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Recent advances in the vibrational spectroscopic diagnosis of non-small cell lung cancer

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ABSTRACT

Lung cancer is the leading cause of cancer deaths worldwide accounting for 1.69 million deaths in 2015. Studies have indicated a 5 year survival rate of 8%–15% in western countries, although a survival rate as low as 1% has been demonstrated for late stage diagnosis. With the advent of targeted therapies, it is imperative to accurately differentiate non-small cell lung cancer (NSCLC) subtypes in order to ensure efficacy of treatment for patients. Immunohistochemistry and molecular techniques for the diagnosis of NSCLC are increasingly part of the diagnostic algorithm and clinical work-up of lung cancer patients, however due to the limitation of small sample size, overlapping morphological features and molecular characterisation, differential diagnosis of NSCLC still proves challenging. Vibrational spectroscopy has shown promising results for the detection of a variety of cancers and a limited number of studies have focused on lung cancer. Yet to date there has been no published evaluation of vibrational spectroscopy on cytology bronchoscopy samples which may eliminate the necessity for an invasive biopsy procedure. Following an introduction to the epidemiology and etiological factors associated with NSCLC, currently used diagnostic methods and their limitations are presented. A thorough review of Raman and FTIR spectroscopic methods in lung cancer diagnosis is then presented. On review of the literature, vibrational spectroscopy offers an alternative or adjunct diagnostic method to be applied in bronchoscopy cytology samples.

1. Lung cancer

1.1. Introduction to lung cancer

With an estimated 2.1 million new cases and 1.76 million deaths in 2018, lung cancer persists as the most common cancer worldwide, and represents the highest cancer incidence and mortality rates in both developed and less developed countries [1,2]. Studies have indicated a 5 year survival rate of 8%–15% in developed countries, although a survival rate as low as 1% has been reported for late stage diagnosis [3–5]. Global lung cancer incidence trends indicate that athough western regions such as Northern America and Europe have the highest rates of incidence, the rate of incidence is declining in these regions, while increasing in less developed regions [6,7]. These trends can be directly correlated with smoking prevalence, as cigarette smoking is responsible for 85% of lung cancer cases. Other risk factors for non-smokers include passive cigarette smoking and air pollution [8].

Lung cancer is divided into two main categories, small cell lung cancer (SCLC) accounting for an estimated 13%–14% of cases, and non-small cell lung cancer (NSCLC), which is the most common form of lung cancer accounting for approximately 85% of cases [9–12]. Furthermore, NSCLC includes different histological subtypes including squamous cell carcinoma (SCC), adenocarcinoma (AC) and large cell carcinoma. AC is more prevalent in non-smokers and accounts for the largest portion of all lung cancer cases at 44%, and accounting for approximately 26% of lung cancer cases, SCC is the second most common subtype [9]. An epidemiological study [13] examining sex differences in lung cancer incidence and survival reported that women are more commonly diagnosed at an earlier stage of lung cancer, and at an earlier age than men. The study also indicated that regardless of age and stage of the disease at diagnosis, women have an increased survival in comparison to men, and this is most evident with the AC subtype. Irrespective of sex, patient prognosis is dependent on the stage of the disease at diagnosis [14]. Table 1 illustrates the approximate 5 year

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Table 1
Progression stage, estimated 5 year survival rate, and appropriate treatments for each stage of NSCLC [15–24].

Stage of NSCLC	Progression stage	Approximate 5 year survival rate (%)	Treatment
Stage IA	Localised (< 3 cm)	75	Lobectomy
Stage IB	Localised (3-5 cm)	55	Lobectomy
Stage IIA	Localised (5-7 cm) OR Spread to nearby lymph nodes (< 5 cm)	50	Lobectomy/adjuvant chemotherapy
Stage IIB	Spread to nearby lymph nodes/diaphragm/mediastinal pleura/parietal pericardium/bronchus (5-7 cm) OR no spreading and > 7 cm	40	Lobectomy/adjuvant chemotherapy
Stage IIIA	Spread to local lymph nodes/heart/trachea/oesophagus/phrenic nerve/ present in more than one lobe (> 7 cm)	10-35	Radiotherapy/chemotherapy/immunotherapy/ tyrosine kinase inhibitors
Stage IIIB	Spread to lymph nodes of opposite lungOR spread to lymph nodes in the mediastinum and one or more of the areas mentioned in stage IIIA or a main blood vessel	< 5	Radiotherapy/chemotherapy/immunotherapy/ tyrosine kinase inhibitors
Stage IV	Metastasised to another body part, other lung, caused malignant pleural effusion/pericardial effusion	< 5	Combination of cytotoxic chemotherapies/ palliative care/ tyrosine kinase Inhibitors

survival rates for different stages of NSCLC [15–19].

