Prognostic factors in resected lung carcinomas

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1. Introduction

A prognostic factor is one which determines or is related to the natural history of a disease, in the absence of disease-modifying therapy. A literature search provides innumerable studies purporting to describe such factors prognostic for patients with lung cancer. The potential significance of virtually every conceivable histopathological feature and molecular biomarker has been reported in thousands of studies. Yet in clinical practice, the only prognostic features which are regularly used in clinical decision-making are the tumour stage and the patient's performance status. This paper will address prognostic factors which are features of the tumour, relating to surgically resected lung cancer. It will not discuss those features of the individual patient which have prognostic significance related to the outcome.

The potential value of efficient prognostication in this particular clinical setting is to enable appropriate selection of patients for adjuvant therapy, determining who should benefit from systemic therapy, with that benefit likely to outweigh potential toxicity. To a lesser extent, knowledge of a prognostic factor before surgery may influence the type or extent of surgery which is carried out, but related practice change is still under trial. Adjuvant treatment is aimed at eliminating clinically undetectable micro-metastatic disease which, if present, may be responsible for tumour relapse. Prognostic factors are therefore predictors of a higher or lower probability of disease relapse and indicators of the likelihood that the surgery alone has cured the patient. Adjuvant therapy is therefore speculative.

Currently, adjuvant cytotoxic chemotherapy is offered to patients with pathological Stage II–III non-small-cell lung carcinoma (NSCLC) and reduces the risk of death by approximately 20% [1]. Trials have demonstrated that surgery effectively cures 64% of patients with p-Stage 1B disease and 39% and 26% respectively of patients with p-Stage II and III disease. Only an additional 3% of p-Stage 1B patients, and 10%/13% respectively of p-Stage II/III patients, will be alive as a result of adjuvant chemotherapy. Adjuvant chemotherapy in p-Stage 1B patients cannot be justified by this modest gain in survival [1–3]. Despite adjuvant chemotherapy, 33% of p-Stage IB, 51% of p-Stage II and 61% of p-Stage III patients succumb to recurrent disease.

The implication of these figures is that current decision-making should be improved to optimise whom and how to treat in the adjuvant setting. Prognostic factors that predict more accurately for postoperative disease relapse could improve selection of those patients most likely to benefit from adjuvant chemotherapy and – equally importantly – where it should be avoided. Factors that predict for effectiveness of individual drugs, which are outside the scope of this review, could be used to decide how to select chemotherapy for those who need adjuvant treatment.

1.1. *Tumour stage*

Tumour stage, a description of the extent of disease, is the only tumour-related prognostic factor regularly used to inform treatment decisions in patients with lung cancer. The latest iteration of the TNM (tumour, nodes and metastasis) system, the 7th edition, is the culmination of over 80 years of historical development and over 10 years of focused project work by the International Association for the Study of Lung Cancer (IASLC) [4]. This work is a 'tour de force' that evaluates a large amount of emerging data, changes in imaging, therapeutic approach and tumour biology, and conflicts between the need for retrospective compatibility with earlier systems and the requirement for better separation of prognostically divergent groups. The project involved analysis of more than 80,000 resected lung cancers, over 68,000 of which were NSCLCs. It amalgamated many international databases, but over half of the cases originated from Europe. Rigorous statistical analysis was applied to the database to produce robust data for all lung cancers, including evidence to support use of the TNM staging system in bronchopulmonary carcinoid tumours and small-cell lung cancer [5–12].

In contrast with the TNM 6th edition, the new TNM 7th edition shows better separation of the Kaplan–Meier survival curves for both clinical and pathological staging [8]. The main changes are (a) the introduction of additional cut-offs of

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Table 1 – Median and five-year survivals (5YS) by stage in resected non-small-cell lung cancer under TNM7 [8].

Stage	Clinical staging (cStage)		Pathological staging (pStage)	
	Median survival (months)	Five-year survival (%)	Median survival (months)	Five-year survival (%)
IA	60	50	119	73
IB	43	43	81	58
IIA	34	36	49	46
IIB	18	25	31	36
IIIA	14	19	22	24
IIIB	10	7	13	9
IV	6	2	17	13

tumour size to refine T-status, (b) movement of tumours >7 cm in diameter from T2 into the T3 category, (c) change in the way additional pulmonary nodules influence T/M status, generally recognising that this is of lesser danger to the patient than previously thought, (d) reclassification of pleural effusion as an M descriptor and (e) reassignment of some T&N combinations to different stages (Table 1). The previously recognised differences in prognosis related to tumour stage are clarified, with 5-year survival ranging from 73% in resected pathological stage IA disease to around 10% for stage IIIB/IV disease.

