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A Translational Approach to Lung Cancer Research

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Abstract

Lung cancer remains the main cause of all cancer deaths in the USA. The gloomy prognosis for non-small cell lung cancer (NSCLC) regardless of advances in current treatment modalities is most disappointing. Traditionally, disinterest and underfunding of research into the pathogenesis of lung cancer compared to other types of malignancies continued until fairly recently; Evaluating the complexity of the socio-politico-economic reasons behind this is beyond the scope of this article. Fortunately, increasing public awareness and current global political and legislative pressure against the tobacco industry is serving as a momentum pushing the study of lung cancer forward. Slowly but readily we are gaining important insights into the molecular pathogenesis of lung cancer, a fascinating and heterogeneous group of diseases; we are starting to understand their genetic and epigenetic anomalies, which seem to occur in a stepwise manner, mainly secondary but not exclusively due to tobacco smoking. Together with this, the emerging power of gene expression signatures for individual lung tumors and with the promising field of stem cell biology and the paradigm of cancer stem cells, we are most certainly paving the way to developing novel tools for the early detection, chemoprevention and treatment of these incredibly morbid pathologies with enormous global human and financial burdens. In this article we will explore all these issues and how we are starting to translate them into real diagnostic, therapeutic and prognostic clinically relevant tools for our lung cancer patients.

Keywords

Non-small cell lung cancer; tobacco; gene expression signatures; lobectomy and sublobar resection; stem cells; novel targeted therapies; chemoprevention; molecular markers; individualized treatment

2 | Introduction

Lung cancer remains the main cause of death both for men and women in the USA. There will be approximately 215,020 new cases diagnosed and 161,840 deaths due to this disease by the end of 2008. Worldwide this disease causes more than 1 million deaths per year [1] with obvious and enormous human and financial impact. Non-small cell cancer (NSCLC) is

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the most common histology in lung cancer accounting to approximately 80% of all cases, the rest being small-cell lung cancers (SCLC). Unfortunately and despite great efforts to improve survival, delayed diagnosis with subsequent late stage disease and high relapse even in patients with early-stage disease ultimately results in dismal prognosis; sadly, overall 5-year survival rates in lung cancer have only slightly changed over the last few decades, with current 5-year survivals being around 15% in the USA and much lower in developing countries [2]. Lung cancer remained until recently the 'black sheep' of human cancers; traditionally, little governmental financial resources were devoted to the study of a disease mainly caused by a lifestyle choice: tobacco smoking, and affecting an older male population. This social prejudice led to stagnation, for many decades, in the understanding of a pathology that is intrinsically fascinating. However, prompted by the staggering epidemiological lung cancer death statistics globally and thank to the work of many persevering outstanding scientists and physicians and due to the advocacy of lung cancer patients and their families there has been a shift of attitude favoring the study of lung cancer with the aim of applying this knowledge in a translational manner to find new clinical tools. Many pulmonary molecular genetic studies have demonstrated that numerous clinically manifesting lung cancers have several genetic and epigenetic anomalies [3]. Interestingly, many research groups have shown that many of these abnormalities are also present in histologically normal and pre-neoplastic adjacent lung epithelial tissue; hinting towards a multistep process of carcinogenesis encompassing the successive accumulation of genetic and epigenetic alterations, that occur contemporary to tobacco smoking; initiating, maintaining and leading to the progression of epithelial malignant conversion in the lung [4]. Recently, generous efforts by many investigators are resulting in the clinical translation of knowledge gained about the specific molecular mechanisms regulating and leading to lung carcinogenesis. Good examples of this translational approach are inhibitors of the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs); gefitinib and erlotinib. These agents are showing clinical benefits alleviating symptoms and extending survival in certain subgroups of lung cancer patients. Recent studies are now showing that the response to gefitinib can be influenced by the presence of certain EGFR mutations. Gefitinib is a good example, of science from the lab bench cycling to the bedside and then to the lab bench again, based on the observations that mutations in the EGFR can be crucial for the long-term efficacy of this drug. We will explore this and more examples, to highlight the importance of translational research in lung cancer. Furthermore, we will investigate how the growing biomedical field of stem cell research is becoming quite relevant and important in the understanding of the carcinogenesis of lung cancer and potential novel therapies against it; we now know that not all lung tumors are the same and there is increasing evidence suggesting that they may derive from the transformation of organ specific-progenitor cells derived from uncommon 'stem cells' that reside in specific pulmonary niches that result in the selective expression of genes enhancing cell fate and cell-renewal. Progenitor and daughter pulmonary cells are thought to represent the bulk-tumor proliferative cell pool that is responsive to chemotherapy, leaving the 'cancer' stem cell subpopulations, unaffected, ultimately leading to disease recurrence [5–9]. Though, translational research is becoming a reality, it is crucial not to forget that approximately 85% of all lung cancers are caused by tobacco inhalation, and therefore effective local, national and global strategies to educate, prevent smoking and help those who already smoke to stop need to be continued [10]; this is particularly important because nearly half of all lung cancers currently being diagnosed occur in ex-smokers. Identifying those ex smokers at higher risk of developing lung cancer becomes, therefore, rather important. Various biomarkers based on the common molecular anomalies, such as hypermethylation of certain genes, found in certain lung malignancies are being exploited and tested clinically [11]. We can see how translating what the molecular biology of lung carcinogenesis dissected and analyzed in the laboratory into useful diagnostic and therapeutic tools is paramount; and it is very exciting indeed to see better communication between basic researchers and clinicians;

