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EGFR mutations in malignant pleural effusions from lung cancer

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Abstract A malignant pleural effusion (MPE) from lung cancer represents stage IV disease and portends a poor prognosis. Routine mutational analysis of tissue samples is the standard of care in advanced lung cancer management because it has treatment implications. Sampling of MPE is minimally invasive, safe, repeatable, and provides both diagnostic and therapeutic value. Mutational analysis on MPE has been shown to be feasible and correlates with a response to targeted therapy with tyrosine kinase inhibitors (TKIs). Guidelines recommend mutational testing in MPE, however there is no one standardized method for testing. There are several testing methods available for mutational analysis in pleural fluid including PCR, mutant-enriched PCR, DNA & RNA sequencing, and immunohistochemistry the sensitivity of which are dependent upon tumor cell heterogeneity. The advantages and disadvantages of each will be reviewed here.

Keywords Epidermal growth factor receptor (EGFR) · Malignant pleural effusion · Non-small cell lung cancer (NSCLC) · Tyrosine kinase inhibitors (TKIs) · Molecular testing

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Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide with an estimated five-year survival rate of 16 % for all comers [1]. Efficacy of traditional platinum-based two drug therapy for advanced stage non-small cell lung cancer (NSCLC) has plateaued with an overall response rate between 25 and 35 % and median overall survival of 8–10 months [2]. Recent advances in molecular testing for *epidermal growth factor receptor* (EGFR) mutations, however, has allowed for the identification of a subset of patients that will be more responsive to therapy with targeted tyrosine kinase inhibitors (TKIs) [3–5]. This evolution toward individualized treatment has made it essential to further differentiate NSCLC based on these molecular aberrations, as it has significant treatment implications including improved survival. TKIs are recommended as first line therapy for stage 4 NSCLC with an EGFR positive mutation [6•, 7] Because patients with metastatic disease are unlikely to undergo surgery, molecular testing of samples obtained using minimally invasive techniques has become important.

Malignant pleural effusion (MPE) from lung cancer is common and signifies advanced disease with median survival of 4 months [8]. Based upon poor prognostic implications, MPE has been reclassified to represent stage IV disease in the most recent iteration of the staging guidelines [9]. This article will review the pathophysiology of MPE, the role of targeted TKIs, methods for EGFR mutational analysis in MPEs, guidelines for mutational analysis testing, and will discuss future directions.

Pathophysiology

A pleural effusion develops when the production of pleural fluid exceeds its removal. One of the most common causes of effusion associated with impaired removal of pleural fluid

is MPE, defined as a pleural effusion that occurs in the context of malignant invasion of the pleura. MPE can be diagnosed by detection of malignant cells in pleural fluid, pleural biopsy, or by direct visualization and biopsy using thoracoscopy/thoracotomy [10]. Tumor cells can invade the pleural space by direct extension from an adjacent tumor, invasion of the pulmonary vasculature with embolization to the visceral pleura, or through hematogenous metastasis from distal tumors to the parietal pleural. Once malignant cells invade the pleural space, they deposit on the parietal pleural membrane and obstruct the lymphatic stomata, disrupting the drainage of pleural fluid [10].

MPE is also related to an increase of pleural fluid production from fluid extravasation through hyperpermeable pleural vasculature. This increased permeability is a result of vasoactive mediators, including vascular endothelial growth factor (VEGF), tumor necrosis factor (TNF), osteopontin (OPN), and chemokine ligand 2 (CLL-2) produced by tumor cells in interaction with the normal mesothelial, endothelial and inflammatory cells [11, 12]. These vasoactive mediators have been targeted as diagnostic, prognostic biologic markers, and as potential targets for treatment [12, 13]. In a recent meta-analysis aimed at determining the overall accuracy of pleural VEGF assays in diagnosing MPE, the sensitivity, specificity, positive likelihood ratio and negative likelihood were 75 %, 72 %, 2.94 and 0.38, respectively [14]. These findings suggest that determining pleural fluid VEGF may improve the accuracy of MPE diagnosis.