It is clear that pathological assessment of tumour stage in the surgically resected case is at least equally important as is full histological typing of the tumour [12] (see below). In order to facilitate an accurate assessment of a submitted specimen, there is an onus upon the surgeon to communicate all relevant information to the pathologist. Important factors include anatomical labelling of all specimens, especially lymph-node samples; details of surgery performed, especially if non-standard surgery has been performed, to help assist the assessment of margins; and information regarding any neo-adjuvant therapy delivered. There is also a duty for the pathologist to prepare properly the specimens in advance of dissection, examination and block-taking since these latter steps are key to determining adequate histological examination and pathological staging. Inflation fixation of resected lung bearing tumour is, in the authors' view, a critical step in preparation. Usually this involves per-bronchial instillation of 10% neutral buffered formalin until the lobe or lung is fully inflated with a smooth pleura. Sub-lobar resections may be inflated by injection. Although some pathologists prefer sectioning down the bronchi, especially for central bronchial tumours, parasagittal sectioning (the authors' preference) or coronal sections give a better view of the parenchyma, and facilitates both examination of peripheral tumours and correlation with radiology.

1.2. *Pathological assessment of lymph nodes*

It is clearly important to assess intrapulmonary, hilar and mediastinal lymph nodes submitted by the surgeon at the time of lung resection for primary carcinoma, since nodal status is a crucial factor in pathological staging. There is, however, debate in the surgical literature regarding how to deal with the mediastinal nodes at thoracotomy, with inspection, node sampling or radical dissection of all tissues at each station location being the three widely different options [13]. Improved staging, better local disease control and improved disease-free survival from more extensive surgery must be set against longer operation times, increased morbidity and no proven overall survival benefit. The concept of sentinel node sampling, a procedure common in the surgical management of other tumour sites, is poorly developed in the lung [14]. The European Society of Thoracic Surgeons guidelines recommend systematic nodal dissection, to include at least three N1 nodes (inter-lobal and hilar) and three nodes from three stations, including the sub-carinal station, in the mediastinum [15]. There is evidence that the number of lymph nodes resected, the number that is positive for tumour and the percentage of resected nodes which are positive have an influence on postoperative outcome [16–18]. Greater clarity is required around these data and the significance of the number of positive lymph node stations, given that true single-station mediastinal lymph-node metastases seem to carry a more favourable prognosis [19,20]. There are practical difficulties relating to assessing lymph node number if nodal fragments rather than whole nodes are delivered to pathology. There is also evidence that inadequate pathological examination may underestimate the degree of nodal involvement [21,22].

Does the degree of nodal involvement matter? Although it is traditionally taught that extracapsular spread of tumour from mediastinal nodes is a poor prognostic factor, some studies have failed to demonstrate a survival disadvantage [23], raising the possibility that this opinion is probably based on assumption rather than on hard data, especially since such spread may render the disease unresectable, rendering information incomplete.

There has been considerably more debate regarding the significance of micrometastatic disease in lymph nodes in patients with surgically resected NSCLC. The fact that a proportion of patients with pStage I (N0) disease relapse and die of tumour recurrence fuels a presumption of undetected micrometastatic disease at the time of surgery. Micrometastatic disease has no clear definition in the context of lung cancer, unlike in some other tumours such as breast cancer where nodal tumour deposits of <2 mm are regarded as micrometastases. Metastatic disease comprising only a few tumour cells may not be apparent on the standard haematoxylin-and-eosin-(H&E-)stained sections but could be detected on immunohistochemistry (IHC) [24]. Various strategies have been employed to detect micrometastases, usually involving immunohistochemistry with or without multiple step-sectioning of lymph nodes [25,26]. Most immunohistochemistry

has used antibodies to a variety of cytokeratins, but p53 and Ber-EP4 proteins have also been sought [24]. More recently, studies have utilised reverse transcription polymerase chain reactions (RT-PCRs) for a variety of mRNA transcripts of numerous genes, including *mucin1, carcinoembryonic antigen* (*CEA*), *p53*, *KRAS*, *FHIT*, *CDKN2A*, *survivin* and *livin* [24,27,28]. These markers are presumed to be sufficiently specific and sensitive to detect metastases of any size.

The outcome of these studies will depend on the adequacy of the 'standard' H&E-based initial assessment which determined N0 status. None of the IHC markers used is specific for tumour cells, and benign intra-nodal inclusions present the risk of a false-positive test. The same lack of specificity applies to most (possibly all) of the mRNA-based studies, although more recent work has used markers which are more specific [27]. Other issues with PCR studies include the following:

- The presence of mRNA does not necessarily mean that tumour cells are present, only that macromolecules have been detected.
- Studies have been based upon the homogenisation and mRNA extraction from fresh/frozen lymph nodes; whilst other nodes from the same location have been deemed negative for metastatses, it is an open question as to whether those homogenised nodes would have been histologically negative if examined in that way.
- There are practical implications in basing a routine test on fresh, frozen material; however, mRNA from formalin-fixed, paraffin-embedded tissue can be obtained and amplified.

