1 was evaluated in the SCC patients and was found in low frequency in patients with *PIK3CA* mutations (25%) and in high frequency in wild-type *PIK3CA* patients (40%). This has also been found in breast and gastric cancers where *PIK3CA* mutated patients had low PD-L1 expression (Gatalica et al., 2014, Böger et al., 2016). These results suggest mutations in the *PIK3CA* could be used as a prognostic biomarker of improved survival for patients with surgically resected early stage SCC. However, in advanced SCC, *PIK3CA* mutations have been found to be associated with aggressive phenotype and poor survival (Paik et al., 2015). The results in Paper I along with those published (above) show mutations in the *PIK3CA* prognosis depends upon stage at diagnosis. Therefore other mechanisms may need to be in place for *PIK3CA* to be oncogenic. Interestingly, YAP has been found phosphorylated on S127 (a target phosphorylation site for protein 14-3-3) and down-regulated in breast cancer patients harbouring exon 9 mutations, and expressed in wild-type *PIK3CA* (Ramirez-Ardila et al., 2017). YAP status was not investigated in Paper I but YAP phosphorylation on S127 along with *PIK3CA* mutations could be used to predict survival and best course of treatment.

## EGFR TKI resistant sub-line characteristics

All cell lines were assessed for their sensitivity to EGFR TKI in the first instance. The HCC827 parental cell line was sensitive to all generation EGFR TKI due to the exon 19 ΔE746-A750. The H1975 parental was resistant to erlotinib and gefitinib treatment due to the EGFR T790M and L858R mutation. This cell line did respond to osimertinib since the drug targets the T790M phosphorylation binding pocket. Osimertinib is a potent drug which took several months to establish the resistant sub-lines H1975/OR and HCC827/OR. Interestingly, generating drug-resistant HCC827 to erlotinib and gefitinib only took a few weeks. This difference in time to resistance in the cell lines may mimic the clinical features of relapse to first generation EGFR TKI. Once established, cross-resistance of HCC827/ER and HCC827/GR were checked to determine if osimertinib could be used to treat first generation EGFR drug-resistant cells. Results from Paper II, figure 1C revealed HCC827/ER and HCC827/GR sub-lines were less sensitive to osimertinib than the HCC827 parental (>200 fold difference in $EC_{50}$). Although, no long term exposure to osimertinib treatment occurred, it would have been interesting to see if the HCC827/ER and HCC827/GR sub-lines would have responded to long term treatment with osimertinib. Osimertinib has a high affinity for the T790M mutation but can also be given to patients with the exon 19 ΔE746-A750 deletion and other mutations. The HCC827/ER and HCC827/GR were tested for cross-resistance against osimertinib. Both HCC827/ER and HCC827/GR were observed to be tolerant to high concentrations of osimertinib suggesting cross-resistance to the third generation EGFR TKI. It has been suggested that additional EGFR mutations outside of the T790M can contribute to relapse of targeted therapy. Such a mutation is the C797S on exon 20 that was responsible for the failure of osimertinib treatment (Thress et al., 2015). All drug-resistant sub-lines were examined for EGFR mutations against their parental cell lines using Sanger sequencing of exons 18-21. No additional mutations were found indicating no secondary EGFR mutations had occurred.

Activation of the AXL RTK has been identified as a mechanism of drug-resistance in a range of cancer types (Zhang et al., 2012, Kariolis et al., 2017, Liu et al., 2009). Over-expression of this RTK was identified in all drug-resistant sub-lines (Paper II, figure 2). Contrary to published reports correlating AXL expression with EMT, and moreover suggesting AXL drives EMT (Asiedu et al., 2013), the results presented in this thesis could

not correlate AXL expression with EMT. This is because both full and partial EMT states were observed between HCC827/ER, HCC827/GR and H1975/OR sub-lines (Paper II, figure 2). The EMT phenotype is characterised with E-cadherin loss and vimentin expression amongst other alterations of slug and snail expression (Singh and Settleman, 2010). The H1975 parental cell line expressed AXL that increased in expression in the H1975/OR sub-line. The EMT biomarker vimentin was expressed in the H1975 parental cells but decreased in expression in the H1975/OR. E-cadherin was not expressed in either H1975 or H1975/OR. It is therefore concluded that the H1975/OR did not undergo full EMT because of the reduced vimentin expression. The HCC827/GR sub-line had AXL expression following drug resistance but was unable to undergo full EMT. The HCC827/GR was positive for vimentin but also maintained expression of E-cadherin. The expression of E-cadherin and the morphology of the HCC827/GR that resembled the HCC827 parental cell line suggested full EMT had not occurred. Further challenging published data, AXL was expressed in the HCC827/GR sub-line but was in partial EMT state. More experiments and biomarkers of EMT (slug, snail etc...) would need to be identified before a full conclusion of EMT status can be confirmed, but in this thesis loss of E-cadherin and vimentin expression suggests HCC827/GR had not undergone full EMT. It can therefore be suggested that EMT is not driven by AXL expression. To support these findings a published report showed the NSCLC cell line H3122 maintained E-cadherin expression along with vimentin after being generated to become resistant to the Alk inhibitor NMS-E628 (Gower et al., 2016). Gower *et al* used siRNA to knockdown AXL which did not affect vimentin expression or alter the EMT morphology, but this did inhibit migration and invasion capabilities. Furthermore, YAP has been shown to drive EMT alterations (Overholtzer et al., 2006). The data from the HCC827/GR characterisation showed that full EMT may not be driven by YAP, but that partial EMT may be regulated by YAP activation. In a separate study in lung metastasis, EMT was not required for invasion as a proportion of tumours negative for EMT markers were able to migrate (Fischer et al., 2015). YAP expression was observed in all sub-lines and at low expression in the parental cells (Paper II, figure 2). The HCC827 parental cell line had a clear expression of YAP in the western blot analysis which increased following EGFR TKI resistance. As YAP increased in expression so did AXL. YAP has been identified as a regulator of the *AXL* gene following interaction with TEAD (Xu et al., 2011). The YAP-TEAD interaction was not evaluated in this thesis following drug resistance, and therefore cannot confirm if YAP regulates AXL in the drug-resistant sub-lines without conducting more experiments.

Following erlotinib and gefitinib resistance, EGFR appeared to be down-regulated in both HCC827/ER and HCC827/GR (Paper II, figure 2). This may be due to internalization and degradation of the RTK following drug binding. This has been observed before in the NSCLC AC cell line HCC4006 following gefitinib resistance where the EGFR was degraded by autophagocytosis (Sakuma et al., 2013). Autophagocytosis may be the cause of decreased EGFR expression in the HCC827/ER and HCC827/GR sub-lines. If this is a true occurrence and not an *in vitro* phenomenon then to what extent the reduced EGFR expression will have on patient outcome and course of treatment would be of interest.

## The role of Yes-associated protein as a mechanism of drug resistance and tumour suppressor

It has been well-documented that YAP plays a role in oncogenesis and also in drug resistance (Hsu et al., 2016, Kim et al., 2015a, Cui et al., 2012b). Paper II and III examined YAP as a mechanism of EGFR TKI resistance in NSCLC AC. YAP was found to be over-expressed in the sub-lines HCC827/ER, HCC827/GR and H1975/OR after western blot identification along with mRNA expression. YAP is only active following translocation to the nucleus where it can interact with one of its nuclear targets. Figure 3 from Paper II shows an immunocytochemistry staining of YAP localization on the HCC827/ER, HCC827/GR and H1975/OR. It was confirmed that YAP was active as positive identification of staining in the nucleus of all drug-resistant sub-lines was seen. This coupled with the results from the western blot show YAP was over-expressed and active in the drug-resistant sub-lines. To understand if YAP was involved in drug-resistance siRNA-mediated knockdown of the co-transcription factor and reintroduction of EGFR TKI was conducted. The results showed re-challenging the drug-resistant sub-lines with EGFR TKI was successful following YAP knockdown compared to the negative siRNA control group (Paper II, figure 4 A-D). These results suggested that YAP was a mechanism of EGFR TKI-resistance in NSCLC cell lines. YAP activation as a mechanism of drug-resistance has also been found in SCLC and in a variety of cells including breast, prostate and ovarian cancer (Su et al., 2012, Kim et al., 2015a, Zhang et al., 2015).

HCC827 was generated to become resistant to osimertinib (HCC827/OR) which was able to express full EMT markers, AXL and YAP over-expression and nuclear localization. Paper III investigated the relationship between PI3K and YAP. It was interesting to find in

Paper II the difference in EMT between each sub-line regardless to YAP and AXL expression. Investigating the PI3K revealed further differences between full and partial EMT. In the first instance four PI3K catalytic subunits were examined for expression in the EGFR TKI-resistant sub-lines HCC827/OR and H1975/OR. Interestingly, HCC827/OR had 20-fold expression of *PIK3CD* and low expression of *PIK3CA*, *PIK3CB*, and *PIK3CG*. The H1975/OR did not show any dominant PI3K mRNA expression. It was hypothesized that *PIK3CD* may only be expressed in cells that had undergone full EMT since this was the only molecular difference observed in Paper II. This hypothesis was true as HCC827/ER also had ~14-fold increased expression of *PIK3CD*, while HCC827/GR did not. Lin, *et al* had shown that YAP can regulate the expression of *PIK3CB* in cardiomyocytes (Lin et al., 2015) and in another study both *PIK3CA* and YAP were required for the development of hepatocellular carcinoma (Li et al., 2015b). To test whether YAP had any regulatory function on the class I PI3K catalytic sub-units siRNA-mediated knockdown of YAP was used. Results presented in Paper III (figure 2) show *PIK3CD* down-regulation following YAP siRNA in the HCC827/ER and HCC827/OR. *PIK3CD* expression did not change in the HCC827/GR and H1975/OR. These results present a novel mechanism of YAP regulation of *PIK3CD*. Furthermore, this regulation only occurred in the sub-lines that had undergone full EMT.

