Quantitative immunohistochemistry in lung cancer: clinical perspective

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Abstract: Quantitative immunohistochemistry remains an important tool in translational lung cancer research with hopes to improve patient outcomes and avoid unnecessary therapies. Present review is aimed to summarize the use of immunohistochemical markers for improved prognostic information and prediction of treatment benefit. Several of these markers are currently explored in phase II – III clinical studies to individualize the treatment of lung cancer.

Key words: lung cancer, immunohistochemistry, clinical trials, protein expression

Introduction

In recent years, traditional treatment modalities have been approaching therapeutic plateau in almost all fields of oncology. In order to improve treatment outcomes, large efforts were undertaken to identify novel therapeutic targets and robust laboratory tests that could identify patients with tumors that are sensitive to particular therapies. We now have a plethora of novel targeted therapies which have modest activity in unselected patient populations but may be very effective in individual patients with particular molecular characteristics of their tumors. Despite rapid improvements in cancer genomic and proteomic technologies, immunohistochemistry remains the most commonly used technique in pathology laboratories worldwide, and is widely applicable for identification of molecular markers of cancer diagnosis, prognosis and prediction of treatment efficacy.

Lung cancer is the leading cause of cancer-related deaths worldwide [1]. Approximately 80% of the lung cancer patients are diagnosed with non-small-cell histological types: squamous cell carcinoma, adenocarcinoma or large cell carcinoma; the remaining 20% of patients are diagnosed with small cell lung cancer. Individualization of treatment strategy is now in the focus of lung cancer research and some of the discovered tests are used in phase II-III clinical trials or in clinical practice. These tests are sought to better define patient prognosis (particularly important after surgical resection), to predict the treatment benefit in the adjuvant (post-surgical) setting, as well as in the first or second line therapy of advanced disease. Prognostic tests are associated with patient's outcome regardless of treatment administered, whereas predictive tests identify subsets of patients who will or will not benefit from administered therapies. In advanced disease the benefit of treatment can be quantified as a response, durable stable disease, or more importantly, prolongation of progression-free or overall survival. Some assays may provide both prognostic and predictive information, as for example tumor estrogen receptor positivity is a weak positive prognostic marker and a strong predictive marker for the benefit of hormonal therapy in breast cancer. Predictive value of the marker can be distinguished from its prognostic value only through analysis of samples and outcome data from controlled phase III clinical trials with the proper control group of patients. In such studies, predictive value of the test for survival benefit can be assessed by comparison of hazard ratios in marker-positive versus marker-negative subsets of patients. The predictive value of the test is indicated by the difference between these hazard ratios, which can be formally compared by the treatment-by-marker interaction test. It is also important to emphasize that single-arm or cohort studies are inadequate to distinguish between prognostic and predictive value of the marker, and thus results of such studies should be interpreted with caution. In this review, we aimed to summarize the current use of quantitative immunohistochemistry markers as prognostic or predictive tools in lung cancer research.

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Quantitative measurement of the observed immunostaining (protein level) by a pathologist is done with numerous scales and there is no consensus which scale is better or more practical. Often, percentage of cells with positive staining of any intensity is recorded. To account for the protein abundance, the percentage of cells with positive staining is multiplied by the staining intensity (graded from 0 to 3 or from 0 to 4) to give the staining index (ranging from 0 to 300 or from 0 to 400, respectively) [2,3]. In other systems, the staining index is composed of percentage of positive cells binned into categories and multiplied by staining intensity (0 to 3 or 0 to 4 scale) [4]. In all classifications, it is very important to note whether the staining is present in the cell membrane, cytoplasm, or nucleus, or in more than one of these cell compartments. Precise quantification of the protein level by the pathologist is aimed to provide accurate data to be analyzed with patient outcome and to select the best prognostic or predictive cut-off points of the test. For example, quantification of epidermal growth factor receptor (EGFR) protein by immunohistochemistry for prediction of therapeutic benefit from EGFR inhibitors was performed in two phase III clinical trials, BR.21 and ISEL. The trials were designed to assess the efficacy of erlotinib or gefitinib versus placebo in non-small-cell lung cancer (NSCLC) patients who failed at least one line of chemotherapy [5,6]. With pre-defined cut-off point of 10% of positive cells of any staining intensity, lack of EGFR protein staining was a predictor of lack of benefit from theses agents [7,8]. In both studies, precise quantitative data on EGFR protein expression were collected, allowing for exploratory analyses aimed to determine the cut-off point of EGFR positivity that provides best predictive value [3,9]. The results of these studies were consistent and indicated that indeed pre-specified cut-off point of 10% of cells with any staining had the best predictive power, as measured by hazard ratios.