A paramalignant effusion is defined as cytologically negative pleural effusion in a patient with a known malignancy in the same lung. This occurs as a result of tumor invasion of the pleural fluid drainage system; anywhere between the lung lymphatic system and the mediastinal lymph nodes. Parapneumonic effusions may also result from complications related to the disease including bronchial obstruction, pulmonary embolism, or decreased oncotic pressure due to severe hypoalbuminemia. They can also arise as a result of therapies, including radiation and chemotherapy [10].

Staging and prognostic implications in NSCLC

Metastatic pleural extension of NSCLC includes the presence of malignant pleural nodules (which can be localized or diffuse-carcinomatosis), and/or malignant pleural effusion. Metastatic pleural extension has been reported in 1–7 % of patients with NSCLC, and in 10 % of patients with metastatic disease. It carries a poor prognosis and precludes a surgical resection [9, 15].

In 2002, the 6th edition of the TNM classification of lung cancer classified malignant pleural extension in NSCLC as T4 (Stage IIIB). The International Association for the Study of Lung Cancer (IASLC) later conducted a large retrospective study using a validated database of 81,015 lung cancer cases between 1990 and 2000 from 46 institutions, 19 countries, and three continents. It was found that patients with malignant pleural extension (N=771) had a median survival of 8 months and a 5-year survival of 2 % (very similar to the patients with intra-thoracic metastasis) [9, 15]. A study of 23,583 patients with NSCLC from the California Cancer Registry demonstrated similar outcomes in patients with malignant pleural extension of NSCLC (5-year survival 3.1 %, median survival 4 months) [16]. Based on these findings, the presence of pleural nodules or malignant pleural effusion in patients with NSCLC was upstaged to M1a (Stage IV) in the 7th edition of the TNM classification of lung cancer, published in 2009.

The presence of MPE also appears to be an independent predictor of worse survival in those with stage IV disease due to extra-thoracic metastasis (median overall survival of 3 months versus 5 months in patients without MPE) [17]. Wu et al. recently found that in patients with stage IV lung adenocarcinoma (N=448), those who present with MPE at the time of diagnosis have worse median overall survival compared to those who developed the MPE later in the course of their disease (14.3 versus 21.4 months, p=0.001) [18].

Tyrosine kinase inhibitors and lung cancer

Over the past several years, advances in molecular and cellular biology have led to a better understanding of the molecular pathogenesis of cancer. This has guided the development of therapeutic agents that target molecular pathways specific to malignant cells and therefore result in less toxicity to normal cells than conventional therapies. One of the best-studied pathways of oncogenic transformation in lung cancer cells is the activation of intracellular signal transduction pathways regulated by tyrosine kinases. Known variants in EGFR, the anaplastic lymphoma kinase (ALK), and Kirsten rat sarcoma (KRAS) are responsible for lung cancer cell proliferation, invasion and dissemination and the molecular targets approved therapeutic agents for the treatment of NSCLC [19].

Several drugs that target and inhibit these tyrosine kinase pathways have been developed and tested in patients with NSCLC. Gefitinib, Erlotinib, and Afatinib are EGFR tyrosine kinase inhibitors (EGFR-TKIs) that have shown significant improvement in progression-free survival compared to conventional chemotherapy in phase 3 trials as first line therapy in those with advanced NSCLC with activating EGFR mutations (Table 1) [20–25, 26•, 28].

The most common toxicity associated to the EGFR-TKIs is manifested in the skin, gastrointestinal (GI) tract and the

Table 1 Phase III studies comparing EGFR TKIs versus platinum-based chemotherapy as first-line treatment of advanced NSCLC