The expression of *PIK3CD* is an important find, as much as YAP over-expression. This PI3K catalytic subunit is mostly found in immune cells and utilized for cell migration (Soond et al., 2010). It is also downstream of RTK and GPCR, relaying signals for cell proliferation (Zhong et al., 2013). Therefore it was of interest to understand if *PIK3CD* was required for cell migration and cell proliferation in the drug-resistant sub-lines. *PIK3CD* knockdown reduced cell migration in the HCC827/ER from 8.8 microns per hour (negative siRNA control) to 6.8-6.9 microns per hour (*PIK3CD* knockdown). This was also observed following YAP knockdown with a velocity of 6.7 microns per hour (Paper II, figure 3). The same reduction in migration velocity was also seen in the HCC827/OR from 15.6 microns per hour (negative control) to 4.6 microns per hour (YAP knockdown) and 7.7-11 microns per hour (*PIK3CD* knockdown). Interestingly, there was also a reduced migration in the HCC827/GR and H1975/OR sub-lines following *PIK3CD* knockdown. HCC827/GR velocity was reduced from 23 microns per hour (negative control) to 12.4-16 microns per hour (*PIK3CD* knockdown). The knockdown of YAP also slowed the migration down to 16.8 microns per hour. The H1975/OR migration slowed from 15.9 microns per hour (negative control) to 11.2 microns per hour following YAP knockdown, and 14-14.4 microns per hour after *PIK3CD* was knocked down. To be confident that cell migration was truly being

observed and not proliferation, a doubling time assay was conducted under the same conditions as the migration. The HCC827/ER doubling time shifted from 54.85 hours (negative control) to 268.7 hours after YAP knockdown. This shift was also observed after *PIK3CD* knockdown to 198-200 hours (Paper III, figure 4). HCC827/OR was also affected by YAP knockdown with doubling time shifting from 45 hours (negative control) to 189 hours (YAP knockdown), and 48-58 hours after knockdown of *PIK3CD*. Increased doubling time was seen in the H1974/OR following YAP knockdown from 40 hours (negative control) to 126 hours (YAP knockdown), and 52-59 hours (*PIK3CD* knockdown). The HCC827/GR did not seem to respond to *PIK3CD* or YAP knockdown with similar doubling times to their negative control. Therefore it was concluded that YAP or *PIK3CD* inhibition can reduce cell migration and doubling time, suggesting two possible therapeutic targets.

It is not clear why HCC827/GR and H1975/OR were unable to undergo full EMT or express *PIK3CD*. A suggestion why may be due to microRNA (mir) regulatory functions. It is documented that *PIK3CD* is regulated by mir-125b, mir-663 and mir-30a (Cui et al., 2012a, Li et al., 2014, Bu et al., 2017, Suda et al., 2014, Zhong et al., 2013). In colorectal cancer, mir-30b has been shown to inhibit p110δ along with KRAS (Kim and Jho, 2017). Interestingly, mir-30b is also able to inhibit EMT by targeting Snail in hepatocellular carcinoma by down-regulating vimentin and up-regulating E-cadherin (Sun et al., 2017). Snail and micro-RNAs were not the focus of this thesis but it may explain the differences between HCC827/ER, HCC827/OR and HCC827/GR. E-cadherin was down-regulated in the HCC827/ER and HCC827/OR sub-lines but not in the HCC827/GR (Paper II, figure 2). It is therefore possible that mir-30b is suppressed following erlotinib and osimertinib resistance in cells that have the exon 19 ΔE746-A750 deletion. Combined with the expression of *PIK3CD* mir-30b is of interest for further study in these sub-lines. However, the fact that H1975/OR was also resistant to osimertinib, and did not have any additional mutations outside the T790M or L858R (namely the C797S mutation) following sequencing, it is doubtful that mir-30b is expressed. The reason to this is that H1975 parental and H1975/OR did not have expression of E-cadherin to begin with. However, western blot showed reduced vimentin expression but no recovery of E-cadherin. Li, *et al* examined the oncogenic properties of mir-19 (mir-19a/mir-19b-1). The cell line HCC827 was transfected with mir-19 that resulted in an EMT phenotype (Li et al., 2015a).

Work performed using macrophage cells identified the membrane-type 1 matrix metalloproteinase (MMP-M1) regulated *PIK3CD* expression (Shimizu-Hirota et al., 2012). Furthermore, MMP-M1 remodels the extracellular matrix (ECM) and also regulates

inflammatory responses, *PIK3CD* being one of them. As well as transcribing *PIK3CD*, MT1-MMP regulates cell migration (Itoh, 2006). Another member of the MMP family is the MMP-7. This protease has been found to be regulated by YAP expression in T84 colorectal cancer cells (Nukuda et al., 2015). MMP-7 has been well-studied and found to regulate metastasis and invasion (Shiomi and Okada, 2003) and has also been found in lung cancers (Safranek et al., 2007, Safranek et al., 2009). Interestingly, microRNA-663 (mir-663) regulates and suppresses *PIK3CD* and MMP-7 (Wang et al., 2016b). It is therefore possible that the difference seen between HCC827/ER, HCC827/OR and the non-mesenchymal HCC827/GR and H1975/OR is the lack of MMP-7 and MT1-MMP. The difference may not be due to drug interaction but more likely drug selection. Similar results were also found in the glioblastoma cell line BS153. Following resistance to erlotinib, BS153 over-expressed *PIK3CD* which was also highlighted as a potential therapeutic target of erlotinib resistance in these cells (Schulte et al., 2013).

In addition to *PIK3CD* expression, Paper III revealed PD-L1 was highly expressed in the HCC827/ER and HCC827/OR. This contrast of *PIK3CA* mutations and low PD-L1 frequencies compared to high *PIK3CD* expression along with YAP nuclear localization may offer a complex relationship between these two PI3K catalytic subunits. Incidentally, PD-L1 was found to be regulated by YAP in NSCLC ACs following drug resistance (Lee et al., 2017). The results in Paper III (supplementary figure 4) did indicate that PD-L1 expression was expressed more in the full EMT sub-lines than the partial. YAP regulation of PD-L1 was not conducted but would have been interesting to observe if this was seen in the sub-lines presented in this thesis.

Many reports have shown YAP to be an oncogene that is associated with poor prognosis (Wang et al., 2010, Kim et al., 2015a, Kim et al., 2015b, Xia et al., 2014). However, some studies have shown YAP to be a tumour suppressor. Yuan, *et al* studied YAP in pre-invasive ductal carcinoma and invasive breast cancer biopsies. Here they reported that loss of YAP was associated with oncogenesis, and the *in vivo* studies confirmed YAP was acting as a tumour suppressor (Yuan et al., 2008). In a more recent study, YAP expression in breast cancer showed to improve patient survival furthering YAP as a tumour suppressor protein (Cao et al., 2017). These results support those in paper IV where YAP high, low and negative expression did not show any statistical difference in survival. It was interesting to see that YAP expression was mostly seen in AC and SCC, while SCLC and lung carcinoids had no YAP expression (Paper IV, figure 1 and Table 1). A previous study into the clinical significance of YAP also found a larger group of patients with lung AC expressed the co-

transcription factor (Wang et al., 2010). There was no association between smoking, age, or gender to YAP expression. Interestingly, although limited to the number of patients in advanced stage lung cancer, a greater number of stage IV lung cancers were seen in the high YAP group. Although no statistical significance was seen between stage and YAP expression, an increase of patient numbers grew (10.8% to 22.2%) from stages I to IV. In a reverse, biopsies negative for yap decreased in patient numbers (21.6% to 11.1%) from stages I to IV. The results from Paper IV suggest that YAP cannot be used as a prognostic biomarker in early stage lung cancers.

## YAP isoform variations and their impact on cancer

YAP has 8 splice variants which can be sub-divided into YAP1-1 and YAP1-2. YAP1-1 is characterised having one WW binding domain and YAP1-2 harbours two WW domains (Sudol et al., 1995). The WW domain locations on the YAP protein depict its range of nuclear target proteins. The WW domain has been shown to interact with TEAD, p73, and ErbB-4 (Vassilev et al., 2001, Downward and Basu, 2008, Komuro et al., 2003). Whilst YAP1-1 can interact with a range of nuclear transcription factors, YAP1-2 has a specific binding to with p73, whereas YAP1-1 does not have this capability (Gaffney et al., 2012). It can by hypothesised that a difference in splice variants may account for the different protein expression between HCC827/OR and H1975/OR. Both had YAP expression but what was not tested was the splice variant expression. This would pose a challenge to determine which isoform is expressed and which is not. Method considerations and limitations have not yet presented a viable standardized. Finch-Edmondson, *et al* studied the splice variants of YAP and concluded that it is very possible that the variation of WW domains coupled with protein-protein interactions may lead to differing biological outcomes and transcriptional activities (Finch-Edmondson et al., 2016). However, all sub-lines responded to YAP knockdown when reintroduced to EGFR TKI indicating that regardless to morphological differences between the sub-lines all can potentially be treated with YAP inhibition. This may account for variations in our data and why some sub-lines could undergo full EMT and express *PIK3CD* and others could not. Furthermore, it may highlight the range of possible outcomes of EGFR TKI failure.

## Potential treatment options for untreatable cancers

As shown in Papers II and III YAP activation may be involved drug resistance to EGFR TKIs. There are no treatment options currently available to target YAP, but some groups have already begun to repurpose current drugs that can be used to inhibit YAP.

Verteporfin is a photosenitizer and was approved in 2000 for the treatment of age-related macular degeneration (loss of vision). Some research groups have tested if this photosensitizer could be repurposed to treat drug-resistant cancers. A study in 2011 showed treating gemcitabine-resistant pancreatic cells lines with verteporfin re-sensitised the cells to the chemotherapy drug inducing cell cytotoxicity (Celli et al., 2011). Another study using verteporfin in a phase I/II clinical trial saw tumour necrosis in advanced pancreatic cancer (Huggett et al., 2014). A more recent study proposed the mechanism of action of this photosensitizer was to inhibit the nuclear localization of YAP by up-regulating the 14-3-3 protein in endometrial cancer cell lines (Wang et al., 2016a). Similar results were also found in glioma cells with an inhibition of YAP-TEAD binding (Al-Moujahed et al., 2017). However, as Wang *et al*, 2016 showed for this inhibition to occur the cell required to have the expression of wild-type p53. It is therefore interesting to note that should cancer be null for p53 or have mutations then verteporfin may not have the desired effect. Verteporfin was tested on the drug-resistant sub-lines in this thesis, in both singular and in drug-drug combination with EGFR-TKIs but not published. The sub-lines did not respond to verteporfin when used as a single treatment or in combination with EGFR TKI. For any clinical study using verteporfin, it is likely essential that patients are carefully selected based upon their p53 status.

Another group of drugs that has been used to inhibit YAP are the statins. Statins are used to lower cholesterol levels and prevent cardiovascular disease (Cannon et al., 2004) but in recent years have also been found to reduce the risk of relapse in patients with breast cancer (Desai et al., 2015) and lung cancer (Cardwell et al., 2015b). This remains controversial as other publications show limited or no protective effects of statin use (Cardwell et al., 2015a, Liu et al., 2017). Statins have been shown to inhibit YAP translocation to the nucleus by targeting the mevalonate cholesterol biosynthesis pathway, particularly the 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA) (Sorrentino et al., 2014). Furthermore, YAP activity was regulated by the mevalonate pathway coupled with mutant p53 expression. This is interesting as it highlights a difference between statins

and verteporfin where statins require mutant forms of p53 and verteporfin requires wild-type. However, because of the conflicting effects on prognosis of long term statin use it is unclear as to how this exposure will benefit cancer patients unless strict clinical trials are conducted with a select group of patients harbouring mutant p53 and YAP.

