Mini Review:

Challenges and strategies in precision medicine for non-small cell lung cancer.

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Abstract

Lung cancer is the most common cause of cancer- related death worldwide, causing over 1.2 million deaths each year. Non–small-cell lung cancer (NSCLC) consists of a group of malignancies that are pathologically and molecularly diverse but that are all characterised by a poor prognosis. Survival rates for lung cancer patients have improved very slowly and only to a modest degree owing partly to poor funding for research into this malignancy and stigma associated with smoking, as well as relative chemo-resistance. However, in recent years, NSCLC has become an exemplar for precision medicine, mainly following development of drugs targeting the receptors of epidermal growth factor and anaplastic lymphoma kinase. While epidermal growth factor receptor and anaplastic lymphoma kinase inhibitors are only applicable to a minority of patients and benefits are almost invariably short-lived, current studies indicate that at least 50% of patients with NSCLC have a targetable mutation. With a growing armamentarium of inhibitors against these targets in development, there is a hope that a greater proportion of patients will benefit from precision medicine and that such benefits will be sustained. However, there remain significant challenges in the development of precision medicine in NSCLC. These include: identification and validation of new targets; ensuring biopsies are fit for purpose; tumour heterogeneity; requirements for serial tumour assessments; and not least cost. In this review, we will discuss the current status of precision medicine in NSCLC as well as how basic and translational research are paving the way towards overcoming the above challenges. In addition, we will pay attention to clinical

strategies in respect to liquid biopsies and the potential use of extracellular vesicles such as exosomes in cancer therapeutics.

Introduction

Cancer is a devastating group of human diseases that result from uncontrolled replication and spread of abnormal cells. There are approximately 14.1 million new cancer cases worldwide with 8.1 million of those diagnosed in 2012 expected to die from cancer [1, 2]. In the EU approximately 1 359 100 cancer deaths are projected in 2015 (592 900 women and 766 200 men), corresponding to a death rate of 138.4 for men and 83.9 for women per 100 000 people, with a decline since 2009 of 7.5% and 6% respectively [3].

Lung cancer is one of the most common forms of malignancies and the most common cause of cancer death worldwide. Lung cancer can be caused by a number of factors that include; lifestyle choices including tobacco use, diet and lack of exercise; genetics, some infections and environmental contact to certain chemicals and radiation. The main causes are smoking, second-hand smoke and alcohol use. The NSCLC risk for smokers is increased by 30-fold on a scale depending upon the persons clinical story. The second most common cause is exposure to radon, which is an invisible radioactive gas produced by the decay of the elements uranium and thorium which is present in the soil and rock. Other causes that are not well documented for one reason or other, cancers affecting the lung, head and neck region such as the oesophagus etc. could be as a result of alcohol use (particularly squamous carcinoma) or release of certain types pollution into the environment such as from the petrochemical and fertiliser industries due to failings or from improperly managed landfill sites.

Non-Small Cell Lung Cancer (NSCLC) is the most common cause of cancer death globally. Although there have been some declines in its incidence in the developed world due to changes in smoking habits, these have been more than

offset by increases in smoking, and thus NSCLC, in developing countries, most notably in China [4]. The majority of patients with NSCLC present with locally advanced or metastatic disease that is not amenable to curative treatment. This is reflected in the poor prognosis of lung cancer and the death of over 1.2 million people worldwide each year [2].

While differences in pathology have long been appreciated, most subtypes of lung cancer were until recently lumped into one of two categories for treatment purposes; small cell lung cancer, or NSCLC, the latter of which we will discuss here. The gold standard chemotherapy for NSCLC is a doublet, including a platinum agent and one of several other agents incuding taxanes, gemcitabine and pemetrexed. While treatment with chemotherapy has been shown to improve survival in NSCLC, a plateau in benefit from chemotherapy appeared to have been achieved around the turn of the century, with one landmark trial showing equivalent outcomes between 4 commonly used regimens [5]. However, since then the importance of histology, and indeed molecular subtyping, has emerged. While this has particularly affected targeted therapies such as the use of EGFR inhibitors, standard chemotherapy agents such as pemetrexed have also been shown to have differential activity in different subgroups.

This has led the evolution of precision medicine in NSCLC. Precision medicine as defined by the National Cancer Institute (NCI) is “a form of medicine that uses information about a person’s genes, proteins and environment to prevent, diagnose and treat disease” [2, 6-9]. In relation to NSCLC, precision medicine has thus far mainly been used in guiding treatment options, however there is additionally great potential to personalise approaches to diagnosis and prevention in future.

Requirements for precision medicine

Precision medicine aims to match the right drug with the right patient, maximising benefit, minimising toxicity and ultimately reducing cost to the health system by reducing use of ineffective treatments. In addition to requiring effective therapies, precision medicine is heavily dependent on robust biomarkers that reproducibly identify patients likely to respond to treatments.

Various factors may be used to stratify treatment, although mostly so far this has been through the use of conventional pathology or molecular markers from the tumour. Notably the treatment options for only a relatively small proportion of patients are truly personalised in NSCLC at present, treatments are often costly, and benefits short-lived. There is thus a great need to further develop precision medicine in lung cancer, and here we will discuss not only the current status of precision medicine in NSCLC in brief, but also potential for the future and how challenges that lie ahead may be addressed.

Use of pathology to guide therapy.

NSCLC is made up of several different histological subtypes, of which adenocarcinoma is more frequent now than squamous cell carcinoma, although other subtypes exist and adenocarcinoma may be divided into further subtypes [10]. In the last decade or so it has become apparent that different subtypes may respond differently to treatment. The clinical development of pemetrexed is a prime example in which the combination of cisplatin and pemetrexed showed improved outcomes in adenocarcinoma but not in squamous cell carcinoma [11]. On the other hand, the angiogenesis inhibitor, bevacizumab, is associated with increased toxicity in

squamous carcinoma due to increased risks of bleeding [12], which has led to its use exclusively in non-squamous carcinomas.

