Minimally invasive diagnostics and immunotherapy of lung cancer

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Chapter 9

Summary and General Discussion

The general introduction of this thesis (**Chapter 1**) gives an overview of epidemiology, classification, the diagnostic process and standard of care treatments of lung cancer. To this day, lung cancer remains a pandemic disease with extraordinarily high morbidity and mortality rates, affecting and killing millions of people around the world[1,2]. Despite this major impact on global health, there is an evident lack of progress in improving the prognosis of this lethal disease, which becomes obvious when examining non-small cell lung cancer (NSCLC) survival rates from the past two decades. In the Netherlands, the five-year overall survival of NSCLC (independent of stage or gender) has improved by a mere 2.6% from 14.8% to 17.4%[3], which reflects a relatively late disease detection of NSCLC patients who generally present with advanced disease[4,5]. Besides the focus on improvements in primary prevention and early diagnosis of NSCLC, research efforts in the field of lung cancer have focused on finding alternative treatment strategies such as immunotherapy[6].

This dissertation deals with two important themes in NSCLC. In **Part I** of this thesis, the main topic is the implementation and optimization of endosonography in the preoperative staging algorithm of NSCLC. In the second part of the thesis (**Part II**), the potential of immunotherapy is assessed, either as an alternative treatment strategy or in combination with standard of care NSCLC therapies.

PART I: ENDOSONOGRAPHY OF LUNG CANCER

Ever since endoscopic ultrasound (EUS), a procedure in which mediastinal lesions are visualized by placing a linear echo-endoscope in the esophagus, was introduced to the field of pulmonology[7,8] several exploratory studies demonstrated promising performance (sensitivity, specificity and accuracy), a strong decline in futile thoracotomies and low complication rates for this new technique[9-12]. However, one common limitation of these studies was that mostly a fixed study design was chosen with preselected patients based on enlarged and/or 18F-fluorodeoxyglucose (FDG) avid lymph nodes on chest computed tomography (CT) and positron emission tomography (PET) scans. There was a lack of knowledge on the impact of EUS when implemented in the routine preoperative staging algorithm of unselected NSCLC patients. To this end, a retrospective study was conducted, the results of which are reported in **Chapter 2**. In a large cohort of consecutive and unselected NSCLC patients (n=152) it was demonstrated that use of EUS prevented futile surgical procedures in 39% of patients and an overall sensitivity of 74%, negative predictive value (NPV) of 73% and accuracy of 85% was found. Based on EUS test characteristics reported from a meta-analysis (pooled sensitivity 83% and NPV 78%) and a recent review (reported sensitivity of 89%) [13,14], one can argue that the EUS test characteristics found in this study are lower than to be expected. This can partly be

contributed to a relatively high proportion of NSCLC patients with small lymph nodes on CT in this study, for whom EUS is known to have a lower sensitivity, and to a relatively low prevalence of mediastinal metastases which is known to diminish NPV[14-16]. Nevertheless, the relatively low sensitivity and NPV, which results in a relatively high false-negative rate, should be considered an adequate reflection of the impact of EUS-FNA on routine NSCLC practice. The fact that in the group of patients, who underwent additional surgical staging procedures (by means of cervical mediastinoscopy), 6 out of 11 mediastinal metastases (55%) were found that went undetected by previous EUS, makes a strong case for confirmatory surgical staging after EUS. Indeed, sensitivity (92%), NPV (85%) and accuracy (95%) were much better in NSCLC patients who underwent both EUS and mediastinoscopy.

The introduction of endobronchial ultrasound (EBUS)[17], a procedure that allows aspiration of hilar, subcarinal and paratracheal lymph nodes under real-time ultrasound guidance from the tracheobronchial tree, was an important step in order to improve the false-negative rate of EUS. By giving access to pre- and paratracheal lymph nodes (station 2 and 4), which are difficult to visualize by EUS due to intervening air in the trachea, EBUS allows tissue staging of the same mediastinal lymph nodes as mediastinoscopy and therefore is complementary to EUS. A recent meta-analysis has indeed confirmed that the combination of EUS and EBUS procedures has higher sensitivity than either procedure alone[18]. An important question that remains is in which cases to perform additional surgical staging. This was addressed in **Chapter 3**, where a study on variables that can predict false negative EUS and EBUS findings is described. A logistic regression analysis of a large retrospective cohort (n = 775) revealed three main predictors for false-negative outcomes for EUS, EBUS and combined EUS/EBUS procedures: central location of the lung tumor, nodal enlargement on CT and FDG-avidity of N2/N3 lymph node stations on PET scans. When combined, several subgroups were identified with low predicted probability for being a false-negative outcome. These determinants can help in guiding clinical decisions on when to perform confirmatory surgical staging procedures in order to decrease the number of futile thoracotomies.