In this thesis a small experiment was conducted to determine if the immune suppressor PD-L1 was expressed following drug resistance, and after *PIK3CD* was found. The data show a difference in PD-L1 expression in HCC827/ER and HCC827/OR and less expression in HCC827/GR. Published data presented earlier showed PD-L1 was regulated by YAP (Lee et al., 2017, Miao et al., 2017, Kim et al., 2018). Patients whom have relapsed from drug therapy and harbour activated YAP may be ideal candidates for immunotherapy.

In Paper III BGB324, an AXL inhibitor currently in clinical trials, was used to inhibit AXL signalling and to determine how this affects downstream targets of PI3K signalling. This was to understand if PIK3CD was able to autophosphoylate or required stimulation. PDK1 is downstream of PI3K activation and therefore was a good biomarker of PI3K inhibition. The results showed EGFR TKI treatment of HCC827/ER and HCC827/OR did not show any inhibition on PDK1 tyrosines$^{9/373/376}$. However, PDK1 phosphorylation on tyrosines$^{9/373/376}$ was inhibited by BGB324 AXL inhibition in the sub-lines HCC827/ER and HCC827/OR. The drug-resistant sub-lines HCC8237/GR and H1975/OR did not respond to either EGFR or AXL inhibition. Phosphorylation sites on 144 proteins were assessed and it was found that BGB324 had a larger off target effect inhibiting the platelet-derived growth factor receptor and p85 (regulatory protein of PI3K). It is possible that AXL was inhibited and that recruitment of p85 to the plasma membrane was inhibited, but because PDGFR also recruits p85 this inhibition could have come from PDGFR inhibition by BGB324. It is possible that BGB324 may be a good treatment option for AXL expressing cells, but as presented, AXL is regulated by YAP in advanced cancers and therefore it may be worth targeting YAP instead.

Understanding more about how YAP functions and the biomarkers of activation pathways may help in identifying candidates for clinical trials.

# Further work

Further understanding how *PIK3CA* mutations offer benefit to patient outcome following surgery is an interesting find that requires further work; and how these mutations on exon 9 and 20 differ in *PIK3CA* downstream signalling. Using advanced stage NSCLC and to understand how these mutations differ between early and late stage cancers will be of interest for further evaluation.

The results presented in this thesis demonstrated the importance of YAP activation in NSCLC EGFR TKI-resistant sub-lines. So far eight isoforms of YAP have been identified and sequenced, but as of yet their biological importance has not been identified. Researchers are actively trying to identify a specific YAP inhibitor. Understanding YAP isoform expression is just one challenge of finding a specific YAP inhibitor. Furthermore, pre-clinical evaluation of off-target effects of using specific inhibitors will need to be addressed as YAP is also required for liver regeneration (Lu et al., 2018). Therefore understanding how targeting YAP will affect other somatic cellular activities will need to be evaluated.

The immune suppressor PD-L1 may be regulated by YAP expression and therefore understanding how this occurs, under what conditions, and which YAP variant is responsible for this expression will need to be addressed. Further work on a large range of cell lines to confirm these findings will need to be conducted in the first instance and prior to any clinical work. It was found that the AXL TKI BGB324 (R428) was successful at inhibiting PDK1 phosphorylation. However, upon further analysis of the heatmap from the phosphorylation assay other receptors outside of the AXL kinase were also being targeted. Therefore this casts doubts as to whether AXL inhibition is really having an impact on downstream signalling. It is also worth returning to YAP regulation of AXL and *PIK3CD*. The results in this thesis along with published work showed AXL and *PIK3CD* expression to be correlated YAP activation following drug-resistance. Therefore, targeting YAP would suggest a better option than targeting AXL if these off-target effects (PDGFR inhibition for example) are also seen in patient biopsies. By targeting YAP with specific inhibitors may circumvent direct AXL therapy and could also down-regulate *PIK3CD*, restricting the PI3K-Akt-mTOR pathway and cell migration. Also, as presented in paper IV, YAP did not have an effect on survival in early stage lung cancer patients. Using more downstream targets of YAP, p73, TEAD and CTGF for example, could offer some biological insight as to how this co-transcription factor is functioning in early stage and advanced stage lung cancers.

# Conclusion

This thesis explored prognostic biomarkers in early stage lung cancer and mechanisms of relapse to EGFR targeted therapy and advanced cancer models.

Early stage lung cancers are treated with surgery as standard care. Even though this is regarded as a curative treatment, patients can still relapse and have recurrence of their tumour. The identification of patients harbouring *PIK3CA* mutations have better prognosis following surgery. These results may help clinicians reduce the use of additional treatments for patients harbouring mutations over those with wild-type.

Even with advances in targeted therapy and patient selection for treatment, recurrence and failure of therapy is inevitable. The Yes-associated protein was shown to be a mechanism of resistance to first and third generation EGFR TKI. Targeting YAP re-sensitized drug-resistant sub-lines to their EGFR inhibitors. Further evaluation of the drug resistant mechanism revealed the PI3K catalytic sub-unit *PIK3CD* was regulated by YAP in the sub-lines that had undergone full EMT. This PI3K expression was potentially active and cells responded to *PIK3CD* silencing with reduced cell migration and doubling time. Therefore two potential therapeutic targets for future drug development were identified in this thesis.

YAP is not associated with prognosis in early stage lung cancer which has highlighted the need for further research in YAP isoform biological mechanisms prior to inhibitor development.

In summary, *PIK3CA* is a useful biomarker for predicting prognosis in early stage SCC. YAP may be a mechanism of drug resistance in advanced NSCLC but not a good biomarker in early stage lung cancer prognosis.

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I

# Paper I

**PIK3CA Mutations as Prognostic Factor in Squamous Cell Lung Carcinoma.**

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# *PIK3CA* mutations as prognostic factor in squamous cell lung carcinoma

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ABSTRACT

*Objectives:* Mutation in the *PIK3CA* gene is reported frequent in squamous cell carcinomas of the lung, but its potential prognostic role is still obscure. We have studied the prognostic importance of *PIK3CA* mutations as well as the relation to other markers in a large number of early stage lung cancers of squamous carcinoma subtype.

*Patients and methods:* Tumour tissue was obtained from 308 consecutively operated lung cancer patients with squamous cell carcinoma in the period 2003–2013. DNA was isolated according to standard procedures, and mutation analysis was done with either the SnapShot method and/or using *PIK3CA* specific primers in the Cobas system. PD-L1-expression was analysed with immunohistochemistry

After thorough follow-up (median 67.6 months), overall survival and time to relapse was calculated.

*Results:* Tumour tissue from 102 females and 206 males were analysed. 167 (54.2%) were in stage I, 96 (31.2%) in stage II and 45 (14.6%) in stage III. *PIK3CA* mutation was found in 35 (11.4%) patients, most frequently in exon 20. There were no differences in sex, stage or smoking behaviour between mutated and non-mutated cases. Patients with *PIK3CA* mutations had a significantly longer overall survival ($p = 0.042$) and time to relapse ($p = 0.030$) than non-mutated cases, and the difference in time to relapse was also retained in stage I-cases ($p = 0.044$). PD-L1-expression was less frequent among mutated cases.

*Conclusion:* Our results indicate that *PIK3CA* mutations may confer a survival advantage in early stage squamous cell lung cancers, but further work is needed to confirm this finding.



## 1. Introduction

Lung cancer remains the leading cause of cancer-related deaths in both genders [1]. Genetic aberrations are regarded both prognostic and predictive in NSCLC, and are found with various frequency and different significance in the subtypes. Hitherto, targets for therapy are found primarily in adenocarcinomas, mainly *EGFR* mutations and *ALK* translocations, but also aberrations with lower frequencies, involving *e.g. BRAF*, *ROS1* and *RET* [2]. *PIK3CA* mutations are potentially therapeutically targetable and are frequently found in squamous cell carcinomas of the lung, which accounts for a third of all the lung cancers, as well as in breast cancer [3], head and neck cancer [4] and other cancers [5], whereas it is relatively infrequent in lung adenocarcinomas [6].

The lipid kinase phosphatidylinositol-3 kinase family (PI3K) is involved in a multitude of normal cellular processes, including the activation of the serine/threonine kinase AKT, which in turn activates a number of factors including mTOR [7]. The PI3K-Akt-mTOR pathway is central in the regulation of multiple cancer-relevant regulatory processes as cell survival, cell growth and cell cycle progression, and somatic mutations in this pathway are frequently found in cancers, hence being attractive targets for therapy [8,9]. PI3K consists of a catalytic (p110) and a regulatory subunit (p85). There are various isoforms of each subunit, and the catalytic subunit are encoded by three genes, *PIK3CA*, *PIK3CB* and *PIK3CD* [10], of which *PIK3CA* is most frequently mutated in cancers.

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The most common mutation hotspots on the *PIK3CA* gene are located in the helical domain, typically E542K and E545K on exons 9, and less frequently in the kinase domain, H1047R on exon 20 [6]. The helical domain mutations are thought to exert their activating effect through "unlocking" the inactive conformation, whereas kinase domain mutations changes the interaction between the protein and the cellular membrane thus providing access to the kinase substrate [11].

The prognostic role of *PIK3CA* mutations is still debated, and some studies have shown *PIK3CA* mutations to confer a survival advantage in some cancers, *e.g.* breast [12,13], whereas studies in other cancers, including lung adenocarcinomas, have indicated a correlation with poor prognosis [14]. So far little if anything is known of the potential prognostic role of *PIK3CA* mutations in squamous cell carcinomas of the lung. Clinical trials targeting PIK3CA are already ongoing [15–17], thus knowing the prognostic role of this aberration is warranted. PIK3CA is a known regulator of PD-L1 in cancer cells, probably involving both transcriptional and post-transcriptional mechanisms [18,19]. Given the new immunotherapies interfering with the PD1-axis, it is of interest to study the correlation of PD-L1 expression and *PIK3CA* mutations.

We have studied the frequency and prognostic importance of *PIK3CA* mutations in a large number of early stage lung cancers of squamous carcinoma subtype, and correlated these findings to clinic-pathological features, including PD-L1 expression.

## 2. Patients and methods

Tumour tissue was obtained from 308 consecutively operated early stage lung cancer patients with squamous cell carcinoma at Oslo University Hospital, Norway, in the period 2003–2013. No pre-defined inclusion or exclusion criteria were defined. None of the patients had undergone neoadjuvant therapy, whereas adjuvant therapy was given according to international guidelines [20]. Smoking information was collected, and categorized as never-smoking (less than 100 cigarettes smoked in lifetime), former smokers (stopped smoking one year or more before diagnosis of lung cancer) or current smokers.