An early finding during the development of epidermal growth factor receptor (EGFR) inhibitors was an increased response rate in adenocarcinomas [13-15]. In addition, other characteristics including; never smokers, female, and Asian ancestry were additionally found to correlate with response to treatment and were then used to stratify treatment in trials [16]. However, these characteristics have subsequently been shown to be surrogate markers for EGFR mutations, which are more reliably associated with response to EGFR inhibitors. Indeed, as discussed below, this shift towards molecular markers has been the driver behind the expansion of precision medicine in NSCLC.

Stratifying by molecular characteristics

Current biomarkers used in cancer diagnosis are either genetic or protein biomarkers which can be detected in biofluids such as in the blood or in surgically removed tissue. Detection of genetic biomarkers is more reproducible than protein biomarkers due to external and internal factors that alter during the progression of cancer [8]. Precision medicine is changing how we approach and tackle certain aspects of oncology. The course of therapy administered and the response of the patient will depend upon understanding individual aspects of molecular carcinogenesis, pharmacogenomics and genetic variances [17, 18]. There are a broad range of cancer biomarkers that can be used to detect for physiological changes such as proteins, sugars, nucleic acids, small metabolites, and cytokinetic and cytogenetic parameters, in addition to tumour cells found in the biofluids.

There is a growing list of biomarkers that have been developed or are in development at the moment for NSCLC patients in the clinical setting. Due to space requirements of this review, we will focus on those biomarkers that are in current use or emerging into the clinic.

Predictive Cancer Biomarkers

Cancer biomarkers can give information on the likelihood of responding to certain treatments [19] and aid in the decision of therapeutic approach [20]. The main predictive biomarkers that are recognised in the clinical setting for the molecular diagnostics of cancer are somatic mutations (point mutations and chromosomal aberrations), of the following genes: EGFR, EML4-ALK, ROS, RET, MET, HER2, KRAS, BRAF, KIT, PDGFRA, and BCR-ABL. Analysis of gene expression or methylation is currently a common approach in research than routinely used for diagnostics, with the exception in breast cancer assessment of specific genes used for predicting treatment response [18]. Nevertheless, there are many NSCLC biomarkers that are emerging from current research- EGFR (overexpression), KRAS, ROS1, RET, AKL, HER2, MET, ERCC, PIK3CA and BRAF. Some mutations that are currently considered to be undrugable, but are important in NSCLC carcinogenesis. The p53 tumor suppressor gene is a prime example as it is mutated in more than 50% NSCLC patients who smoked and used alcohol [21, 22]. Here, we discuss a few of the key NSCLC biomarkers, their frequency and associated-therapy (Table 1).

Epidermal Growth Factor Receptor (EGFR)

The EGFR family of receptor tyrosine kinase, EGFR (ErbB1/HER1), ErbB2 (HER2/neu), ErbB3 (HER3), and ErbB4 (HER4), are activated by the binding of

ligands such as EGF functioning in cell signalling events at the cell surface. The classical RTKs are composed of a large extracellular domain, a hydrophobic transmembrane domain, and an intracellular domain. The association of the RTKs and their respective ligands leads to the homo- or hetero-dimerisation of the receptors [23]; consequently, the dimerised RTK can be activated by autophosphorylation of free tyrosine amino acids present in the cytoplasmic tail of the receptor [24, 25]. The dimerised and activated RTKs are able to recruit other signaling molecules prompting many downstream signaling cascades [26-28] that regulate cellular proliferation, differentiation, and programmed cell death [24, 29]. Subsequently, the numerous signaling transduction pathways activated include the MAPK signaling cascade, the phosphoinositide-3- kinase (PI3K) pathway brings Akt/PKB to the plasma membrane, and the phospholipase Cγ pathway associates directly with EGFR resulting in the protein kinase C (PKC) activation [30].

EGFR activating mutations are considered to instigate cells to grow rapidly. Notably, the EGFR gene is mutated in approximately 10% of NSCLC cases overall and ~50% of patients who have never smoked [31]. It has been demonstrated the somatic EGFR mutations in exons 19 or 21 are linked to tumour sensitivity to tyrosine kinase inhibitors (TKIs) such as gefitinib and erlotinib. The survival rate has been observed to improve when exon 19 is deleted than compared to exon 21 point mutation at position L858R [32]. A Polish cohort study discovered that these mutations (exon 19 and exon 21 L858R substitution) in ~12% of cases with adenocarcinoma through the study of exon 19 deletion and L858R substitution [33] as well as in 13% of patients with the same diagnosis through the study of 29 mutations in exons 18, 19, 20 and 21 of EGFR gene [18, 34].

Regrettably, NSCLC patients acquire resistance to TKI treatment with time. Approximately 50% of adenocarcinoma cases have the somatic mutation T790M in the kinase domain of EGFR in exon 20 with the exception of mutations that sensitize the tumour to TKI treatment [35]. The main mechanism of TKI resistance occurs as a result of substitution of a threonine residue for a methionine residue at position 790 in EGFR which thus enhances adenosine triphosphate (ATP) affinity [36]. Furthermore, it is likely that drug resistance in ~20 % of patients are a consequence of MET gene amplification which is independent of T790M EGFR mutation. This acquired resistance to treatment could be triggered by an activation of the EGFR signalling cascade pathways. For progression in EGFR mutation patients under TKI (first generation), we observed T790 mutation, cMet, but also transformation in small cell lung cancer and nothing else to detect. The clinical development of EGFR inhibitors in NSCLC dates back over two decades, and initially involved assessment in an unselected population of NSCLC. In the BR.21 trial for example, erlotinib was compared to chemotherapy as secondline therapy for NSCLC [14]. In this trial, erlotinib improved overall survival from 4.7 to 6.7 months leading to its approval in this setting. However, evidence of improved responses in EGFR mutant patients emerged.

