Review Article

Ancillary Techniques for Early Detection of Lung Carcinoma in Sputum: An Update

Neema Tiwari[1], Mukta Tiwari[1], Anand Narain Srivastava[1] and Rajendra Prasad[2]

Departments of Pathology[1] and Pulmonary Medicine[2], Era's Lucknow Medical College, Lucknow, Uttar Pradesh, India

Abstract

Lung cancer is the most rapidly spreading cancer worldwide as well as in India**.** The major cause of development of such malignancy is the cigarette or *bidi* smoke being inhaled by the smokers as well as their family members passively, leading to predominantly squamous or the small cell variety of tumours. Lung cancer is regarded as one of the most common cancers in the world.[1] In 2012, lung cancer occurrence worldwide was approximately 1.8 million patients with an estimated mortality of 1.6 million.[1]

Immunohistochemistry (IHC) and fluorescence *in-situ* hybridisation (FISH) are molecular techniques involved in the diagnosis of particular mutations in lung cancer which would affect the therapeutic outcome of the tumour. The earliest known sputum cytology was performed in the year 1970, when it was mooted as non-invasive screening methodology for lung cancers. This review is an attempt to elucidate the role of sputum cytology to diagnose lung cancer as well as the ancilliary techniques available to improve the sensitivity and specifity of diagnosis of cancer by sputum cytology. **[Indian J Chest Dis Allied Sci 2016;58:241-246]**

Key words: Sputum, Lung cancer, Detection techniques.

Introduction

Lung cancer is slowly rising, especially in the developing countries due to increasing levels of smoking patterns. The major cause of development of such malignancy is the cigarette or *bidi* smoke being inhaled by the smokers as well as their family members passively, leading to predominantly squamous or the small cell variety of tumours.

Incidence and Prevelance

Lung cancer is regarded as one of the most common cancers in the world.[1] In 2012, worldwide occurrence of lung cancer was approximately 1.8 million cases, with an estimated mortality of 1.6 million.[1] Approximately 225,000 new cases of lung cancer and over 160,000 deaths are reported annually in the United States.[2] Lung cancer is considered to be a predominant cause of death among patients in today's world.[3]

Genetics

The most investigated methodology in detecting carcinoma lung and getting an idea about tumour grading and prognosis is fluorescence *in situ* hybridisation (FISH) and immunohistochemistry (IHC). The two most common markers being studied and used are epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK). Tumour cells contain genetic abnormalities also called as driver mutations which are essential for tumour cell survival. The EGFR, KRAS (V-ki-ras2 Kirsten rat sarcoma viral oncogene homolog) and ALK are considered prototypical driver mutations in carcinogenesis of lung cancers. Mutation and amplification leads to activation and formation of oncogenes. These oncogenes affect the tyrosine kinase which is responsible for cell cycle and cell proliferation. The uncontrolled cell proliferation due to constant growth signals from these mutated genes leads to formation of a neoplasm. Hence, these mutations confer a clonal growth advantage to tumour cells. Among the non-smokers who develop adenocarcinomas, most common mutation is EGFR and ALK, while in smokers it is KRAS. Hence, the complex pathobiological process involved in the genesis of lung carcinoma involves activation of oncogenes by mutation in EGFR, KRAS, BRAF (serine/threonine-protien kinase B-raf), ERB2 (tyrosine kinase receptor), translocations (ALK, ROS1, RET), and amplification (MET, EGFR1).[4]

Aetiology

The major cause of development of such a malignancy is the cigarette or *bidi* smoke being inhaled by the

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Correspondence and reprint requests: Dr A.N. Srivastava, Professor and Head, Department of Pathology, Era's Lucknow Medical College, Lucknow-226 003 (Uttar Pradesh), India; E-mail: ans4csmmu@gmail.com

smokers as well as their family members passively, leading to predominantly squamous or the small cell variety of tumours. The nicotine present in the smoke is responsible for inflammatory damage to the respiratory epithelium which further leads to carcinogenesis in the damaged epithelium. Cigarettes, cigars, and pipe tobacco are made from dried tobacco leaves with substances being added for flavour. The smoke from these products is a complex mixture of chemicals produced by the burning of tobacco and its additives. Tobacco smoke is made up of more than 7000 chemicals, out of which over 70 known to cause cancer (carcinogens). Some of the chemicals found in tobacco smoke include: nicotine (the addictive drug that produces the effect people are looking for and one of the harshest chemicals in tobacco smoke), cyanide, benzene, formaldehyde, methanol (wood alcohol), cetylene (the fuel used in welding torches), ammonia. The poisonous gases inhaled are carbon monoxide and nitrogen oxide, vinyl chloride, ethylene oxide, arsenic, chromium, cadmium, nitrosamines, polynuclear aromatic hydrocarbons.[4] These are carcinogenic alkaloids that binds to nuclear material forming addicts causing mutations which drive the carcinogenesis pathway to form a tumour.[4]