DNA was isolated from fresh frozen tumour tissue stored in a dedicated biobank (n = 91) or archival paraffin embedded formalin fixed tissue samples (n = 217) according to standard procedures, and mutation analysis was done with either the SnapShot method (Life Technologies, Foster City, CA, USA), or using *PIK3CA* specific primers in the Cobas 4800 platform (Roche Diagnostics, Basel, Switzerland). The tumour cell content in the specimens was found to be more than 70% in most samples. The SnapShot-analysed samples were also analysed on the Cobas-platform with identical *PIK3CA*-results. The SNaPshot assay for evaluation of multiple oncogenic mutations in *APC, AKT1, BRAF, CTNNB1, EGFR, FLT3, JAK2, KIT, KRAS, MAP2K1 (MEK1), NOTCH1, NRAS, PIK3CA, PTEN*, and *TP53* was performed by amplification using 13 multiplexed PCR reactions followed by single nucleotide base extension reactions. The products were separated by capillary electrophoresis and analysed using GeneMapper 4.0 as has been previously described [21,22].

Immunohistochemical staining for p40 (Calbiochem, Cat.No PC373) was performed in order to verify the squamous histology by using the EnVisionTM FLEX + detection system from Dako (Dako, Glostrup, Denmark) according to manufacturer's protocol. Nuclear staining was evaluated and intensity scoring reported as none (0), weak/intermediate (1+) and strong (2+), the latter two regarded positive. PD-L1-staining (Ventana SP142 assay) was evaluated on tumour-infiltrating immune cells (IC) or tumour cells (TC). Positivity was defined as detectable staining on ≥5% of either IC or TC.

**Table 1**
Distribution of mutations found in the SnapShot-analysed cohort (n = 91).

| | N | % |
|---|---|---|
| No mutation | 70 | 76.9 |
| PIK3CA | 10 | 11.0 |
| TP53 | 7 | 7.7 |
| KRAS + TP53 | 1 | 1.1 |
| PTEN | 1 | 1.1 |
| JAK2 | 1 | 1.1 |
| BRAF (G469A) | 1 | 1.1 |

**Table 2**
Patient characteristics of the SnapShot cohort.

| | No mutation (n = 70) | *PIK3CA* mut (n = 10) | Other mut (n = 11) |
|---|---|---|---|
| Age (median, range) | 66.4 (43.2–82.4) | 71.0 (65.1–81.3) | 66.0 (45.9–81.0) |
| | *N* (%) | *N* (%) | *N* (%) |
| *Sex* | | | |
| Females | 20 (28.6) | 3 (30.0) | 7 (63.6) |
| Males | 50 (71.4) | 7 (70.0) | 4 (36.4) |
| *Smoking* | | | |
| Never | 1 (1.4) | 0 (0.0) | 0 (0.0) |
| Former | 49 (70.0) | 5 (50.0) | 8 (72.7) |
| Current | 20 (28.6) | 5 (50.0) | 3 (27.3) |
| Packyears | 32.0 | 32.5 | 39.0 |
| *Stage* | | | |
| I | 38 (54.3) | 9 (90.0) | 6 (54.5) |
| II | 22 (31.4) | 0 (0.0) | 4 (36.4) |
| III | 10 (14.3) | 1 (10.0) | 1 (9.1) |

After thorough follow-up (median 67.6 months, range 18.0–109.6), time to relapse (TTR) and overall survival (OS) was calculated in GraphPad Prism 6 software (GraphPad Inc., La Jolla, CA) with the Kaplan-Meier method using Log-rank for significance assessment. The study was approved by the Regional Ethics Board who granted a waiver of informed consent.

## 3. Results

Fresh-frozen tumour tissue samples from 91 resected early stage non-small cell lung cancer patients with unequivocal squamous cell carcinoma were analysed with the SnapShot method which detects mutations in hot-spot regions of 15 different cancer genes. *PIK3CA* mutations were most frequently found, in 10 cases (11%), whereas a *TP53* mutation (only minor parts of the *TP53* gene are analysed in SnapShot) was found in 8 cases, of which one also harboured a *KRAS* mutation (Table 1). None of the *PIK3CA* mutated cases had additional aberrations. More of the *PIK3CA* mutated cases were in stage I (9 of 10 versus 38 of 70 of non-mutated cases, based on the 7th edition of TNM-classification), otherwise there were no major differences in clinical characteristics between the non-mutated, *PIK3CA* mutated and otherwise mutated cases respectively (Table 2).

In this primary data-set, there was a difference in time to relapse regarding mutation types, with only one relapse (47.8 months after surgery) among the ten *PIK3CA* mutated cases, while 7 of the 11 patients with other mutations relapsed (Fig. 1).

We therefore decided to study *PIK3CA* mutated squamous cell carcinomas more extensively, and in addition to the 91 samples, another 217 resected squamous cell carcinoma cases were analysed with the Cobas *PIK3CA* mutation test kit. The SnapShot-analysed cases were re-tested with the Cobas method. There was a 100% concordance between the two methods (not shown). Approximately 20% of these cases were stained for the squamous cell carcinoma marker p40, and 92% were deemed p40 positive, the rest were diag-

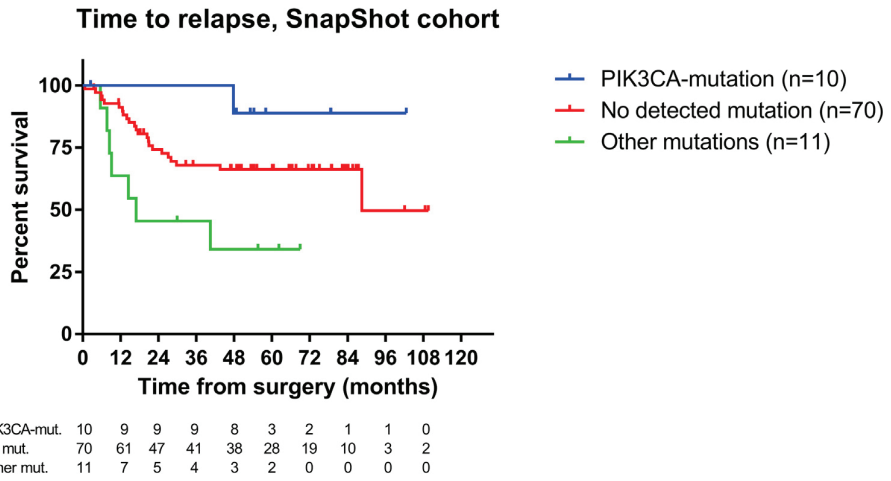


**Fig. 1.** Time to relapse after surgery for early stage lung squamous cell lung cancer differs depending on mutational type detected by the SnapShot method. Cases with *PIK3CA* mutations (n = 10) conferred better prognosis and cases with other mutations (n = 11) associated with a worse survival compared to cases without mutations (n = 70) (p = 0.012).

**Table 3**
Patient characteristics of the total cohort (n = 308).

| | *PIK3CA* wt (n = 273, 88.6%) | *PIK3CA* mut (n = 35, 11.4%) |
|---|---|---|
| Age | 66.9 (43.2–82.4) | 67.6 (53.6–81.3) |
| | *N* (%) | *N* (%) |
| *Sex* | | |
| Females | 91 (33.3) | 11 (31.4) |
| Males | 182 (66.7) | 24 (68.6) |
| *Smoking* | | |
| Never | 4 (1.5) | 0 (0.0) |
| Former | 111 (40.7) | 16 (45.7) |
| Current | 153 (56.0) | 19 (54.3) |
| Unknown | 5 (1.8) | 0 (0.0) |
| Packyears | 39.9 (n = 212) | 36.4 (n = 30) |
| *Stage* | | |
| Ia | 74 (27.1) | 9 (25.7) |
| Ib | 71 (26.0) | 13 (37.1) |
| IIa | 62 (22.7) | 4 (11.4) |
| IIb | 26 (9.5) | 4 (11.4) |
| IIIa | 39 (14.3) | 5 (14.3) |
| IIIb | 1 (0.4) | 0 (0.0) |
| *p40-IHC* | | |
| Stained | 59 | 7 |
| Positive | 55 (93.2) | 6 (85.7) |
| *PD-L1-IHC* | | |
| Stained | 59 | 8 |
| Positive | 24 (40.1) | 2 (25.0) |

**Table 4**
Mutation status dependent on eligibility for adjuvant therapy (n = 308).

| | *PIK3CA* wt | *PIK3CA* mut |
|---|---|---|
| | *N* (%) | *N* (%) |
| Eligible for adj. therapy (<70 years and stage II–III) | 77 (28) | 9 (26) |
| Non-eligible for adj. therapy (>70 years or stage I) | 196 (72) | 26 (74) |

*p = 0.76.*

nosed with squamous cell histology based on routine p63-staining or morphological features alone. Most cases were males with a significant smoking history, and 54.7% of the cases were stage I (Table 3).

*PIK3CA* mutation was found in 35/308 (11.4%) patients, and there were no differences in median age, sex distribution, or smoking behaviour between non-mutated and *PIK3CA* mutated cases (Table 3). There was a numerical, non-significant, difference in stage distribution, with 53.1% of *PIK3CA* non-mutated versus 62.8% of *PIK3CA* mutated cases being in stage I. There was a tendency of a higher frequency of T2N0 tumours (stage Ib) among *PIK3CA* mutated cases. We have no detailed information on the extent of adjuvant therapy given to the patients, but in Norway there is high adherence to guidelines. We have thus analysed mutation frequency in patients eligible vs non-eligible for adjuvant therapy. There was no statistical difference between the groups (Table 4).

A random subset of cases (n = 67, based on tissue availability) were stained with PD-L1 (Ventana SP142 assay), and a numerical, though non-significant, lower percentage of mutated cases (25.0%) expressed this ligand compared to in non-mutated cases (40.1%) (Table 3). None of the PD-L1-positive mutated cases relapsed (data not shown).

The most frequently found *PIK3CA* mutations were in the helical domain of exon 9; either E545K (21 cases, 60.0%) or E542K (n = 7) (20.0%). Mutations in exon 20 (H1047) were found in 5 cases (14.3%) (Fig. 2).

