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Original Research Article

Detection of Genetic Mutations in Lung Cancer Patients with the Help of Liquid Biopsy in Madurai, India: A Cross Sectional Study

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Abstract

Background and Objectives: Current biopsy procedures are invasive and especially in NSCLC, often produce too few cells or tissue sections for extensive analysis, multiple biopsies are not feasible for many patients, such as the elderly and those with comorbidities. Based on the detection of circulating free DNA, liquid biopsy is a minimally invasive procedure for determining the genetic status of cancer. Because blood samples can be easily obtained, the concept of a blood- based biopsy has long held promise as a less invasive complement to traditional biopsy techniques.

The present study aimed to detect the incidence of Epidermal growth factor receptor (EGFR), Kirsten rat sarcoma viral oncogene (KRAS) in the newly detected lung cancer patients with the help of Liquid Biopsy.

Methods: 20 Patients with Non-Squamous Non-Small Cell Lung Cancer (NSCLC) who were recently diagnosed and were planned for initial therapy underwent blood sampling (10 to 15 ml) and plasma droplet digital Polymerase Chain Reaction(ddPCR) for EGFR & KRAS mutations was done.

Results: 40 % of the patients in the study population were positive for EGFR mutation. In 7 out of 10 females with adenocarcinoma histology (70 %), EGFR mutation was found to be positive. KRAS Mutation was not detected in the female cohorts. 3 out of 10 patients (30%) among the female cohorts were negative for KRAS and EGFR mutations. One patient was positive for KRAS and EGFR mutations each among the male patient population.

Conclusion: In this study, we found that approximately one third of NSCLC patients harbour an EGFR mutation. Patients who are female, non-smokers, and have adenocarcinoma are more likely to harbour an EGFR mutation, which is consistent with previous studies. Detection of EGFR mutation from blood sampling can be done rapidly, reliably and conveniently in lung cancer patients whereas obtaining the tissue through invasive procedure is associated with delay in diagnosis and morbidity.

Keywords: Epidermal Growth Factor Receptor, Droplet Digital Polymerase Chain Reaction, Kirsten Rat Sarcoma Viral Oncogene, Liquid Biopsy.

Introduction

Cancer is a leading cause of death globally which requires rapid and reliable diagnostic methods. Lung cancer is one of the most common cancers worldwide with <15% five-year survival rates. Smoking is the main risk factor and other risk factors are environmental tobacco exposure, radon gas, asbestos, arsenic and genetic predisposition. Lung cancer is subdivided into Non-Small Cell Lung Cancer (NSCLC) and Small Cell Lung Cancer (SCLC). NSCLC in further subdivided into Squamous cell carcinoma, Adenocarcinoma and other types. Several mutations are detected in the subtype of adenocarcinoma histology, such as Epidermal growth factor receptor (EGFR), Kirsten rat sarcoma viral oncogene (KRAS). [1] Approximately 35% in East Asia have tumor associated EGFR mutations. [2] These mutations occur within EGFR exons 18–21. Such markers often guide treatment decisions in choosing the appropriate targeted therapies.

Currently tissue biopsy is the gold standard for detection of lung cancer. Small biopsies and cytological samples are the sources of tissue for diagnosis in majority of patients, as surgery specimens are not easily available in lung cancer. Therefore, liquid biopsies are of great value in noninvasive detecting methods. [3] Liquid biopsies could monitor the real time dynamics of cancer. [4] Determination of genetic mutations using serum samples which often contains circulating cell free DNA (cfDNA) or circulating tumor cells (CTC) has emerged as a new strategy for tumor genotyping. [5]

