



Sensitive molecular testing methods can demonstrate NSCLC driver mutations in malignant pleural effusion despite non-malignant cytology

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Abstract: Malignant pleural effusion (MPE) may be diagnosed by cytologic evaluation of pleural fluid, though false negative results can occur. Pleural effusions may provide a source of tumour material for genotyping in lung cancer patients. Detection of MPE may be improved through use of highly sensitive molecular techniques. We identified five patients with non-small cell lung cancer (NSCLC) with initial pleural fluid samples that were non-malignant on cytology, but were subsequently clinically confirmed to have MPE. Tumour mutation status was confirmed via routine testing of diagnostic clinical specimens. Cytologically negative pleural fluid cell-block specimens were analysed by amplicon-based parallel sequencing (APS) for somatic mutations commonly detected in NSCLC, and selected cases by improved and complete enrichment CO-amplification at lower denaturation temperature PCR (ICECOLD PCR) for known mutations. Mutations were detected in three out of three (sensitivity 100%) cytologically non-malignant pleural fluids from patients with a known mutation: two patients with known Kirsten rat sarcoma (*KRAS*) mutation demonstrated the same *KRAS* mutation in their pleural fluids by APS, both at approximately 2% mutant allele frequency. In one patient with a known *KRAS* mutation, ICECOLD PCR detected the same *KRAS* variant at 0.7% frequency. No mutations were detected in patients with wild-type findings from reference samples (specificity 100%). Sensitive DNA sequencing methods can detect cancer-driver mutations in cytologically non-malignant pleural fluid specimens from NSCLC patients with MPE. Our findings demonstrate the feasibility of sensitive molecular diagnostic techniques for improvement of diagnostic assessment of pleural effusions in patients with lung cancer.

Keywords: Pleural effusion; molecular testing; driver mutation; lung cancer; diagnostic sensitivity

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Introduction

Cytology is currently the standard method of diagnosing malignant pleural effusion (MPE), however the sensitivity is approximately 60–70%, and is dependent upon tumour load, volume of fluid assessed and cytopathologist experience (1,2). Lung adenocarcinoma is the commonest cause of MPE and following cytologic diagnosis, molecular

testing of MPE cell block specimens is feasible (3–5).

The recent introduction of highly sensitive molecular testing techniques, such as amplicon-based parallel sequencing (APS) (6) and improved and complete enrichment CO-amplification at lower denaturation temperature PCR (ICECOLD PCR) (7–9) is rapidly changing the application of DNA sequencing for lung

cancer patients. These techniques are currently applied only to specimens demonstrated to be malignant by cytologic/histologic examination. The sensitivity of these methods suggests molecular testing could also provide an alternative approach for detection of MPE.

We investigated whether the sensitive molecular testing methods of APS and ICECOLD PCR could detect tumour-

derived mutations in cytologically non-MPEs from patients with non-small cell lung cancer (NSCLC).

Methods

This project was approved by the Human Research Ethics Committee of the Royal Melbourne Hospital (LNR/15/MH/289).

Patient selection

Retrospective review of institutional records identified 61 patients with a final diagnosis of MPE in 2014 (1). In five of these patients, initial pleural centesis specimens demonstrated non-malignant results on cytologic examination (including both morphologic examination and immunohistochemical examination with TTF-1 and calretinin). Subsequent clinical investigation in these patients confirmed malignant pleural involvement by NSCLC (Figure 1). These five patients (Table 1) form the basis of this report.

Mutation testing—reference specimens

Tumour mutation status was established by amplicon

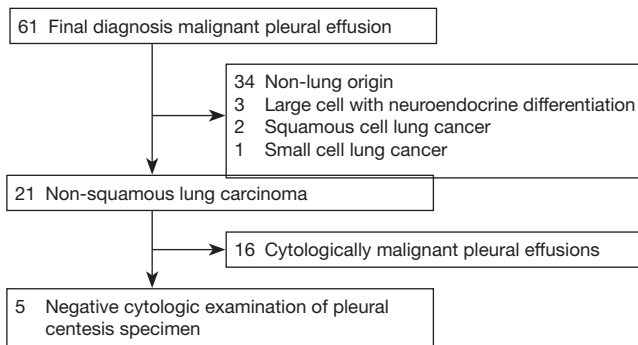


Figure 1 Flow diagram demonstrating process to identify five patients comprising the basis of this report. Initial 61 patients were identified following retrospective review of institutional records for all patients with final diagnosis of MPE between January–December 2014.