There was a significant difference in overall survival between *PIK3CA*-mutated and non-*PIK3CA*-mutated cases (log rank p = 0.042, HR 0.62 (95% CI 0.39-0.98)) in the complete cohort (Fig. 3a). Cohorts were similar, and analysis stratified by SnapShot versus non-SnapShot cohort is performed, yielding a log rank p = 0.041. As lung cancer patients are prone to suffer from non-cancer related deaths, the time to relapse (TTR) might be a better parameter for studying biological factors, and we found a highly significant difference in TTR between mutated and non-mutated cases (p = 0.028, stratified by cohort) (Fig. 3b). To avoid a potential bias due to stage distribution differences, stage I only-cases were analysed, and a difference in TTR was also retained in these cases (p = 0.044) (Fig. 3c).

Furthermore, we analysed if there were prognostic differences among mutation subtypes. Interestingly, none of the 14 cases with non-E545K-mutation relapsed, whereas all relapses were seen in the E545K-positive group (Fig. 3d).

In Cox regression multivariate analysis, including age, stage, sex and *PIK3CA* mutation status, *PIK3CA*-status remained significant, with p = 0.050, whereas the p-values for age, stage and sex were 0.047, 0.038 and 0.43 respectively.

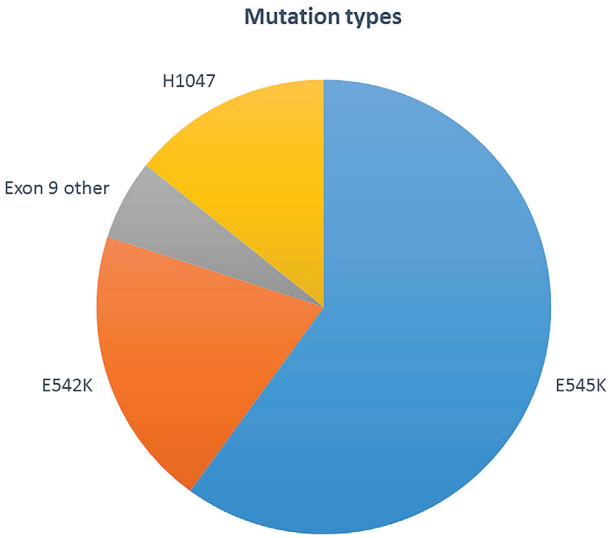


**Fig. 2.** The most prevalent subtype of *PIK3CA* mutations in squamous cell carcinomas were found in exon 9 (E542K and E545K) followed by mutations in exon 20 (H1047R).

Of the 5 *PIK3CA* mutated patients with relapse, three had lung metastases, one had skeletal and one had metastases to adrenal and liver. None were diagnosed with brain metastases.

## 4. Discussion

In a cohort of over 300 early stage squamous cell carcinomas of the lung we found a frequency of *PIK3CA* mutations of 11.4%. Other studies, including The Cancer Genome Atlas (TCGA), have reported similar or somewhat lower frequencies in squamous cell carcinomas of the lung [6,23,24], whereas the frequency in adenocarcinoma is much lower, in the range of 3% [6,25]. Furthermore, our data agree with the literature, also including other tumour types, in that E545K- and E542K-mutations in exon 9 (helical domain), along with H1047R in exon 20 (kinase domain) were the most common mutation types [6,9,26].

In contrast to other specific mutations in lung cancer (*ALK*, *EGFR* and *BRAF*), there was no association with lower smoking exposure. Virtually all patients in this cohort were current or former smokers, as expected [27], and the number of packyears was similar among mutated as non-mutated cases. On the other hand, the fact that *PIK3CA* mutations are prevalent with even higher frequencies in a number of cancers with no strong relation to smoking (*e.g.* breast and colon cancers) [3] might indicate that this aberration is not induced by smoking.

The *PIK3CA* mutated cases tended to be detected in earlier stages, but a higher fraction of the stage I-cases were found to have larger tumours, T2, indicating a lower propensity for metastatic spread. This is in line with findings in breast cancer, where *PIK3CA* mutations are associated with lower tumour stages and improved DFS and OS, especially mutations in the kinase domain [13]. Furthermore, breast cancer cell lines expressing kinase-domain mutations have been shown to have reduced capacity for blood intravasation and lung extravasation in vivo, compared with mutations in the helical domain [28]. Interestingly, abnormal progressive localized growth, the congenital mosaic overgrowth syndrome, is also found to be correlated to *PIK3CA* mutations, leading to a temping speculation of a correlation with this mutation and cellular growth with a lesser malignant potential [29].

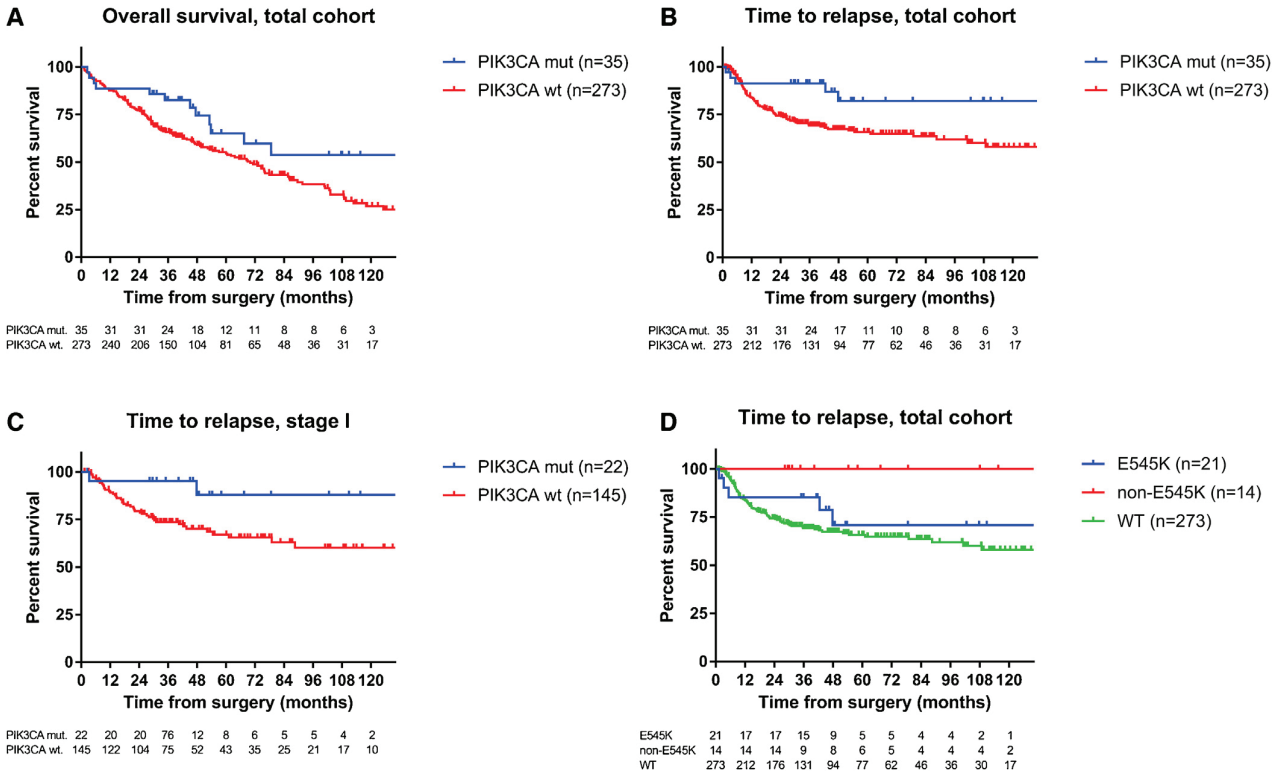


**Fig. 3.** Squamous cell lung cancer cases with PIK3CA mutations had an improved overall survival ($p = 0.042$) (A), which also translated to an improved time to relapse in the total cohort ($p = 0.030$) (B) as well as in stage I-cases only ($p = 0.044$) (C). Mutations in E545K, which was the most prevalent, conferred only slightly improved prognosis, the major contribution for the better time to relapse was related to the non-E545K-mutated subgroup ($p = 0.040$) (D).

Using the SnapShot method, which analyses for hot-spot mutations in 15 different cancer-related genes, none of the *PIK3CA* mutations were found to co-occur with other mutations, in contrast to what has been published for adenocarcinoma or non-specified non-small cell lung carcinoma [6]. If this is a true biological phenomenon in squamous cell carcinomas, or if using other more extensive and/or sensitive methods, or a higher sample number would detect co-existing mutations is unknown. However, in the TCGA cohort, most of the *PIK3CA* mutations were private [24], and as the most frequent co-existing mutations (in EGFR and kRAS) are very infrequent in squamous cell carcinoma, this may well be a true biological phenomenon in squamous cell carcinoma.

The positive prognostic impact of *PIK3CA* mutations, as seen both in overall survival, and even more significant in time to relapse, is somewhat contrasting to previously published data in lung cancer. There might be a number of possible explanations for these apparently opposing results. Firstly, it might simply be due to low number of cases in other studies. Secondly, most of these studies have been conducted on adenocarcinoma samples. It is conceivable that the histological subtype, with the intrinsic molecular differences, may be responsible for differing prognostic value. For instance, the PI3K/Akt factor AKT2 seems only to be co-amplified in squamous cell carcinomas, and complete *PTEN*-loss is also most often seen in squamous cell carcinomas. *KRAS* mutations are more frequent in adenocarcinomas, as is loss of *LKB1* [30]. Of note, no prognostic data are available in the TCGA cohort [24].

Furthermore, it is possible that *PIK3CA* mutations in advanced cancers are associated with an aggressive phenotype, exemplified by a study showing such mutations in squamous cell carcinoma being associated with brain metastases and a dismal prognosis [31]. Interestingly, none of the relapsed *PIK3CA* mutated cases in our cohort were diagnosed with brain metastases. It is also known that *PIK3CA* mutations are associated with drug resistance in breast cancer [32], thus the predictive value of the mutation may be opposite of the prognostic value. Ultimately, this may indicate that *PIK3CA* mutations are both indicator of a favourable prognosis, and that adjuvant therapy may be of less benefit in this patient group and may be omitted. Of note, in our study, of the 35 mutated cases 26 (74%) did not receive adjuvant chemotherapy either due to stage (22 patients in stage I) or age (four patients aged above 70 year).

Our finding that PD-L1 expression was less frequent in *PIK3CA* mutated tumours is intriguing since activation of the PI3K/Akt pathway is a regulator of PD-L1 in cancer cells, both via transcriptional upregulation and other mechanisms in a tissue-dependent manner [18,19,33]. A correlate to this is observations from breast and gastric cancer where *PIK3CA*-mutations also were associated with lower PD-L1-positivity [34,35]. This could indicate the PD1/PD-L1 axis to be involved in the favourable prognosis in *PIK3CA* mutated cancers including lung squamous cell carcinomas, explained by a lesser immune-evading capacity in these tumours. Our finding should be interpreted with caution due to the low number of analysed samples, but is also consistent with the fact that PD-1/PD-L1 positivity in NSCLC is negatively associated with known driver-mutations in *EGFR* and *ALK* and in general correlated with increased mutational burden [36].