Review of literature

In lung cancers, the current standard of tissue biopsy at the time of diagnosis and progression is not always feasible or practical and associated with morbidity and underestimate the intra tumoral heterogeneity. With increased specificity and sensitivity, tumor-derived somatic changes can now be detected in urine, cerebrospinal fluid (CSF) and plasma owing to recent technological advancements in the sequencing of circulating tumour DNA (ctDNA). [6] It is simple to acquire liquid biopsies from plasma (10-20 mL of blood), lumbar puncture (1-2 mL of CSF), or urine (urinary volume needed: 30–50mL). A variety of methods can be used to extract and examine the cell-free DNA (cfDNA) for tumor-specific changes. [7,8] The two main techniques for ctDNA analysis are next generation sequencing (NGS) and digital polymerase chain reaction (PCR). BEAMing and Droplet Digital PCR (ddPCR) are two of the prominent digital PCR-based techniques. Both of these techniques make advantage of emulsion PCR, which produces droplets containing distinct DNA fragments and enables independent amplifying of DNA molecules. The droplets containing wild-type alleles of interest or mutant are subsequently separated using sequences distinguishing fluorescently tagged probes. To enrich the yield of the test, hybrid capture NGS, on the other hand, identifies the regions of the genome harbouring reference oncogenic mutations before sequencing. Digital PCR is quick, affordable, and enables quantification of mutant alleles at very low concentrations. [9] It is difficult to do multiplex analysis of more than a few mutations. It necessitates prior knowledge of the precise mutations of interest. Additionally, it is unable to detect rearrangements unless the precise genomic breakpoint is known. In addition to single nucleotide variants and brief insertions/deletions, hybrid capture-based NGS enables multiplex investigation

of thousands of genomic locations and can quickly spot rearrangements and copy number variation.

Circulating Tumor RNA

Cancer patients' plasma contains RNA from tumour cells (ctRNA), which is similar to ctDNA and can be used in identification of clinically significant RET, ROS1, and ALK fusion genes as well as the MET14 splicing variation. Genetic analysis in cfRNA, however, pose unique difficulties and are not commonly used. After blood extraction, cfRNA must be swiftly purified since, in contrast to cfDNA, it degrades very quickly.

Circulating Tumor DNA

Circulating free DNA (cfDNA) can be found dissolved in plasma and serum, at variable amounts. A portion of the cfDNA in cancer patients comes from the tumour, and this portion ranges from <0.1 per cent to >10 per cent of the total cfDNA. This percentage depends on stage, tumor burden, vascularization of the tumor, biological features like apoptotic rate and metastatic potential of the cancer cells, and response to therapy. Active mechanisms and passive methods such as the digesting of tumour cells by macrophages or the lysis of necrotic and apoptotic cells are used to release ctDNA. The ctDNA can be utilised in the identification of clinically important mutations like those in the EGFR or KRAS genes since it bears the same somatic changes as the tumour itself. When no sample is available for genetic analysis, this is especially helpful. In this situation, the European Medicines Agency advises using liquid biopsies to screen patients for tyrosine kinase inhibitor (TKI) medication. Due of low sensitivity, several common approaches for mutation identification are ineffective for ctDNA analysis. To find low abundance mutations in cfDNA, highly sensitive approaches or modifications of current methodologies have been devised.

Exosomes

Exosomes are tiny vesicles that are found in bodily fluids like blood. Many cells, including tumour cells, constitutively release them by exocytosis when under normal and pathological conditions. They have a diameter of 30–100 nm. Exosomes' composition, which includes lipids, proteins, mRNA, several kinds of non-coding RNAs, and double-stranded DNA, is somewhat similar to that of the parental cells. Exosomes are typically obtained by immune-bead isolation techniques [10], like magnetic activated cell sorting, or sucrose gradient ultracentrifugation. Commercial kits are also available. Exosomes are examined using techniques such as transmission electron microscopy, Western blot, FACS, and other techniques after they have been isolated.