Smoking, is the major causative factors implicated in the development of the carcinoma, and that leads to the development of squamous cell carcinoma which is more central or hilar in position and is prone to metastasise more than other histological subtypes.

Other factors being implicated in pathogenesis of lung carcinoma is the biomass gas exposure, especially in the rural population where women still use wood and cow-dung to cook on smoke *chulhas*. Another major cause of pulmonary pathology is the occupational exposure of compounds, like arsenic, asbestos beryllium, coal, coal tar, radon due to indoor exposure, gamma radiations, etc.[4-6]

Diagnosis of Lung Cancer

Radiological

The key for good survival for lung cancer patients is the early diagnosis and prompt treatment of the disease. However, this is not easily achieved, especially in cases of lung cancer, where tumour is unfortunately detected at a very advanced stage. The common screening procedures are the routine radiological investigations, like chest radiography, where an opacity or collapse or effusion might point towards a malignancy. The radiographic findings can be confirmed on computed tomography (CT) and high resolution computed tomography (HRCT) and exact position, extent of spread and size of the lesion can be assessed.[7,8]

Early detection of lung cancer can improve the 5-year survival rate by 60%.[9-12] Patients with radiographical documentation of stage I lung cancer have a 5-year survival rate of 40% to 80%, whether discovered by screening or accident. However, mortality in lung cancer worsens rapidly with advancing stage at the time of diagnosis.[13]

Patients can live with lung cancer for many years before it becomes apparent. Early lung cancer is largely asymptomatic due to internalisation of tumours, hence the patients are not alerted by obvious physical changes that leads to delay in the diagnosis. Squamous cell carcinoma, the commonest variety of lung cancer, takes around eight years to reach a size of 30mm when it can be commonly diagnosed so, and by the time symptoms arise, the risk of metastasis is considerable.[14,15] Once symptoms appear these are often ignored by the patients, delaying the diagnosis and treatment. Other major reasons for delay in diagnosis are poorly understood.[16]

Newer methods of detecting lung cancer and its progression includes positron emission tomography (PET). This modality is useful in the initial staging to identify sites of tumour involvement. Integrated PET-CT has been shown to improve staging over PET scanning alone.[17]

PET-CT measures the metabolic activity of the tumour using the standardised uptake value (SUV) to assess the tumour uptake of fluorodeoxyglucose (FDG). In a meta-analysis of 21 retrospective studies[18] include 2637 patients with stage I to IV non-small cell lung cancer (NSCLC), it was seen that a high SUV was associated with a poor prognosis. Another meta-analysis[19], limited to patients with stage I NSCLC, also found that a lower FDG uptake was associated with a better prognosis. The PET (or PET-CT) may also be useful in predicting response to chemotherapy.[20-22]

Radiological suspicion of lung carcinoma needs to be confirmed by a pathological diagnosis, either by cytological or by histopathological investigations.

The cytopathological investigations involve microscopic inspection of sputum, pleural fluid, bronchoalveolar aspirate and fluid. However, a tissue diagnosis is necessary to determine whether a lung cancer is a NSCLC or an small cell lung cancer (SCLC), as well as to rule out the possibility that disease represents a non-malignant process or lung metastasis from a primary tumour at another site.