1.2. Current treatment options

Therapeutic strategies and patient management are dependent on the stage, subtype, and mutation status of the malignancy [25]. Patients diagnosed with early stage (stage I and stage II) lung cancer may avail of potentially curative treatment through surgical resection. Although patients with early stage lung cancer have a 5 year survival rate of over 70%, and chemotherapy post-surgery has been shown to benefit some stage II patients, the risk of metastatic recurrence after surgery remains as high as 70% [12,14,26]. However, over half of patients are diagnosed at advanced stage and are not eligible for surgical resection. Treatments available for advanced stage disease include platinum based chemotherapies, radiotherapy, immunotherapies and molecular targeted therapies, including tyrosine kinase inhibitors (TKIs) [15–19,27]. In recent years combining chemotherapy with the appropriate TKIs has been shown to increase progression free survival of patients with advanced stage AC, although there is currently no cure [27].

Understanding the underlying molecular pathways involved with cancer cell progression drives the development of novel therapeutics, and identifying and targeting molecules that drive neoplastic proliferation has become a major tool for combatting lung cancer. Specific therapies that inhibit carcinogenic pathways are now available for NSCLC patients with genetic aberrations such as epidermal growth factor receptor (EGFR) mutations, BRAF mutations and anaplastic lymphoma kinase (ALK) fusion translocations [18,25,28]. The EGFR gene encodes a receptor tyrosine kinase and mutations of this gene are commonly observed in lung AC. EGFR mutations are reported in approximately 50% of Asian AC patients, and 20% of Western patients with AC [29]. Patients that are EGFR mutation positive have exhibited sensitivity to TKIs, such as Gefitinib, which has been implemented in treatment regimens since 2003 [30]. Another molecular targeted therapy, Crizotinib, is recommended as first-line treatment for patients with ALK gene rearrangements, which are present in approximately 3–6% of AC patients, particularly in non-smokers [18,31–33]. The BRAF gene encodes a protein in the serine threonine kinase family, and mutations are present in 1–3% of NSCLC patients [34,35]. Two therapies that target proteins in the serine threonine kinase family, dabrafinib and trametinib, have been approved for patients with the BRAF mutation, and have been shown to slow tumour growth [36,37].

As EGFR, ALK and BRAF mutations are targetable aberrations observed in AC, and as TKIs are associated with increased progression free survival and a lower toxicity than chemotherapeutic agents, molecular testing for these mutations on bronchoscopy cytology samples has now become a major part of the diagnostic work up of NSCLC, and is considered imperative for formulating treatment strategies [25,38–40].

Chemotherapy remains the cornerstone treatment strategy for most patients with SCC, as targeting the potentially actionable mutations currently shows little therapeutic benefit for patients with SCC. Although, the recent approval of the monoclonal antibody, Necitumumab, as first-line therapy in metastatic SCC has been shown to improve overall survival of patients with EGFR mutations [41].

The use of immunotherapeutics for patients with SCC is increasing. With the recent approval of three immune checkpoint inhibitors, nivolumab, atezolizumab and pembrolizumab, for SCC patients with PD-L1 positive tumours, patients receiving immunotherapy demonstrate prolonged overall survival, increased objective response rate and progression free survival, compared with systemic chemotherapy [20–24,42–44]. Testing for PD-L1, which is an immune molecule capable of inhibiting an anticancer immune response, is now part of the routine diagnostic algorithm of SCC [45–48], and expression is measured by the tumour proportion score. Patients with a proportion score greater than 50% may avail of first-line treatment with immunotherapy.

The increasing preference for personalised therapeutics for the treatment of NSCLC, is not only due to the negative side effects imparted on patients by non-selective chemotherapy, but also the increased progression free survival exhibited by patients in receipt of targeted therapies compared to chemotherapy [49,50]. Research for novel therapeutic markers is ongoing and includes studies investigating targetable molecules that drive carcinogenic progression such as FGFR, VEGFR-2, DDR2, P13 Kinase and PDGF, as well as novel immunotherapies [48,51]. As more targets and subsequent therapies are discovered, retaining adequate sample during the diagnostic work up of NSCLC for molecular testing will be imperative for future treatment planning.

1.3. Current methods for differential diagnosis of NSCLC subtypes

With the introduction of targeted therapies and immunotherapies, distinction between NSCLC and small cell lung cancer (SCLC) although paramount, is no longer adequate and further sub-classification of NSCLC is required. Although the most common symptoms associated with lung cancer are cough, dyspnea, chest pain and haemoptysis, lung cancer often presents with no symptoms until it has reached advanced stages, resulting in the majority of patients presenting with distant metastasis of the disease at diagnosis [15,52]. The following paragraphs detail the current methods used to acquire lung samples for analysis and the diagnostic tests used.

1.4. Bronchoscopy

Bronchoscopy is employed as an investigatory technique subsequent to the detection of an abnormality within the lung using CT scanning or X-rays [53]. Flexible bronchoscopy was first developed during the 1960s and remains one of the principal diagnostic methods in pulmonology. In the case of suspected lung cancers, the main focus of

bronchoscopy is to attain a sample of the suspicious lesion for diagnostic analysis [54]. At present, morphological evaluation of small specimens is the principal method used to differentially diagnose lung cancers. Accordingly, flexible bronchoscopy has evolved with the addition of adjunct applications including navigational bronchoscopy, endobronchial ultrasound, and bronchial thermoplasty, making it an effective method for obtaining minimally invasive cytology samples [55].