Study	Treatment	HR for progression (95 % CI)	RR, %
IPASS (*N*=1,217) [6•]	Gefitinib vs. carboplatin/gemcitabine	Overall: 0.74 (0.65–0.85)[a]	Overall: 43.0 vs. 32.2[e]
		EGFR-mut pos: 0.48 (0.36–0.64)[a]	*EGFR*-mut pos: 71.2 vs. 47.3[e]
		EGFR-mut neg: 2.85 (2.05–3.98)[a]	*EGFR*-mut neg: 1.1 vs. 23.5[b]
First-SIGNAL (*N*=309) [22]	Gefitinib vs. cisplatin/gemcitabine	Overall: 0.737 (0.580–0.938)[b] Gefitinib (*EGFR*-mut neg vs. *EGFR*-mut pos): 0.385 (0.208–0.711)[b]	Overall: 53.5 vs. 42.0[c]
		CT (*EGFR*-mut neg vs. *EGFR*-mut pos): 1.223 (0.650–2.305)[c]	
WJTOG3405 (*N*=172) [24]	Gefitinib vs. cisplatin/docetaxel	0.489 (0.336–0.710)[a]	62.1 vs. 32.2[a]
NEJ002 (*N*=228) [23]	Gefitinib vs. carboplatin/ paclitaxel	0.30 (0.22–0.41)[d]	73.7 vs. 30.7[d]
OPTIMAL (*N*=154) [27]	Erlotinib vs. carboplatin/ gemcitabine	0.164 (NR)[a]	83 vs. 36[a]
EURTAC (*N*=174) [26•]	Erlotinib vs. platinum-based chemotherapy	0.37 (0.25–0.54)[a]	64 vs. 18[a]
LUX-Lung 3 (*N*=345) [28]	Afatinib vs. pemetrexed/cisplatin	0.47 (0.34–0.65) [a]	56 vs. 23 [a]

CI confidence interval; *CT* chemotherapy; *EGFR* epidermal growth factor receptor; *EGFR-mut pos* *EGFR*-mutation positive; *EGFR-mut neg* *EGFR*-mutation negative; *HR* hazard ratio; *NR* not reported; *TKIs* tyrosine kinase inhibitors

[a] $P<0.0001$

[b] $P<0.01$

[c] $P>0.05$

[d] $P<0.001$

[e] $P=0.0001$

lungs. In the skin, an acneiform rash and dry skin are the most frequent reported side effects (up to 55 % in patients taking gefitinib) [29]. In the GI tract, diarrhea is reported in up to 60 % of patients taking erlotinib [30, 31]. In addition, there are case reports of GI perforation associated with the use of erlotinib, as well as hepatic failure in patients treated with erlotinib [30, 31]. Less commonly, erlotinib and gefitinib have been associated with interstitial pneumonitis.

Unfortunately, almost all patients who initially respond to an EGFR- TKIs subsequently develop resistance and experience progression of the disease [32]. Secondary mutations in EGFR (T790M, D761Y, and L747S) and amplification of the hepatocyte growth factor (cMET) oncogene appear to be responsible for the majority of cases of acquired resistance to EGFR-TKIs [32–35]. The T790M mutation is the most common secondary mutation, detected in approximately 50 % of patients with acquired EGFR-TKI resistance [32]. Amplification of the cMET oncogene has been associated with resistance to EGFR TKIs in 5–20 % of disease progression while on treatment with erlotinib or gefitinib [35].

Despite the eventual progression of disease with EGFR-TKIs, treatment guidelines recommend molecular testing for EGFR mutations in those diagnosed with advanced (stage 4) adenocarcinoma NSCLC, because targeted therapy significantly prolongs progression-free survival. While larger tissue samples are preferable, the majority of patients with stage IV NSCLC will not undergo surgical resection, and thus diagnostic approaches have shifted toward minimally invasive procedures [36]. Techniques have therefore been developed whereby molecular testing can be performed on lesser amounts of tissue, and specific criteria for sample type, size, collection and storage have previously been published [36]. Cytologic analysis of pleural fluid is an attractive alternative to tissue acquisition, as it has the potential to simultaneously yield a diagnosis and a stage. Tumor cells obtained from the pleural fluid may also be tested for EGFR mutations. Furthermore, a higher rate of EGFR mutations have been demonstrated in patients with a MPE associated with lung adenocarcinoma [37].