Cytological

A large number of diagnostic modalities are available in diagnosing the lung carcinomas, e.g., cytological examination of sputum, specimens collected at bronchoscopic examination like bronchial washing, lavages, tracheobronchial and transthoracic fine needle aspiration. Yet there is a delay in diagnosis

and treatment that could be the result of many factors, including socio-economic, cultural and health care. The reasons for late detection of tumour and delayed therapy is that the symptoms are too non-specific, especially in the context of co-existing respiratory diseases, a cause of concern.[23] The most common evidence that a patient has, is an x-ray as a means of radiological evidence of lung carcinoma. However, for a pathological confirmation one has to undergo a number of procedures, like bronchoscopy, fine-needle aspiration cytology (FNAC), thoracoscopy/ thoracotomy, which are invasive, painful and expensive while, sputum cytology, an easier, better and cost-effective method of diagnosis is readily ignored.[23] Chest physicians prefer to use costlier techniques, like bronchoscopy etc, in hope of getting a better diagnostic rate. However, very few studies have been conducted on the usefulness of sputum cytology as compared with other diagnostic modalities.[15]

Bronchoalveolar lavage and bronchial brushings

Bronchoalveolar lavage principally relies on exfoliative cytology of the malignant lesion. The diagnostic efficacy of BAL depends on many factors, like degree of differentiation of the tumour, preservation quality of the cells and technical skills of the pulmonologist in obtaining the sample. Another more refined method is bronchial brushing where the surface of the tumour is carped by a brush attached to the bronchoscope in order to dislodge the cancer cells, for a better yield of cancer cells. This method is more sensitive and specific than BAL. In a study,[24] diagnostic efficacy of BAL and brushings was very high, not only in radiologically detectable carcinomas of the lung but also in the small carcinomas missed by the radiologists.[24] However, in another study,[25] diagnostic accuracy of BAL over bronchial brushings was more sensitive (87.3%) as compared to BAL (39.4%).

Despite such advancements in the field of cancer screening, sputum cytology still remains the mainstay, especially at peripheral health centres/primary health centres (PHCs) and hospitals catering to the economically poor population.

Sputum as Screening Tool

The most important criteria for labelling a methodology as screening test, is that first, it should reduce mortality due to the disease in screened groups as compared to unscreened groups, and secondly, the cost, specificity and sensitivity should be within desirable limits.[26] The earliest known sputum cytology was performed in the year 1970, when it was mooted as non-invasive screening methodology to diagnose for lung cancers. Multiple studies[27-29] were carried out to establish the role of x-rays in diagnosis, however, none was efficient enough to be established as a screening methodology. Saccomano *et al*[30] were the pioneers in the field of sputum cytology as they harvested sputum from uranium miners and analysed it for carcinogenic cells. Since then, many studies have analysed the role of sputum cytology as a screening methodology.[31,32]

A study[33] reported that diagnosis of cancer could be made in approximately 50% of sputum samples and 65% of lavage fluids and further studies should be performed on the cytological samples. Raab *et al*[34] in 1997, highlighted the need of low cost-effective diagnostic modality, like sputum cytology as the need of the hour. In another study[35], use of newer modalities, like cytometry combined with conventional cytology increased the specificity of diagnosis between 90% to 95% and sensitivity to 70% to 80%. However, other studies advocate combination of sputum cytology with radiology and advanced screening techniques for a better diagnosis.[36,37] Some studies reported about reluctance of clinicians to use sputum cytology as a diagnostic modality and advocate use of sputum cytology as first-line test to be done in suspected cases of lung carcinoma.[38-40] Bhatia *et al*[41] in their study published in 2004 mentioned that definitive treatment strategies can be based on sputum cytology alone. A cytologist needs to use well established criteria to differentiate between NSCLC and SCLC. Another study[38] showed that the low sensitivity of the sputa can be attributed to degenerating cells in the sputa. They also suggested that multiple (at least 3) sputum samples be done for diagnosis.[38] Studies done on sputum cytology prove that it is the poor man's bronchoscopy, with a sensitivity of 60% which increased with the number of samples screened.[39] This fact was condoned by Xing *et al* in their study in 2010.[42] Deffebach and Humphrey[43] advocate the use of screening methods in lung carcinoma because of highly unpredictable progression of lung carcinoma with high mortality and morbidity rates. A study showed that sensitivity of bronchoscopy is high for tumours placed centrally and greater than 2cm in size; however, sputum cytology can be used for tumours less than 2cm with exfoliating cells as a screening tool for the diagnosis.[48]

Sputum Cytology: What is New

Few studies highlighted the use of advanced techniques in simple sputum diagnosis for better sensitivity and specificity of the techniques.