Circulating Tumor Cells

CTCs are the most extensively studied component of liquid biopsies of cancer patients, along with ctDNA. [11] They are cancer cells that have broken away from the solid tumour mass and are now known as circulating tumour micro emboli, which circulate in the blood and lymphatic system as single cells or as clusters. CTCs are very uncommon in advanced NSCLC patients, averaging 1–10 per mL against a background of 106–107 peripheral blood mononuclear cells. The creation of reliable and delicate enrichment techniques will face enormous obstacles as a result of this low abundance. Some CTC capture techniques require a label and are based on particular epithelial cell surface markers, such as EpCAM for positive selection or CD45 for negative depletion. [12] T790M resistance mutation and EGFR-sensitizing mutations have been found in the CTCs of EGFR-positive patients at presentation and following progression to Tyrosine kinase inhibitors (TKI) therapies, respectively, using NGS and modified PCR techniques. [13,14]

Objectives

To prospectively validate the plasma droplet digital PCR (ddPCR) for the rapid detection of common Epidermal growth factor receptor (ECFR), Kirsten rat sarcoma viral oncogene (KRAS) in the newly detected lung cancer patients.

Material & Methods

Type of Study: Cross Sectional study

Study duration: 2 months

Ethical clearance was obtained from Institutional Ethics Committee before initiating the study. (IEC)

Study Population:

Patients with Non-Squamous Non-Small Cell Lung Cancer (NSCLC) who were recently diagnosed and were readied for initial therapy underwent blood sampling (10 to 15 ml) and plasma droplet digital Polymerase Chain Reaction(ddPCR) for EGFR & KRAS mutations was done.

Sample size:

20 subjects who were diagnosed as a case of NSCLC in the Medical College hospital -Madurai, were enrolled for the study.

Data collection methods and tools

10 to 15ml blood will be collected from the peripheral vein of the study subjects, plasma will be separated from it and sent for droplet digital Polymerase Chain Reaction (ddPCR) determination at the central laboratory. Droplet A water-oil emulsion droplet mechanism is used in digital PCR technology, which is a digital PCR technique. The partitions that separate the template DNA molecules are created by the formation of droplets in a water-oil emulsion. Although in a considerably smaller format, the droplets perform roughly the same role as individual test tubes or wells of a plate where the PCR reaction takes place. A crucial component of the ddPCR process is the large sample splitting. The PCR amplification is done inside each droplet created by the Droplet Digital PCR System, which divides nucleic acid material into thousands of nanoliter-sized droplets. Compared to other commercially available digital PCR systems, this method requires fewer samples, which lowers costs and preserves priceless samples.

The secret to droplet digital PCR is sample partitioning. A single sample provides only one measurement in standard PCR, whereas with droplet digital PCR, the sample is divided into 20,000 nanoliter-sized droplets. This segmentation makes it possible to measure thousands of distinct amplification events simultaneously in a single sample.

Results and Discussion

The study population was equally divided between 50% male & 50 % female. Majority of the study population were from rural area (55%). Middle income group were the majority (60%). Semi-skilled workers were the majority with 35%.

Table 1: Profile of study population (n=20)

Variable	Groups	Number	Percentage
Sex	Female	10	50.0%
	Male	10	50.0%
Area	Rural	11	55.0%
	Semi Urban	4	20.0%
	Urban	5	25.0%
Income	High	1	5.0%
	Low	7	35.0%
	Middle	12	60.0%
Edu Qualification	Graduate	2	10.0%
	HSC	6	30.0%

	Illiterate	5	25.0%
	SSLC	7	35.0%
Family Size	>5	4	20.0%
	3	6	30.0%
	3 TO 5	10	50.0%
House Dwelling	Cement Home	4	20.0%
	Indiv House	8	40.0%
	Thatched Home	8	40.0%
Occupation	Semi-Skilled	7	35.0%
	Skilled- Agri	3	15.0%
	Skilled- Office	4	20.0%
	Skilled-Office	1	5.0%
	Unskilled	5	25.0%

Smoking is one of the major risk factors for the carcinoma of lung.[14]All the Male cohorts were smokers & the female cohorts were nonsmokers. Cigarette & Beedi smokers were equally distributed among the cohorts. Majority of the patients had more than 20 pack years of smoking among the smoking population.