1.5. Tumour classification utilising cytology specimens

Small biopsy and cytology samples are the primary methods used for NSCLC diagnosis as these inflict less discomfort on the patients and still allow accurate identification of malignancy. Abrasive and exfoliative methods such as bronchial washing, brushing and bronchioalveolar lavage (BAL) are frequently utilised to attain samples from bronchoscopy for cytological analysis [56–58]. According to international multidisciplinary classification systems approximately 70% of lung cancer histological subtypes are diagnosed by assessing the predominant cytological pattern of small biopsies and cytological specimens, making cytological analysis a commonly implemented diagnostic method for NSCLC [59–61]. When a tumour does not show standard morphologic criteria for SCC or AC, immunohistochemical markers are used for further classification. Current guidelines suggest a vigilant use of immunohistochemstry (IHC) for diagnosis as limited cell sample remains for molecular testing for therapeutics, such as Fluorescent in situ hybridisation (FISH) and molecular sequencing [60].

1.6. Immunohistochemistry

IHC is concerned with the identification and distribution of cellular components through pinpointing specific antigens [62]. The application of IHC can be used to identify specific cell types by labelling tissue specific biomarkers. The method is based on antigen-antibody interactions, where the location of antibody binding is visualised by direct or secondary antibody labelling methods [63]. This is an effective technique used to discriminate between the different subtypes of NSCLC, and double IHC staining has been shown to be effective in the detection of AC [64].

A systematic review by Desai et al. [28] analysed data published between 2000 and 2012 and identified IHC as the most commonly used molecular testing technique for identifying biomarkers for the differential diagnosis of SCC and AC. The most frequently used biomarkers for this distinction are thyroid transcription factor 1 (TTF-1), EGFR, and the tumour suppressor gene p63 [28,48,49]. TTF-1 modulates genes responsible for pulmonary morphogenesis and differentiation, and has exhibited elevated immunoreactivity in ACs [65,66]. The genomic profile of AC has also displayed an association with EGFR mutations [67], therefore the high prevalence of EGFR mutations in AC, alongside the recent development of molecular targeted therapies, makes it an effective marker for diagnosis as well as treatment strategy [68]. In contrast, p63 has been noted to display significantly higher positivity in SCCs than in ACs, making it a useful diagnostic indicator for SCC [65].

While IHC may be required to aid diagnosis in some cases, the technique is becoming increasingly applied in the realm of therapy prediction. It is not only imperative for the detection of treatment sensitive tumours, but also for the identification of those patients who may suffer ill effects where specific agents may be contraindicated. Despite the efficacy of IHC for the differential diagnosis of NSCLC subtypes, this diagnostic technique has multiple limiting factors. Although dual staining methods are now applicable with IHC, an additional slide is still required, significantly reducing the scant cellular material available for subsequent molecular testing. IHC also relies on the use of large molecules such as antibodies to label molecular structures, which interfere with further investigation of intrinsic molecular properties [69,70].

1.7. Fluorescent in situ Hybridisation hybridisation

FISH is an effective molecular testing technique for identifying fusion genes and has been approved by the FDA as the gold standard method for the detection of ALK rearrangements in NSCLC [71,72]. As IHC does not directly detect the ALK fusion gene, not all fusion positive tumours are positive using IHC [73,74]. FISH however has the ability to directly detect ALK fusions and also has an overall sensitivity and specificity higher than IHC for the identification of ALK gene aberrations in NSCLC [73,75]. Despite being a powerful technique needed for ALK testing and identifying patients that may respond to molecular targeted therapy, FISH requires the limited residual sample left over from diagnosis.

1.8. Current research on genomic profiling

In the era of personalised therapies and as new molecular markers are discovered, molecular testing is becoming part of the routine diagnostic workup of NSCLC. Current research is investigating potential molecular markers which may act as therapeutic targets or predictors of response to therapy [76–78].

Genomic and molecular profiles of AC and SCC are being developed using techniques applicable on archival cancer samples such as mass spectrometry and next generation sequencing. These techniques have been used to investigate the genomic profile of AC and have identified some high frequency mutations associated with the AC subtype, such as EGFR, MET, FGFR and HER2 [38,76–78].

Genomic hybridisation has been used in a number of studies to explore the gene-phenotype relationship of NSCLC subtypes, and research using this technique has identified entire chromosomal regions and individual genes which are applicable for the discrimination of AC and SCC. Using residual bronchial brush samples, Su et al. [79] identified 19 genes that are applicable for the discrimination of SCC and AC, many of the genes associated with epidermis development and cell adhesion and may present as potential therapeutic markers. Lockwood et al. [80] identified an entire chromosomal region (8p12) which may be valuable for profiling NSCLC subtypes. A gene within this region, BRF2, was identified as a SCC specific genetic amplification, and indicates that this gene is a promising marker for SCC. As BRF2 encodes a protein involved in transcription initiation and facilitates in transcribing proteins required for cell growth, it may present as a novel target for future therapies.

