Review of the use of pretest probability for molecular testing in non-small cell lung cancer and overview of new mutations that may affect clinical practice

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Abstract: This article considers the use of pretest probability in non-small cell lung cancer (NSCLC) and how its use in EGFR testing has helped establish clinical guidelines on selecting patients for EGFR testing. With an ever-increasing number of molecular abnormalities being identified and often limited tissue available for testing, the use of pretest probability will need to be increasingly considered in the future for selecting investigations and treatments in patients. In addition we review new mutations that have the potential to affect clinical practice.

Keywords: EGFR mutation, molecular testing, non-small cell lung cancer, pretest probability

Introduction

Pretest probability is used in medicine on a daily basis. Clinical guidelines detailing the selection of patients for a particular test are often established based on the pretest probability of having a particular disorder. The importance of pretest probability is increasingly being recognized, particularly with the recent discovery of an increasing number of molecular alterations in patients with non-small cell lung cancer (NSCLC). This is particularly relevant because many of these recently identified gene anomalies [c-ros Oncogene 1, Receptor Tyrosine Kinase Gene (ROS1), v-Raf Murine Sarcoma Viral Oncogene Homolog B1 (BRAF), Human Epidermal Growth Factor Receptor 2 Gene (HER2), MNNG-HOS Transforming Gene (MET), Rearranged During Transfection Gene (RET)] have been detected in small subsets (1–7%) of patients, and with limited tissue available it is often necessary to use pretest probability to identify patients who are candidates for potential new treatments. In this review we discuss the use of pretest probability and how its use in EGFR testing has helped establish clinical guidelines on selecting patients for EGFR testing. We also discuss new mutations in NSCLC where ongoing clinical trials are establishing targeted therapies. As newer treatments come on-stream for specific molecular abnormalities, the principles of pretest probability testing used in EGFR testing may be modified for this use. Recent advances in immunotherapy also need to be now considered when assessing treatment options.

In the last decade, the introduction of molecularly targeted therapies in a wide range of cancers has led to increasing demands on health care resources. Molecular profiling of tumours has aided in the identification of molecular subsets of cancer with distinct biological and clinical characteristics that guide treatment with targeted therapies. This is preferable to traditional chemotherapy, targeting cell cycle, which non-selectively interferes with rapidly dividing cells. The decision to perform molecular testing on a patient is influenced by a number of factors.

A literature search was conducted using PubMed, the proceedings of the American Society of Clinical Oncology annual meeting, the World Conferences on Lung Cancer and www.ClinicalTrials.gov to identify relevant clinical trials.

Pretest probability definition

Pretest probability is the chance of having a disorder before a diagnostic test result is known.
can help decide whether molecular testing should be performed, help select the appropriate test and aid in the interpretation of results. Pretest probability can be calculated from the formula: \( P(D^+) = \frac{D^+}{D^+ + D^-} \), where \( P(D^+) \) is the probability of the target disorder, \( D^+ \) equals the number of patients with the target disorder, and \( D^- \) equals the number of patients without the target disorder. The pretest probability depends not only on the prevalence of a disease, but also on individual factors such as pathologic subtype, environmental and lifestyle factors, ethnicity. The threshold for testing in a clinical setting also depends on several factors, including geographical variation in prevalence, and available funding for testing. It is important to recall that the pretest probability of disease is an evolving estimate and can change depending on the results of other investigations. For example, if a patient is being investigated for metastatic NSCLC, the pretest probability of having an epidermal growth factor receptor (EGFR) mutation would be 5%–15% in an unselected population. However, if following pathological confirmation a diagnosis of metastatic pulmonary adenocarcinoma is made and the patient is Asian, the pretest probability of having an EGFR mutation will increase to 36%–60%. The decision to consider molecular testing depends not only on the pretest probability, but also on the sensitivity and specificity of a test.