There are several limitations to this study, foremost the retrospective nature may have led to missed information, secondly even though over 300 cases were analysed a limited number of mutation-positive cases were found. There has not been performed any external validation of these findings, the SnapShot method analyses only relatively few amplicons, and the PI3K-axis beyond *PIK3CA* mutations has not been analysed. Finally, the potential PD-L1-association is based on few cases only.

There are also some strengths that should be noticed—this is to our knowledge the largest cohort of this kind that has been analysed for *PIK3CA* mutations, the follow-up has been thorough, providing reliable time to recurrence data (no patient lost to follow up), and the findings were internally validated in two parts of the cohort and with two different mutational analyses.

In conclusion we have confirmed a relative high frequency of *PIK3CA* mutations in early stage squamous cell carcinoma of the lung. Our results indicate that these mutations may have a positive prognostic impact in squamous cell carcinomas of the lung, similar to what is seen in breast cancer. Due to the low frequency, further work on larger cohorts is needed to confirm whether a prognostic role of PIK3CA mutations exists in this tumour type.

## Funding

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## Conflict of interest

The authors declare that there are no conflicts of interest.

## Acknowledgements

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# Paper II

**NSCLC Depend Upon YAP Expression and Nuclear Localization After Acquiring Resistance to EGFR Inhibitors.**

Marc McGowan, Lilach Kleinberg, Ann Rita Halvorsen, Åslaug Helland, and Odd Terje Brustugun

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II

# NSCLC depend upon YAP expression and nuclear localization after acquiring resistance to EGFR inhibitors

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## ABSTRACT

**Yes-associated protein (YAP) is a downstream target of the Hippo pathway and has been found to be oncogenic driving many cancers into developing metastatic phenotypes leading to poor survival outcomes. This study investigated if YAP expression is associated with drug resistance in two non-small cell lung cancer (NSCLC) lines (HCC827 and H1975) generated to become resistant to the EGFR tyrosine kinase inhibitors (EGFR TKI) erlotinib, gefitinib or the T790M-specific osimertinib. We found that acquired EGFR TKI resistance was associated with YAP over-expression (osimertinib-resistant cells) or YAP amplification (erlotinib- and gefitinib-resistant cells) along with EMT phenotypic changes. YAP was localized in the nucleus, indicative of active protein. siRNA-mediated silencing of YAP resulted in re-sensitizing the drug-resistant cells to EGFR TKI compared to the negative siRNA controls ($p = <0.05$). These results suggest YAP is a potential mechanism of EGFR-TKI resistance in NSCLC and may presents itself as a viable therapeutic target.**

## INTRODUCTION

Innon-small cell lung cancer (NSCLC), responsible for the highest death toll among cancers [1], targeted therapy has been a remarkable success. Both EGFR-, ALK-, ROS1 -directed therapies are approved, and about a fifth of all metastatic NSCLC patients may be offered such therapies with median responses of around a year [2]). However, progression due to acquired resistance is virtually inevitable, and a number of different resistance mechanisms are described [3]. In EGFR-mutated tumours treated with the first (erlotinib, gefitinib) or second (afatinib) generation EGFR tyrosine kinase inhibitors, the most frequent mechanism of resistance is a secondary mutation in exon 20 of the EGFR-gene; T790M [4]. Recently a drug targeting T790M-positive tumours, osimertinib, was approved after studies showing prolongation of progression free survival in EGFR-pre-treated and progressed patients harbouring T790M [5]. Still, a substantial fraction of tumours harbour other resistance mechanisms of which some, as AXL over-expression [6] and *MET* amplification [7, 8] are known, but others are still unknown and where no targeted therapies are available.

Yes-associated protein (YAP) has been found to both regulate the expression of Axl [9] and also drive the required phenotypic changes to cause epithelial to mesenchymal cell transformation (EMT) after binding with its transcriptional co-activator TEAD [10]. YAP expression is associated with reduced survival and relapse trends in NSCLC patients [11] which further highlights this co-transcription factor as an interesting target for drug-resistance research. But not much is known about YAP's role in drug-resistance. This study presents a new view of YAP using the HCC827 (exon 19 E746-A750 deletion) NSCLC cell line generated to become resistant to first generation TKIs and H1975 (harbouring both the T790M and L858R mutation) to osimertinib.

## RESULTS

### Drug-resistant sub-lines

After proliferation was observed in the HCC827 gefitinib (GR) and erlotinib-resistant (ER) sub-lines they were isolated using cloning cylinders and expanded in individual colonies. Twenty-four sub-lines were generated and analysed for *EGFR* mutations outside of the exon 19 in frame deletion. Sequencing results showed no alterations from the HCC827 parental and drug-resistant sub-lines (data not shown). We randomly selected one erlotinib and one gefitinib sub-line for all further experiment. We determined the HCC827/ER and GR sub-lines were drug-resistant following a cell viability assay showing a shift in $EC_{50}$ values from the HCC827 parental line (Figure 1 A and B). We tested whether the third generation EGFR inhibitor osimertinib (formally AZD9291) was able to inhibit growth in the drug-resistant sub-lines compared to the parental controls using the cell viability assay. The results show a shift in $EC_{50}$ value from the drug-resistant cells compared to the parental (Figure 1 C).

The H1975 cell line is characterized by harbouring the EGFR gatekeeper mutation T790M in exon 20 that prohibits signalling inhibition by erlotinib and gefitinib. We generated three sub-lines resistant to osimertinib (referred to as H1975/OR), whereof one sub-line was selected at random for all further experiments. The cell viability showed a shift in $EC_{50}$ from the H1975 parental and H1975/OR – from 0.01 µM to ~2.5 µM – (Figure 1 D). We sequenced exons 18-21 of the *EGFR* gene for additional mutations but our results showed no alterations from the parental line (data not shown).

### Resistant sub-lines promote EMT changes and Expression of YAP

The HCC827/ER sub-line showed markers of EMT (vimentin expression and loss of e-cadherin) and AXL expression after acquiring resistance to erlotinib. HCC827/GR sub-lines also showed AXL and vimentin expression but still some e-cadherin expression (Figure 2) compared to the HCC827/ER sub-line. The morphology of the sub-lines also differed where HCC827/ER were mesenchymal and differed from the parental, and HCC827/GR resembled their parental and did not appear to have undergone EMT. Interestingly, EGFR appeared to be down-regulated in the HCC827/ER sub-line when compared to HCC827/GR and parental line. We observed

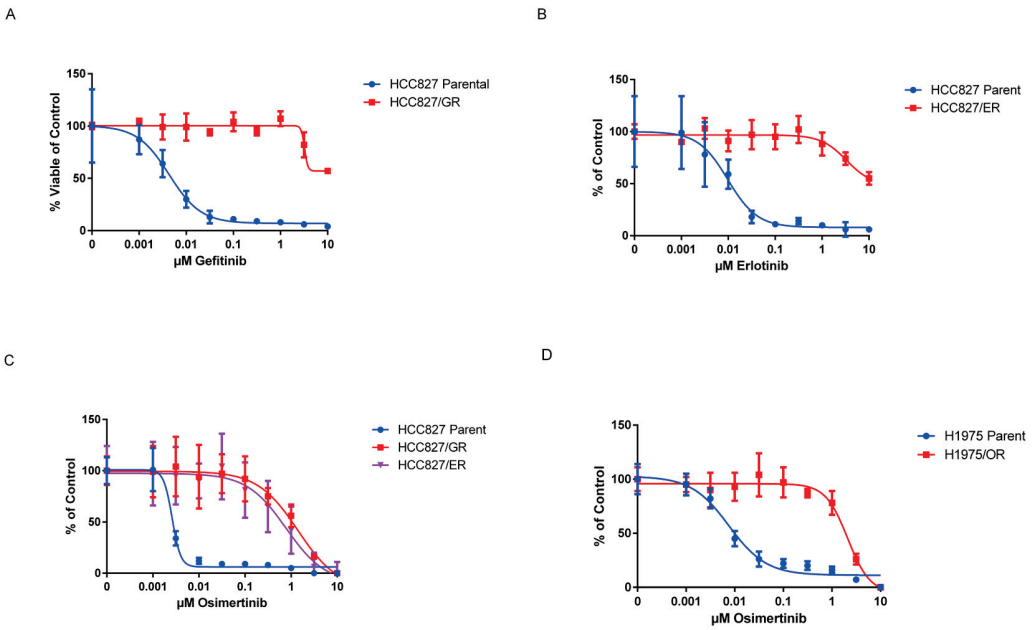


**Figure 1: $EC_{50}$ of HCC827GR, HCC827/ER, and H1975/OR sub-lines.** A. There was a clear difference between drug-sensitive HCC827 parental ($EC_{50}$ = 0.004 µM) and gefitinib-resistant (GR) sub-line ($EC_{50}$ >10 µM). B. Erlotinib-resistant HCC827/ER showed the ability to proliferate in high concentrations of drug ($EC_{50}$ >10 µM) compared to the parental ($EC_{50}$ = 0.001 µM). C. The HCC827/ER, GR levels of tolerance to osimertinib. The HCC827/GR sub-line showed a greater tolerance to osimertinib ($EC_{50}$ = 1.4 µM) while the HCC827/ER had a lower tolerance ($EC_{50}$ = 0.8 µM). It was concluded that the cells not being completely resistant to osimertinib did show the ability degree to proliferate in higher concentrations than the parental line ($EC_{50}$ = 0.003 µM). D. The H1975/OR shows resistance to osimertinib ($EC_{50}$ = ~2.5 µM) compared to the H1975 parental ($EC_{50}$ = 0.008 µM).

YAP over-expression after acquiring resistance to the first generation EGFR inhibitors. Using RT-qPCR confirmed amplification of YAP at the mRNA level (data not shown). We then analysed if the overexpression of YAP resulted in the increased expression of its inhibitor Merlin. We found Merlin was not expressed in any of the HCC827 parental or drug-resistant sub-lines. The H1975/OR sub-line was tested for known resistant proteins (AXL, EGFR) as well as YAP expression (Figure 2). From the Western Blot the H1975 parental cells harboured AXL which was over-expressed in the H1975/OR sub-line. We saw YAP expression in the H1975/OR sub-lines and not in the parental. We further evaluated these findings with RT-qPCR which confirmed expression differences (data not shown). We then assessed if Merlin was also expressed in these cells. Interestingly, we observed co-expression of both YAP and Merlin in the H1975/OR drug-resistant sub-line. This was not observed in the HCC827 drug-resistant cells (ER or GR) and warranted further experimentation to determine if YAP was active.