each with a different perspective but with a common goal. We will now move on to evaluate the issues highlighted here in an attempt to emphasize the importance of translational research in lung cancer. We will firstly explore relevant aspects in the pathogenesis of lung cancer to then evaluate the role of newer scientific tools to study regions of genetic instability in this disease; we will also explore the role of genome wide studies and microarray gene expression profiling to then evaluate the importance of lung cancer murine models to finally assess the concept of cancer cells in lung neoplasia.

3 | The Molecular Basis of Lung Cancer

The concept of oncogenesis is a multi-step process during which genetic mutations sequentially accumulate leading to carcinoma in situ and subsequently, upon breaking through the basement membrane in an invasive lesion. Good examples of this model are breast and colorectal cancer [12–14]. Similarly, recent studies suggest that lung carcinogenesis also follows a multistep oncogenic process. Bronchioalveolar carcinoma (BAC) and atypical adenomatous hyperplasia (AAH), a pre-malignant lesion thought to be a precursor to BAC is frequently found adjacent to invasive adenocarcinoma [15–18]. The elaborate collection of genetic abnormalities and redundancy of disrupted pathways is caused by many substances present in tobacco and other environmental carcinogens resulting in the heterogeneous nature of lung cancers. It is not surprising that many tumor suppressor genes and oncogenes exert an important role in the development of lung cancer [19,20]. Single allele mutations in a proto-oncogene can frequently be enough to initiate and maintain the malignant transformation of critical, potentially progenitor cells within the lung leading to various malignant lesions. Amplification, translocation, re-arrangement and point mutations in dominant oncogenes assist this transformation. Homozygous loss of function in tumour suppressor genes, by deletion, mutation or both leads to abnormal regulation of transcription. The equilibrium between oncogenes and tumour suppressor genes has an effect on cell proliferation. Similarly, abnormalities in essential signaling developmental pathways such as Wnt, Hedgehog (Hh) and Notch leading to their activation during adulthood leads to the initiation of lung cancer. We will explore some of their aspects later on with a translational research approach, but we will now focus on the role of EGFRs in the treatment of lung cancer.

4 | Epidermal Growth Factor Receptor

The epidermal growth factor receptor (EGFR) signalling pathway although tightly regulated in normal cells it becomes abnormally active in cancer. The epidermal growth factor (EGF) was isolated from the murine salivary gland more than 4 decades ago. It was found to be involved in eyelid opening and incisor eruption in newborn mice[21]. Two decades later, the EGFR receptor, which has tyrosine kinase activity, was characterized[22]. The EGFR (HER1 or erbB1) is part of a bigger family of transmembrane receptors that include HER2 (EGFR2 or erbB2), HER3 (EGFR3 or erbB3) and HER4 (EGFR4 or erbB4). EGFRs share a common protein molecular structure of 170-kilodaltons; although they share homology in the tyrosine kinase (TK) region and common biochemical features include an amino-terminal extracellular domain involved in ligand binding, a single hydrophobic trans-membrane-anchoring region and a carboxyl-terminal cytoplasmic region with tyrosine kinase activity [23], each has their specific characteristics. For example, HER2 does not have a ligand-binding domain and HER3 is kinase deficient. Subsequent to binding of the relevant ligand, inactive monomers shape into homodimers or heterodimers, resulting in the autophosphorylation of the intracellular tyrosine kinase domains, that in turn results in the activation of a complex network of downstream signaling pathways, influencing cellular proliferation [23]. Examples of signaling transduction pathways activated by the EGFR include the mitogen-activated protein kinase (MAPK) pathway, regulating gene