Threshold approach
The threshold approach, originally described by Pauker and Kassirer in 1980, uses the physician’s estimate of the probability that a patient has a particular disease to determine whether to withhold treatment, perform further testing, or treat without subjecting to further testing. The upper threshold describes a probability of disease greater than the treatment threshold, where no further investigation is required. The lower threshold describes the probability of disease as lower than the treatment threshold. Between the two thresholds lies the testing zone, where testing is performed and treatment is dependent on the test outcome. The testing zone is a function of the test properties, risk attributes of the test relative to the disease, and the risk–benefit profile of available treatment options. Although the underlying principle continues to be useful today and helps aid with the interpretation of diagnostic tests, initiating targeted treatment usually requires confirmation of the target molecular aberration. This ensures better identification of potential responders to treatment, is often used for reimbursement of testing and treatment, and avoids the potential for harm of ineffective or inappropriate therapy. For example, advanced NSCLC patients with a high pretest probability of having a tumoral EGFR mutation (such as never-smoking Asians with pulmonary adenocarcinoma), but that in fact have EGFR wild-type tumours, have inferior progression-free survival and quality of life if treated with initial EGFR-tyrosine kinase inhibitor (EGFR-TKI) instead of platinum-based chemotherapy. In addition, consideration of the potential harm of missed diagnoses should be considered, as well as the risks and cost that additional biopsies and molecular testing entail. In molecular oncology, clinicians rarely consider that the test result may not reflect the disease status, with little available information on false-positive rates of testing, and more known about the potential for false-negative results.

Prediction models can be useful as a guide to pretest probability; however, including all potential variables that may affect outcome is a limitation of this approach. Rather than single-genotype testing, multiplex-genotype testing will facilitate assessment of multiple simultaneous genes. However, this will also lead to unique challenges as not all mutations are found in the same patient subgroups. For example, not all are seen in non-smokers. Therefore, testing guidelines are being revised to reflect the increasing knowledge that we are gaining regarding molecular subtypes, and incorporate the use of immunotherapy.

Setting parameters
Medical guidelines advising on pretest probability parameters must also consider the potential harm that a missed diagnosis carries. For example, in one study determining testing threshold for computed tomographic angiography (CTA) and D-Dimer in the evaluation of thoracic aortic
dissection (TAD), it was found that CTA should be considered for patients with diagnostic probability >0.3–2.1%, and D-Dimer testing is recommended for pretest probability of 0.01–0.6%.22 The testing threshold for TAD is low, reflecting the large mortality associated with a potential missed diagnosis. In molecular testing in NSCLC, threshold levels are not quantified to the same extent, perhaps reflecting lack of available prediction models. In the molecular testing guidelines of lung cancer, treatment thresholds are not quantified.23 Prior to proceeding with EGFR mutation testing, physicians consider several variables, including histology, sex, smoking history and heritage. These factors when taken together can affect the pretest probability of having an EGFR mutation. However, no pretest probability model currently exists which comprises all possible variables that may impact EGFR mutation prevalence. However, prevalence parameters are provided and these help guide the recommendations. For example, EGFR mutation prevalence is approximately 5% in squamous cell histology, and the guidelines do not recommend testing for EGFR mutation unless certain clinical and historical features are present.23 In the acute medical setting, where a missed diagnosis can carry high acute mortality, guidelines often suggest testing at a much lower frequency. Defining an exact threshold for pretest probability in molecular oncology may not be feasible, not only due to multiple variables that can affect outcome, but also due to the financial implications of testing large cancer subpopulations.

Multiplex-genotype testing will add additional considerations beyond EGFR testing and guidelines are currently being revised to reflect these changes.

Real-life examples

While the examples presented here focus on EGFR mutations, there is an increasing number of actionable mutations being identified and therefore patient selection will become even more important.

Physicians assessing a patient for molecular testing have guidelines available that have been established by international organizations.23,24 However, with the accelerated pace of scientific data, guidelines often lag behind, leaving physicians to decide on testing without the support of established consensus on best practice. In addition, guidelines do not account for individual diversity among patients.25 Difficulty often arises in establishing acceptable pretest probability parameters. For example, in metastatic adenocarcinoma, the pretest probability of a patient having an EGFR mutation is approximately 25%. Therefore, EGFR genotyping of all pulmonary adenocarcinoma cases is recommended.25 Whereas, in squamous cell carcinoma, the pretest probability is lower, at 5% [pretest probability (squamous cell carcinoma) = squamous cell carcinoma EGFR mutation positive (5%)/squamous cell carcinoma EGFR mutation positive (5%) + squamous cell carcinoma EGFR mutation negative (95%)]. Testing for EGFR mutations in adequate squamous cell carcinoma samples is not routinely recommended due to the low pretest probability, but it could be argued that some patients who may benefit from EGFR-TKIs are being excluded from potentially beneficial treatment, particularly those with characteristics that may increase the pretest probability, such as non-smoking status.