Table 1 Histocytological and molecular findings for reference specimens and cytologically non-malignant pleural fluid specimens

Patient	Reference specimen			Cytologically non-malignant pleural fluid		
	Histocytological diagnosis (IHC)	Specimen type	Molecular result (mutation frequency)	Cytological diagnosis	Molecular test method	Molecular result (mutation frequency)
1	Adenocarcinoma, lepidic pattern (CK7+, TTF-1 -)	Lung biopsy	KRAS G12C (16%)	Negative for malignancy	APS	KRAS G12C (2.2%)
2	Adenocarcinoma (CK7+, TTF-1 +)	Chest wall biopsy	KRAS G12C (77%)	Negative for malignancy	APS	KRAS G12C (2.0%)
3	Pleomorphic lung carcinoma (CK7+, TTF-1 +)	Pleural biopsy	KRAS G12C (58%)	Inconclusive for malignancy	APS ICECOLD	No mutation identified KRAS G12C (0.7%)
4	Adenocarcinoma (CK7+, TTF-1 -)	Pleural fluid	Wild-type*	Inconclusive for malignancy	APS	No mutation identified
5	Adenocarcinoma (CK7+, TTF-1 -)	Pleural fluid	Wild-type*	Inconclusive for malignancy	APS	No mutation identified

* , wild-type genes identified for all of *KRAS*, *EGFR*, *NRAS*, *BRAF*, and *PIK3CA*. TTF, thyroid transcription factor; CK, cytokeratin; KRAS, Kirsten rat sarcoma; APS, amplicon parallel sequencing; ICECOLD, improved and complete enrichment CO-amplification at lower denaturation temperature; ER, estrogen receptor; p63, p63 oncogene; PSA, prostate specific antigen.

parallel sequencing of separate diagnostic clinical specimens (see *Table 1*).

Cell blocks and DNA extraction

Archival formalin-fixed paraffin embedded (FFPE) cell-blocks of the non-malignant pleural fluid samples were obtained. Five unstained sections of 10-micron thickness were cut onto glass slides, dewaxed, then macro-dissected. DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany).

Amplicon parallel sequencing

All non-malignant pleural fluid samples were analysed in triplicate using APS. Targeted regions of *EGFR* exons 18, 19, 20, 21, *KRAS* and *NRAS* exons 2, 3, 4, *BRAF* exon 15, and *PIK3CA* exons 9 and 20 were amplified by multiplex polymerase chain reaction (PCR) and sequenced on a MiSeq Next Generation Sequencer (Illumina, San Diego, USA). Mutations were detected using MiSeq Reporter Software (Illumina, San Diego, USA). Only mutations at >1% frequency in at least two out of three assays were recorded.

ICECOLD PCR

The more sensitive ICECOLD PCR method was used for samples in which a patient's known mutation was not detected by APS. After the multiplex reaction the samples were Pico-tested for double-stranded DNA content, with those having concentrations over 30 ng/ μ L considered to have passed primary amplification, followed by dilution to a uniform 4 ng. Transgenomic ICE-me kit (Transgenomic, Omaha, USA) reagents were used for the nested ICE COLD enrichment reaction, with the manufacturers thermal cyclers protocols. Verity thermal cyclers used were stepped down to a 38.4% ramp rate (1.5 degrees/second). Samples underwent BigDye 3.1 cycle-sequencing on a 3730xl DNA analyzer. Sequencing traces were analysed on Sequencher 3.5 software. Variant detection was determined by dual comparison—wild-type control (WTC) and sample-peak under peak (PuP) levels. Variants were deemed genuine if they were;

- (I) Higher by >3 \times than any PuP in the WTC at the same nucleotide location, and,
- (II) Higher by >2 \times than any PuP in the surrounding region of the tested sample. WTC DNA was

derived from FFPE material.