transcription, and the phosphatidylinositol 3,4, 5 kinase (PI3k)/protein kinase B (PKB) signaling pathway, which plays a role in cell maintenance and survival. Disruption of these pathways in mouse models leads to abnormal angiogenesis, anomalies in epithelial development and malignant transformation in organs such as the skin, liver, eye and gastrointestinal tract [24–26]. Interestingly, high levels of EGFR RNA and protein expression have been shown in a wide array of human cancers such as cancer of the lung colon, the ovary and the esophagus. The role if these pathways in cancer has been further reinforced by in vitro experiments where transfection of high levels of EGFR and its ligands led to malignant transformation [23]; and by evidence that EGFR transcription and activation can be caused by certain viruses [27–31].

4.1 | EGFRS in Lung Cancer

Studies have shown that EGFR is overexpressed in around 70% of NSCLCs, while HER2 seems to be overexpressed in around 30% of NSCLC. In contrary, SCLC seldom over-expresses EGFR or HER2 [3,32]. In physiological pulmonary conditions EGFR localizes to the proliferative basal layer of the epithelium. When lung epithelium is exposed to toxic tobacco-related substances, hyperplasia followed by subsequent metaplasia and eventually dysplasia ensues. It has been shown that severely dysplastic lung tissue has increased EGFR expression compared to hyperplastic and metaplastic lesions again pointing towards the involvement of this pathway, in a stage-dependent manner in lung cancer [33–35]. Ironically, whether overexpression of EGFR in lung cancer correlates with poorer prognosis, as initially thought remains equivocal; a meta-analysis of 11 studies failed to show a clear relationship between EGFR overexpression and survival [36–52].

4.2 | The Clinical Impact of Mutations in the Tyrosine Kinase Domain of EGFR

It has been recently observed that mutations in the intracellular EGFR tyrosine kinase domain are common in patients who respond well to gefitinib and less so in those with reduced response [53–55]. An analysis summarizing nine separate studies estimated that EGFR mutations occur in about 24% of NSCLCs [56]. These mutations are divided into three different categories: 1 - missense point mutations occurring in exon 21 and accounting for approximately 41 of all mutations; 2 - in-frame deletions in exon 19 responsible for 44% of all mutations; and 3 - insertions. Interestingly it was observed that these mutations were more commonly found in tumours from specific patient subpopulations: that is females of East Asian origin, regardless of the continent where they lived, and who had responded better to treatment and had never smoked [57]. The frequency of EGFR mutations is thought to be 39%, and 48% among Japanese and Taiwanese patients respectively. In contrast, that rate is between 3 and 9% in non-Asian US patients and the molecular basis to explain this remains unclear. Although there seems to be enough scientific evidence linking mutant EGFR to clinical response to TKIs, it has been shown that there are certain populations with mutant EGFRs that actually fail to adequately respond to treatment and vice versa; why patients ‘without’ EGFR mutations do respond to TKIs may be partially explained by the relative insensitivity of current diagnostic tests to detect such mutations. To tackle the issue of why some patients with EGFR mutations fail to respond to TKIs, two studies in lung cancer patients with EGFR mutations were conducted. A new TK domain mutation was found in four out of the seven patients studied [58,59]. This mutation consisted on the substitution of methionine for threonine at position 790 (T790M). However in the remaining recurrence patients this particular mutation was not found suggesting an alternative mechanism for drug resistance development. An EGFR irreversible inhibitor of phosphorylation, CL-387785 functions successfully even in the presence of the T790M mutation [58]. Interestingly, the T790M mutation is analogous to a secondary mutation in bcr-abl causing resistance to imatinib in CML patients [60–62]. All this illustrates the need for prospective studies to determine the exact relationship between RTKI activity and EGFR