Immunocytochemistry staining (ICC) was chosen to visualize if YAP was active (localized in the nucleus) or inactive (withheld in the cytoplasm). ICC results showed YAP was distributed in both cytoplasm and nucleus in the HCC827/ER and GR sub-lines. In the H1975/OR sub-line YAP was more predominantly localized to the nucleus than in the cytoplasm (Figure 3).

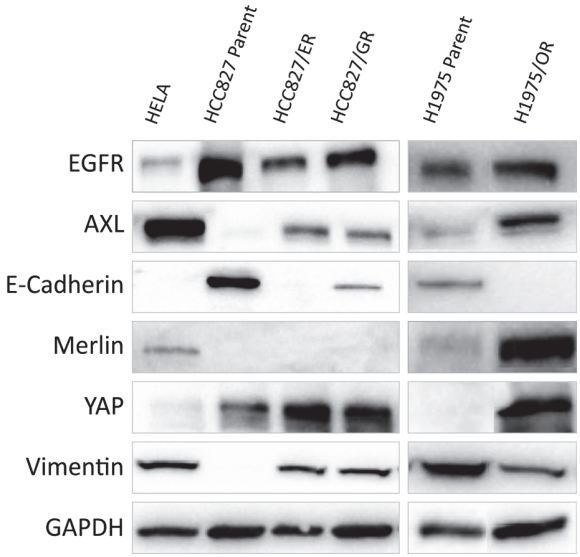


**Figure 2: A Western Blot of HCC827 and H1975 parental and drug-resistant sub-lines characterisation.** EGFR fluctuated slightly from HCC827 parental and both ER and GR sub-lines. AXL expression was observed in the drug-resistant sub-lines along with EMT marker Vimentin and loss of E-Cadherin. YAP was observed in parental cells and was amplified in the drug-resistant cells. EGFR remained consistent between H1975 parental cells and H1975/OR sub-line. AXL expression was also observed in parental and appeared to increase in expression as cells acquired resistance to osimertinib. EMT marker vimentin was down-regulated but remained expressed as e-cadherin was lost in H1975/OR. Merlin expression was increased in H1975/OR sub-line with co-expression of YAP.

### Silencing of YAP restores drug-resistant cells sensitive to EGFR inhibitors

To further understand and evaluate YAP overexpression in relation to drug-resistance in HCC827 ER/GR and expression in H1957/OR we conducted knockdown using siRNA targeting YAP. Western Blot confirmed knockdown prior to evaluating the effects of reintroducing drug to the three drug-resistant cells (Figure 4).

H1975/OR sub-line (Figure 4 A) responded to osimertinib after silencing YAP ($p = <0.05$). Only 34% of the H1975/OR sub-line cells with YAP silencing siRNA were viable after normalizing to their 10 μM osimertinib negative siRNA controls. HCC827/ER sub-line (Figure 4 B) responded to lower concentrations of erlotinib after silencing YAP ($p = <0.05$). However, HCC827/GR sub-line showed a reduced response to gefitinib compared with erlotinib-resistant cells but did show significant reduction in cell viability after YAP silencing ($p = <0.05$) (Figure 4 C).

## DISCUSSION

Drug resistance is a major cause of cancer treatment failure. Targeted EGFR-directed drugs show effect duration of around one year, where after resistance is inevitable. In this report we show that induction of YAP is a possible mechanism of drug resistance to EGFR tyrosine kinase inhibitors in NSCLC adenocarcinomas, and that inhibiting this co-transcription factor can re-sensitize the cells to EGFR inhibitors.

We exposed the HCC827 cell line to two first generation EGFR inhibitors, erlotinib and gefitinib, and H1975 to osimertinib (AZD9291) and found AXL, a known mechanism of EGFR-TKI-resistance [6] to be expressed in all sub-lines. This was an interesting observation as previous studies have shown that YAP may regulate AXL expression in lung adenocarcinomas [9] and in hepatocellular carcinoma by way of TEAD binding to the promoter region of the *AXL* gene [12]. Thus YAP expression and activation may be a reason for AXL induction in drug-resistant adenocarcinomas and some other cancer types; though we did not evaluate AXL expression following YAP knockdown, which would have been interesting. Future studies should investigate if YAP inhibition contributes to the reduced capability of AXL signalling which could be clinically exploited in drug-resistant cells.

We also observed a reduced band intensity of EGFR in the HCC827/ER and GR sub-lines compared to the parental (figure 2). This may indicate internalization and

degradation of EGFR after erlotinib and gefitinib binding. Sakuma *et al* observed the down-regulation of EGFR after generating gefitinib-resistant HCC4006 adenocarcinoma cells – also harbouring the exon 19 E746-A750 deletion – but not in the HCC827 gefitinib-resistant sub-line [13]. This was concluded to be due to autophagocytosis and may explain why we also see this in our results after acquiring resistance to erlotinib and gefitinib. If this is also observed in the clinic it would be interesting to determine if this is also a prognostic marker of patient outcome.

We noted that the H1975/OR sub-line had a suppressed e-cadherin expression along with reduced expression of vimentin, while HCC827/ER and GR sub-lines expressed vimentin and down-regulated E-cadherin (figure 2). E-cadherin has been found to regulate the phosphorylation of YAP and promote its degradation by way of activating protein 14-3-3 localizing it to the cytoplasm and preventing the binding to TEAD [14]. Loss of the trans-cellular E-cadherin results in epithelial-mesenchymal transition and contact independent growth, migration and invasion. Vimentin induces morphological changes and increases cell motility [15]. It is conceivable that osimertinib may have off targets effecting the transcription of vimentin.

We further evaluated YAP expression and amplification in the HCC827/ER, GR and H1957/OR sub-lines. YAP is only active once it has sequestered to the nucleus where it binds to TEAD and begins transcription of cell survival genes and EMT capabilities [16]. We confirmed YAP expression via western blot and RT-qPCR and found YAP was distributed in both cytoplasm and cell nucleus in the HCC827/ER and GR sub-lines, but more localized to the nucleus in the H1975/OR sub-line (figure 3). We therefore suspected that YAP was both over-expressed and active in all drug-resistant sub-lines. This question, however, was limited as we only used one method of identifying YAP localization. We plan to further investigate YAP-TEAD downstream transcription targets such as amphiregulin that YAP-TEAD downstream transcription targets such as amphiregulin, which would also help confirm YAP activation. We will employ these in all further experiments evaluating YAP activity in drug-resistant NSCLC.

We then wanted to determine if YAP was involved in drug-resistance or merely acted as a biomarker. We found that siRNA-induced YAP silencing restored the negative effect of EGFR inhibitors on cell viability. Our results highlight YAP as a possible mechanism of drug resistance also for the third-generation EGFR-inhibitors, and thus confirm and extend the findings by Hsu *et al* [17] whose group showed HCC827 erlotinib-resistant and H1975 cells to become re-sensitized to erlotinib after YAP

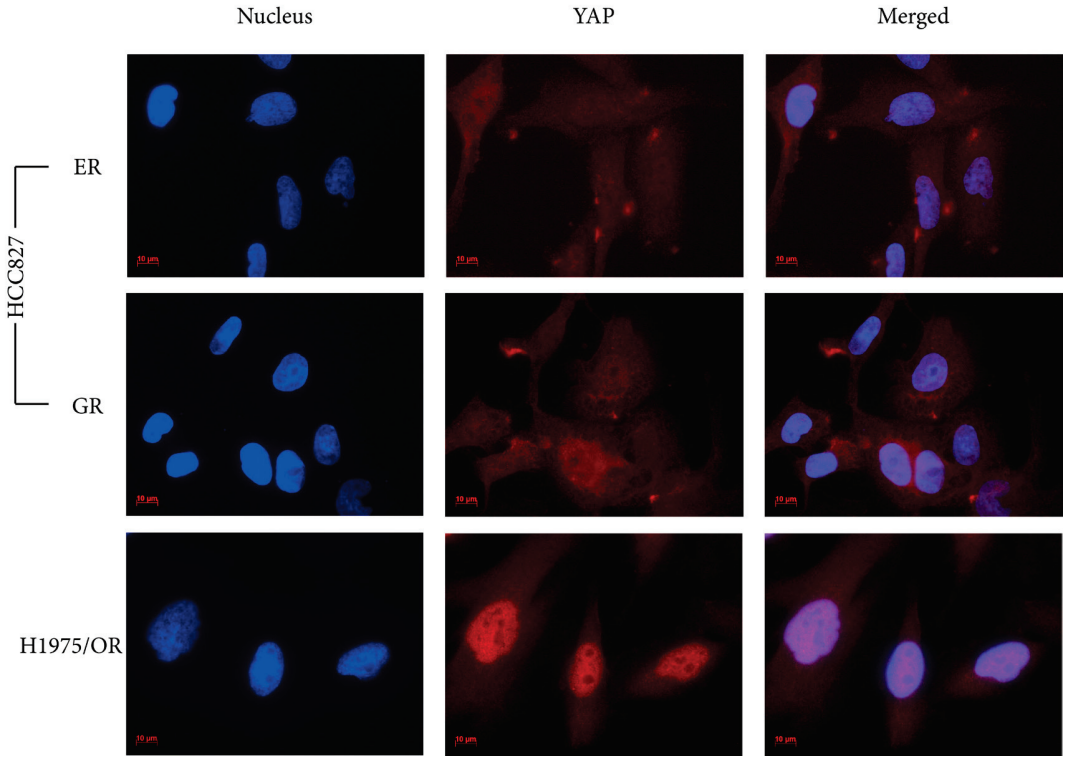


**Figure 3: An immunocytochemistry staining showing the localization of YAP in drug-resistant sub-lines.** Both HCC827/ER and GR sub-lines show YAP expression distributed in the cytoplasm and nucleus. The H1975/OR sub-line shows YAP staining more focused in the nucleus than the cytoplasm. Scale bar was 10 μm.

was silenced. Another group also found that inhibiting YAP re-sensitized breast cancer cells to chemotherapeutic agents demonstrating YAP's ability to orchestrate drug-resistance [18]. These, along with our results, demonstrate an emerging role of YAP in drug resistance and increase the possibility of a new therapeutic target of relapsed EGFR tyrosine kinase inhibitors. The use of statins has been studied and shown to be a potent inhibitor of YAP sequestering to the nucleus in breast cancer cell lines [19]. This study showed potential in pre-existing drugs already approved for use in the clinic which can be revaluated into a new role speeding up the use in YAP-driven cancers. We did not evaluate the expression of EMT markers and AXL post YAP silencing, which has limited our results to suggest YAP is involved with orchestrating EMT.