PART II: IMMUNOTHERAPY OF LUNG CANCER

As an introduction to the second main topic of this thesis, namely active immunotherapy of NSCLC, an overview of recent research efforts in the field of therapeutic peptide vaccination is given in **Chapter 4**. Many clinical trials have been conducted in which peptide vaccines were utilized for the treatment of cancer patients, although the number of trials that have been undertaken specifically for NSCLC is limited. Numerous early phase trials managed to induce a vaccine-specific immune response in cancer patients, but few

trials managed to achieve clinical success and a clear link of clinical parameters with the vaccine-boosted immunity was rarely made. Several explanations for these findings are discussed, such as the abundance of early phase trials performed in late-stage cancer patients together with a lack of information on general parameters of adaptive immune status of these patients. Perhaps one underrated aspect is the negative effect of the tumor microenvironment, one of the hallmarks of cancer[19], on immunological parameters and clinical outcomes of therapeutic vaccination of NSCLC. This is reflected by the fact that clinical studies have rarely, if at all, combined vaccine treatment with interventions aimed at depletion of tumor-associated macrophages (TAMs), myeloid derived suppressor cells (MDSC) or regulatory T cells (Tregs). Strategies on how to exploit the tumor microenvironment as a target for cancer therapy are discussed in two recent reviews[20,21].

In **Chapter 5**, the prognostic impact of tumor infiltration by CD8+ T cells in the context of expression of classical and non-classical HLA molecules was assessed. Tumor-infiltration by CD8+ T cells was shown to be correlated to classical HLA class I expression, and a positive effect on overall survival (OS) was demonstrated for tumors with high influx of CD8+ T cells together with high expression of classical HLA class I molecules (HLA-A and HLA-BC). As a singular determinant, a dense CD8+ infiltrate in NSCLC stroma was associated with improved OS, but this effect vanished when tumors had high expression of non-classical HLA-E, which had an expression rate of 70% in this study.

Another noteworthy aspect of the review outlined in **Chapter 4** is that only one study was published in which NSCLC patients were treated with a peptide vaccine combined with a conventional anticancer therapy between 2011 and 2013[22], and only a few of such combinatorial studies since 2014[23-25]. This might be related to scarcity of studies that report on the effect of standard of care NSCLC therapies on the human immune system, which is remarkable since knowledge on this subject is crucial when considering combinatorial approaches of active T cell based immunotherapy with standard of care NSCLC therapies. We have addressed this in **Chapter 6** where we report on a study of advanced NSCLC patients to see how treatment with conventional doublet chemotherapy or chemoradiotherapy influenced systemic immunity. Whereas chemotherapy had no manifest effects on antigen presenting cell (APC) and T cell function as well as on immune cell composition, treatment with irradiation caused a negative functional effect on APCs and T cells which coincided with a persistent drop in lymphoid cells. These findings are in contrast with the predominantly immune-potentiating effects of radiotherapy that are documented in animal models and *in vitro* studies[26]. However, most preclinical studies have examined the effects of radiation on immunity under different circumstances, testing a single high dose radiation as opposed to our clinical study in which patients were treated with multiple fractions of low dose radiation, which is known to induce markedly different gene expression profiles[27,28]. It seems that, to enhance active T cell based immunotherapy, an adjustment of radiation dose and fraction should be con-

sidered. In this light, it is particularly interesting that stereotactic radiotherapy, which utilizes high radiation doses in a few fractions, is now increasingly being used in stage III and IV NSCLC patients, whereas its capacity to cure early stage NSCLC was already well known[29]. Future studies are needed to answer the question whether successful immunotherapeutic approaches such as PD-1/PD-L1 targeting antibodies, now mainly used in advanced NSCLC patients, can be enhanced by radiotherapy, and if so, what the optimal dose and fraction should be.