Current strategies and future developments in anti-EGFR therapies

The first-generation EGFR tyrosine kinase inhibitors (EGFR-TKIs) are reversible inhibitors such as gefitinib and erlotinib, have already been extensively studied in the treatment of advanced NSCLC patients. Tumours harboring activating EGFR mutations in particular the deletion in exon 19 (EGFRdel19) and exon 21 L858R mutation (EGFRL858R), are more than likely and are predicted to respond to EGFR-

TKI treatment [37-39]. Numerous phase II and III studies validate the function of first-line EGFR-TKI treatment in advanced EGFR-mutated lung cancer [16, 40-47]. The second-generation of EGFR-TKIs are irreversible inhibitors such as afatinib and dacomitinib, with the goal to be more potent in their inhibition of EGFR tyrosine kinase and to specifically target other ErbB-family members, including the HER2 receptor. These receptors can be catalytically activated by a cascade of intracellular pathways that play a role in cellular differentiation, migration and proliferation. As yet, no HER2 ligand has been identified, even though HER2 shows structural similarity to other ERBB receptors. The HER2 receptor is commonly and the preferred dimerising partner of EGFR displaying an increased potential compared to EGFR homodimers [48].

The second-generation EGFR tyrosine kinase inhibitors (EGFR-TKIs) are irreversible inhibitors such as afatinib and dacomitinib. Afatinib is an inhibitor selective against EGFR, HER2, and HER4. A number of reports have demonstrated the efficacy of afatinib in first line treatment of advanced EGFR-mutated lung cancer [49, 50]. Afatinib has been shown to be active against cancer cells displaying the activating EGFR mutations (EGFRL858R and EGFRL858R/T790M) [51, 52]. Dacomitinib is a selective inhibitor against EGFR, HER2 and HER4. *In vitro* studies with cell lines and xenograft studies show that dacomitinib is active against EGFR mutations and EGFRT790M [53, 54].

The third-generation EGFR tyrosine kinase inhibitors (EGFR-TKIs) are irreversible inhibitors such AZD9291, rociletinib, and HM61713. Both afatinib and dacomitinib have activity against both EGFRT790M and wild-type EGFR. However, the third-generation inhibitors were specifically designed to inhibit only EGFRT790M not wild-type EGFR [55]. The compound AZD9291 (AstraZeneca; [56]) is structurally different

to the first- and second-generation EGFR-TKIs. Early preclinical trials show that AZD9291 inhibits the phosphorylation of EGFR in cells with the EGFR mutations (EGFRdel19 and EGFRL858R) and EGFRT790M. Recently, AZD9291 entered phase I clinical trials in patients with advanced NSCLC with EGFR mutation T790M [57]. The results of the study concluded that AZD9291 was highly reactive in lung cancer patients with the EGFR T790M mutation [57]. AZD9291 has been shown to cause significant and unremitting tumour regression in transgenic mouse models and tumour xenograft displaying the EGFR mutation T790M. A number of studies are entering clinical trials using large cohorts with other third-generation compounds (Table 2).

An alternative strategy to irreversible and mutation-specific EGFR inhibitors is a combination therapy approach. This type of strategy overcomes acquired resistance to EGFR inhibition with dual treatment with afatinib and the anti-EGFR monoclonal antibody cetuximab. Currently, this therapy combination is in phase IB trials advanced NSCLC patients with EGFR-mutation with acquired resistance showing promising clinical response irrespective of T790M status [58]. However, trials with combination therapy of cetuximab with gefitinib or erlotinib of patients displaying acquired resistance to erlotinib showed no response [59]. These results implicate that irreversible EGFR inhibition and HER-family inhibition are required for the mechanism of action in the case of afatinib and cetruximab combined treatment.

ALK

Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase with unknown function normally expressed in the testes, central nervous system, and small intestine [60]. The oncogenic activity of ALK in anaplastic large cell lymphoma was

reported more than 20 years ago [60]. A recent screen to identify ALK arrangements in NSCLC showed that the genes encoding echinoderm microtubule associated protein like 4 (EML4) and ALK are found as a fusion oncogene located to chromosome 2 [61]. The ALK-EML4 fusion oncogene is important in up to ~7% of advanced stage of NSCLC [62]. Likewise, ~6.7% of the Japanese population with NSCLC patients were positive for EML4–ALK fusion oncogene [61]. In contrast, some other studies demonstrate a variable frequency of EML4–ALK fusion [63-65]. Generally, the EML4-ALK oncogene is more frequent in young, male and non-smoker patients with adenocarcinoma histology [61, 66-68].

Fortuitously a drug with activity against the ALK kinase (crizotinib) was already available at the time of discovery of the EML4-ALK translocation, allowing the rapid instigation of a clinical trial of crizotinib in NSCLC patients with ALK rearrangements [62, 69]. The trial demonstrated response rates of approximately 60% and a 6-month PFS of 72%, with these promising results confirmed in subsequent trials including a first line trial in comparison with chemotherapy in which crizotinib was superior [62, 70]. A second inhibitor of ALK, ceritinib, has also shown activity in ALK rearranged lung cancer, including in patients who had progressed on crizotinib [71]. Both agents have now been approved by the FDA and are in wide clinical use. Recently, for ALK patients, there is a new drug called Alectinib. This drug is for patients who have no response to crizotinib and ceritinib. However, the problem for ALK patients is that brain metastasis occurs in these patients with treatment (B. Melloni, Personal communication).