With the expanding demand for genomic profiling there is an increased demand on limited cell sample. The optimisation of diagnostic processes is therefore paramount to retain cellular material for subsequent molecular analysis such as genomic profiling.

1.9. Limitations of current diagnostic methods

Although IHC is a highly accurate technique for discriminating SCC and AC, this diagnostic technique significantly reduces the amount of sample available for subsequent molecular testing, and as previously discussed, molecular profiling of the tumour is paramount in order to ensure efficacy of treatment. The demand for limited samples is ever increasing, this drives the need for an alternative diagnostic method which can be applied to small tissue and cytology samples without compromising subsequent applications [69,70]. In response to the need for new diagnostic methods, the investigation of alternative methods such as vibrational spectroscopy has been growing in recent years. The following section will discuss vibrational spectroscopy methods, such as Fourier Transform Infrared (FTIR) spectroscopy and Raman spectroscopy, as alternative techniques for the diagnosis of lung cancer.

2. Vibrational spectroscopy

Vibrational spectroscopy uses electromagnetic radiation to probe

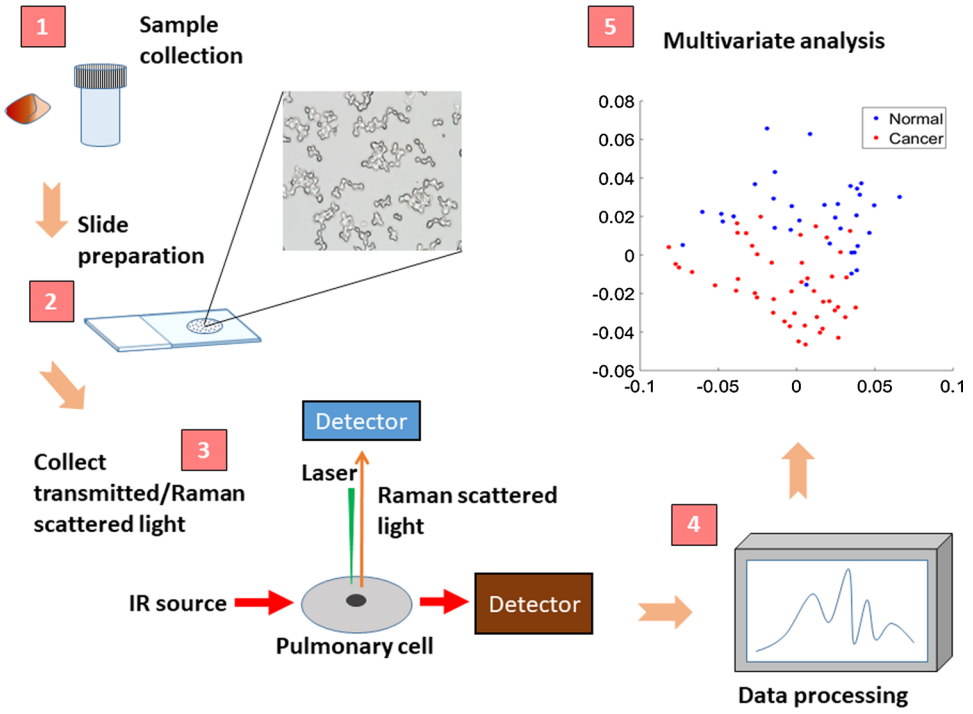

Fig. 1. Flow diagram of the process involved in applying vibrational spectroscopy to bronchoscopy obtained specimens for cancer detection. After obtaining the cytology/biopsy sample the biological material is stored in a preservative. The tissue section or cellular material is then fixed to a substrate such as a glass slide and left unstained for spectroscopic analysis. Using a laser or an infrared source, spectra are obtained by collecting the Raman scattered light or transmitted light. The spectral data are then pre-processed to minimise noise and spectral contaminations. Diagnostic features are then extracted from the data with multivariate analytical techniques.

vibrations within molecules, and allows analysis of such molecular vibrations [81]. Molecular vibrational energies are routinely investigated using Fourier transform infrared (FTIR) spectroscopy and Raman spectroscopy. Fig. 1 demonstrates the process involved in applying vibrational spectroscopy to biological specimens for cancer detection.