Pretest probability must consider multiple variables in a clinical scenario when making a decision to determine the lower threshold for testing guidelines. For example, consider the pretest probability of having an EGFR mutation in three new patients presenting to a thoracic oncology clinic: Patient A, a 40-year-old never-smoking Asian female with a diagnosis of stage IV adenocarcinoma; Patient B, a 68-year-old Caucasian male with a 50 pack per year smoking history diagnosed with metastatic NSCLC, squamous cell histology; and Patient C, a 40-year-old never-smoking Asian female with a diagnosis of stage IV lung squamous cell carcinoma (large tissue sample). The additive effect of each variable in Patient A’s case – histological subtype, Asian, female, never-smoker – increases the pretest probability of having an EGFR mutation. Guidelines clearly state that EGFR mutation testing should be performed to select patients for EGFR-TKI in those with stage IV adenocarcinomas. EGFR mutation testing is performed by direct sequencing or real-time quantitative PCR-based approach. For Patient B, when we consider the additional variables along with squamous histology – male, Caucasian, heavy smoking history – the probability of having an EGFR mutation is further reduced. The lower threshold for testing is not felt to be breached in Patient B, and patients would therefore not be tested and treated with an EGFR-TKI.
In the case of Patient C, when we assess the individual variables that comprise the pretest probability of this patient: $P(D+) = \frac{D+}{(D+ + D-)}$, female (58/58 + 42) + age (38/38 + 62) + never-smoker (58/58+42) + squamous (5/5 + 95) = 40% – the pretest probability in this clinical scenario would be 40%. In this situation, most clinicians would consider testing for an $EGFR$ mutation.

**Discussion**

Although the threshold for testing cannot always be defined on an individual basis, recommended guidelines often highlight the need to account for additional clinical parameters when making a decision on molecular testing, particularly if this will increase the probability of a positive test. One example of this is when a limited tissue sample exists and where adenocarcinoma cannot be excluded; guidelines suggest: ‘$EGFR$ and $ALK$ testing may be performed in cases showing squamous or small cell histology but clinical criteria (eg, young age, lack of smoking history) may be useful in selecting a subset of these samples for testing.’

Therefore, the physician must consider all variables that may impact on the pretest probability prior to ordering a molecular test.

The published guidelines on the use of molecular testing for $EGFR$ and $ALK$ in lung cancer aid the oncologist in the decision-making process around molecular testing. However, adequate tissue samples, qualified laboratories and economic resources differ across regions, and so international guidelines must often be adapted for use in the local clinic.

Several other biomarkers have been investigated and may affect clinical practice. Therefore the concepts of pretest probability should be considered. Some of these biomarkers include the following.

**Overview of other mutations affecting clinical practice**

Several other biomarkers have been investigated and may affect clinical practice. Therefore the concepts of pretest probability should be considered. Some of these biomarkers include the following.

**ALK**

The $EML4$–$ALK$ fusion gene results when echinoderm microtubule-associated protein-like 4 ($EML4$) gene is fused to the anaplastic lymphoma kinase ($ALK$) gene. $ALK$ translocations have been described in 2–7% of NSCLC patients and is more commonly seen in adenocarcinoma histology and never- or light smokers. Two phase III studies in the first- and second-line setting of crizotinib versus chemotherapy in $ALK$-positive patients have demonstrated an improvement in progression-free survival (PFS) and response rate (RR) in patients treated with crizotinib. No difference in overall survival (OS) was seen, which was likely a result of patients being allowed to cross over to crizotinib following progression.