Another important aspect of successful immunotherapeutic strategies is the identification of novel tumor antigens[30,31]. One such tumor antigen is XAGE-1b, a member of the cancer-testis antigen group which is expressed mainly in pulmonary adenocarcinoma. A study on spontaneously induced XAGE-1b specific local and systemic immune responses in adenocarcinoma patients is presented in **Chapter 7**. In 10% of patients, XAGE-1b specific T cells were detected in the primary tumor or draining lymph nodes and a XAGE-1b specific humoral response was present in 7.5% of patients. All antibody positive patients demonstrated the presence of circulating XAGE-1b specific T cells (both CD4+/ CD8+ T cells secreting both type I and II cytokines). This is the first European cohort of NSCLC patients in which both local and systemic XAGE-1b specific tumor immunity were demonstrated, supporting the notion of XAGE-1b as a novel, tumor specific and immunogenic tumor antigen. In order to translate these preclinical data, a phase I clinical trial is currently recruiting patients with advanced (stage III/IV) NSCLC for treatment with a XAGE-1b based synthetic long peptide vaccine. Safety and immunogenicity of the new XAGE-1b vaccine will be assessed. The study protocol and preliminary results are discussed in **Chapter 8**. How a XAGE-1b vaccine can contribute to cure is discussed below.

FUTURE PERSPECTIVES

What does the future hold with regards to the place of endosonography in lung cancer staging? In spite of claims that mediastinoscopy remains the gold standard of mediastinal nodal tissue staging[32,33], an overwhelming body of evidence points to the opposite. Nowadays, combined EUS and EBUS procedures achieve sensitivity, predictive values and accuracy that can match or even outperform mediastinoscopy[9,34,35] while being more cost-effective[36,37]. It would be much more sensible to consider combined EUS and EBUS as the new gold standard of pathologic staging of the mediastinal lymph nodes, as is reflected by the prominent place of endosonography as the first test of choice in mediastinal tissue staging in recent international guidelines [38-40]. An important prerequisite to maintain this position in the NSCLC staging algorithm is to adequately train pulmonology residents to learn and gain experience in performing EUS and EBUS procedures[41,42]. Furthermore, future studies should attempt to further reduce the number

of false-negatives, either by further improving EUS/EBUS sensitivity or by optimal allocation of NSCLC patients for additional surgical staging procedures. Several false-negative endosonography predictors were reported in this thesis, but nevertheless these results should be externally validated, preferably in a prospective multi-center study.

While endosonography has now been well-positioned in the landscape of pulmonary oncology, this is not yet the case for immunotherapy of lung cancer, which makes research efforts in this field all the more exciting, especially given the recent success of PD-1 blocking antibodies in the treatment of advanced NSCLC[43]. Currently, NSCLC patients are recruited to a multitude of studies testing PD-1 and PD-L1 blocking antibodies in a variety of clinical settings and in combination with conventional therapies and (peptide) vaccines to produce synergistic antitumor responses [44]. These much anticipated trials obviously hold high hopes for the near future. One patient group which is expected to benefit specifically from PD-1/PD-L1 antibody treatment are early stage NSCLC patients. This patient group is treated with curative intent by surgical resection but nevertheless 5-year recurrence rates as high as 24% are reported[45]. Most probably, these patients have minimal but undetected residual disease after resection (e.g. irradical resection or unknown nodal or distant metastasis), eventually leading to recurrence. Adjuvant treatment with PD-1/PD-L1 antibodies could lead to more effective anti-tumor immune responses especially since the tumor burden is low, and hence a suppressive tumor microenvironment is likely absent. It is noteworthy that early stage NSCLC patients have slightly higher rates of HLA class I expression than patients of higher stages (intermediate to high HLA expression, 71-89% stage I vs 61-76% stage II-IV) and, more importantly, stage I NSCLC patients with high HLA class expression have improved overall survival[46-49]. Thus, it makes sense to select resected early stage NSCLC patients based on HLA class I status in order to improve clinical effect of adjuvant PD1-PD-L1 antibody treatment.