2.1. FTIR spectroscopy for lung cancer diagnosis

Fourier transform infrared spectroscopy (FTIR) spectroscopy is a label free vibrational spectroscopic technique, which necessitates minimal sample pre-treatment [81–83]. When applied to biological tissue, the FTIR spectrum reveals comprehensive detail about the molecular content of the tissue. Intracellular changes induced by cancer states may be detected as variations from the spectral profile of normal tissue. The use of FTIR spectroscopy for lung cancer diagnosis has been investigated for over 20 years, with previous reports examining the applicability of FTIR spectroscopy for lung cancer investigation using *in vitro* models and *ex vivo* samples [84,85]. The *in vitro* studies included exploring the ability of FTIR spectroscopy to discriminate normal and cancer cells, and assessing lung cancer cells response to therapy [86,87]. Lee et al. [86] differentiated normal and lung cancer cell lines and reported differences in nucleic acid and phospholipid bands in the malignant lines. Sulé-Suso et al. [87] demonstrated the ability of FTIR spectroscopy to detect therapeutic response in lung cancer cell lines after the addition of a chemotherapeutic agent. The FTIR spectra indicated an increase in phosphate groups in the cells after the addition of the chemotherapeutic agent. The spectral changes detected were correlated with cell survival, showing that FTIR spectroscopy may potentially identify treatment sensitive cells. The study also demonstrates the suitability of FTIR spectroscopy for single cell analysis.

Although cell line studies provide proof of concept, stronger evidence to support the feasibility of using FTIR spectroscopy for lung cancer diagnosis are provided by applying the technique to surgically resected samples. A pilot study conducted by Xiaoliang et al. [88] used surgically resected tissue from 60 patients and applied a variation of FTIR spectroscopy, attenuated total reflection (ATR) FTIR spectroscopy to collect spectra from malignant and non-malignant tissues. The study showed the ability to detect the presence of lung malignancy with a sensitivity, specificity and accuracy of 96.7%. In addition to allowing the classification of neoplastic changes with high diagnostic accuracy, analysis of FTIR spectra reveals the molecular differences between malignant and non-malignant lung tissue. Yano et al. [84,89] described the increase in glycogen content of NSCLC tissue sections compared to non-cancerous tissue sections.

Akalin et al. [90] used a machine based learning approach to show that spectral profiles can distinguish between normal and cancerous tissues with high accuracy. They also reported the ability of IR spectroscopy to accurately classify SCLC, as well as the NSCLC subtypes AC and SCC. The authors indicated that this method may potentially be used to identify clinically significant subtypes of AC, although training and test data sets of adequate size were not available for their study. Further classification of AC into prognostically different groups was achieved by Grosserueschkamp et al. [91]. Using representative spectra for each subtype a supervised classifier was constructed. The first and second level random forests separated between normal tissue, diseased tissue and lung cancer subtypes. The third level random forest enabled further classification of AC into prognostically relevant subtypes, the favourable non-mucinous lipidic subtype, less favourable papillary and acinar subtypes, and the poor solid and micropapillary subtypes. A discrimination accuracy of 97% for cancer subtypes and 95% for AC subtypes was reported [91].

As cytological samples are the least invasive samples to attain for the diagnosis of NSCLC, several studies have assessed the ability of FTIR spectroscopy to characterise lung cancer using pulmonary cytological samples. Ghosal et al. [92,93] have demonstrated the feasibility of applying FTIR spectroscopy to sputum samples to detect lung cancer. Using two wavenumbers (1031.7, 1409.7 cm^{-1}) to develop a predictive model, lung cancer cells were identified with a sensitivity and specificity over 91%. Lewis et al. [94] generated FTIR spectra from sputum cell pellets, and identified an increase in glycogen in the lung cancer cells. This finding is in accordance with multiple studies and signifies that elevated glycogen content may be a useful diagnostic biomarker for lung cancer using FTIR spectroscopy [84,85,89]. These studies demonstrate that applying this powerful diagnostic technique to cytological specimens for the detection of lung cancer in a clinical setting is a realistic prospect. In addition, FTIR spectroscopic analysis of sputum

samples may be a cost effective and fast tool for lung cancer screening due to the non-invasive procedure of obtaining sputum samples and high throughput FTIR technology. In order for the application of FTIR spectroscopy to progress from a research setting to preclinical or clinical trials, an interdisciplinary approach is required to validate the performance of spectral biomarkers with a sufficient number of patients. The complexity and current availability of these validated models is a significant limitation for the application FTIR for lung cancer diagnosis.