MPE and EGFR

Although the literature concerning EGFR-positive NSCLC and its response to TKI therapy is broad, the number of studies focusing on EGFR positivity in pleural involvement has been less robust [38]. Based upon data from ten such studies, the incidence of EGFR mutation rates in MPE appears to range from 24–68.7 %, with a median rate of 34 % [37, 39–47]. Additionally, several of these studies indicate a statistically significant response rate to TKI therapy within the EGFR mutated patients [37, 40, 42–44, 46]. These results indicate that EGFR positive pleural metastases have a similar response to TKI therapy as seen in other EGFR positive metastatic sites. It is, therefore, reasonable to manage patients with EGFR positive MPEs with TKI

therapy and look for resolution before considering invasive procedures such as indwelling pleural catheter placement and/or pleurodesis. A lack of response or recurrence of a MPE following initial resolution with TKI therapy, however, should prompt repeat pleural fluid sampling to assess for change in EGFR mutation status and consideration of alternative therapies.

EGFR detection in MPEs

Patients with pleural effusion in the setting of lung cancer often require thoracentesis for diagnosis, staging and symptom management. Thoracentesis therefore provides a convenient, less-invasive, easily repeatable and relatively safe means of cell sampling that yields important clinical and therapeutic results. Although pleural fluid cytology is routinely performed when there is suspicion of malignancy, initial sampling is positive in only 50 % of cases of MPE. Cell heterogeneity within the pleural fluid likely contributes to this low diagnostic yield, as inflammatory and mesothelial cells also contribute to the milieu. As such, it has been shown that malignant cell concentration in pleural fluid is only around 40 % [48]. Diagnostic yield, however, increases by 17 % with subsequent sampling, and is therefore the recommended approach to a suspected MPE by the American College of Chest Physicians (ACCP) [49]. In addition to routine cytology, it has been shown that molecular testing for specific gene mutations on very small samples of pleural fluid is feasible, and helpful clinically to identify patients who may benefit from targeted TKI therapy.

Certain EGFR mutations are associated with an increased response or lack of response to EGFR TKIs in NSCLC patients. *EGRF* gene exon 19 deletions, exon 21 mutation L858R (c.2573T>G), exon 21 mutation L861Q (c.2582T>A) and exon 18 mutation at amino acid location 719 (G719A, c.2156G>C; G719S, 2155G>A; and G719C, 2155G>T) are the most common mutations associated with increased response to TKIs [50, 51] The most common EGFR mutations associated with lack of response or resistance to EGFR TKIs are exon 20 mutation T790M (c.2369C>T), exon 20 mutation S768I (c.2303G>T), and identified insertions in exon 20 [52]. Certain patient characteristics are associated with EGFR mutations in NSCLC including females, East Asian descent, never smokers, and adenocarcinoma subtype [53]. This same population of patients has also been associated with a favorable response to TKI therapy [22]. Mutational analysis of pathologic specimens for EGFR, however, remains the only reliable way to determine the presence of EGFR mutations. Huang, et al. reported the first case of successful EGFR mutation determination from MPE sampling in 2005 using polymerase chain reaction (PCR), clinically correlated with a positive response to Gefitinib therapy [54]. Subsequent research has shown that DNA in pleural fluid can be used to detect EGFR mutations, and that mutation status predicts response to TKIs. [40, 42, 44, 55, 56].

In addition, discordance between primary tumor and metastatic lesions including MPE represents a unique challenge in terms of disease behavior, progression, and recurrence. A recent study comparing the EGFR mutation status between 37 primary NSCLCs and their corresponding metastatic (synchronous and metachronous) sites showed a discordance rate of 16.2 % for all metastatic sites, and 14.3 % amongst the pleural metastases specifically [57•]. This study further demonstrated that positive response to TKI inhibitors in the discordant pleural cases was associated with the EGFR mutation status of the pleural metastasis, not the primary tumor. Although this is a retrospective study involving a small cohort of patients, it highlights several important aspects of metastatic tumor biology and its potential clinical implications for repeat molecular testing of MPE following disease progression.