ROS1

C-ros (ROS1) is an oncogene encoding for a tyrosine kinase receptor part of the insulin receptor family. The rearrangement of ROS1 was first observed in glioblastoma [72-74]. Recently, the ROS1 rearrangement was discovered in NSCLC cell lines and tumoural tissue [75]. The rearrangement of ROS1 often involved in the fusion with other genes such as CCDC6, SDC4, CD74, EZR, KDELR2, LRIG3, SLC34A2 and TPM3 [76]. The ROS1 rearrangement in NSCLC occurs in between 0.7%–1.7% cases [76-78]. The ROS1 translocation is mainly observed in patients with adenocarcinoma pathology with a young age and never smoked. Nevertheless, ROS1 rearrangement is also demonstrated in patients with squamous pathology [77]. The ALK and MET inhibitor crizotinib is active in NSCLC with ROS1-rearrangement [78]. A recent cohort crizotinib clinical trial (PROFILE 1001) of patients with ROS1 translocation observed approximately 72% overall response rate (ORR) with 3 patients in remission (median response 17.6 months and median progression-free survival (PFS) 19.2 months) [79]. On the other hand, in an European study of NSCLC patients positive for the ROS1 gene treated with crizotinib (Mean age 50.5 years with ~65% women and ~68% never-smoked), the ORR was 80%, disease control rate (DCR) at ~87% and PFS was ~9 months. These cohort studies highlight that the patients' response to crizotinib vary according to the geographical location and that other factors may contribute to the progression of NSCLC in patients harbouring the ROS1 rearrangement. There are a number of drugs in development for lung cancer patients positive for ROS1 including AP26113, ceritinib, foretinib, PF-06463922 and inhibitors against HSP90 [80].

RET

The receptor tyrosine kinase, RET (rearranged during transfection) was an oncogene originally identified in thyroid cancer that is epigenetically controlled through translocations and activating mutations [24, 81]. RET translocations can be detected in about 1-2% of NSCLC patients with adenocarcinoma histology and poorly differentiated tumours who are often young and never smoked [82].

KRAS

KRAS was the first oncogene to be characterised and is part of the RAS oncogene family along with HRAS and NRAS [83]. The KRAS mutations in lung cancer are found in approximately ~25% of adenocarcinoma and ~4% of squamous cell carcinoma [84]. The KRAS gene is more common in smokers and infrequently detected in East Asian patient cohorts [85, 86]. KRAS mutations are normally present along with other types of oncogenic mutations in EGFR, BRAF, and HER2 or rearrangements in ALK and ROS1 [87]. The KRAS mutations are more common in NSCLC than HRAS and NRAS [84]. The predictive role and prognostic value of KRAS mutations remains controversial. For early stages of lung cancer, KRAS mutations have no prognostic or predictive value for chemotherapy treatment. At an early stage of lung cancer, KRAS mutations have no prognostic relevance or predictive to be used in adjuvant chemotherapy [88]. Conversely, in a NSCLC meta-analysis data review of the literature revealed negative correlation of the prognostic impact of RAS mutations mainly in adenocarcinoma cases [89]. While the KRAS mutations in patients with a form of metastatic NSCLC, these patients showed no response to chemotherapy [90, 91]. Nevertheless, the response to EGFR TKIs is negatively predicted through KRAS mutations [35, 92, 93].

ERCC1

The ERCC1/xeroderma pigmentosum, complementation group F (XPF) is found as a heterodimer and is a member of the XPF/MUS81 family. The ERCC1 gene is located on chromosome 19 and is present as four isoforms. It plays a major role in DNA repair pathways such as in UV-induced lesions and intra- or interstrand cross-links. A number of studies suggest that ERCC1 at the level of DNA, mRNA or protein, could be a prognostic or predictive biomarker for NSCLC patients treated with platinum doublets [94-99]. A well-established mechanism of cisplatin-resistance is related to ERCC1 overexpression. The levels of ERCC1 can also predict both patient survival and response to cisplatin therapy after resection in NSCLC patients [100, 101]. Surgical resection of NSCLC tumours receiving no additional treatment display an improved survival with ERCC1-positive as opposed to ERCC1-negative. Hence, the presence of the ERCC1 marker provides favourable prognosis and there is no benefit from adjuvant platinum chemotherapy. Though, the absence of ERCC1 from the tumour in NSCLC patients without treatment gives a poor prognosis and these patients would benefit from adjuvant cisplatin-based chemotherapy. Therefore, high levels of ERCC1 is a negative predictive marker, signifying to how well the patient will respond to certain types of treatment [97, 102].

Other biomarkers

c-MET is a receptor tyrosine kinase, is a receptor to hepatocyte growth factor which activates many downstream signalling pathways. This receptor has a global cellular function in proliferation, motility, migration and invasion. The c-MET receptor has a normal physiological control of tissue homeostasis but in cancer it becomes

deregulated as result of epigenetic and proteic changes such as mutations and amplification, or protein overexpression respectively. Interestingly, around 20% of cases with MET gene amplification that is independent of T790M EGFR mutation could be a potential cause of drug resistance. The deregulation of cMET receptor could be a source of resistance to treatment through activation of the ERBB signalling pathway. Some studies have demonstrated that ERBB signalling requires MET activity using the promising small molecule multikinase inhibitor (XL880) that is highly specific of MET or RNA interference to block MET expression [103].