Results

The histo-cytological findings and corresponding molecular results of reference specimens are presented in *Table 1*, alongside cytologic and molecular findings from non-malignant pleural fluid specimens.

Molecular testing results—reference specimens

Two patients had known lung adenocarcinoma diagnosed by core-biopsy, with *KRAS* c.34G>T G12C mutation detected on molecular testing of the clinical biopsy specimens. One patient had pleomorphic carcinoma with a known *KRAS* c.34G>T G12C mutation detected in a pleural biopsy specimen. The remaining two patients demonstrated wild type *EGFR*, *KRAS*, *NRAS*, *BRAF* and *PIK3CA* on testing of *cytologically malignant* pleural fluid samples.

Molecular testing results—cytologically non-malignant pleural fluid specimens

In all three patients with known *KRAS* mutations detected in reference diagnostic specimens, the expected mutation was detected by the highly sensitive molecular testing techniques in cytologically negative pleural effusion specimens.

For the two adenocarcinoma patients with *KRAS* c.34G>T G12C mutation, APS testing of the cytologically non-malignant pleural fluid specimens demonstrated the same *KRAS* mutation at approximately 2% mutant allele frequency.

In the patient with pleural biopsy demonstrating *KRAS* c.34G>T G12C mutation, APS testing of cytologically non-malignant pleural fluid did not demonstrate a mutation. However, further molecular analysis of the same pleural fluid with the more sensitive method of ICECOLD PCR detected the *KRAS* variant at a mutant allele frequency of 0.7% (*Figure 2*).

There was no mutation detected by APS on the cytologically non-malignant pleural fluid samples from the two patients with wild-type findings from the reference samples (specificity 100%).

Discussion

We demonstrate that highly sensitive mutation detection

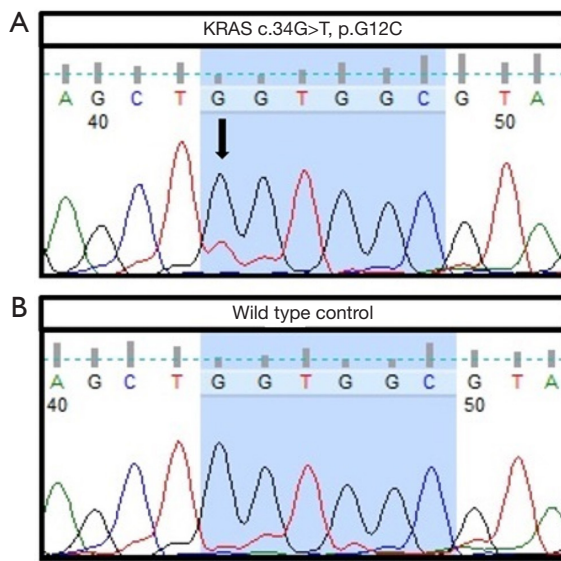


Figure 2 ICECOLD PCR Sanger sequencing traces. (A) Cytologically non-malignant pleural fluid. A clear peak is seen at codon 34 above the baseline noise (arrow) indicating a *KRAS* codon 34 G>T substitution mutation (p.G12C), with a calculated mutant allele frequency of 0.7%, in keeping with the presence of rare malignant cells; (B) FFPE wild type control, with expected baseline noise due to FFPE processing. ICECOLD, improved and complete enrichment CO-amplification at lower denaturation temperature; *KRAS*, Kirsten rat sarcoma; FFPE, formalin-fixed paraffin embedded.

methods can detect driver mutations in cytologically non-malignant pleural fluids. Using APS and ICECOLD-PCR we detected mutations in all three cytologically non-malignant pleural fluids for which a separate diagnostic clinical reference specimen demonstrated a mutation. In each case the detected mutation was identical to that reported in the reference sample. Importantly, there were no false-positive mutations detected in any samples, including two samples with known wild type tumours. Our findings demonstrate the feasibility of molecular diagnostic techniques for improvement of diagnostic assessment of pleural effusions in patients with lung cancer.