2.2. Raman spectroscopy for lung cancer diagnosis

Raman spectroscopy has been discussed as a promising tool for cancer diagnostics for over 20 years [95]. Multiple reviews have illustrated how this spectroscopic technique has demonstrated favourable results for the identification of cancerous tissue and has the potential to improve cancer diagnostics in a variety of cancers including brain, breast, skin, lung and gastrointestinal cancer [96–99]. As with FTIR spectroscopy, Raman spectroscopy is a form of non-invasive, label free vibrational spectroscopy, however Raman spectroscopy relies on the detection of inelastically scattered light [82,100,101]. The Raman spectrum is a plot of the intensity of the scattered light versus the change in energy given in wavenumbers (cm^{-1}), and represents a detailed biochemical fingerprint of the cellular components, as shown in Fig. 2. As Raman spectroscopy provides comprehensive details about the composition of tissues, biochemical changes in cancerous tissue may be detected in the Raman spectra. Combining Raman spectroscopy with multivariate analysis can enable a highly accurate classification of tissue types and malignancy. In recent years, many studies have shown the efficacy of Raman spectroscopy for detecting lung cancer *in vitro*, *ex vivo* and intraoperatively. Work by Jess et al. [102] showed the potential of Raman spectroscopy for grading of lung neoplasia in cellular samples. The cell lines utilised in the study were representative of three groups, normal cells (primary normal bronchial epithelial cells HBEpCs), cells with extended lifespan (HBEpCs retrovirally transduced with either human papillomavirus (HPV) type 16 E7 or CDK4), and immortalised or malignantly transformed cells (BEP2D and AsbTB2A). BEP2D is a human bronchial epithelial cell line expressing HPV18, and AsbTB2A is a transformed cell line derived from BEP2D following exposure to asbestos. A multivariate classification model based on linear discriminant analysis classified the cell types with accuracies ranging from 58%–77%. Normal cells were discriminated from all abnormal cell types with a sensitivity of 91% and specificity of 75%. The Raman spectra indicated that normal cells, cells with extended lifespan, and immortalised and transformed cells, could be characterised by their DNA, protein, amide and lipid content, suggesting that Raman spectroscopy may identify stage of lung cancer development [102].

The efficacy of Raman spectroscopy for the *in vitro* discrimination of normal and cancerous cells was also investigated by Oshima et al. [103]. Five lung cell lines from different histological origins were analysed (MRC-5 from normal, RERF-LC–MS from AC, EBC-1 from SCC, Lu-65 from large cell undifferentiated, and RERF-LC-MA from small cell carcinoma). The study showed that step-wise linear discrimination analysis of the Raman spectra allowed the label-free detection of each histological cell type with an accuracy of 100%, validating the effectiveness of Raman spectroscopy for cancer cell diagnosis.

In addition to the assessment of *in vitro* models, the applicability of using Raman spectroscopy for the assessment of surgically resected samples has been explored. Using a 1064 nm laser to eliminate background fluorescence, Kaminaka et al. [104] investigated the molecular differences between normal and malignant lung tissue. Spectra with a high signal to noise ratio were obtained and distinct Raman bands at 1448 and 1666 cm^{-1} differentiated the normal and cancer spectral profiles. This demonstrated that an increase in collagen content in lung cancer tissue is detectable with Raman spectroscopy.

In 2012 Pavićević et al. [105] applied Raman spectroscopy to tissue samples from an array of different tumour types, which included lung SCC, and AC. Using PCA and a neural network algorithm, the SCC and AC subtypes were correctly classified with accuracies of 83.3% and 91.7% respectively. Normal and tumour tissue spectra were also discriminated with an overall sensitivity of 95%. Huang et al. [106] applied Raman spectroscopy to biopsy samples to differentiate normal, AC, and SCC tissue and discovered significant differences between the spectra of normal and cancerous tissue. The Raman spectra indicated that the malignant tissue was characterised by an increase of nucleic acids, tryptophan and phenylalanine, in comparison to the normal tissue, while the AC and SCC subtypes were discriminated by a different ratio of peak intensities at 1455 cm^{-1} and 1655 cm^{-1}, corresponding to a variation in lipid to protein ratio.

Gao et al. [107,108] applied another form of Raman spectroscopy, Coherent anti-Stokes Raman Scattering (CARS), to frozen lung tissue samples, differentiating normal and benign tumours, cancerous and non-cancerous tissues, and subsets of lung cancers. The authors developed a highly accurate classification system which delineated malignant tissue from normal tissue with classification accuracy over 91%, and successfully differentiated small cell and NSCLC. An investigation by Magee et al. [109] using surgically resected samples achieved a sensitivity of 84% and a specificity of 61% for the discrimination of normal and cancer tissue, reporting that tumour tissue could be characterised by an increase in DNA and a decreased level of porphyrin in comparison to the normal tissue. In addition to showing the effectiveness of Raman spectroscopy to detect cancerous tissue, this study was