mutations; this ideally should happen before routine screening for EGFR mutations becomes incorporated into clinical practice. Adding more complexity to the situation is the discovery of several other predictive markers additional to the EGFR mutations; they include EGFR protein expression (not unequivocal as described above), amplification of the EGFR gene as well a various other markers. The degree of correlation of these markers with TKI response is diverse and as such, unsurprisingly, there is currently no standard procedure to determine good candidate NSCLC patients for TKI treatment although it is generally accepted that those ethnically Asian female non-smokers and adenocarcinoma histology should receive TKI treatment. Proposed prospective studies should also take into consideration the patients' clinicopathological characteristics as well as the specific molecular and biological features of the lung lesions: EGFR mutation status, expression level and the presence of other mutations such as for example K-ras. K-ras and EGFR mutations are mutually exclusive and they tend to present in about 30% of lung adenocarcinomas of smoker females. These patients fail to respond to TKIs and tend to have worse survival rates [63–66]. It is imperative to dissect all these factors both individually and in unison in order to advance our ability to make significant improvement to the management of our lung cancer patients.

4.3 | Inhibitors Against EGFR Tyrosine Kinase

Gefitinib and erlotinib are ATP competitive inhibitors of the EGFR tyrosine kinase domain. Phase-I trials of gefitinib demonstrated benefit in NSCLC. Two large phase-II trials, IDEALs 1 and 2 in patients previously treated with one or more chemotherapy regimes [67] showed response rates in the range of 9–19%, compared to only 7% for docetaxel. Symptomatic improvement occurred in 40% of patients on a 250 mg dose of the drug. A correlation was observed between developing of a rash and the response to treatment, and the main side-effect apart from this was diarrhea [68], although interstitial pneumonitis in lung cancer patients has been linked to gefitinib. A difficulty to ascertain this side effect is that in patients with advanced NSCLC the diagnosis of parenchymal lung disease is complicated. The overall world incidence of interstitial lung disease-type events in patients on gefitinib is approximately 0.34% (<0.1% in North America) compared to 1.9% incidence in Japan [69]. Difference in incidence may be explained by environmental factors, clinical practice and population differences (single nucleotide polymorphisms). The efficacy of erlotinib increasing NSCLC patient survival was illustrated in the Br.21 trial [70]. The trial consisted of 731 patients previously treated with one or two lines of chemotherapy and they were randomized to a 150 mg dose of erlotinib daily or placebo. Erlotinib patients were found to have a higher median survival compared to placebo (6.7 months vs. 4.7 months) as well as a higher 1-year survival (31.2% vs. 21.5%). The hazard ratio for death in the erlotinib group was 0.73 (95% CI, 0.61–0.86; $P < 0.001$). The FDA approved the use of Erlotinib in 2004 metastatic NSCLC. In contrast, the Iressa Survival Evaluation in Lung Cancer Trial (ISEL), did not show a significant difference in the primary median survival between patients in the gefitinib group compared to the placebo group in advanced NSCLC [5.6 months compared to 5.1 months (P=0.11) respectively]. Although there is encouraging evidence from several phase-II trials about the beneficial role of combining multiple targeted with little extra side-effects there is still a lot of work to be done, as illustrated by two large randomised trials, INTACT 1 and 2. When gefitinib was used together with a first line platinum-based agent, no survival advantage over chemotherapy alone was shown [69,71]. This may be explained by the antagonism between the cytotoxic effect of chemotherapy and the cytostatic (causing G_1 arrest) effects of the TKI. The results of trials of sequential chemotherapy and RTKIs will certainly help advance this field. Combining erlotinib with conventional cytotoxic drugs, like in the case of Gefitinib, did not prove to be useful. However, when erlotinib was combined with traditional chemotherapy in non-smokers, a survival benefit was observed [68,69]. As well as the examples discussed so far,

there are many other trials translating basic research into clinical practice; TOPICAL, looking at the role of erlotinib in patients not suitable for chemotherapy, etc.

4.4 | Monoclonal Antibodies Against EGFR Tyrosine Kinase

Lynch *et al.* showed that cetuximab conferred clinical benefit by disease control in 24.13% of the EGFR-expressing NSCLC patients treated [72]. Data from phase-I/II trials indicate that treatment with a first line platinum-based agent and Cetuximab is well-tolerated in patients being rash the only side-effect [73–75]. The LUCAS randomised study suggests that in the first-line treatment of advanced NSCLC the combination of cetuximab with cisplatin/vinorelbine shows beneficial clinical response with reasonable safety profiles compared to chemotherapy alone [76]. Furthermore, there are ongoing trials evaluating the combination of cetuximab with radiotherapy and chemotherapy in stage-III disease.