Critically, the three patients in this cohort where *KRAS* mutations were detected on cytologically non-malignant specimens were subsequently demonstrated clinically to have MPE, indicating their molecular findings were not false-positive results. Carcinoma cells are recognised in cytology samples on their morphology, and therefore must be intact and well preserved to be recognized on cytological examination (10). We hypothesise that the mutation-

positive but cytologically non-MPE samples had small numbers of poorly visualised, degenerate, fragmented, or entirely lysed tumour cells, as the source of tumour-derived DNA.

There is significant potential for sensitivity of cytologic detection of MPE to be improved by molecular diagnostic methods such as those used in the current study. Benefits may include obviating need for more invasive procedures, and avoiding delayed diagnoses. Patients with known early stage lung cancer who develop a cytologically negative pleural effusion may benefit from molecular testing prior to radical-intent treatment to avoid potentially futile therapy.

The ability of sensitive PCR-based techniques to detect a range of driver mutations in NSCLC within cytologically malignant pleural fluid specimens is well established (11,12). However, to our knowledge only two prior studies have applied molecular diagnostic methods to *cytologically negative* pleural fluids in patients with confirmed driver mutations. Buttitta et al. used next-generation sequencing to detect activating *EGFR* mutations in patients with known *EGFR* mutated cancers. An activating *EGFR* mutation was detected in only one of five pleural fluid specimens examined (13). As part of a larger study on pleural fluids that were mostly cytologically malignant, Akamatsu et al. used a combination of a combination of methods to identify mutations in three of 17 (18%) cytologically non-malignant pleural fluids from patients with lung carcinoma (14). However, they did not report which mutations were found in these particular samples, the mutant allele frequency, which method was used, or whether the same mutations were confirmed in another tumour sample. It therefore remains unclear whether the detected variants were from pleural spread of known tumour, from a benign proliferative process in the pleura, or potential artefacts.

The high sensitivity for detection of mutations in our study (100%) likely reflects the higher analytical sensitivity of the methods we used. The APS method we used has a limit of detection of 1% mutant allele frequency, and ICECOLD PCR has a reported limit of detection of 0.05% (9,15), therefore our sequential testing method should have detected mutations to the level of 0.05%. There are more sensitive methods of mutation detection now available, with analytical sensitivity below this level, such as BEAMing, digital droplet PCR, and SAFE-SeqS (16), and these could be even more effective at detecting tumour derived mutations, although false positives could arise from contamination with circulating tumour DNA or circulating tumour cells.

Limitations

This is a small retrospective study. Larger studies using various high sensitivity mutation detection methods are required. The clinical significance of cytological negative/mutation-positive effusion also requires further consideration. Other approaches to enhancing detection of MPE may complement PCR-based detection of tumour markers. Metabolic-based assays for rapid detection of rare metabolically active tumor cells in cytologically non-malignant pleural fluid specimens are reported (17). These demonstrate “live” tumour cells, though the clinical predictive significance of this finding, similar to that for detection of circulating tumour cells in early stage NSCLC, remains unclear. Detection of driver mutations is likely to be highly significant/specific, though negative predictive value is not established.

Recent animal studies suggest *KRAS* mutations may promote development of MPE (18), suggesting such patients are at higher risk of development of MPE. Future studies including patients with other driver mutations are required to confirm findings.

Use of highly sensitive molecular techniques may also be of diagnostic utility in other lung cancer specimen types, such as bronchoscopic specimens (19). Such specimens may be non-diagnostic or not suitable for molecular testing (20,21), due to small specimen volume, or due to presence of a significant proportion of non-malignant cells (22). Molecular testing even on a single bronchial brushing specimen may significantly enhance diagnostic sensitivity of minimally invasive investigation (23,24), though this remains to be examined in future studies.

Conclusions

The sensitive molecular methods Amplicon parallel sequencing and ICECOLD PCR may detect cancer-driver mutations in cytologically non-malignant pleural fluids from patients with lung adenocarcinoma. Molecular testing of pleural effusions demonstrates significant potential utility in improved diagnosis of pleural effusions in NSCLC patients.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest

to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This project was approved by the Human Research Ethics Committee of the Royal Melbourne Hospital (LNR/15/MH/289).

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