There is a growing trend across several institutions and groups that has instigated genomic profiling contributes to the therapeutic direction for patients diagnosed with NSCLC [87, 104, 105]. A large cohort US collaborative study called 'The Lung Cancer Mutation Consortium' involving 14 academic institutions (ClinicalTrials.gov id: NCT01014286) was recently initiated with the view to genotype ten mutations associated to adenocarcinoma histology. This study uses the multiplex PCR 'SNapShot' technology that can identify potential molecular targets in lung cancer from genomic DNA derived from small amounts of formalin-fixed paraffin-embedded tumour samples. In addition, along with the analysis for ALK rearrangement and MET amplification using the FDA-approved fluorescent in-situ hybridization [106]. This assay is widely used within the cancer research community and is showing promise in the clinical setting [107]. This study reveals an actionable driven mutation in 54% of tumours analysed with 22% KRAS mutation, 17% EGFR mutation and 7% EML4-ALK rearrangement [106].

These biomarkers can be used before and during cancer treatment management for the risk assessment, diagnosis, prognosis, and for the prediction of outcome such toxicity and recurrence.

Identifying biomarkers for response to immune therapy

Recent trial results have confirmed the emergence of an exciting new class of therapy for lung cancer, the immune checkpoint inhibitors [108, 109]. These agents target inhibitory receptors on cancer or immune cells which would normally have an immunosuppressive effect, with the checkpoint inhibitors thereby 'releasing the breaks' on the immune system and allowing it to recognise and eliminate malignant cells [110]. The most exciting agents at present target the interaction of programmed cell death protein 1 (PD-1) (lymphocytes) with its ligand programmed death-ligand 1 (PD-L1) (cancer cells). Although relatively early, the data support activity for anti-PD-1 and anti-PD-L1 agents in both adenocarcinoma and squamous carcinoma. Notably only a relatively small proportion of patients appear to benefit significantly, however these may have prolonged responses to treatment. Therefore, there is a clear need to identify markers that predict response. Prime among candidate biomarkers is PD-L1 expression on cancer cells, with the seminal Keynote-001 study of pembrolizumab in NSCLC showing higher response rates and improved PFS in patients with high PD-L1 expression [109]. However, on the other hand, in the randomised phase III study of nivolumab there was no correlation between either PFS and OS and PD-L1 expression [108]. The reason for this disparity is unclear, although a recent analysis of multiple studies suggests that PD-L1 expression is predictive for response to both nivolumab and pembrolizumab. It is likely that the performance of different assays also contributes to differences in apparent correlation. That said, correlation between PD-L1 expression and response is imperfect, with responses likely also occurring in a proportion of PD-L1 low. In view

of this, there is active investigation into alternative biomarkers, with the main examples being mutational burden and gamma interferon signatures.

Liquid biopsies- the future?

A liquid biopsy is a biomarker isolated easily from body fluids such as blood, saliva, urine, ascites, and pleural effusion. Liquid biopsy was originally used to describe circulating tumour cells (CTCs) [111]. Presently, circulating tumour DNA (ctDNA) and exosomes are also used [112-114] (Figure 1). Liquid biopsy is an emerging field in oncology that could be useful in predicting cancer progression and determining the route of therapy to utilise in the clinic. They could be used at different stages including for early diagnosis; prediction of metastasis; monitoring efficacy of treatment; identifying potential new targets and mechanisms of resistance; and following metastasis in the patient [111]. In fact, analysis of CTCs, ctDNA and exosomes will provide a clearer picture concerning the information of both the epigenetic and proteomic profile than a tissue biopsy. Furthermore, the liquid biopsy is a non-invasive procedure and can be performed on most body fluids.

CTCs are derived from the primary tumour site by cell detachment and migration to distal secondary sites accessed through the lymphatic and blood system. These cells can be found in the blood of patients with solid tumours and their presence is linked to a poor outcome in metastatic cancer for lung and other solid tumours [115-117]. The analysis of CTCs can be used to measure the progression of the cancer and the metastatic process. A majority of studies have concentrated their efforts towards the epigenetic analysis such as circulating miRNAs, mRNA and mainly cell free DNA (cfDNA). This analysis of miRNA has provided a clear signature profile, which can determine the presence of the cancer [118]. A number of studies are

emerging of novel biomarkers used to identify cancer using the non-coding RNA (ncRNA) and the long-non-coding RNA (lncRNA) [119]. cfDNA is released from both healthy and cancer cells but in disease the levels increase with progression of cancer [120]. The analysis of ctDNA from a liquid biopsy can provide both information concerning the emergence of mutations acquired during treatment and be a non-invasive method to monitor outcome of treatment [114].

Cancer changes according to progression and treatment, which is often molecularly not stable. In the case of lung cancer, the most common EGFR mutations or ALK rearrangements, patients have benefits with TKI treatment such as afatinib, gefinib, elotinib, crizotinib, and ceritinib [126, 127]. Unfortunately, these benefits to treatment are short-lived due to acquired resistance to treatment [128, 129]. Liquid biopsy provides information from a simple and non-invasive blood test. In addition, tumour dormancy can be studied through ctDNA analysis. A number of studies have demonstrated that T790M EGFR mutations can be detected in the blood sometime before the disease can be found with conventional methods such as radiological [79, 130, 131]. However, at the present liquid biopsy is a challenge for the analysis of cancer originating from the nervous system, the lung and the bone. It is also important to provide a new biopsy at the time of progression.