However, some critical hurdles leading to non-responsiveness to immunotherapy of NSCLC need to be overcome. Firstly, the high presence of immune suppressive myeloid cells in NSCLC patients is a major impediment to successful immunotherapy of NSCLC. Not only do these cells promote tumor growth and progression, but it is also well known that immune cells with a major role in anti-tumor immunity (such as DCs, NK cells and CD8+ T cells) are inhibited by these immune suppressive myeloid cells. Furthermore, there is evidence that these cells can impair the efficacy of chemotherapy and immunotherapy in treating lung cancer[50]. Depletion of these immune suppressive myeloid cells (or alteration of their function) might therefore be a valuable addition to immunotherapeutic strategies to treat NSCLC. An example of this suppressive effect of myeloid cells was given in **Chapter 7** in a patient whose serum clearly showed XAGE-1b IgG antibodies but in whom we managed to detect XAGE-1b specific T cells only after removal of the abundant CD14+ myeloid population in the PBMC. There is also evidence of this concept of myeloid cell depletion in patients with other types of cancer. In advanced cervical carcinoma patients,

high frequencies of circulating myeloid cells normalized after treatment with carboplatin and paclitaxel which resulted in stronger recall T cell responses[51]. Furthermore, in a recent randomized phase II trial in patients with extensive stage small cell lung cancer (SCLC), treatment with a DC vaccine pulsed with wild-type p53 combined with all-trans-retinoic acid (a metabolite of vitamin A which promotes MDSC differentiation into mature, non-suppressive cells) induced a more than twofold decrease in MDSC, which was not observed in patients treated with the DC vaccine only. Importantly, this decrease in MDSCs resulted in a higher frequency of p53 specific immune responses[52].

Another important focus of future research efforts should be to identify new immunological checkpoints on cancer cells that are able to regulate anti-tumor T cell responses. One potential new checkpoint molecule, non-classical HLA-E, is discussed in this thesis in **Chapter 5**. The HLA-E molecule is expressed in 70% of pulmonary adenocarcinoma specimens. Its ligand is CD94/NKG2A and by engaging this receptor it has an inhibitory effect on T cells and NK cells. Hence, when assessing the clinical effect of peptide vaccines or other forms of active T cell-based immunotherapy targeting NSCLC, HLA-E should be considered as a biomarker and its expression should be taken into account given its potential inhibitory effect on tumor-infiltrating CD8+ T cells. Like its ligand CD94/NKG2A, for which an anti-NKG2A antibody is produced and currently tested in a phase I/II trial with head and neck cancer patients (ClinicalTrials.gov, Identifier: NCT02331875), HLA-E might in the future also become an attractive target for treatment with inhibitory antibodies.

Finally, the quest for novel and highly immunogenic tumor antigens will play an important role on the road to success (or failure) when it comes to immunotherapy of NSCLC. The role of CT antigens in general and XAGE-1b specifically has been discussed, but perhaps a bigger impact will be made by identification of tumor neoantigens which are not encoded in the genome, but arise as a consequence of somatic mutations (either driver or bystander mutations)[53]. Due to advances in next-generation sequencing and epitope prediction, these neoantigens will be increasingly investigated, particularly since treatment of NSCLC patients with PD-1 blocking antibodies improved clinical outcomes when a high nonsynonymous mutation and neoantigen burden was present, and a correlation of clinical response with neo-antigen specific CD8+ T cell response was found[54]. One can envisage that treatment of NSCLC patients with a XAGE-1b peptide vaccine can also lead to a cytotoxic T cell response to neoantigens by means of epitope spreading, since a case report showed that treatment of a metastasized melanoma patient with adoptive T cell transfer of a NY-ESO-1–specific CD4+ T-cell clone led to a T cell response to unrelated tumor antigens MART-1 or MAGE-3[55]. Another possibility would be to design multi-peptide vaccines containing a mix of CT antigen based peptides and personalized peptides based on tumor neoantigens. Either way, in spite of challenges ahead of us, the road ahead is exciting and perhaps can lead us to a better future for the treatment of this devastating disease.

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