Whatever the pros and cons of the technical approach, it is the outcome that ultimately matters. Can these techniques upstage – in a clinically significant way – patients otherwise regarded as having pN0 disease? Such studies are prone to reporting bias, with several using a range of approaches 'upstaging' 20–30% of patients who were considered to be pN0. It has been suggested that upstaging to pN1 may not be clinically significant, unlike upstaging to pN2 [25]. A very detailed original study of over 4000 lymph nodes from 266 Stage I resections, plus a meta-analysis of published work up to 2008, demonstrated that identified micrometastatic disease did not significantly decrease postoperative survival [29]. Subsequent publications, however, based upon mRNA PCR, continue to report significantly poorer postoperative survival in patients who are pN0 by histological examination but molecularly N1 or 2 on those nodes examined by PCR [27,28,30]. Notwithstanding the many technical issues around this approach to detect metastatic disease, and the biological significance of the findings, there is still a lack of trial evidence that patients would benefit from adjuvant therapy based upon a molecular upstaging of their tumour.

1.3. *Bronchial resection margins*

The status of the bronchial resection margin assessed in the resected specimen has been a matter of some controversy, and it is difficult to analyse due to limited and heterogeneous data. The presence of macroscopic disease at the resection margin (R2) is a poor prognostic factor [31]. R1 disease is also a poor prognostic factor although there are variables which need to be considered: the presence of extrachondral disease at the margin, or lymphangitis carcinomatosa, seems to be particularly poor prognostic factors, as both are associated with N2 disease [32–34]. Invasive disease within the mucosa also determines an R1 resection but may indicate a slightly smaller risk of recurrence, especially in the context of Stage I/II disease [32,34]. The significance of carcinoma in situ at the bronchial resection margin is less clear [33,34]. Unless the disease is extensive and involving bronchial glands as well as the mucosal surface [33], there may be insufficient risk of recurrence to warrant any further therapy [32,34].

2. Tumour histology

Although there is an extensive literature on the subject of tumour histology and prognosis, some studies lack statistical power, and it is difficult to determine whether any factor is significant in multivariate analysis, especially in controlling for Stage and rare tumour types. The use of neo-adjuvant or adjuvant therapy may also bias the outcomes of analyses.

2.1. *Squamous versus adenocarcinoma*

Is there a significant difference in postoperative survival between squamous-cell carcinoma and adenocarcinoma when controlling for Stage? Even this simple question provides issues to debate, but the probable answer is either 'very little' or 'no difference' (Table 2). A large German series of 2376 cases found squamous-cell carcinoma patients had a better 5-year survival (5YS) than adenocarcinoma: 53.6% compared with 48.2% [35]. A Japanese Lung Cancer Registry study of 13,010 cases found the opposite: 5YS for squamous carcinomas was surprisingly similar to that of the German study at 52.5%, but the 5YS for all adenocarcinomas was significantly

Table 2 – Five-year survivals (5YS) in resected non-small-cell carcinoma subtypes (all resected stages).

	Squamous-cell carcinoma (%)	Adenocarcinoma (%)	Large-cell carcinoma	Adenosquamous carcinoma
Pfannschmidt et al., 2007 [35] $n = 2376$ cases	53.6	48.2	45.8	–
Asamura et al., 2008 [36] $n = 13{,}010$ cases	52.5	67.3[a]	45.5	42.1
Chansky et al., 2009 [12] $n = 9137$ cases	43	44[b]	41	29

[a] These cases would include adenocarcinoma in situ (AIS).
[b] This is the 5YS for adenocarcinomas excluding those diagnosed as 'BAC'(see text). Given variations in stage distribution and other potential confounding factors, comparison between cell types within studies are probably more meaningful than those between studies.

better at 67.3% [36]. The likely explanation for this difference is the inclusion of significant numbers of cases of adenocarcinoma in situ or minimally invasive adenocarcinoma in this cohort (see *Types of adenocarcinoma*, below). These lesions are more common in Japanese studies, and until the publication of the new IASLC/ERS/ATS recommendations of adenocarcinoma classification [37] these cases were often classified as well-differentiated adenocarcinoma. It is now known that they pose no metastatic risk and show 100% 5YS. In the IASLC staging study cohort of 9137 cases, cases reported as 'bronchioloalveolar carcinoma' (BAC) were separated out from other adenocarcinomas and showed a 5YS of only 61%. The low figure suggests that this was still a pathologically heterogeneous group, comprising true 'BAC' i.e. adenocarcinomas in situ, and other invasive adenocarcinomas incorrectly classified as BAC (see below). The effect of this separation was to leave the non-BAC adenocarcinoma group with a 5YS of 44%, not significantly different from the 43% 5YS for squamous-cell carcinomas [12].