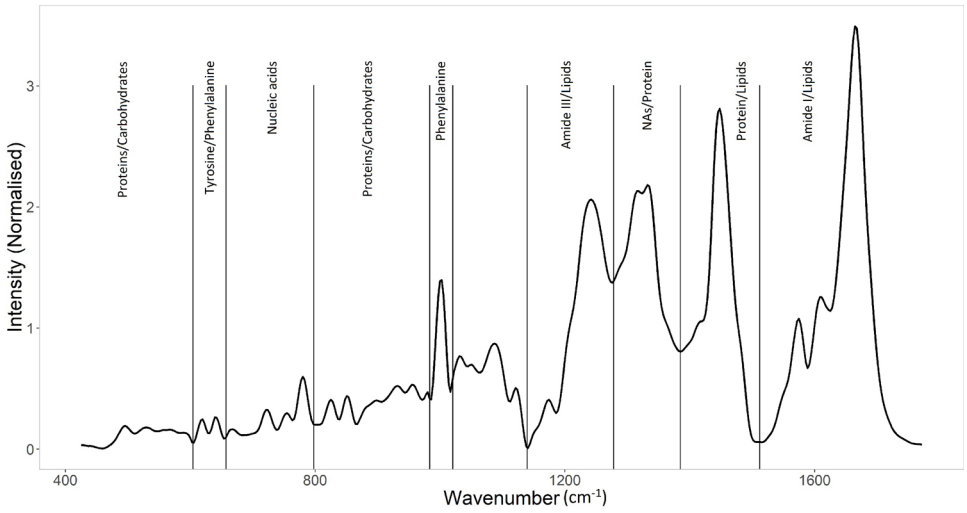

Fig. 2. Depiction of a Raman spectrum showing the different spectral regions and the corresponding biomolecules associated with each region. The spectrum which represents a bronchial epithelial cell nucleus was baseline corrected and the non-negatively constrained least squares method was used to remove spectral contaminants from the substrate.

the first to demonstrate a prognostic ability of Raman spectroscopy to predict cancer recurrence in patients with a sensitivity and specificity of 73% and 74% respectively [109].

The CARS variant of Raman spectroscopy utilises multiple pulsed laser beams that interact with the sample through a wave mixing process and produces a signal that is much higher than the spontaneous Raman signal. With no interference from fluorescence, it has been shown to be a suitable method for the differential diagnosis of NSCLC, while preserving tissue specimens for follow up diagnostic tests [110–112]. Xu et al. [111] applied CARS as part of a multimodal imaging technique to unfixed, unstained lung tissue to effectively differentiate normal lung tissue, cancer tissue, and desmoplastic tissue. In another study by Gao et al. [112], a label free diagnostic system was developed by applying a 3D imaging strategy in conjunction with CARS to differentiate NSCLC subtypes. Human AC and SCC cell lines were grown in mouse models, and the tumours were excised after 2 weeks of growth. By acquiring individual 2D images of the same field of view from different imaging depths, the authors created a 3D data structure of the tissue. The system enabled a more accurate analysis of whole cells and nuclear size, overcoming the limitations of 2D sections. The NSCLC subtypes were differentiated with an accuracy of 97%, proving the ability of vibrational spectroscopy to improve the current diagnostic algorithm with minimal sample consumption and without hindering additional molecular testing.

Surface enhanced Raman spectroscopy (SERS) has also been applied to biological samples including saliva and cell derived exosomes to characterise biomolecular differences between normal lung and lung cancer [113,114]. Zhang et al. [115] examined the prospects of implementing SERS in lung cancer diagnostics by applying SERS technology to lung tissue sections and developing a PC-LDA classifier to analyse the data. The study identified characteristic changes in the cancer spectra associated with increased DNA, carotenoids, lipids and proteins. After performing PC-LDA, a ROC curve was constructed to evaluate the performance of the developed classifier, and a diagnostic sensitivity of 95.7% and specificity of 95.7% was achieved for the discrimination of normal and malignant lung tissue.

The use of Raman spectroscopy to improve the *in vivo* diagnosis of lung cancer has also been investigated and presents as an exciting clinical application for the real-time diagnosis of NSCLC, which may prevent the need for surgical biopsy procedure. In 2008 Short et al. [116] designed an endoscopic Raman probe and acquired the first *in vivo* lung Raman spectra. With 1–2 second integration times and filters to reduce noise and fluorescence, quality Raman spectra were acquired in the high frequency spectral range from normal, dysplastic, and tumour sites. Analysis of the spectral profiles showed large variations between the SCC and normal tissue, although due to the low number of patients involved in the study the authors could not rule out inter-patient variability as a potential cause. The following year a pilot study by Magee et al. [117] then provided proof of concept for the suitability of applying Raman spectroscopy for the *in vivo* diagnosis of lung cancer. Using a biomedical filtered fibre optic probe and a method to reduce background fluorescence, clear Raman spectra were acquired from *ex vivo* normal and malignant lung tissue. Spectral variation analysis identified increased peak intensities for amide I (1655 cm^{-1}), amide III (1260 cm^{-1}), and phenylalanine (1002 cm^{-1}) in the tumour spectra. The normal and tumour spectra were also classified with 100% accuracy using PCA leave one out cross validation. In 2016, a single centre clinical investigation with 80 patients explored the adjunct application of Raman spectroscopy with bronchoscopic methods for the *in vivo* detection of lung cancer [118]. Real time point laser Raman spectroscopy (1 s per spectrum) was performed on 280 sites from non-malignant and malignant lung lesions. Multivariate analysis of the spectra differentiated the normal, benign and malignant tissue with a sensitivity of 90% and specificity of 65%.