Table 2: Profile of smoking study population (n=10)

Variable	Group	Count	Column N %
Smoking	No	10	50.0%
	Yes	10	50.0%
Active/Passive	Active	10	50.0%
	N	10	50.0%
Cigarette/Beedi	Beedi	5	25.0%
	Cigarette	5	25.0%
	Nil	10	50.0%
Filtered/Unfiltered	Filtered	4	20.0%
	Nil	15	75.0%
	Unfiltered	1	5.0%
No of Cigar/Day	<10	1	5.0%
	>20	8	40.0%
	10 to 20	1	5.0%
	Nil	10	50.0%
Pack Yrs	>20	7	35.0%
	10 to 20	2	10.0%
	20	1	5.0%
	Nil	10	50.0%

All the patients had cough as the one of the predominant symptoms. Productive cough was the predominant symptom among the study population. Chest pain & Haemoptysis were equally distributed among the study population. Majority of the patients also had breathlessness.

X ray investigation was negative in majority of the patients. Sputum was negative in all the patients for malignant cells. CT scan was able to detect lung cancers in all 20 patients. (Table III).

Table 3: Symptom and Investigation profile of study population (n=20)

Symptom/ Investigation	Report	Male		Female		Overall	
		N	%	N	%	N	%
Symptoms- Cough	Yes	10	100.0%	10	100.0%	20	100.0%
P/Non-Productive	Non-Productive	3	30.0%	4	40.0%	7	35.0%
	Productive	7	70.0%	6	60.0%	13	65.0%
Chest Pain	No	5	50.0%	5	50.0%	10	50.0%
	Yes	5	50.0%	5	50.0%	10	50.0%
Haemoptysis	No	5	50.0%	6	60.0%	11	55.0%
	Yes	5	50.0%	4	40.0%	9	45.0%
Breathlessness	No	4	40.0%	4	40.0%	8	40.0%
	Yes	6	60.0%	6	60.0%	12	60.0%
X Ray	NEG	7	70.0%	7	70.0%	14	70.0%
	POS	3	30.0%	3	30.0%	6	30.0%
Sputum	NEG	10	100.0%	10	100.0%	20	100.0%
Ct Scan	Yes	10	100.0%	10	100.0%	20	100.0%
Histology	Adenocarcinoma	10	100.0%	10	100.0%	20	100.0%

40% of the patients in the study population were positive for EGFR mutation. EGFR mutation was positive in 7 out of 10 females with adenocarcinoma histology (70%). KRAS Mutation was not detected in the female cohorts. 3 out of 10 patients (30%) among the female cohorts were negative for EGFR and KRAS mutations. One patient was positive for EGFR & KRAS mutations each among the male patient population. (Table IV).

Table 4: Frequency of mutation in study population

Mutation	Male		Female		Overall	
	N	%	N	%	N	%
EGFR	1	10.0%	7	70.0%	8	40.0
KRAS	1	10.0%	0	0.0%	1	5.0
NIL	8	80.0%	3	30.0%	11	55.0

Conclusion and Summary

In this study, we found that approximately one third of NSCLC patients harbor an EGFR mutation. Patients who are female, non-smokers and have adenocarcinoma are more likely to harbor an EGFR mutation, which is consistent with previous studies. [15] Detection of EGFR mutation from blood sampling can be done rapidly, reliably and conveniently in lung cancer patients whereas obtaining the tissue through invasive procedure is associated with delay in diagnosis and morbidity. The gold standard of care for patients with lung cancer is genomic profiling of the tumour for diagnosis and monitoring treatment response. [16] The new paradigm for diagnosing lung cancer is liquid biopsy employing circulating tumour cells (CTC), cell free DNA (cfDNA), or exosomes.