Laser induced fluorescence endoscopy (LIFE). Kusunoki *et al* observed that the accuracy of diagnosis by white-light bronchoscopy in histopathologically confirmed cases was 48.6%. However, accuracy of the same specimen by LIFE was 72.7%. The sensitivity of conventional bronchoscopy for the detection was 61.2% and specificity was 85.0%. Corroborating with the

results by LIFE, these values were 89.8% and 78.4%, respectively. Generally, the red/green intensity of cancers on histograms of LIFE images was greater than the ratios for metaplasia or normal bronchial wall.[5]

Micro-RNA's. Theses are a large class of evolutionary non coding small RNA's (ribonucleic acid) regulating post-transcriptional genetic modifications. Xing *et al* in 2015 have studied presence of miRNAS (types 205, 210, 708) in the sputum to diagnose SCLC with a sensitivity of 75% and specificity of 96%. Thunnissen in 2003[48] advocates combining simple sputum, cytology with DNA, RNA testing, autoflouresence and nuclear imaging studies to obtain a more specific diagnosis. Similar findings were reported by Balkan *et al* in 2002 where they have studied miRNA in sputum of cancer patients.

Immunocytochemistry. Sputum diagnosis can further be enhanced by molecular genetics, immunocyto-chemistry, including the monoclonal antibody staining of antigens expressed by lung cancer cells.[23,24] Hence, the role of sputum cytology as a cheap, cost-effective modality in the diagnosis of lung carcinoma has to be emphasised, especially in lower socio-economic sub-group population where advanced diagnostic modalities are out of the patients' reach and budget.

Other newer techniques in future will be CT screening combined with sputum cytometry and autofluoresence study to detect flat lesions and dysplasias.

White light bronchoscopy. In 40% of patients, squamous cell carcinoma *in situ* can be detected by this method. Lesions appearing as polypoidal masses of 1-2 cm can be detected easily. However, flat lesions upto 5-10 mm might appear as mere indurations or might even be missed. Herein comes the role of autoflouresence.[4]

Autofluorecence. Pre-invasive lesions are detected by this methodology using a violet or blue light. As the lesion progresses towards malignancy there is a progressive decrease in green autoflouresence, but a very small decrease in the red autoflouresence. Lesions as small as 0.5mm can be detected by this method.

The standard methodology followed in diagnosing lung carcinoma is staining the sputum with Haematoxylin and Eosin, Papinacoulau (PAP) and Giemsa (MGG) for cytology. However, newer modalities as mentioned before are improving the specificity and accuracy of diagnosis. As these modalities are still not available in smaller pockets of developing countries, making diagnosis to be a difficult to establish at an early stage of the disease.[4]

Cell block. Preparing cell blocks and cell buttons from sputum and BAL and applying IHC and immunocytochemistry on these samples is a boon in the field of diagnosis of lung cancer by sputum examination. It provides an easy, non-invasive way of acquiring good amount of sample for the investigation.

Histopathological

Despite the above screening procedures tissue diagnosis remains the mainstay of confirmation of diagnosis. A biopsy can be obtained through various routes involving common endobronchial biopsy, transthoracic biopsy, transbronchial biopsy etc. Histopathologically, the most common tumour in smokers is squamous cell carcinoma and small cell carcinoma. Other types of lung carcinoma are glandular tumour and adenocarcinoma. Atypical glandular proliferation, another variety of adenocarcinoma, have no association with smoking. Histopathological diagnosis can be made on simple staining with Haematoxylin and Eosin, accompanied by specific IHC markers like p63 and p40 in squamous cell carcinoma and TTF1 and Napsin a in adenocarcinomas. Chromogranin, synaptophysin are common neuroendicrinal markers used for the diagnosis. IHC markers are useful in categorising the previously un-categorised, poorly-differentiated large cell carcinomas in specific subtypes.