Quantitative evaluation of protein levels in tumor samples presents several challenges, including technical issues related to immunohistochemistry procedure and subjectivity of assessment. Small tumor specimens, such as those frequently obtained in advanced lung cancer, may not be representative of the tumor due to its significant heterogeneity [10]. Protein expression in primary versus metastatic tumor sites may vary by as much as 30%, and clinical significance of this difference is unknown. Small samples are more susceptible to artifacts, such as the "edge effect" with non-specific staining observed in the very edge of the specimen. Choice of the primary antibody and antigen retrieval conditions are of great importance. In our recent study, we obtained completely different results for two tested primary antibodies against insulin-like growth factor-1 receptor (IGF1R) in NSCLC primary tumor samples. We confirmed the association of IGF1R protein level with IGF1R mRNA expression measurement for one but not for the other antibody, which suggests that one of these antibodies does not detect its target protein [Dziadziuszko *et al.*, submitted]. Ideally, specificity of the primary antibody against the target protein should be confirmed by other techniques, such as Western blot. Positive controls (specimens with known expression of the target protein) and negative controls should always be included in the procedure. To account for the variability of some of the above problems, automated staining procedures were developed and are in use in many laboratories.

The subjectivity of quantitative measurement of protein levels remains an important consideration. It is generally recommended that at least two pathologists should independently score each specimen, with the consensus meeting to solve significant discrepancies. The concordance between the readings of two (or more) observers provides a good estimate of the robustness of the scoring system and provision of such information is highly recommended in research protocols and publications. Automated scoring systems are in development to avoid inter-observer variability and facilitate the quantification. Such systems are based on optical density measurements [11] or the quantification of fluorescence measurements from the secondary antibody [12]. Before these systems become widely accepted, validation studies with standard immunohistochemistry scoring methods are needed.

Quantitative immunohistochemistry studies as prognostic tests

The information on prognosis of patients who underwent curative pulmonary resection for NSCLC is based on pathological TNM staging system and other criteria, such as vessel invasion or patient's comorbidity indices. This information is not precise, as for example up to 30-40% of patients with very early stage NSCLC will later present with local or distant relapse and die from their disease [13]. Thus, novel prognostic indices are needed to better stratify patients who are likely to relapse following surgery alone. A number of reports proposed "molecular staging" using several immunohistochemical markers together with clinical parameters to refine prognosis of early stage NSCLC. Kwiatkowski *et al.* [14] analyzed the expression of several proteins in primary tumors from 244 surgically treated stage I NSCLC patients. After elimination of insignificant markers, the authors included p53 staining and H-ras p21 staining together with other features (*K-ras* mutations, mucin producing tumors, lymphatic invasion and tumor diameter of 4 cm or more) in their final prognostic model, which separated stage I NSCLC patients into three markedly different prog-

nostic groups [14]. In a study of 408 consecutive stage I patients, D'Amico *et al.* proposed erbB-2, p53, CD-44 and factor VIII protein expression as independent prognostic markers stratifying patients who have excellent or poor outcome [15]. Despite demonstration of additional prognostic information, these systems were not validated in other patient cohorts nor widely accepted for the use in the practice. Currently developed molecular prognostic models in operable NSCLC explore other technologies, which are mainly based on gene or micro-RNA expression quantification using gene array or quantitative RT-PCR platforms [16-20]. The main advantages of these technologies include wide dynamic range of gene expression quantification and automated simultaneous measurement of up to hundreds of genes in one reaction. The most promising molecular prognostic signatures are now tested in prospective clinical trials in early operable NSCLC to answer the question of whether patients in the poor risk category may benefit from adjuvant chemotherapy. Due to the above mentioned advantages of gene expression evaluation, it is unlikely that multiple immunohistochemistry based markers will find their practical application to refine prognosis of operable NSCLC patients. Nevertheless, it is important to know the prognostic value of any immunohistochemical marker evaluated for other purposes in tumor samples of NSCLC patients.

Quantitative immunohistochemistry studies as predictive tests

Chemotherapy represents current standard of care in advanced unresectable or metastatic NSCLC and as an adjunct to surgery in stage II-IIIA patients. In both settings, the survival benefit from this treatment is relatively modest (survival hazard ratio in the order of 0,8) at the expense of significant toxicity and costs.

Sensitivity to chemotherapy appears to inversely correlate with the activity of several enzymes responsible for the DNA damage repair. Low level of excision repair cross-complementing-1 (ERCC1), a rate limiting enzyme of nucleotide excision repair pathway, is associated with sensitivity to alkylating agents *in vitro* and in samples from patients treated with platinum-based chemotherapy [21]. Low level of human MutS homolog 2 (MSH2), a mismatch repair enzyme, was linked with sensitivity to cisplatin [22]. Low expression of regulatory subunit of ribonucleotide reductase (RRM1) was associated with sensitivity to gemcitabine in cell lines as well as in a clinical setting [23]. Low expression of BRCA1 seems to predict sensitivity to cisplatin [24], whereas high expression may be associated with sensitivity to antimicrotubule drugs such as taxanes and vinorelbine [25]. Many of the studies with predictive markers for chemotherapy were done with quantitative immunohistochemistry, whereas other studies evaluated gene expression with quantitative RT-PCR in fresh-frozen or paraffin-embedded tumor samples. Tests based on both platforms are being explored in prospective clinical studies aiming at individualizing chemotherapy according to the sensitivity profiles of the tumors.