2.2. *Types of squamous-cell carcinoma*

The WHO classification of lung tumours [38] describes a papillary variant of squamous-cell carcinoma that generally has a good prognosis, probably because it demonstrates limited invasion and tends to be of low stage. Similarly, so-called 'creeping' squamous-cell carcinoma [39], an invasive tumour confined to the mucosa, demonstrates relatively indolent biology and a relatively good prognosis. Peripherally located squamous-cell carcinoma, arising from third-order or greater bronchi, may be increasing in prevalence. The growth pattern of these tumours may be infiltrative and destructive or non-infiltrative with preservation of lung architecture, the so-called alveolar filling growth pattern [40–42]. When this latter pattern is prominent, tumours tend to be of lower stage, show less vascular invasion (see below), and patients survive for longer [40–42]. Despite the relatively poor prognosis demonstrated by basaloid carcinoma (see *Other histological types*, below), the basaloid variant of squamous-cell carcinoma has been shown to be no more aggressive than poorly differentiated squamous-cell carcinoma [43,44].

2.3. *Types of adenocarcinoma*

The proposed changes in adenocarcinoma reporting and classification for surgically resected cases – authored by a multidisciplinary group of experts representing the IASLC, the European Respiratory Society (ERS) and the American Thoracic Society (ATS) – are largely based upon significant differences in prognosis demonstrated by different histological subtypes of adenocarcinoma [37]. This work acknowledged published descriptions of bronchioloalveolar carcinoma (BAC) and how that diagnosis is often associated with a better postoperative outcome. It also noted that there was enormous variation in type of tumour classified as BAC, in many instances that were clearly not BAC as defined in the 1999 and 2004 WHO classification. This led to the strong recommendation that the use of the term BAC be discontinued; that cases fulfilling criteria for BAC (small, localised lesions lacking invasion and showing only lepidic growth around alveolar walls) be reclassified as adenocarcinoma in situ (AIS), since such lesions pose no metastatic risk and have 100% 5YS [45], and that other lesions with evidence of invasion be classified as invasive adenocarcinoma, even if there is widespread lepidic pattern disease.

In resected invasive adenocarcinomas, the degree of invasion in a lesion which is otherwise AIS with a lepidic growth pattern may be very limited in extent. Assuming that some (most?) adenocarcinomas arising in the lung develop in this way, such lesions would be expected. If the focus of invasion in such a lesion is <5 mm in maximum diameter, there is still no metastatic risk and patients have 100% 5YS [46,47]. Such lesions are classified as minimally invasive adenocarcinomas (MIAs). If the focus of invasion, characterised by one or more of the other four invasive adenocarcinoma patterns (acinar, papillary, micropapillary, solid with mucin), is >5 mm across, the resected tumour is classified as invasive adenocarcinoma and a qualifier should be added to the classification when the report is issued by the pathologist, indicating which pattern of disease is the predominant one. This is also strongly recommended because of the notable prognostic effect: several studies have shown that resected adenocarcinomas with a predominantly lepidic pattern have a relatively good prognosis, independent of stage. Conversely, cases which are predominantly micropapillary or solid in pattern have a relatively poor prognosis [48–52]. Some studies show poor prognosis for papillary predominant disease [50], whilst others do not [48], possibly due to differences in interpretation of the papillary pattern. Although these patterns can be reliably and consistently identified, some are more difficult than others, notably papillary patterns [53].

2.4. *Multiple tumours*

The presence of multiple synchronous carcinomas was traditionally considered a poor prognostic factor for both squamous-cell carcinomas and adenocarcinomas [54], presumably reflecting intrapulmonary metastases in many cases. A better understanding of carcinogenesis in these two distinct tumour types, and recognition that multiple synchronous primary tumours – especially adenocarcinoma – are not uncommon, has modified this view. Multifocal disease undoubtedly reflects a biologically heterogeneous group of cases, making generalisations unhelpful. Unusual cases of mucinous or non-mucinous multifocal, predominantly lepidic pattern adenocarcinomas (the mucinous form now referred to as mucinous adenocarcinoma) were formerly considered to be BAC, despite these cases not fulfilling the post-1999 definition. Although demonstrating relatively indolent growth behaviour, these tumours carry a relatively good prognosis, with less propensity to spread widely outside the thorax, although they are invasive and do represent advanced, potentially fatal disease.

2.5. *Other histological types*

Large-cell carcinomas and sarcomatoid carcinomas appear to be aggressive, often large lesions [38]. Whether the associated poor prognosis is independent of stage is less clear. In the large studies presented in Table 2, large-cell carcinomas

appear to have a consistently and significantly lower 5YS. Sarcomatoid carcinomas are rare lesions which may or may not demonstrate components of differentiated squamous-cell or adenocarcinoma. These tumours are renowned for a poor prognosis and aggressive behaviour, although published series of cases are generally small [55–58]. There are two variants of large-cell carcinoma which are notable for their poor prognosis: basaloid carcinoma and large-cell neuroendocrine carcinoma (LCNEC). Case series of basaloid carcinomas are few, but data suggest an aggressive tumour, often of high stage at presentation, and a propensity for brain metastases [59,60]. LCNEC is a high-grade neuroendocrine carcinoma sharing many epidemiological and genetic features with small-cell carcinoma. This is a highly invasive tumour type prone to widespread metastases [61–63].