Extracellular vesicles (EV) ranging in size from 30 to 1000 nm such as exosomes, ectosomes, microvesicles, oncosomes, and shedding bodies i.e. apoptotic bodies [121]. Exosomes (ranging in size 30 to 100 nm) are the smallest class of EVs found in most body fluids, are derived by an inward budding event of the late endosome or multivesicular body [122, 123]. Exosomes contain a plethora of signalling proteins, miRNA, mRNA and lipids [124, 125]. They are released from both healthy and cancer cells but their numbers increase exponentially with

progression of cancer. Exosomes are believed to provide the first communication network between tumour cells, its microenvironment and at distal sites transported by the blood circulation. In NSCLC patients, they can be detected in many body fluids including the plasma and bronchoalveolar lavage [126]. Their function may change during the progression of cancer in the earlier stages they could provide a means to evade immune detection, providing nutrient support from the microenvironment through release of growth factors and a communication system at later stages crucial for assisting the metastatic process [127]. These changes could be undetectable in the tumour-alone with current procedures whilst in EVs could provide a clearer picture for progression and outcome [128]. Exosomes also contain double-stranded DNA (dsDNA or exoDNA), which also could be used in genetic profiling to identify potential cancer biomarkers [129]. Numerous researchers have systematically investigated the content of EVs using proteomic approaches and miRNA microarrays. Molecules specific to disease identified in EVs are now being developed as novel diagnostic biomarkers and therapeutic targets for a range of human disorders including several lung diseases.

In the last 10 years, EVs and exosomes have been shown to be enriched and provide a protective environment for mRNA and miRNAs [124]. miRNAs are small non-coding RNAs with many functions and believed to control various genes by weakly binding to the 3'UTR of their target mRNA causing the deregulation of its target genes [130, 131]. The expression of several genes in neighbouring cells and within the microenvironment can be influenced by a single miRNA. The miRNAs have the power to effect both the secretion at their primary site and at distant sites through the circulation [132]. In different cancers, circulatory miRNAs are being extensively investigated as potential cancer biomarkers [133]. It is well-established

that exosomal miRNAs control intercellular communication [134]. In 2007, Valadi and colleagues showed for the first time that miRNA could be transported to neighbouring cells via exosomes which gave birth to the idea of genetic intercellular communication [124]. This study demonstrated that mouse exosomal RNA could be transferred and expressed in human mast cells. In later studies, some groups showed that the activity of miRNA could be regulated by exosomal transport [135]. Exosomes provide a protective cushion encapsulating miRNA such as in extreme environments (fecal matter) especially observed in the case of colon cancer [136]. It is suggested that mesenchymal stem cells (MSC) secrete mainly pre-miRNAs as opposed to mature miRNAs [137]. In MSC, RNA silencing was undetected indicating that pre-miRNAs in MSC exosomes have the potential to be active in the neighbouring cells. Nevertheless, both pre- and mature miRNAs have been isolated from different types of tumour cells. In a Drosophilia model, exosomes have been shown to induce miRNA splicing [138]. In later studies, a number of groups have proposed to use exosomal miRNAs for diagnostic and prognostic purposes for several cancers such as breast [139], lung [140], colon [141], esophageal squamous cell carcinoma [142], glioblastoma [143], kidney [144], leukemia [145], ovarian [146], prostate [147, 148], and for other cancers [149].

A number of studies have shown deregulation of exosomal-miRNA levels as a potential biomarker for cancer [150]. For example, exosomal levels of miR-141 and miR-375 can be used to predict metastasis or the recurrence of disease in prostate cancer [151]. There are diverse changes to the exosomal miRNA profiles of ovarian cancer compared to benign ovarian tumours. In addition, exosomal miRNA in normal controls is absent and can be used to determine healthy patients from patients with disease [152]. Likewise, in lung cancer, there are significant differences in the total

exosomal RNA levels of patients versus healthy control [153]. Whilst at the same time, in lung cancer patients there was a similarity between exosomal miRNA found in the circulation and the miRNA profiles observed in the tumour [140]. In particular, levels of specific exosomal miRNAs (miR-17-3p, miR-21, miR-106a, miR- 146, miR-155, miR-191, miR-192, miR-203, miR-205, miR-210, miR- 212, miR-214) are elevated in lung cancer [140]. In lung adenocarcinoma patients, the circulating exosomes were significantly higher compared to control [140]. This study and in several other studies, the identity of the purified exosomes is validated by electron microscopy and Western blot with exosome markers. Hence, relying solely on these techniques to identify exosome populations is invalid and highlights the need to develop new technologies to detect EVs. In fact, it's necessary to analyse the whole population of EVs to be certain not to exclude certain populations. The sizes of vesicles can be altered through modification of the lipid content and the exosome formation processes occurring at the multivesicular body [125]. In patients with lung adenocarcinoma, circulating exosomes were significantly higher than in control group [83]. Ultimately, serum harvested exosomal miR-1246 was elevated in esophageal squamous cell carcinoma (ESCC) patients, and is considered to be a strong impartial risk factor for poor survival [154]. Together, these studies confirm that the analysis of circulating exosomal miRNAs would be an extremely useful tool in cancer diagnostics.

Exosomes contain a plethora of more than 400 proteins; most of these proteins have diverse biological functions within the cell and microenvironment. Some of these proteins are involved in cellular metabolism or have a structural function and are associated to the cytoskeleton although, most importantly exosomes contain proteins that are potentially involved in cell signaling pathways.