In conclusion, YAP may be a potential central factor in acquired EGFR-TKI resistance, and further work studying in-depth mechanisms of regulation, role in clinical prognostication and response predication as well as potential interventional approaches are warranted and ongoing. More work will need to be conducted to confirm YAP activation using the limitations from our experiments to unravel more about this co-transcription factor

## MATERIALS AND METHODS

### Cell lines and Reagents

HCC827 and H1975 were purchased from ATCC (CRL-2868 and CRL-5908 respectively) and were maintained in RPMI1640 media (R8758, Sigma) supplemented with penicillin-streptomycin (15140-122, Gibco), L-glutamine (G7513, Sigma), and 10% v/v foetal bovine serum (S1810-500, VWR) in a 95% humidified

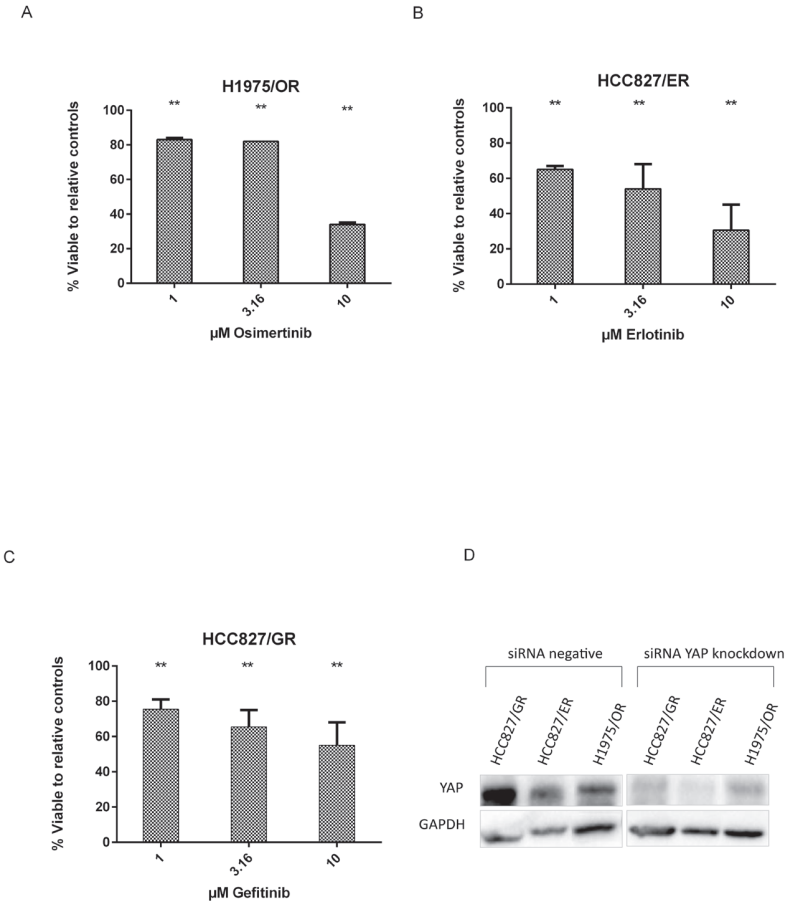


**Figure 4: YAP siRNA knockdown allows re-sensitization to EGFR inhibitors.** A. H1975/OR sub-line shows reduced cell viability when re-introduced to osimertinib after YAP silencing, especially evident at doses around 10 µM. B. We observed HCC827/ER respond to erlotinib after siRNA silencing of YAP from 1 µM drug, with gradually pronounced difference from non-silenced cells up 10 µM. C. HCC827/GR sub-line showed decrease in cell viability after the introduction of drug and silencing of YAP. All siRNA knockdown wells were normalised to their negative siRNA drug counterpart and standard error was calculated based upon two repeats. D. siRNA knockdown was confirmed using Western Blot with GAPDH as a loading control. ** p = <0.05.

5% $CO_2$ atmosphere at 37°C. Erlotinib was purchased from LC Laboratories (E-4007), osimertinib (AZD9291) was bought from MedChem Express (HY-15772), and gefitinib was a kind gift from Solveig Pettersen at the Radium Hospital. All drugs and compounds were diluted in DMSO (D2650, Sigma) and kept at 0.1% v/v or lower in cell culture media.

HCC827 erlotinib-resistant (ER) and gefitinib-resistant (GR) sub-lines were generated by culturing early passage HCC827 parental cells stepwise from their $EC_{50}$ to above their $C_{max}$ values. HCC827/ER sub-lines were cultured to 3.5 μM Erlotinib and HCC827/GR sub-lines cultured to 2.5 μM Gefitinib; their $C_{max}$ concentrations are 2.5 μM erlotinib [20] and 0.3 μM gefitinib respectively [21]. H1975/OR sub-lines took much longer to establish due to the response to osimertinib. These cells were cultured to 2.5 μM osimertinib stepwise over several months. The $C_{max}$ value for osimertinib is between 2-3 μM [22] and thus sustaining the sub-line at 2.5 μM was deemed acceptable. Analysis of drug-resistance was determined using $EC_{50}$ data prior to further experiments.

## YAP silencing

YAP silencing was achieved using Qiagen Flexitube siRNA (Qiagen, S104438651) and negative control siRNA (Qiagen, 1022076) diluted in Lipofectamine RNAiMAX (Thermo Fisher, 13778075) and Opti-MEM (Thermo Fisher, 31985070) used as described in the manufacturers specifications. Protein knockdown was assessed using Western Blot.

## $EC_{50}$ cell viability assay

Parental cells were assessed for sensitivity to TKI along with their respective TKI-resistant sub-lines. Cells were seeded at a concentration of 5 x $10^3$ into 96 x well plates with their respective media and allowed to adhere overnight. After 24 hours the media was removed and replaced with media containing varying concentrations of erlotinib, gefitinib or osimertinib and DMSO only for control wells at half-log dilutions. DMSO was kept consistent at 0.1% v/v for all treatment groups and controls. A resazurin metabolism assay (R7017, Sigma) was performed by making a 1/20 dilution of 2.3 mg/ml resazurin stock in sterile Dulbecco's phosphate buffered saline (DPBS) (14190094, Thermo Fisher) and using a 1/10 dilution directly in the wells after 120 hours drug incubation. Resazurin sodium salt was allowed 4 hours incubation at 37°C in a 95% humidified, 5% $CO_2$ atmosphere. Plates were assessed for active viable cells using fluorescence imager BioTek Synergy 2 with software Gen5 with filters for excitation at 544 nm and emission at 590 nm. The $EC_{50}$ curve was created using GraphPad v.6 software by normalising the average treatment wells to the DMSO controls. Data was presented as percent of controls.

For siRNA-erlotinib/gefitinib/osimertinib combination, we loaded each well of a 96 well plate with 5 x $10^3$ cells per well and left them to adhere overnight in media with drug. The following day media was changed to media with siRNA YAP or negative control siRNA and allowed to incubate for 48 hours without drug. After 48 hours we added drug dilutions directly onto the siRNA loaded cells and incubated for a further 72 hours before assessing viable cells using the resazurin assay. We normalized each siRNA drug/compound group to the negative groups, for example 10 μM erlotinib YAP siRNA was normalized to 10 μM negative siRNA control. Results were presented as percentages of the negative siRNA in a bar graph with ± SEM and statistical differences between controls and YAP knockdown were conducted using a two-tailed, unpaired and unequal variance student's T-Test and presented on the graphs.

## Immunocytochemistry of YAP

Cells were trypsinized and seeded at a concentration of 10 x $10^4$ onto frosted-coated glass slides and left to adhere overnight at 37°C in a 95% humidified 5% $CO_2$ incubator. Once adhered, slides were rinsed twice with sterile D-PBS and treated with 0.3% v/v Triton-X100 (T8787, Sigma) for five minutes to permeabilize the cells. Slides were washed three more times before blocking with 5% Goat serum (16210, Thermo Fisher) for 30 minutes before incubating with either primary YAP antibody (PA1-46189, Thermo Fisher) or 5% Goat serum (staining control) for 1 hour at room temperature. Slides were washed three times and treated with Goat Anti-Rabbit IgG Alexa Fluor® 594 conjugated secondary antibody (A-11037, Thermo Fisher) for 60 minutes in the dark at room temperature. Slides were washed another three times and stained with ProlLong® Gold Antifade Mountant with DAPI nucleus dye (P36931, Thermo Fisher) for 10 minutes. Slides were mounted and used immediately for imaging and then stored at -20°C in the dark.

## Western Blot

Cells were harvested for protein by using Ripa lyses buffer (89901, Thermo Scientific) with 50 mM sodium orthovanadate (S6508, Sigma), 50 mM Pefabloc (1.24839.0500, Merck), 50 mM sodium fluoride (S6508, Sigma), PhosSTOP (04906845001, Roche) and Protease inhibitor cocktail (05892970001, Roche). Protein concentration was measure using the Pierce BCA kit (23227, Thermo Scientific) at the manufacturer's specifications. A concentration of 15 μg protein lysate was boiled with Laemmli sample buffer (161-0747, Bio-Rad) with 100 mM DTT (A3668, AppliChem) for

five minutes at 95°C then loaded into a 12% SDS mini-PROTEAN® TGX™ gel (456-1046, Bio-Rad) and ran for 60 minutes at 200 v. Gels were transferred to a Midi Format nitrocellulose membrane (1704159, Bio-Rad) using a Turbo blot transfer module form Bio-Rad. The membranes were blocked for 60 minutes in (1706404, Bio-Rad) 0.1% Tween 20 (1610781, Bio-Rad) diluted in Tris buffered saline (1706435, Bio-Rad) containing 5% v/v fat-free milk. Membranes were washed for five minutes three times in 0.1% TTBS before sectioning and incubating overnight at 4°C rotating in primary antibodies: Axl (8661, Cell Signalling), EGFR (4267, Cell Signalling), Merlin (PA5-35316, Thermo Scientific), YAP (PA5-13504, Thermo Scientific), E-Cadherin (ab1416, Abcam), Vimentin (ab92547, Abcam) and GAPDH for loading control (MAB374, Millipore). Membranes were washed three times for five minutes in 0.1% TTBS before incubating with secondary antibodies for 60 minutes at room temperature in either peroxidase-conjugated Goat anti-Mouse (31431, Thermo Scientific) or Goat anti-Rabbit (31466, Thermo Scientific). Membranes were washed another three times for five minutes in 0.1% TTBS and developed using SuperSignal® (34095, Thermo Scientific) and being viewed using the Bio-Rad Chemi Doc ™ MP imager running Image Lb v4.1 software.

### Abbreviations

Epidermal growth factor receptor (EGFR), Epithelial to mesenchymal transition (EMT), Yes-associated protein (YAP).

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## CONFLICTS OF INTEREST

The Authors declare no conflict of interests.

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