2.6. *Other histological features*

Certain histological features, independent of histological tumour type, have been shown to be independent prognostic factors. Features such as vascular invasion, lymphatic invasion, pleural invasion, tumour necrosis and poor differentiation have been so reported. The first three features are intuitive, and relate to key factors in the TNM system which correlate with poor prognosis. Vascular invasion within the tumour is a feature consistently associated with early relapse featuring distant metastatic disease [64]. Lymphatic invasion is associated with increased risk of lymph-node metastases [65]. The poor prognostic effect of pleural invasion is reflected in this feature, upstaging tumours in the TNM classification [66]. Tumour necrosis is usually associated with larger tumours, more poorly differentiated lesions and greater proliferative activity being indicative of an aggressive phenotype. Poor differentiation has long been associated with aggressive tumour behaviour and most of the other features determining higher tumour stage [38]. Criteria for grading tumours in this way have been poorly described and undoubtedly inconsistently applied by pathologists. However, there is increasing interest in tumour grading as an important factor in lung tumour pathology [67].

Tumour cell proliferation deserves particular consideration. High mitotic activity has long been recognised as an indicator for the presumption of relatively rapid tumour growth, and high mitotic indices are certainly associated with poorly differentiated tumours and tumours which have recognised aggressive biology (small-cell and large-cell neuroendocrine carcinomas). It is also a diagnostic defining feature for carcinoid versus atypical carcinoid tumour, and *de facto*, of large-cell neuroendocrine carcinoma with most assessments made on resected tumour specimens. This differential diagnosis carries recognised prognostic significance [38].

There are several problems in relying upon mitotic index as an indicator of likely tumour growth rate:

- Mitoses may be difficult to recognise on pathological specimens.
- The mitotic (M) phase of the cell cycle is relatively short so may poorly reflect overall cell cycle activity.
- Actual tumour growth is dependent on the balance between cell production and cell loss, the latter being very difficult to assess in tumours.

Proteins expressed during part or all of the cell cycle have been used as proliferation markers, although strictly speaking they only indicate cell cycle 'activity' and do not provide unequivocal evidence of cell division. These markers include proliferating cell nuclear antigen (PCNA), Ki67, a variety of the minichromosome maintenance proteins (MCMs) and histone-H3. MCMs may have an advantage over Ki67 in being evenly expressed throughout all phases of the cell cycle, whereas Ki67 accumulates later in G1, persisting through S, M and G2. PCNA has not shown convincing prognostic significance in NSCLC [68]. By far the greatest literature has been concerned with Ki67 expression in both early, surgically resected and advanced NSCLC, mostly as measured by the MIB1 antibody [68,69]. Two reviews, including publications up until 2006, described 46 reports of Ki67 as a prognostic factor in NSCLC [68,69], of which only 19 (41%) show 'over-expression' of Ki67 as a poor prognostic marker. Most found no independent effect on prognosis.

Actual tumour growth rates may be derived from preoperative imaging measurements and expressed as a volume doubling time (vDT) [70,71]. This parameter has been related to postoperative survival, some studies demonstrating an association between short vDT and poorer prognosis, although the relationship is not clear cut [72–74]. vDT is also used as a factor in predicting malignancy during nodule follow-up, often in the context of lung cancer screening [74,75].

3. Tumour molecular pathology

There is probably more literature on the putative prognostic effects of molecular markers in lung cancer than exists for other prognostic features in this disease. This is not surprising since molecular changes are the fundamental factors driving each tumour, making it behave in a unique way. Molecular markers are perceived to be more objective assessments than are some other pathological features. They are also considered to be more easily measured, numerous and to possess 'scientific' and 'topical' cache.

Studies have ranged from single marker investigations to pan-genomic works using a variety of approaches. Comparing studies of the more commonly investigated biomarkers is hampered by enormous heterogeneity of study design, variation in techniques, case mix and interpretation of data. Contradictory conclusions are frequently drawn, and perhaps because of this – and despite the enormous amount of data available – there is not a single molecular prognostic biomarker in regular clinical use for managing patients with lung cancer. A review of the topic in 1995 identified this issue and proposed trials of biomarkers in selecting patients for adjuvant therapy on the basis of claimed prognostic significance [76]. To date, very little progress has been made. It is only recently that mRNA-based gene signatures have been seriously investigated in this context (see below).'Single gene' studies have tended to use immunohistochemistry (IHC) to identify the protein product of the gene(s) of interest, although there are many studies looking at gene mutation, fusion or amplification using a variety of techniques. Gene transcription products (mRNA) have also been used, either for specific genes or using a more global approach using array techniques. Global

genetic studies looking for mutations, or gains and losses using comparative genomic hybridisation (CGH), are now frequent, although they have been less often studied from a prognostic perspective.

One of the most critical issues regarding tumour biomarkers concerns methodology. Techniques for carrying out the test, the reagents used, methods used to score/quantify the result, the analysis and interpretation of the results are all critical yet prone to variability and error. Some are more subjective than others; many are simple and readily available, others are complex, expensive and less accessible. Complexity does not guarantee accuracy, greater reliability or relevance. In terms of biomarker testing of tumour samples, the handling and processing of the tissues prior to testing are of critical importance yet difficult to standardise, but these factors are often ignored or overlooked [77,78].