The impact of these facilitators on the neighbouring or distant cell signaling pathways essentially remains to be elucidated [155]. The EGFR signaling cascade regulates several pathways involved in cell survival, adhesion, migration, differentiation, and proliferation [156]. The EGFR signaling pathway is linked to 3 main mitogen-activated protein kinase (MAPK)-pathways and the phosphoinositide 3-kinase (PI3K)/AKT survival pathway. The MAPK pathway is composed of the ERK1/2 part, the p38 MAPK part, and the JNK part. Microenvironment communication through neurotrophin signalling could lead to the progression of cancer [157-159] while an imbalance in expression of neurotrophin receptors such as sortilin is strongly linked to different types of cancer [160-163]. In a previous study, we discovered that the EGFR signalling pathways are induced between 3 to 50-fold in endothelial cells exposed to NSCLC exosomes enriched for the neurotensin receptor 3, also known as sortilin [125].

A number of proteomic studies use cell culture media and biofluids to analyse tumour-derived EVs [164]. Proteomic EV analysis of malignant pleural effusion from NSCLC patients reveals that the EVs contain over 900 proteins including EGFR, RAB family proteins, K-RAS, carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6), basigin (CD147, EMMPRIN), claudin-1 and claudin-3 [165]. A number of potential biomarkers found in exosomes have been reported such as leucine-rich α2-glycoprotein (LRG1), EGFR, CD91, CD317, and antigen A-kinase anchor protein 4 (AKAP4) [165-168]. In a recent study, NSCLC patients were distinguished from control subjects via differential display of 30 EV protein biomarkers from plasma using an ELISA-based method [169]. We have reported that a specific marker of NSCLC exosomes, sortilin forms a trimeric complex with two tyrosine kinase receptors TrkB (also known as NTRK2) and EGFR called the TES

complex [125]. Altogether, these data indicate the need to identify specific biomarkers and to develop specific EV technology for lung cancer. This will allow improvements to be made to the sensitivity of early diagnosis and to assess the progression of cancer, which is necessary to enhance patient outcome and survival.

EVs derived from the tumour site are believed to act as biological heralds in human disease such as in cancer and are thought to transfer the tumoural growth signals. Presently, it is implicated that the release of EVs-derived from the tumour site could symbolize a prospective target for cancer therapy [170]. A number of studies have reported that by perturbing EV release from cells through specifically targeting Neutral sphingomyelinase 2 (nSMase2) may decrease tumoural growth and metastasis at distant site [171, 172]. In NSCLC cells, other factors regulate EV secretion such as Rab27A [125] and p53 [173]. Though inhibiting these factors has been demonstrated to be effective against cancer, it will be necessary to study the molecular mechanisms of how specifically inhibiting EVs-derived from the tumour site are released from lung cancer cells. Moreover, the elimination of tumoural EVs from the circulation could be a possible therapeutic approach. This approach has recently been suggested by extracorporeal hemofiltration of a specific population of circulating of metastatic cancer patients [174].

While the function of proteins identified in the cargo of EV-derived from the tumour remains to be elucidated in most cases, those tumoural EV-derived proteins might stimulate the pathological-related mechanisms associated with disease. Likewise, EV-derived proteins could be used as biomarkers in therapy and thus could predict the response to treatment in NSCLC. In recent NSCLC proteome studies, EVs phospholipid content were modified significantly with gefitinib resistance

[175, 176]. This study suggests that EVs phospholipid metabolism could signify a tumoural phenotype and is associated to gefitinib resistance. In addition, cisplatin treatment of A549 cells sufficiently reduces the sensitivity of non-exposed A549 cells to cisplatin [177]. While the actual mechanism of EV-mediated cisplatin resistance remains to be decided. This study implicates that by modifying EV formation and release could be a future strategy for lung cancer therapeutics.

Conclusions

Precision medicine has already made a significant impact in NSCLC, and its remit is likely to grow. Through an improved understanding of its biology we have an unprecedented opportunity to develop the field, and overcome the many challenges that are outlined above. The key for future treatment is early detection of the cancer, which would improve the chance of survival and reduce mortality as a result of cancer. Many current efforts are devoted to developing new technologies steered towards early diagnosis. Liquid biopsies have the potential to overcome many of the shortcomings of current clinical management of NSCLC.

Figure legends

Figure 1

Liquid biopsy approaches and their potential use in lung cancer. CTCs can be found in the blood circulation and are generated by detaching from the tumour mass. The ctDNA originates from the tumour during necrotic events while cfDNA can be released from both healthy and tumour cells. Exosomes are actively released from most cell types and their numbers increase in cancer.

Table 1

A comparison of key NSCLC biomarkers, their frequency and associated-therapy.

Table 2

A comparison of first-, second- and third-generation TKIs against ErbB family members and their half maximal inhibitory concentration (IC50).

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Author Contributions

Cornelia Wilson and Joseph Sacco wrote the paper.

Abbreviations

ALK, Anaplastic lymphoma kinase; CTC, circulating tumour cells; cfDNA, cell free DNA; ctDNA, circulating tumour DNA; EGFR, Epidermal growth factor receptor; EV, extracellular vesicle; EML4, echinoderm microtubule associated protein like 4 ; ESCC, esophageal squamous cell carcinoma; MAPK, mitogen-activated protein kinase; MVB, Multivesicular body; NSCLC, Non-Small Cell Lung Cancer; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; RET, rearranged during transfection; RTK, Receptor tyrosine kinase.

Conflicts of Interest

The authors declare no conflict of interest.