Current National Comprehensive Cancer Network (NCCN) guidelines recommend testing for EGFR mutations in NSCLC, but do not specify which method should be used to do so. Several tests currently exist, some of which have been studied either comparatively or in combination in attempts to identify a superior detection methodology (Table 2). A significant challenge specific to pleural fluid analysis is the heterogeneity of the sample, including varying ratios of normal and malignant cells. This makes the sensitivity of a chosen mutation detection assay critical so as to avoid high false negative rates. A standardized method for EGFR mutation detection in MPE has yet to be determined. Polymerase chain reaction (PCR), direct DNA sequencing, direct RNA sequencing, and immunohistochemistry are some of the methods that are currently being utilized and have been tested in isolation or in combination. In Fig. 1, we propose a testing algorithm for the evaluation of malignant pleural effusions.

Methods for EGFR mutational analysis in MPE

Polymerase chain reaction (PCR) and direct sequencing (Sanger)

One method used to directly sequence a designated target involves amplification by PCR to generate amplicons of the target gene. PCR exponentially amplifies a given target using forward and reverse primers. The PCR product of the given gene targeted area are then sequenced using direct sequencing, which involves dye-terminating fluorescent dideoxynucleotides (ddNTPs) that identify the order of the individual nucleotides for the given target. This method offers full characterization of the designed PCR product.

Table 2 Pleural fluid variant analysis

Methodology*	DNA requirements (ng)**	Detect<5 % mutation***	Pros and cons
Real-Time PCR	1.0–100+	Yes	Very sensitive method. Will only detect variants that have been designed targets of the assay.
Direct Sequencing (Sanger)	3.0–3,000+	No	Can detect all potential variants within the designed amplicon. Lower sensitivity than the other two methodologies.
Massively Parallel Sequencing (NGS)	10–400+	Maybe	Can detect all potential variants for designed amplicon. Sensitivity has been shown to be greater than direct sequencing, but may not be as high as RT-PCR.

*Reproducibility of each methodology is dependent upon the individual laboratory it is performed in, and will vary slightly between laboratories

**DNA requirements are based on the assay used; assays will vary in starting material required. For direct sequencing, DNA requirements will vary based on the size of the sequence and the number of cycles needed

*** Variant detection of less than 5 % mutation in wild-type background

The size of the sequences fragment can vary based on design, but is on average relatively short, around 300 to 700 nucleotides long. In addition, due to primer binding issues, the first 15–40 bases of the sequence is often poor. Also, the quality of the DNA sequence can deteriorate at the long-range nucleotides. The sensitivity of Sanger