A comprehensive review of prognostic biomarkers in lung cancer is beyond the scope of this article, but there follows a selective commentary on some important issues.

3.1. *Immunohistochemistry*

Following others' methodology in reviewing the literature [79], in 2006 Zhu and colleagues published an excellent and extensive review of 462 original papers and 12 reviews on immunohistochemical markers of prognosis in NSCLC published between 1987 and 2005 [68]. These studies focused mainly on resected NSCLC. Their data were helpfully grouped according to Hanahan and Weinberg's original six hallmarks of cancer [80] and accounted for 50 different markers. They identified five markers (EGFR, HER2, Ki67, p53 and bcl2) which had been extensively studied and were the focus of meta-analyses. For Ki67 and p53, higher levels of expression showed a weak but significant poor prognostic effect whilst high bcl2 showed a weak but significant poor prognostic effect. The authors suggested that 'over-expression' of cyclinE and VEGF, and p16, p27 and beta-catenin, were 'promising' as poor and good prognostic factors respectively. They also highlighted hepatocyte growth factor (HGF) and MET as potentially important, given in vitro data. Only MET has subsequently emerged as being clinically relevant due to its providing a therapeutic target and predictive factor rather than being a poor prognostic factor [81]. One of the most telling aspects in the review of Zhu et al. is that for almost every marker that is the subject of more than two publications, the prognostic effect claimed by some is absent in others. On occasion there are studies claiming good, poor and no prognostic effect for the same marker [68]. The authors highlight the differences in use of antibodies and definitions of over-expression as the probable explanation for such variation in outcome and emphasise the need for a consistent, planned approach to execution of such studies.

A more recent review of 111 reports took a very similar approach to that of Zhu et al. but concentrated on biomarkers relating to three of the six hallmarks of cancer: cell cycle activity, apoptosis and angiogenesis [82]. The authors' conclusions were similar, in that cyclin E, VEGF, p27 and p16 showed some prognostic effect, although bcl2 did not. Cyclin B1, p21, survivin and collagen VIII were also identified to have sufficient potential as independent predictors of patient outcome. The potential for use of combined panels of markers as prognostic predictors was also emphasised in this review.

The plethora of literature and inconsistency of data were highlighted in a further review [83] which also suggested that, given the molecular heterogeneity of lung cancer, it was unlikely that a single marker would emerge as universally useful. Essentially this is true, although a decision to treat or not could be based upon a simple binary evaluation of a reliable marker at a certain threshold, including patients with no expression. Such a marker has yet to emerge. Meta-analyses have suggested that TTF1 is a good prognostic factor in resected adenocarcinoma [84], whilst COX2 may be a weak, poor prognostic factor in stage I disease [85].

The determination of the best threshold (cut-off) for a quantifiable biomarker is also frequently unexplained or poorly executed. Simplistic approaches such as present/absent or above/below a median may ignore the biology of the system under study and will fail if the effect sought varies around a point elsewhere in the range. It is much better to use a statistical approach to determine the most effective threshold [86]. This is just one of the methodological factors which requires to be standardised if real progress is to be made with tumour biomarker testing and application [68,87].

3.2. *Gene mutation and copy number*

Gene mutations potentially have the same pitfalls as single IHC biomarkers, in terms of being ubiquitous and yet adequately discriminating in order to be clinically useful. Unlike with IHC biomarkers, where NSCLC subtype has largely been ignored, mutation studies have demonstrated prognostic effects for some mutations which are mostly found in lung adenocarcinomas.

TP53 mutations are the commonest mutation found in lung cancer and do appear to be associated with poor prognosis, but they are associated with positive smoking status, squamous cell as opposed to adenocarcinoma histology, male gender, poor tumour differentiation and higher stage disease at presentation [88,89]. Analysis of the effect of the mutation, as opposed to other associated factors, is therefore challenging. In multivariate analyses, *TP53* mutations have not been reliably independent prognostic factors in two surgical series [88,90], despite being associated with shorter postoperative survival in one of these studies [88].

Mutations in codons 12, 13 and 61 of *KRAS* are relatively frequent in lung adenocarcinomas, being found in up to 40% of European and North American cases but in around 10% of Japanese cases [88,91,92]. Individual studies and meta-analysis have demonstrated a poor prognostic effect of *KRAS* mutation [88,90,91,93–95] but some of these associations were rather weak and have not stood up to multivariate analysis [88,90,94,96]. *KRAS* mutation is associated with positive smoking status, poor tumour differentiation and higher stage, again probably confounding the prognostic effect. The presence of increased gene copy number as well as mutation in *KRAS* has been associated with poor prognosis [95].

Mutations on the tyrosine kinase domain of *EGFR* occur in around 50% of adenocarcinomas in East Asian patients and around 15–20% of European/North American patients [92].