4.5 | Translational Research on Other EGFR Mutations

It is estimated that HER2 mutations occur in approximately 2% of NSCLCs [77,78] and they were found to be present in the same subpopulation as those with EGFR mutations (Never smoker, female, Asian and adenocarcinoma histology). Unfortunately, there is no identified physiological ligand to HER2, yet there is some evidence that it may play a role as a growth factor. Furthermore, heterodimerisation between HER2 and other EGFR receptors seems to potentiate signaling transduction [79]. Trastuzumab (Herceptin, Genentech), a monoclonal antibody against HER2, failed to show any advantage for NSCLC patients with HER2 mutations [80,81]. An important explanation could lie in the fact that subsequent immunohistochemical analyses of the tumours were negative for HER2, this would have obviously flawed the study and highlights the need for more robust studies to assess whether specific small molecules targeting HER2 receptors that express activating mutations in their tyrosine kinase are useful for the management of specific subgroups of NSCLC patients [78].

Soung *et al.* described that HER4 mutations were present in 2.3% of a 217 NSCLC patient cohort. Surprisingly, the majority of these patients were smoker males [82], indicating the complexity of this field and the need for additional studies that will hopefully eventually translate into novel therapies for lung cancer. The potential to exploit EGFR mutations as diagnostic biomarkers comes from the observation that often histologically benign lung epithelial tissue adjacent to malignant lesions harbor EGFR mutations [83]. Various groups are currently pursuing those observations by using mouse transgenic models [84,85]. The examples described herein demonstrate the heterogeneity of EGFR mutations; and that only through their careful understanding, will we be able to exploit them for the development of diagnostic biomarkers; targeted agents that can be offered individually or in combination with traditional chemotherapeutic or radiotherapeutic agents [86].

5 | Angiogenesis in Lung Cancer

Folkman *et al.* elegantly observed that tumors fail to grow beyond 2 mm without the presence of supporting vascularisation [87] and it is now well established that the growth a solid malignant lesion depends on appropriate remodeling and vascularisation of the tumor itself and its microenvironment [88]. In lung cancer, the role of angiogenesis has been evaluated and described by the measurement of micro-vessel density and its significant association with decreased survival rates [89].

5.1 | Vascular Endothelial Growth Factor (VEGF) in Lung Cancer

Endothelial cells produce VEGF: a mitogenic factor with a myriad of physiological functions[90], during lung development and homeostasis later on during adulthood. It is

unsurprising that abnormalities in the VEGF pathway can result in acute and chronic lung disease. The fact that tumours are dependent on the delicate balance between pro and anti-angiogenic factors, released by both tumour and their microenvironment has led many groups to exploit the role of targeting angiogenesis as a therapeutic tool to control cancer. Various VEGF families of factors have been characterized: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental factor. VEGF ligands activate upon binding three structurally similar type III receptor tyrosine kinases: VEGF-receptor (VEGFR-1), VEGFR-2 and VEGFR-3. Alternative gene splicing as illustrated in the case of VEFG-A leads to six different identified isoforms [91], these in turn may combine with the various receptors, highlighting biological functional heterogeneity. This heterogeneity is being exploited to develop targeted anti-angiogenic therapies [92] that with minimal side effects to non-cancerous cells.