Raman technology also has the potential to help clinicians decide which lesions are suitable for biopsy, as the adjunct use of *in vivo* Raman spectroscopy with bronchoscopy methods has been shown to detect preneoplastic lesions with sensitivity and specificity above 90% [119]. These studies show that Raman spectroscopy is a highly sensitive method for the *in vivo* diagnosis of lung cancer, and now with the ability to detect tumours in the peripheral lung with novel miniature Raman probe technology [120], and the capacity to produce clear spectra from weak Raman signals, further multicentre clinical trials are warranted.

In addition to *in vivo* and *ex vivo* studies, others have explored the feasibility of applying Raman spectroscopy combined with data mining methods to body fluid samples for lung cancer diagnosis. These are promising, cost effective methods and would allow simple and reliable screening to detect early stage malignancy. Analysing the serum of patients with NSCLC, Wang et al. [121] were able to obtain distinct Raman spectral profiles for stage I, stage II, and advanced stage NSCLC. Comparative analysis of the mean spectral profiles for each stage of NSCLC revealed multiple peaks involved in the carcinogenic progression of NSCLC. Cancer progression corresponded with a decrease in all of the analysed peak intensities. The authors noted that protein and phospholipid content was significantly reduced in the serum samples of NSCLC patients, evident by a decreased peak intensity at 1658 cm^{-1}. With PCA and discriminant analysis, spectra taken from the sera of healthy people and patients with stage I- IV NSCLC were differentiated with an overall accuracy of 92%. Other studies have shown that Raman spectroscopy also has the potential to characterise lung cancer and detect malignancy with high diagnostic sensitivities and specificities with non-invasive samples such as saliva and urine [114,122,123]. Applying Raman spectroscopy to these minimally invasive specimen types may be an effective tool for NSCLC screening and staging without the need for bronchoscopy. The translation of these technologies into a clinical setting now requires successful randomised clinical trials before they are accepted.

As discussed in this review, applying Raman spectroscopy to tissue sections has shown the ability to discriminate between non-malignant and malignant lung tissue, and between NSCLC subtypes. Raman technology has also been successfully applied to single cells, effectively identifying malignancy. However, based on a comprehensive review of the literature it appears no studies have investigated the use of Raman spectroscopy on bronchoscopy cytology samples, which would align with the current sampling techniques for lung cancer. In light of the research to date, applying this technique to cytology samples may present as a minimally invasive method for identifying and discriminating NSCLC subtypes and treatment sensitive tumours. This method may be used to provide an accurate diagnosis and retain sufficient sample for ancillary molecular tests, maximising the use of limited samples.

2.3. Summary and future perspectives

Small tissue and cytology samples are often the only specimens available for the diagnosis and molecular analysis of NSCLC. Current diagnostic methods for NSCLC consume much of the available sample, and with an increasing demand for the sample for ancillary molecular tests, an alternative accurate diagnostic method is desirable. As vibrational spectroscopic techniques are label free, highly sensitive and allow the rapid detection of intracellular biochemical information, they addresses the limitations of current diagnostic methods and are a potential option for the differential diagnosis of NSCLC. As vibrational spectroscopy is non-destructive, the unstained slides can be used for subsequent analysis.

Numerous studies have demonstrated the ability of vibrational spectroscopic techniques to accurately detect and discriminate lung cancer subtypes using tissue and cell samples. As the process of obtaining cell specimens is minimally invasive, future research may investigate the viability of using vibrational spectroscopic methods on bronchoscopy cytology samples. A previous study has already shown the ability of FTIR spectroscopy to characterise lung cancer cells using

cytological samples, and although Raman spectroscopy has been investigated on lung carcinoma samples *in vitro*, *ex vivo*, and *in vivo*, to our knowledge it has never been used on bronchoscopy attained cytology samples [94,103,106,108,119].

As probe based Raman spectroscopy is developing and is a promising technique for the *in vivo* diagnosis of cancer, development in the application of Raman spectroscopy for the *in vivo* diagnosis of NSCLC may present with many promising advantages for clinical setting [118,124,125]. It can be used at a lower cost than other imaging techniques including magnetic resonance imaging (MRI) and ultrasound, in addition to giving real time information on biochemical composition in high resolution with no stains or labels [98].

3. Conclusion

As the identification of NSCLC subtype is pertinent for the development of treatment regimens, an enhanced label-free, rapid, and even automated diagnostic technique would be largely beneficial. Integrating vibrational spectroscopic methods into the diagnostic algorithm for lung cancer offers the ability to provide an accurate diagnosis and discover new molecular markers for the discrimination of NSCLC subtypes. Vibrational spectroscopic methods may be useful for the non-destructive analysis of small unstained bronchoscopy attained samples, without compromising subsequent ancillary molecular tests. These techniques have the potential to reduce the need for surgical biopsies, and may be economically advantageous by saving medical resources.

Declaration of Competing Interest

The authors declare no conflict of interest.

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