Resistance to crizotinib eventually develops, with secondary $ALK$ mutations being one of the main mechanisms of resistance. Second-generation $ALK$ inhibitors (e.g., ceritinib, alectinib, brigatinib) have demonstrated promising results in patients treated following progression on crizotinib in $ALK$-positive patients, with median PFS greater than 5.7 months. These second-generation $ALK$ inhibitors have greater specificity than crizotinib and do not inhibit MET and ROS1. In addition, they are able to cross the blood–brain barrier.

Brigatinib, an oral tyrosine kinase inhibitor, has demonstrated promising results in a phase I/II
trial. Patients with prior crizotinib exposure demonstrated RR 72% (95% CI, 60–82%) and median PFS 13.2 months (95% CI, 9.2–NR).\textsuperscript{37} Trials are ongoing comparing these second-generation ALK inhibitors in the first-line setting with crizotinib in ALK-positive patients. Significant PFS has been demonstrated in a phase III Japanese trial comparing alectinib with crizotinib in the first-line setting.\textsuperscript{39} However, we will have to wait for the completion of trial data to assess survival outcomes in order to know the optimal timing of treatments in the first- and second-line setting of ALK-positive patients and those who develop resistance to treatment.

**KRAS mutation**
The RAS oncogene family, including KRAS, NRAS and HRAS, are membrane-bound intracellular GTPase which act as central mediators activating multiple downstream pathways including RAF (MAP kinase pathway), PI3K (AKT/MTOR pathway), ERK, RLIP and RALGDS, which regulate cell proliferation, apoptosis, angiogenesis and cellular metabolism.\textsuperscript{40} KRAS mutations are the most common mutation in lung cancer and are more frequently seen in adenocarcinoma (25%) versus squamous histology (5%), non-Asian ethnic origin and in current or ex-smokers.\textsuperscript{41–43} Studies to date have not conclusively identified KRAS as a prognostic or predictive marker, with the exception of negative predictive value of KRAS mutations and response to EGFR-TKI.\textsuperscript{44–45} The majority of KRAS mutations in NSCLC are single amino acid substitutions in codon 12 and to a lesser extent in codon 13 and 61.\textsuperscript{46}

Therapeutic targets to date inhibiting KRAS have largely been discouraging, likely as a result of its functional complexity. KRAS mutations can be associated with other genetic alterations and these different genetic profile combinations may require alternative therapeutics.\textsuperscript{42}

Phase II trials of the MEK inhibitor selumetinib plus docetaxel demonstrated promising survival results in KRAS-mutant NSCLC.\textsuperscript{47} However, in the phase III, double-blind, randomized SELECT-1 trial, selumetinib plus docetaxel did not significantly improve PFS, OS or objective response rate (ORR) versus placebo plus docetaxel.\textsuperscript{48}

While KRAS remains an elusive target for therapy, it could potentially affect the testing algorithm as many mutations are mutually exclusive with these mutations.

**MET amplification or mutation**
MET is a tyrosine kinase receptor for the ligand HGF.\textsuperscript{49} Activation of the receptor results in downstream signalling pathways [mitogen-activated protein kinase (MAPK), PI3K (phosphoinositide 3-kinase protein kinase B)/AKT, signal transducer and activator of transcription proteins (STAT) and nuclear factor-κB].\textsuperscript{50} MET abnormalities can result from gene amplification, exon 14 splice mutations of the MET receptor gene or decreased degradation.\textsuperscript{51} MET amplification occurs in approximately 2–4% of lung cancer cases, and in addition might contribute to progression after treatment with EGFR inhibitors in 5–20% of patients (www.mycancergenome.org/content/disease/lung-cancer/met/59). Exon 14 MET splice mutations occur in 3% of patients with lung cancer.\textsuperscript{52,53} MET inhibitors include monoclonal antibodies targeting HGF or the MET receptor (e.g. onartuzumab, ficlatuzumab) or MET TKIs (foretinib, crizotinib, tivantinib, and cabozantinib).\textsuperscript{41,51} A phase II trial comparing onartuzumab plus erlotinib versus erlotinib alone in pretreated patients with advanced NSCLC demonstrated an improvement in PFS and OS in the MET-positive population.\textsuperscript{54} However, a phase III trial was stopped early due to futility, as the addition of onartuzumab to erlotinib did not improve PFS, ORR or OS, even in patients with high expression of MET.\textsuperscript{55}