A prolonged pre-clinical phase in lung cancer has been seen. There are clones of endobronchial cell populations, that accumulate genetic mutations leading to more malignant, and finally, an invasive malignant state.[17] These may only roughly reflect the Saccommano morphologic criteria (normal cells; hyperplasia; metaplasia; mild, moderate, and severe dysplasia; carcinoma *in situ*; and invasive carcinoma), as these evolve into malignancy. Even though a number of studies are being conducted yet no study has been able to establish the critical number of mutations, critical combinations of mutations, or a necessary order of events till now. Hence, study of these parameters is the most exciting prospect in the current environment of clinical research. However, lack of scientific surveillance in high-risk population ails the field of research right now.[36,37]

Newer modalities and discoveries in the field of research will lead to the development of new detection technologies, identification of key risk factors, and/or the validation of interventions for pre-cancer or early cancer. Half of the new cases of lung cancer occur in ex-smokers.[49-51] More women die annually of lung cancer than breast cancer, but lung cancer is not considered a woman's disease predominantly.

Treatment Modality: Old and New

Treatment of lung carcinoma is not only dependent on the grading and staging of the tumour but also on the molecular status of the tumour. The two most common molecular markers being studied are the EGFR (good prognosis in adenocarcinomas), ALK and KRAS in squamous carcinomas. It was observed in earlier

studies that the time from onset of symptoms to treatment was shorter in patients with stage IV lung cancer (median 3.4 months) than in those with stage I/II disease (median 5.5 months).[21,22] Tyrosine kinase inhibitors and drugs like cisplatin and taxanes have been the mainstay of lung cancer treatment for long time. However, now drugs like erlotinib, dasatinib, nilotinib, gefitinib are opening newer avenues in the field of cancer chemotherapy. Specifically targeting the molecular mechanism of carcinogenesis is gaining momentum. Whether surgical resection has to be done or not and at what stage is being determined by clinical staging, radiological finding and pathological grading. The future of treatment lies in the role of cancer stem cells in carcinogenesis.

Role of Cancer Stem Cells and Tumour Markers

Although, lung carcinogenesis is a multi-step process involving mutations, inflammatory cytokines, etc, but of late, the role of stem cells in initiation and propagation of cancer and its control has opened up doors for new research in the field of oncopathology. Despite the significance of this interplay, alterations in protein composition underlying tumour-stroma interactions are largely unknown.[42,43] These stem cells exist in a safe micro-environment referred to as stem cell *niche* and the degree of their stemness is responsible for the rate of tumour growth and degree of tumour resistance. These markers are inducible by exposure to anti-cancer agents. This finding highlights not only the potential fluidity of the cancer stem cells compartment, but also the functionality of these markers.[43] Instead of looking solely at the marker expression in these population, it is important to clarify the biologically significant roles of these markers in tumour progression, metastases, and role of these stem cells as possible targets for cancer therapy. It is critical to find specific biomarkers to identify and isolate cancer stem cells as well as to predict patient prognosis. Major markers seen in lung cancer studies are CD133, ALDH, ABCG2, CD44, etc. Till now major molecular markers studied in lung cancer, commonly by FISH and less commonly by IHC are EGFR and ALK re-arrangements. According to a popular newer concept, cancer stem cells are a rare population of undifferentiated cells driving tumour initiation, maintenance and spreading.[42,43] Most therapies are directed at the bulk of rapidly dividing tumour cells but not the slow dividing cancer stem cells. Expression of these stemness factors results in genetic plasticity that allows these cells to remain in a dormant, drug-tolerant state. Eradicating cancer stem cells, in addition or instead of the fast growing tumour mass seems to constitute a promising approach to achieve a long-lasting response and thereby to improve cancer therapy. A large body of evidence is in favour of the cancer stem cells concept; several aspects of its foundations were questioned. For example, the proof and enumeration of cancer stem cells in xenograft transplantation experiments is subject to the degree of immune system incompetence of the host and appropriate micro environmental conditions.[45,46] In comparison to the rapidly advancing research on breast and colon cancer cancer stem cells, investigations in the area of lung cancer is lagging behind. Identifying these stem cells in lung cancer and finding ways for their elimination may lead to new clinical approaches to delay or prevent disease recurrence.[44]

Future Aspects

Although the future of cytological diagnosis and advancements in the field to aid cytologists like molecular studies, is bright, yet it is important to stress the need of simple sputum cytology especially in the villages and Primary Health Centres considering it as a cost-effective, reliable, cheap mode of screening with good patient compliance. However, with progressing times where more stress is being laid on treatment on principle of evidence-based medicine, pathologists have to become more innovative and try and merge the old diagnostic modalities, like simple sputum cytology with more advanced techniques, like IHC, autofluoresence, mina, etc for a better life of patients with lung carcinoma.

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