Several markers have shown promising predictive value in the adjuvant chemotherapy. Immunohistochemical analysis of ERCC1 expression in tumor samples from participants of the IALT study investigating the role of postoperative chemotherapy indicated that the survival benefit from chemotherapy was confined to protein-negative subset (adjusted hazard ratio, HR=0.65 as compared with surgery alone) in contrast to the protein-positive subset of patients (HR=1.14; p-value for interaction with treatment =0.009) [4]. Of several cell-cycle regulatory proteins studied, tumoral expression of p27^{Kip1} was predictive for survival benefit in patients treated with adjuvant chemotherapy in the IALT trial [26]. Low protein level indicated the subset of patients who benefited from treatment (adjusted HR=0.66) and high level – those who did not (adjusted HR=1.09, p-value for interaction = 0.02). P27^{Kip1} is a member of cyclin-dependent kinase inhibitory proteins preventing transition from G1 to S phase of the cell cycle. High levels of the protein result in accumulation of cells in G1 phase, which is relatively insensitive to phase-specific chemotherapy, and protection from apoptosis. A recent report presented at 2009 annual American Society of Clinical Oncology meeting showed that negative immunostaining for MSH2 protein is associated with sensitivity to chemotherapy (adjusted HR=0.76) whereas positive immunostaining associated with resistance (adjusted HR=1.12, p-value for interaction= 0.06) [27]. A subset analysis of JBR.10 study testing the efficacy of adjuvant cisplatin and vinorelbine showed that patients with p53-positive tumors by immunohistochemistry achieved survival benefit from chemotherapy (HR=0.54) as opposed to patients with p53-negative tumors (HR=1.40, interaction test p=0.02) [28]. Untreated p53-positive patients had shorter survival than p53-negative patients, confirming previous observations of negative prognostic value of p53 expression in NSCLC. The predictive value of these and other markers by immunohistochemistry for adjuvant chemotherapy in NSCLC is presented in table 1.

Most of the predictive markers for chemotherapy also carry prognostic information in early-stage NSCLC. High levels of ERCC1 or RRM1 (indicative of chemoresistance to cisplatin and gemcitabine respectively) are associated with better prognosis of surgically-treated patients through increased genomic stability of the tumor cells [12]. It appears that the analysis of these markers would indicate the subset of

Table 1. Immunohistochemistry markers for survival benefit from adjuvant chemotherapy in placebo-controlled trials in operable NSCLC

Author [reference]	Marker	Clinical study	Hazard ratio in marker negative group (95% confidence interval)	Hazard ratio in marker positive group (95% confidence interval)	Interaction test p value
Olaussen [4]	ERCC1	IALT	0.65 (0.50-0.86)	1.14 (0.84-1.55)	0.009
Filipits [26]	p27^{Kip1}	IALT	0.66 (0.50-0.88)	1.09 (0.82-1.45)	0.02
Filipits [26]	cyclin D1	IALT	0.87 (0.64-1.18)	0.85 (0.65-1.11)	0.90
Filipits [26]	cyclin D3	IALT	0.83 (0.63-1.09)	0.88 (0.65-1.19)	0.78
Filipits [26]	cyclin E	IALT	0.77 (0.57-1.02)	0.94 (0.71-1.25)	0.33
Filipits [26]	Ki-67	IALT	0.79 (0.59-1.04)	0.92 (0.69-1.22)	0.45
Filipits [28]	MRP1	IALT	0.79 (0.60-1.04)	0.95 (0.71-1.28)	0.37
Filipits [28]	MRP2	IALT	0.93 (0.71-1.21)	0.82 (0.60-1.12)	0.56
Fouret [22]	MSH2	IALT	0.76 (0.59-0.97)	1.12 (0.81-1.55)	0.06
Tsao [27]	p53	JBR.10	1.40 (0.78-2.52)	0.54 (0.32-0.92)	0.02

patients with relatively good post-surgical outcome in whom adjuvant chemotherapy would be unnecessary and ineffective. Prospective clinical studies to test this hypothesis are ongoing in Europe (TASTE phase II feasibility study, led by the French Lung Cancer Cooperative group) and in North America (Southwest Oncology Group SWOG 0720 phase II trial).

Conclusions

Quantitative immunohistochemistry remains a very important and practical platform and is being evaluated in multiple clinical trials in lung cancer with hopes to improve patient outcome and avoid unnecessary therapies. Importantly, these tests are relatively unexpensive and easily applicable in virtually all pathology departments. Test standardization and the choice of optimal cut-off points to define a positive result remain the most important issues before such tests are widely adopted. Currently initiated controlled phase III randomized clinical trials should include properly defined translational part. Quantitative immunohistochemical assessment of putative prognostic and/or predictive markers remains essential component of these trials.

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