EGFR mutations, associated with female gender and never smoking, are generally perceived to be a good prognostic factor. Although some studies have shown a good prognostic effect in surgically resected adenocarcinomas [97], there are many studies in which this effect does not survive multivariate analysis [88,90,94,96,98,99]. *EGFR* mutations are associated with lower stage disease [98], well-differentiated adenocarcinomas [88] and tumours with a predominantly lepidic component [100], all factors known to carry a good prognosis. In adenocarcinomas, high copy number of *EGFR* was reported as a good prognostic factor in one study [94] but another found no effect [99]. In studies looking at 'NSCLC', *EGFR* polysomy/amplification has been reported as a poor prognostic factor overall [101,102], or in squamous-cell carcinoma but not in adenocarcinoma [103]. The overall impression is that whilst *EGFR* mutations are associated with a better prognosis, this effect is not independent of the other good prognostic factors with which this mutation is associated.

To expand on the statement regarding HGF as a poor prognostic factor, MET is the HGF receptor and increase in *MET* gene copy number is associated with poorer survival through more aggressive tumour biology, higher tumour stage and histological grade [81,104,105]. *ALK* fusion genes and *BRAF* mutations are targetable oncogenic drivers in advanced adenocarcinomas. *ALK* fusion may be a good prognostic factor in surgically resected and advanced-stage adenocarcinomas [106–108], even although *ALK* fusion is associated with solid and cribriform adenocarcinomas with signet ring cells, aggressive histological features [37,108]. ROS1 and RET fusion both also appear to be good prognostic factors [108]. This may be because tumours bearing these various gene fusions are not associated with tobacco carcinogenesis. *BRAF* mutations are associated with micropapillary adenocarcinoma histology, a poor prognostic factor [109].

3.3. *Pan-genomic studies*

Global chromosomal disarray, often reflected in tumour-cell nuclear pleomorphism, has long been associated with aggressive tumour behaviour. More extensive genetic gains and losses shown by comparative genomic hybridisation (CGH) are associated with higher tumour stage, poor differentiation and tumour progression [110–113], and early relapse of resected adenocarcinoma [114].

Oligonucleotide and cDNA expression microarrays can be used to determine the expression of thousands of genes from mRNA extracted from resected tumour samples [115]. This technology has been used extensively to characterise resected lung carcinomas. The clustering of tumours into different groups that share patterns of gene expression has led to subdivisions and molecular classifications of lung adenocarcinomas in particular [116–120]. These subdivisions have been associated with differential patient survival, but a closer examination of the categories with better or worse prognosis suggests many of these molecular subdivisions are recapitulating histological factors already recognised as prognostic [37]: well and poorly differentiated tumours, or lepidic predominant tumours [119–121].

There is also an extensive literature investigating the potential for mRNA-based gene expression profiles to predict overall survival in surgically resected lung cancer [122–129], disease recurrence in stage I patients [130–133] and lymph-node metastatic disease [134–136]. Panels (signatures) ranging from 2 to 8644 genes were identified for squamous–cell carcinoma, adenocarcinoma or all histological types, but it is striking that there is almost no overlap in the genes identified between studies. Also, depending on the statistical methods applied, it is possible to generate different predictive signatures from the same data set [137]. The extent to which investigators undertook validation of their signature is variable. One large study did attempt multi-institutional validation but essentially failed to produce a robust, consistent signature, although the molecular data did appear to enhance the prognostic value of the clinical data available [138]. One PCR-based study did validate a ten-gene prognostic signature for Stage I adenocarcinoma between a European and a North American centre with 75% accuracy [139], whilst another validated a 14-gene expression assay in two North American and one Chinese institution [140], generating three risk groups in resected stage I–III non-squamous carcinomas ranging from 74.1% to 44.6% 5YS. There is no overlap between the 10- and 14-gene sets used by these groups, and neither of these studies included squamous-cell carcinomas.

The appeal of such positive signatures is obvious, but there are many similar and all claim more or less the same prognostic power. None of these has been prospectively tested as a means to select patients for adjuvant therapy, but trials are ongoing and the outcomes are awaited with interest. Whether any of these trials will risk (allow?), in case of adenocarcinomas, any comparison with the prognostic stratification by histology [48–52] remains to be seen.

Other molecular signatures have been related to prognosis. In studies of squamous-cell carcinoma, a panel of five microRNAs' (miRNA) expression has been related to increased mortality risk in squamous-cell carcinomas [141], whilst miRNA expression was found to be superior to an mRNA signature in predicting overall survival [142]. Lu et al. reported two prognostic miRNA signatures in resected stage I lung cancers, one for all NSCLC types, and a different one for adenocarcinoma only [143]. Promoter methylation of the *P16* gene as a mechanism of gene silencing has been suggested as a poor prognostic factor in NSCLC in one meta-analysis [144]. In a case–control study of resected Stage I NSCLC, promoter methylation of *P16*, *CDH13*, *RASSF1A* and *APC* was associated with early relapse due to tumour recurrence, an effect independent of stage, tumour histology and patient characteristics [145].