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References:

1. Jang TW, Oak CH, Chang HK, Suo SJ, Jung MH. EGFR and KRAS Mutations in Patients With Adenocarcinoma of the Lung. Korean J Intern Med. 2009 Mar;24(1):48–54.
2. Zhou W, Christiani DC. East meets West: ethnic differences in epidemiology and clinical behaviors of lung cancer between East Asians and

Caucasians. Chin J Cancer. 2011 May;30(5):287–92.
3. Guibert N, Pradines A, Favre G, Mazieres J. Current and future applications of liquid biopsy in nonsmall cell lung cancer from early to advanced stages. Eur Respir Rev [Internet]. 2020 Mar 31 [cited 2022 Oct. 23];29(155). Available from: https://err.ersjournals.com/content/29/155/190052
4. Diaz LA, Bardelli A. Liquid Biopsies: Genotyping Circulating Tumor DNA. J Clin Oncol. 2014 Feb 20;32(6):579–86.
5. Alix-Panabières C, Schwarzenbach H, Pantel K. Circulating tumor cells and circulating tumor DNA. Annu Rev Med. 2012;63:199–215.
6. Tivey A, Church M, Rothwell D, Dive C, Cook N. Circulating tumour DNA — looking beyond the blood. Nat Rev Clin Oncol. 2022 Sep;19(9):600–12.
7. Offin M, Chabon JJ, Razavi P, Isbell JM, Rudin CM, Diehn M, et al. Capturing Genomic Evolution of Lung Cancers through Liquid Biopsy for Circulating Tumor DNA. J Oncol. 2017;2017:4517834.
8. Elazezy M, Joosse SA. Techniques of using circulating tumor DNA as a liquid biopsy component in cancer management. Comput Struct Biotechnol J. 2018 Oct 9;16:370–8.
9. Coccaro N, Tota G, Anelli L, Zagaria A, Specchia G, Albano F. Digital PCR: A Reliable Tool for Analyzing and Monitoring Hematologic Malignancies. Int J Mol Sci. 2020 Apr 29;21(9):3141.
10. Muller L, Hong CS, Stolz DB, Watkins SC, Whiteside TL. Isolation of Biologically-Active Exosomes from Human Plasma. J Immunol Methods. 2014 Sep;411:55–65.
11. Saarenheimo J, Eigeliene N, Andersen H, Tiirola M, Jekunen A. The Value of Liquid Biopsies for Guiding Therapy Decisions in Non-small Cell Lung Cancer. Front Oncol [Internet]. 2019 [cited 2022 Oct. 25];9. Available from: https://www.frontiersin.org/articles/10.3389/fonc.2019.00129
12. Eslami-S Z, Cortés-Hernández LE, Alix-Panabières C. Epithelial Cell Adhesion Molecule: An Anchor to Isolate Clinically Relevant Circulating Tumor Cells. Cells. 2020 Aug 5; 9(8): 1836.
13. Sundaresan TK, Sequist LV, Heymach JV, Riely GJ, Jänne PA, Koch WH, et al. Detection of T790M, the acquired resistance EGFR mutation, by tumor biopsy versus noninvasive blood-based analyses. Clin Cancer Res Off J Am Assoc Cancer Res. 2016 Mar 1;22(5):1103–10.
14. What Are the Risk Factors for Lung Cancer? | CDC [Internet]. 2022 [cited 2022 Oct. 23]. Available from: https://www.cdc.gov/cancer/lung/basic_info/risk_factors.htm
15. Ha SY, Choi SJ, Cho JH, Choi HJ, Lee J, Jung K, et al. Lung cancer in never-smoker Asian females is driven by oncogenic mutations, most often involving EGFR. Oncotarget. 2015 Feb 28;6(7):5465–74.
16. Kenfuni M. M., Gallouo M., alafifi mahmoud Tsikambu A. C. D., Alafifi R., Moataz A., Dakir M., Debbagh A., & Aboutaieb R. Pyonephrose: Risk factors, clinical, para-clinical and anatomopathological profile about 19 cases. Journal of Medical Research and Health Sciences. 2022; 5(2): 1770–1773.