5.2 | Monoclonal Antibody Against Angiogenesis

Research has demonstrated that VEGF is overexpressed in many cancers such as gliobastoma multiforme (GBM) and carcinogenic processes such as lymphangiogenesis in gastric and lung cancer [93–96]. Interestingly, expression of VEGF-C in maicecrophages around lung tumors was found to significantly correlate to prognosis; also, poorly differentiated and hyper-vascular lung tumours contained higher VEGF levels [97]. All these observations led to the development of antibodies to modulate angiogenesis during the process of carcinogenesis. Bevacizumab, a recombinant humanized monoclonal VEGF antibody has displayed favorable synergism in combination with chemotherapy in various preclinical and clinical scenarios. The survival advantage was exemplified in colorectal cancer, leading to the approval of bevacizumab by the FDA [98]. A phase-II trial studying the synergistic effects of bevacizumab and paclitaxel/carboplatin in lung cancer showed a significant improvement in the response rate to treatment [99]. In the 99 randomized and previously untreated patients, an overall response rate (ORR) of 31.5% was observed when bevacizumab was administered, contrasting to an ORR of 18.8% in patients who received chemotherapy alone. Equally, the bevacizumab group of patients demonstrated a longer disease-free interval (7.4 vs. 4.2 months) and they had an overall survival time of 17.7 months compared to 14.9 months in the paclitaxel/carboplatin group of patients. Hemoptysis and haematemesis were observed in six patients with centrally located squamous-cell tumors nearing major blood vessels; of those four died. For this reason and until clearer data becomes available lung cancer patients with squamous-cell have been excluded from most of the larger bevacizumab controlled randomized clinical trials. The Eastern Cooperative Oncology Group (ECOG) phase-III trial of paclitaxel/carboplatin with or without bevacizumab in untreated stage IIIB or metastatic NSCLC [99] showed a 27% response rate in patients who received bevacizumab combined with paclitaxel/carboplatin. However patients who were treated with paclitaxel/carboplatin alone had a 10% response rate. Progression-free survival for the former group was 6.4 months versus 4.5 months and the median survival 12.5 and 10.2 months respectively. This modality of treatment is currently ECOG's golden standard treatment for patients with advanced NSCLC. Potential side-effects include an increased 7.6% risk of neutropaenia, an 0.8%-increased risk of thromoboembolism and a 3.1%-increased risk of hemorrhage [100]. Other studies are underway to assess the role bevacizumab in neoadjuvant therapeutic regimes and in the context of combined regimes with EGFR TKIs [101].

5.3 | Miscelaneous Anti-Angiogenesis Agents

The number of molecules under current clinical investigation include the platelet-derived growth factor (PDGF), the platelet derived endothelial-cell growth factor (PD-ECGF) produced by many tumours and their infiltrating macrophages [102,103], fibroblast growth

factors (FGF)-1, FGF-2, various integrins and angiopoetins. Below is a brief description of the most relevant ones for lung cancer.

5.3.1 | SU11248 (Sunitinib)—This is an FDA approved single molecule administered orally. Its clinical benefit in the management of renal cell carcinoma was shown in two single-arm multi-center studies. Sunitinib targets various tyrosine kinase domains, including those of VEFGR, platelet-derived growth factor receptor (PDGFR) and c-Kit [104]. Due to the above and the promising data from a mouse small-cell lung cancer model various studies evaluating the role of SU11248 in NSCLC patients are underway [105].

5.3.2 | ZD6474—This is a low molecular weight inhibitor of the tyrosine kinase domain of VEGFR-2 and EGFR. The benefit of this agent in lung cancer was shown in a phase-I/II study in patients with metastatic tumors when used in conjunction with docetaxel [106]. Further trials will help elucidate the role of ZD6474 as first line therapy, either as single agent or in combination with other chemotherapeutic agents.

5.3.3 | BAY43-9006(Sorafenib)—This agent is a potent tyrosine kinase inhibitor of VEGFR-2, VEGFR-3, B-Raf PDGFR-β. In various lung cancer cell lines expressing B-Raf mutations, administration of BAY 43-9006 induced tumor growth inhibition [107]. Several phase-III clinical studies are assessing this agent in the context of NSCLC; as a single agent or in combination with other agents (Study ID Numbers: 050049; 05-C-0049: National Cancer Institute).

5.3.4 | AG-013736—AG-013736 is an anti-angiogenesis agent administered orally. It has activity against a variety of receptor tyrosine kinases such as VEGFR-1, VEGFR-2, VEGFR-3, c-Kit, and PDGFR-beta. In lung cancer, it seems to show clinical benefit as demonstrated in a phase-I trial were patients with advanced disease showed signs of disease stabilization and various phase-II trials have been designed [108].