There is no specific conclusion to be reached with regard to resected NSCLC genetics and prognosis. It stands to reason that more aggressive tumour behaviour, with the propensity for postoperative disease relapse, is likely to be driven by genetic changes in tumours. Given the diversity of NSCLC, it seems unlikely that any such 'genetic signature' will comprise only one or two altered genes. Combinations of genetic alterations making an individual tumour more aggressive are highly likely to vary from case to case, depending upon histology, aetiology and other factors. It remains to be seen whether clinically useful prognostic genetic signatures can be identified, and what forms of genetic alteration these will be.

4. Tumour immunology

The importance of the tumour immune response in tumour progression has been recognised by the inclusion of both tumour-promoting inflammation and mechanisms to avoid immune destruction in the next generation of hallmarks of cancer [146].

Chronic inflammatory cell infiltrates (lymphocytes, plasma cells and macrophages) are common in resected NSCLC but global histological assessment of these infiltrates has failed to show prognostic significance [147]. If, however, the microlocation (stroma versus amongst the tumour cells) and cell content of these infiltrates are taken into account, there is an effect on prognostics in resected NSCLC. Intra-tumoural infiltrates rich in $CD4^+$ lymphocytes and $S100^+$ Langerhans cells are associated with better postoperative survival [147]. Uncommon examples of resected NSCLC showing marked immune cell destruction reminiscent of immunological regression seen in renal and skin cancers have been reported [148]. These cases had a superior postoperative survival, showed evidence of radiological shrinkage prior to resection, and were characterised by infiltrates rich in Langerhans cells, $CD4^+$ and $CD57^+$ lymphocytes and macrophages.

More recent studies have been better able to characterise the nature of intra-tumoural immune-cell infiltrates, and there are several reviews and many reports of tumour-infiltrating lymphocytes (TIL – B cells, $CD4^+$ and $CD8^+$ T cells, natural killer cells), macrophages, plasma cells and others, generally demonstrating that immunological reactions seem to indicate a more favourable prognosis in resected NSCLC [149–152]. There are reports, however, of certain TIL cell types, such as $FoxP3^+$ T cells and macrophages over-expressing IL10 or TREM-1, which seem to be pro-tumourigenic and associated with shorter survival [150]. Prognostic immune gene profiles have also been derived from tumour mRNA extracts [150], supporting the histological data on intra-tumoural immune-cell infiltrates. In an interesting evolution of this argument, mRNA gene signatures derived from circulating blood mononuclear cells have been shown to be prognostic in NSCLC patients [153,154].

5. Tumour metabolism

Tumour metabolism, as assessed by ^{18}F-fluorodeoxyglucose positron emission tomography (FDG-PET), has been shown to correlate with tumour stage, lymph-node involvement and postoperative survival [155]. Higher PET positivity (SUV_{max}) indicates higher tumour metabolism and is a poor prognostic factor which also correlates with central tumour location, squamous-cell rather than adenocarcinoma subtype, poor tumour differentiation, larger tumour size, pleural invasion, lymph-node metastases and higher stage. SUV_{max} has been shown to be an independent prognostic variable in resected NSCLC in multivariate analysis [156–158]. In resected stage I adenocarcinomas, patients at high risk of disease recurrence could be identified on the basis of lymphovascular invasion and by SUV_{max} [158]. High SUV_{max} also correlates with high tumour-cell density and high cell cycle activity (Ki67 assay) [157]. In a meta-analysis, 11 out of 13 studies concluded that high SUV_{max} was a poor prognostic factor in resected NSCLC [159]. The threshold SUV_{max} described by various authors separating good from poor prognostic cases is very variable, probably the result of case and histological mix, but also variations in scanners used.

6. Conclusion

There is a clinical need for better prognostic markers which more effectively identify patients with resected NSCLC who are at most risk of disease relapse/recurrence, in the hope that more efficient selection will lead to better outcomes from adjuvant therapy. Many studies have identified prognostic factors relating to the tumour type, extent, histopathological features, individual molecular characteristics and more global, multiplex genetic assessments as well as factors related to tumour immune responses and metabolism. Of these, only tumour stage is currently used in clinical decision-making, but the relatively poor survival gains from adjuvant therapy suggest that this approach to patient selection could be improved upon. Given the multiplicity of NSCLC types, frequent intra-tumoural heterogeneity and the biological differences between the two major subtypes of squamous-cell carcinoma and adenocarcinoma, it is unlikely that a single histological feature or molecular change will provide the required finer discrimination. It is also likely that any solution will differ between squamous-cell carcinoma and adenocarcinoma. More complex, multiplex assessments of genetic change may prove more effective, but we should not ignore histopathological classification which, ultimately, is a morphological reflection of the myriad genetic changes present in the lesion. Prospective trials to select adjuvant therapy based upon proven prognostic factors are needed, but these should embrace validated histopathological assessment as well as molecular profiles.

Conflict of interest statement

No conflicts of interest declared in relation to this article.

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