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Figure 1

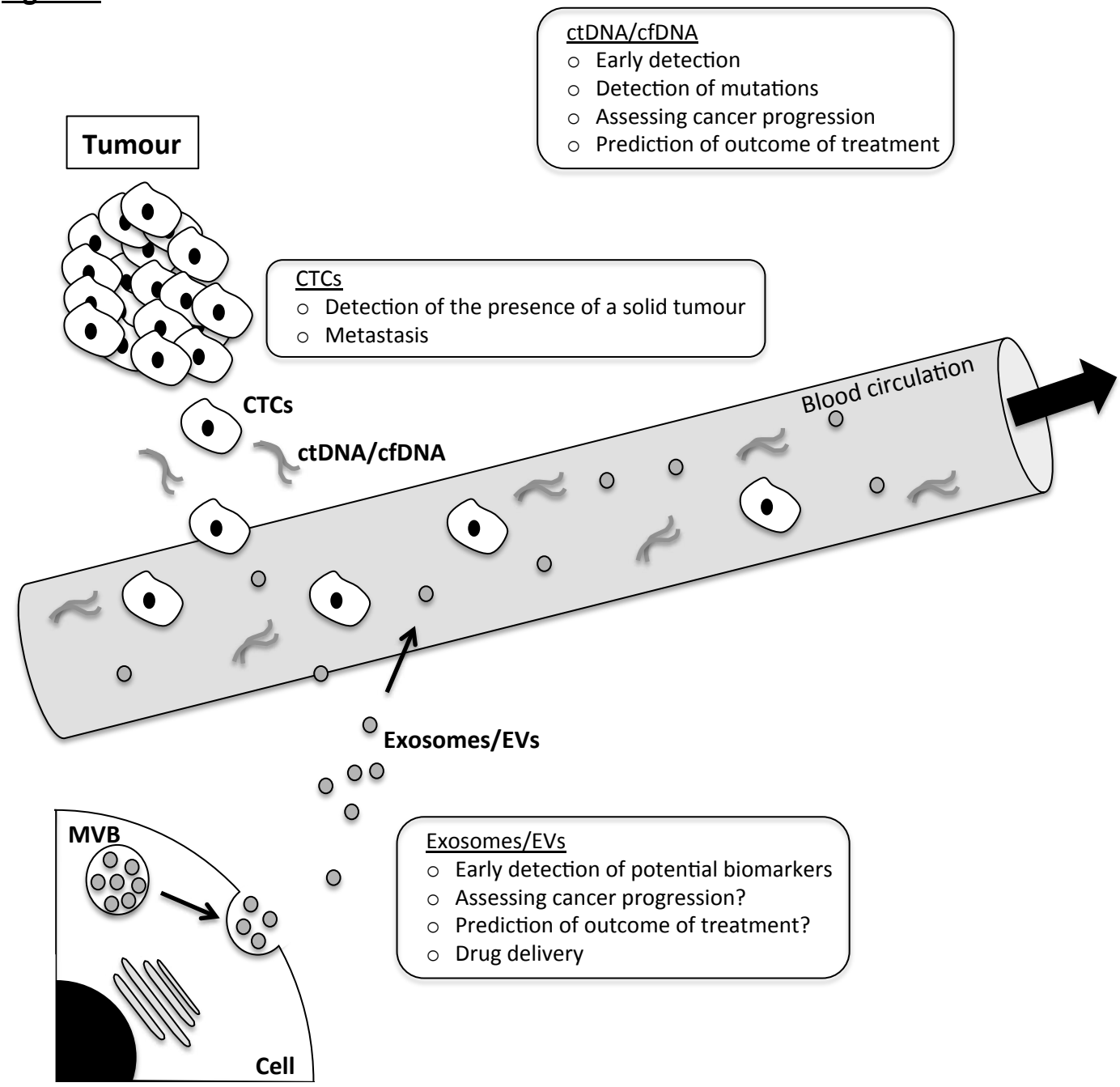
ctDNA/cfDNA
Early detection
Detection of mutations
Assessing cancer progression
Prediction of outcome of treatment
Tumour
CTCs
Detection of the presence of a solid tumour
Metastasis
CTCs
ctDNA/cfDNA
Blood circulation
Exosomes/EVs
MVB
Cell
Exosomes/EVs
Early detection of potential biomarkers
Assessing cancer progression?
Prediction of outcome of treatment?
Drug delivery

Table 1

Biomarker	Aberration	Frequency of aberration (%)	Therapy
EGFR	Mutation or amplification	10-25	Erlotinib, gefitinib, cetuximab
EML/ALK	Fusion	5-13	Crizotinib
ROS	Fusion	2	Crizotinib
RET	Mutation or translocation	1	Vandetanib, Sorafenib, and Sunitinib (clinical trials)
KRAS	Mutation leading to activation	15-25	Selumetinib and docetaxel combined
cMET	Mutation or amplification	2-4	Crizotinib

Table 2

Generation	Inhibitor	Type of therapy	Target (IC_{50}, nM)
First	Gefitinib (AstraZeneca and Teva)	Reversible TKI	EGFR (3) HER2 (1830)
	Erlotinib (Genentech, OSI Pharmaceuticals and Roche).	Reversible TKI	EGFR (0.5) HER2 (512)
	Icotinib (Beta Pharma)	Reversible TKI	EGFR (5)
Second	Canertinib (CI-1033) (Selleck)	Irreversible TKI	EGFR (0.8) HER2 (19) HER4 (7)
	Neratinib (HKI-272) (Puma Biotechnology)	Irreversible TKI	EGFR (92) HER2 (59)
	Dacomitinib (PF-00299804) (Pfizer)	Irreversible TKI	EGFR (6) HER2 (46) HER4 (74)
	Afatinib (BIBW 2992) (Boehringer Ingelheim)	Irreversible TKI	EGFR (0.5) HER2 (14)
Third	AZD9291 (AstraZeneca)	Irreversible TKI	EGFR mutation (100)
	Rociletinib (Clovis)	Irreversible TKI	EGFR mutation (21)
	WZ4002 (Selleck)	Irreversible TKI	EGFR (8)
	HM61713 (Hanmi)	Irreversible TKI	EGFR mutation (10)