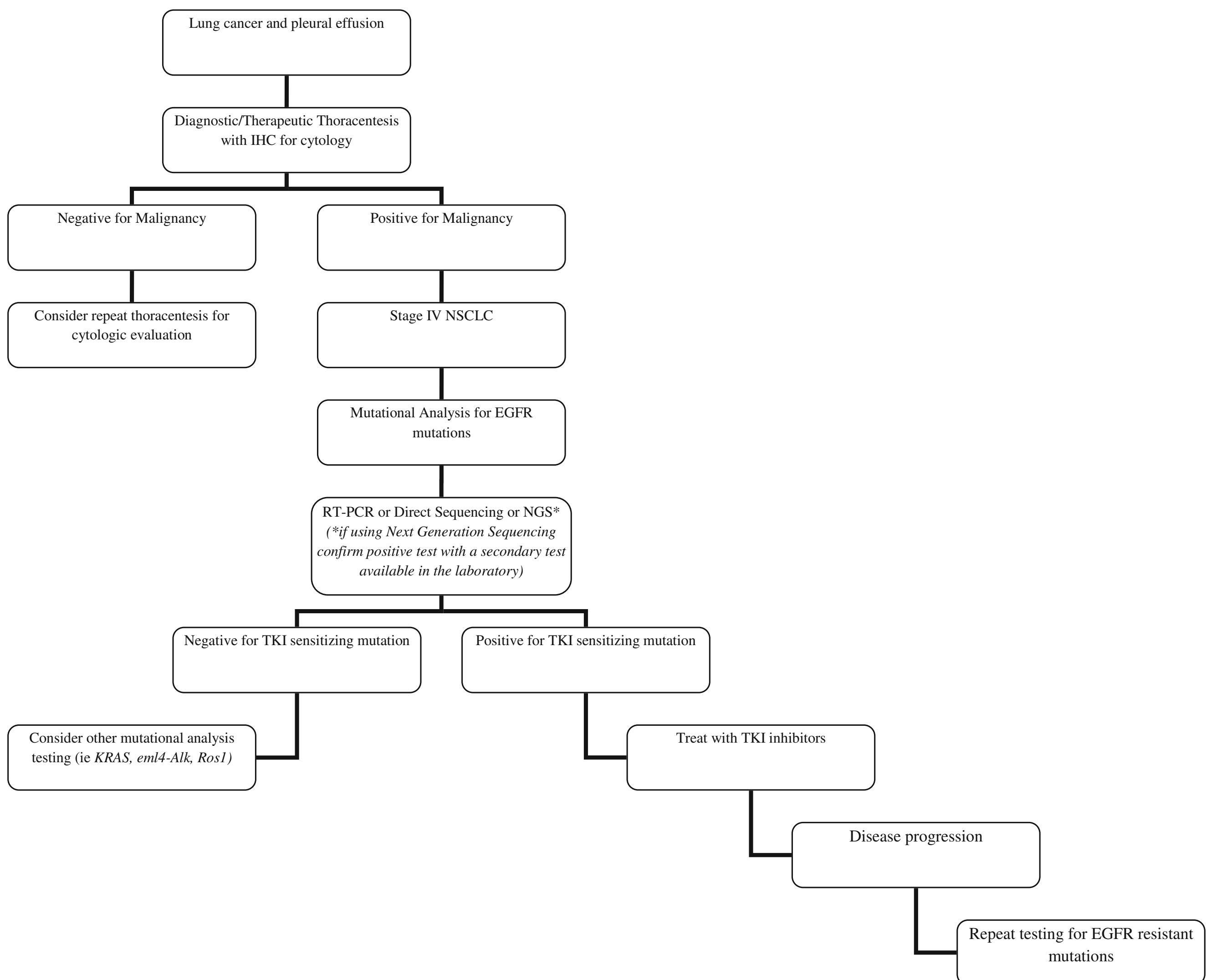

Fig. 1 Proposed algorithm for testing of malignant pleural effusion (MPE)

sequencing is often lower, around 20 %, suggesting it may not be the ideal method for heterogenous samples of normal and malignant cells, such as those obtained from pleural fluid [58, 59].

Sequencing of cell-derived RNA has also been investigated and shows increased sensitivity to detect EGFR mutations (67.3 % vs. 44.7 %) when compared to genomic DNA sequencing [46]. The sequencing technique is the same for both methodologies, except one uses RNA as a template as opposed to DNA, thus avoiding the additional cost and procedural complexity associated with highly sensitive mutant-enrichment PCR modalities. Disadvantages of using RNA targeting include its inherent lability as well as the abundant presence of RNase in biologic samples [56].

Massively parallel sequencing

Massively parallel sequencing, also known as next generation sequencing (NGS) or second/third generation sequencing, is a high throughput technology that allows for sequencing of millions of clonally amplified DNA templates at the same time. Depending on the platform used, read lengths can range from 35 to 700 nucleotides. This technology has been used for analysis of whole genomes, single genes, and panels of targeted gene regions. NGS sequencing has been found to be sensitive in the identification of EGFR mutations compared to Sanger sequencing for both bronchoalveolar lavage and pleural fluids. The sensitivity is dependent on the number of sequences generated for each sample [60–62]. While NGS was previously only used in research studies, it is becoming increasingly available in clinical laboratories.