6 | Epigenetic Changes in Lung Cancer and Their Therapeutic Potential

CpG islands refer to those DNA regions in which CpG dinucleotides are clustered and it is estimated that CpG dinucleotides are clustered in the promoter regions of about 50% of protein coding genes. When hypermethylation of cytosine occurs in the promoter regions of genes involved in tumor suppression cancer initiation ensues [109]. In lung cancer, it is estimated that around 80 genes are hypermethylated, and often these epigenetic changes occur simultaneously to multiple genes within a cancer [110]. These epigenetic changes can be usefully exploited and translated into practical tools for the early diagnosis of lung cancer. An example is the detection of hypermethylated DNA of the $p16^{INK4a}$ gene in the sputum of patients with higher risk to develop lung cancer. Great efforts using various approaches including the genome-wide strategy to identify a panel of genes specifically hypermethylated in lung tumors aims to create novel tools for the early diagnosis of lung cancer [111]. Efforts beyond the diagnostic ones are being employed to exploit the reversible nature of gene promoter methylation in order to design novel targeted therapeutic agents against lung cancer. Another epigenetic process currently being studied is that of histone deacetylation inhibiting gene expression. Therefore molecules capable of inhibiting this epigenetic process by reversing the silencing of essential tumor suppressor genes are currently being pursued by the pharmaceutical industry as potentially novel drugs against lung cancer. Examples of inhibitors of DNA methylation and histone deacetylation include azacitidine, and depsipeptide respectively [112].

7 | Immunomodulation in Lung Cancer and its Therapeutic Potential

William Coley, a surgeon in New York was one of the pioneers linking cancer to the immune system. He described how a recurrent cheek sarcoma successfully regressed upon concurrent infection with erysipelas [113]. Unfortunately, his rather innovative hypothesis, not solidly supported by evidence and only some anecdotal success, was met with great skepticism from the medical community and his views were not explored further for many decades until it was observed that subsequent to solid organ transplantation, immunosuppressed patients were at increased vulnerability of developing skin neoplasia as well as hematological malignancies such as lymphoma [114]. The field of cancer immunology has significantly moved forward since those early days, and the specificity of the immune system to target tumors makes it a very attractive research field with great therapeutic potential. There are currently various trials evaluating the role of vaccine therapy in the management of lung cancer. Two of the current strategies exploiting this field focus on active vaccination and adoptive T-cell transfer. Vaccination, however, has many challenges because of the ability of many lung malignancies to evade the immune system and the sketchy knowledge of lung antigens. Despite these difficulties a recent phase I/II multicenter clinical trial using granulocyte macrophage colony-stimulating factor in patients with advanced staged NSCLC demonstrated promising results that provide not only proof of principle but also the basis for further studies [115,116]. Adoptive T-cell transfer has been shown to play a role in the treatment of malignant melanoma; it is a process that entails the isolation and subsequent expansion of tumor infiltrating T cells *in vitro* followed by their reinfusion into the patients [117,118]. The challenge of this focus relies in the fact that it can be difficult to isolate enough tumor-reactive T cells from lung cancer patients. A summary of clinical trials evaluating the role of immunotherapy in lung cancer is shown in [Table 1].

8 | The Cancer Stem Cell Hypothesis

Interactions between cancer and the surrounding stroma rely on deregulated feedback mechanisms that in physiological circumstances are involved in cellular homeostasis [119–122]. Tissue maintenance stem cells must have three features; the power to self-regenerate allowing the maintenance of a population of undifferentiated stem cell pool throughout adulthood; a precise regulation of stem-cell numbers; and the capacity to differentiate in order to clonally repopulate functional cells within an organ [123]. Stem cells can diverge in their intrinsic ability to differentiate and self-renew [124]. The hypothesis of ‘cancer stem cell’ proposes the existence of a cancer cell that has the intrinsic ability to self-renew into another malignant stem cell as well as a cell responsible for the diverse cancer cell phenotypes [125–127]. According to this hypothesis, cancer recurrence is thought to occur because of the rarer stem-cell like cell populations evading traditional therapies via the acquisition of permanent mutations that render them resistant [127]. The model of cancer stem cells has been extensively explored in the context of blood malignancies where only a fraction minority of cells extensively proliferated [128–131]. There is evidence that disruption of self-renewal regulatory genes within the specific micro-environment harvesting the stem cells may provide the necessary signals needed for the stem cells to continue escaping the constrains that normally restrict their capacity to self-renew and allows them, upon exit from their niche to undergo differentiation.