Crizotinib, which inhibits cMET, ALK and ROS-1, demonstrated promising anti-tumour activity in patients with cMET amplified NSCLC in a small (n = 13) pilot study.\textsuperscript{56} In the expansion cohort of the PROFILE 1001 study, 10 of 15 patients with MET exon 14-altered NSCLC demonstrated anti-tumour activity.\textsuperscript{57}

**MET exon 14 skipping mutations**
MET exon 14 skipping mutations occur in 3% of patients with NSCLC. Early studies have demonstrated promising results in patients with MET exon 14 skipping mutations treated with crizotinib and cabozantinib.\textsuperscript{58,59}

cMET dysregulation is seen in 15–20% of NSCLC patients with acquired EGFR-TKI resistance. Capmatinib (INC280) is a highly selective cMET inhibitor.\textsuperscript{60} A phase Ib/II trial investigated the safety and efficacy of capmatinib plus gefitinib in EGFR mutant, cMET positive
NSCLC who progressed on gefitinib, erlotinib, or afatinib. In the phase II expansion phase, capamatinib plus gefitinib was well-tolerated and demonstrated encouraging clinical activity, particularly in patients with high cMET GCN (gene copy number) [partial responses in 12/65 evaluable patients (ORR 18%) and 40/65 (62%) patients had stable disease (SD); 10/53 patients with IHC 3+ or IHC 2+ and GCN ≥5 had PRs (ORR 19%) and 7/23 patients with GCN ≥6 had PRs (ORR 30%)].

ROS-1 rearrangements
The ROS-1 oncogene encodes an orphan receptor tyrosine kinase with structural similarity to the ALK. It occurs in approximately 2% of NSCLC patients. Characteristics of ROS-1 patients are similar to those with ALK-rearrangements – young, never-smokers and adenocarcinomas. ROS-1 rearrangements have been reported to be mutually exclusive with other oncogenic driver alterations (EGFR, KRAS, ALK, HER2, RET). Nine fusion protein variants have been described in NSCLC; fluorescence in-situ hybridization (FISH) assay is currently considered the gold standard for ROS-1 fusion detection. A phase I expansion phase cohort study – 50 patients with advanced NSCLC who were pretreated with chemotherapy – demonstrated an ORR of 72% (95% CI, 58–84) and median PFS of 19.2 months (95% CI, 14.4 not reached). A retrospective European study of 31 patients with ROS-1 rearrangement treated with crizotinib demonstrated a median PFS of 9.1 months. Other ROS-1 inhibitors are being investigated.

Conclusion
Pretest probability by definition is a concise number. However, in clinical practice it often cannot be determined precisely due to individual variation. The concept of using pretest estimates of disease probability is widely used in practice, including in molecular oncology. This allows a broad identification of the most suitable patients to test, but pressing questions remain in the current economic climate. These include questions prior to ordering molecular testing, such as whether a positive test result will impact a treatment decision. And if a test is not performed (due to a low estimated pretest probability), could a missed positive result have an adverse effect on the individual patient’s outcome?

Traditionally, diagnostic testing has been performed when it will change medical management or provide further information regarding a patient’s prognosis. However, in many cancer centres, multiplex genotyping or sequencing assays that test a large number of genes simultaneously are offered to oncology patients. Turnaround time for results with these assays may be several weeks, and many mutations identified have no proven therapeutic options. Although the identification of such genomic abnormalities may add scientific merit to future research, molecular testing where potential therapeutic interventions are available should take precedence. In those jurisdictions where testing resources are limited, pretest probabilities of positive results may be important to factor into policy decisions about molecular testing. An increasing number of molecular abnormalities have been identified in NSCLC patients, and also there has been an increasing interest in using circulating DNA for the diagnosis and monitoring of disease. Currently no guidelines have been established to guide the clinician on which patients to test for these new anomalies. In situations where limited tissue is available, it is likely that results from clinical trials will provide information on pretest probability and allow the establishment of clinical guidelines. This will enable the clinician to advise patients of the potential benefits, risks and limitations of testing.

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