Real-time polymerase chain reaction (PCR)

Real-time PCR is a molecular method used to amplify and quantify a targeted DNA. This technology is based on PCR, with the added advantage of the detection and quantification of the intended target. There are many techniques used to identify the target generated by the PCR. The two most common detection techniques include a fluorescent dye that intercalates into the intended DNA target generated by the forward and reverse primer and sequence specific fluorescent probes that hybridize to the DNA target amplicon and emit their fluorescence. This method tends to have a high sensitivity; however, it will only detect the variants specified through probe design and PCR enrichment strategies, such as amplified refractory mutation systems (ARMS), PCR-restriction fragment length polymorphism (PCR-RFLP), or peptide nucleic-acid locked nucleic-acid (PNA-LNA) PCR. ARMS technology has been shown to increase sensitivity for detecting EGFR mutations and may be used in conjunction with other methods such as direct sequencing [42, 63]. A comparative study of DNA sequencing and PCR-RFLP on both pleural fluid samples and cell-free supernatant demonstrated that 19 % more EGFR mutations were found with a highly sensitive PCR assay [47].

Immunohistochemistry

Immunohistochemistry (IHC) is the process of detecting specific proteins within cells of a tissue sample using the concept of antibody/antigen binding. It is a well-established, easily performed, low-cost technique that may have an important role in the detection of EGFR mutations in MPEs. Mutant specific antibodies for the exon 19 deletion (E746-A750del) and the exon 21 mutation (L858R) are currently available for immunostaining [64]. This technique uses sections cut from cell block of pleural fluid that are prepared and incubated with the EGFR mutant-specific antibodies. Immunostaining is then evaluated for reactivity and graded based upon intensity. This modality was recently evaluated to determine its feasibility using RNA sequencing as the standard procedure for comparison. Results showed the combined sensitivity and specificity for the two mutant-specific antibodies to be 74 % and 82 % [56]. Additionally, those patients with mutations identified by IHC showed a similar response rate to TKIs when compared to those identified by the molecular method (67 % vs. 72 %, respectively). However, a significant number of subjects with negative IHC staining (42 %) also showed a high response rate, highlighting the limited sensitivity of this modality. Although the available antibodies may identify the two most commonly encountered EGFR mutations, they do not target other less common mutations that may benefit from TKI treatment. Interobserver variability in assay interpretation also poses a problem in terms of increasing false positive rates. Similar limitations have been noted in tissue samples other than MPE, indicating that IHC should not be considered as first line testing for EGFR mutations at this time [65].

Pleural fluid specimen preparation

There are two common fixation methods for pleural fluid including ethanol fixation usually performed on slide smears and formalin fixation of cell blocks made from pleural fluid. Any of the above mentioned methods can be performed on pleural fluid from a slide smear or cell block. Due to the heterogeneous samples of normal and malignant cells, it is common practice to use additional tumor enrichment techniques, such as macrodissection, manual microdissection, or laser capture microdissection of the smear or cell block, in order to ensure sufficient malignant cell material for analysis. The use of formalin fixation is commonly

used to preserve the structure and architecture of tissue or a cell; however, this causes DNA fragmentation and cross-links DNA to other molecules. Formalin fixation leads to fragmentation of DNA whereas fixation with ethanol does not cause fragmentation resulting in longer strands of DNA for amplification or analysis.

Conclusion

As personalized medicine for NSCLC continues to evolve, the effective integration of cytologic, molecular and histologic testing to determine the most appropriate therapy for each individual patient will be of the utmost importance. While molecular testing is increasingly becoming the standard of clinical practice, there remains a need to standardize the method of EGFR detection in MPE to ensure a test or testing algorithm that maximizes sensitivity and specificity. This may be accomplished via a combination of testing modalities. Testing for EGFR is only the beginning as samples are also currently being tested for EML4-Alk translocation and there are a number of new molecular targets being investigated, such as ROS1, BRAF, KRAS, HER2, and PIK3CA [66]. As new therapies are developed directed at specific mutations, more testing of MPE will be required.

Conflict of Interest Clayton J. Shamblin declares no conflict of interest.

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