8.1 | Identification of Pulmonary Cancer Stem Cells

The presence of a clonogenic population of cells in human lung cancer was described almost 3 decades ago. Clinical specimens from SCLC and adenocarcinoma patients were found to contain a small subpopulation of cells (<1.5%) that possessed the ability to form colonies when grown on agar. Upon their intracranial injection into athymic nude mice, they yielded cancers with features identical to those of the original specimens. This supports the notion of

cancer stem cell (CSC) populations within some lung cancers[165]. Elevated expression of ABC transporters is associated with an increased resistance to chemotoxic agents compared to non-SP cells[166,167]. Side-populations (SP) cells isolated by the efflux of Hoeschst 33342 by ABC transporters with stem cell characteristics have been characterized in NSCLC and in clinical specimens of lung cancer cell lines. Further evidence supporting that CSCs have features of immortality and quiescence is demonstrated by the fact that certain SP cells contain high levels of telomerase mRNA, and decreased levels of MCM7, a proliferation marker [125,168]. Purified SP cells were cultured *in vitro* and showed a greater invasion potential. They produced not only more SP subpopulations but also non-SP subsets, repopulating the original presorted cell line. Furthermore *in vivo* inoculation of these SP cells into mice proved that smaller number of cells (inoculums) were required to generate malignant xenografts in non-obese diabetic-severe combined immunodeficiency mice, suggesting that these cell populations had increased tumorigenecity compared to non-SP cells[168].

9 | Conclusion and Future Perspectives

Lung cancer, a heterogeneous group of malignancies, remains to be the leading cause of cancer death worldwide with an estimated toll of over 1 billion lives by the end of the 21st century. Despite advances in surgical techniques and traditional chemoradiotherapeutic modalities, 5-year survival rates have remained unchanged for many decades. A great challenge in the treatment of heterogeneous cancers is their intrinsic resistance to conventional therapies demonstrated by the stem and progenitor cells responsible for the initiation, sustained growth and survival of the cancers. However there is light at the end of the tunnel and despite the grim facts surrounding lung cancer, recent scientific efforts to dissect and understand the molecular and biological processes of the disease are paving the road to more efficient and precise methods for early diagnosis and prevention; some of this knowledge is being utilized for the design of novel therapeutic agents targeting specific biological and histological subtypes of lung cancer. In this review we have highlighted the most illustrative examples of pulmonary basic research being translated from the laboratory bench into the clinic and vice versa. A good example of unexpected clinical results requiring further study in the laboratory is demonstrated by the case of EGFR-TK mutations and response to TK-Is in specific patient subpopulations. Fascinatingly, recent advances in stem-cell technology, have led to the characterization of stem cells in several cancers such as those of the mammary gland and the intestinal system. Studies in these organs have revealed how cancer stem cells though related to differ from their corresponding non-malignant homeostatic stem cells in the originating tissues. We have therefore devoted extra effort in this review to highlight the concept of tissue maintenance stem cells and cancer stem cells; because even though the field is still rather immature in the case of lung cancer, the cancer stem-cell hypothesis presents us with essential repercussions for the early detection and prevention of this pathology, and more importantly it facilitates the notion of novel therapeutic strategies to target those lung cancer cells responsible for resistance to traditional chemoradiotherapy and cancer recurrence, which in turn result in decreased survival rates. We hope that not too far in the future we will be able to integrate and improve our ability to use tools such as microarray and proteomic techniques to profile the blood and/or individual lung cancer patients in order to deliver the most efficient single or combined therapeutic agents relevant to the particular tumor biology; the achievement of such individualized targeted therapies will represent the ultimate example of effective and successful translational lung cancer research.

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Table 1

Immunotherapy in Lung Cancer

Trial	Year	Antigen Type	Notes	Clinical Outcome	Reference
Phase I/II ALVAC (n=3 lung, 15 gastrointestinal)	2000	CEA	Non-replicating canarypoxvirus (ALVAC) constructed to express both human carcinoembryonic antigen (CEA) and the B7.1 co-stimulatory molecule	SD in 3/18	[169]
Phase I (n=4 lung, 1 mesothelioma and 8 other)	2003	MUCI	TG4010 is a viral suspension of a recombinant vaccinia vector (MVA) containing DNA sequences coding for the human MUC1 antigen and interleukin-2 (IL-2)	SD in 4/13	[170]
Phase I (n=10 lung and 16 other)	2004	WT1	HLA-A*2402-restricted, natural, or modified 9-mer WT1 peptide emulsified with Montanide ISA51	SD in 2 and PR in 12	[171]
Phase I (n=19 NSCLC)	2004	Allogenic cell lines	NSCLC modified to express B7.1	SD in 5 and PR in1	[122]
Phase II (n=171 NSCLC)	2005	MUCI	MUCI peptide with liposome adjuvant	Evidence of survival benefit	[172]

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