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Full length Research Paper

Mutational Assay Using Liquid Biopsy in Metastatic Non Small Cell Lung Cancer by BEAMing Versus RT PCR Technique

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Abstract

Metastatic brain tumors may be the first radiological presentation of advanced lung cancer that may be suspected clinically mainly by optic disc edema. The most recent treatments for advanced non-small cell lung cancer (NSCLC) harboring activating EGFR gene mutations is epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) . Biobsy using bronchoscopy is the standard method to obtain tumor material for genetic assay to determine mutation for further prospection of new treatment by TK inhibitors. However repeated biopsy may carry the risk of bleeding. So it was mandatory to replace it by more accessible and safe sample which is the Liquid biopsy. Liquid biopsy is the cell- free tumor DNA (ct DNA) that is considered to be the best marker for diagnosis and follow up of genetic alteration in blood of patients with NSCL cancer. Different molecular methods could be used to assay mutation like RT PCR, sequencing and BEAMing Technique. Present study was to assess the correlation between serum level of ct DNA of patients with metastatic NSCLC with histopathological state of tumour and to assess immunohistochemistry and the BEAMing Technique in detection of EGFR mutation compared to RT PCR. Histopathology for 40 patients with advanced NSCLC & Blood samples, DNA extraction, RT PCR, immunohistochemistry and BEAMing of EGFR on both peripheral ct DNA and tumour cells. EGFR mutation was detected in 24/40 in tumour cells while 16/40 mutations was detected in ct DNA by RT PCR and immunohistochemistry. By BEAMing technique 24/40 EGFR mutation was detected in tumour cell biobsy while 23/40 mutation was detected in ct DNA. ct DNA carries genetic information from whole tumour genome could be used as a biomarker for diagnosis and follow up of mutational assay of EGFR in NSCLC.

Key words: *Liquid biopsy, NSCLC, BEAMing and RT-PCR.*

Introduction

Lung cancer is the most common cause of annual morbidity all over the world, causing about 1.2 million deaths annually. Approximately 85% of lung cancers can histologically be defined as non–small cell lung cancer. About 50% of NSCLC is diagnosed in their terminal stage and for the majority of these patients prognosis remains poor. About 20% of cases of non small lung cancer contain mutation in gene encoding EGFR and this determine the prognosis and response to treatment (1,2). Expression of tumor markers as CEA and CA19.9 may aid in cancer diagnosis but they are not specific for lung cancer. Circulating tumour Cells (CTC) are free cells which are liberated from tumour tissue and circulate freely in blood it could be isolated from blood and be used as a good target for genetic assay. But its technique of isolation is difficult because it depends on dimension of cells and the type of gene expressed on its surface (3).

Circulating Free Tumour DNA (ct DNAs) are Fragments of free DNAs that are released from lysis, necrosis and apoptosis of tumour tissue. Studies found that the amount of ct DNA is significantly higher in cancer patients than in healthy volunteers. Apoptotic and necrotic cells are cleared by infiltrating phagocytes and cell-free DNA levels are relatively low. However, this mechanism does not act effectively in the tumor mass. Free ct DNA is a short nucleic fragment 166bp (4). massive amount of

tumor DNA is released into blood stream during turnover or apoptosis where it can be isolated and analyzed. Therefore, the level of ct DNA in plasma is affected by tumour size, locality and vascularity (5) Advanced non small cell lung cancer that harbor EGFR gene mutation is charachterized by being very virulent because it can produce metastasis very rapidly and can grow easily. Its 1st clinical presentation is brain metastasis. There are three lines for the treatment of NSCLC which are: chemotherapy , immunotherapy and EGFR TK inhibitors. Epidermal growth factor Tyrosine kinase inhibitors is promising therapy for NSCLC. Mutation in EGFR encoding gene determine the therapeutic response to EGFR tyrosine kinase inhibitors (TKIs). TKI registered as the first line therapy for patients with EGFR mutated NSCLC because it is more tolerable than chemotherapy. But evaluation of genetic status of tumour before the start of treatment is mandatory (6). Cancer harbors somatic genetic mutations and these tumor-specific alterations can be detected in ct DNA. Different techniques are used to assay the EGFRTK mutation in NSCLC as RT PCR, Sequencing and genotyping. Recently detection of EGFR mutation is used for diagnosis and follow up of patient with advanced metastatic lung cancer(7).

Patients and method

The study was carried out on 40 patients who were diagnosed to have metastatic tumours in the brain secondary to primary tumour in their lungs. Those patients were found among 75 patients who were examined in ophthalmology Department for optic disc edema then they made MRI with contrast on brain and multiple metastatic lesions were found in 48 of them. Those reported radiologically to have metastatic brain lesion were further investigated for the primary lesion by Plain Chest x-ray A-P and lateral view and CT chest in most cases and by Mass Spectrometry and by U/S on neck for thyroid assay and also abdominal U/S. Forty cases were proven to have primary tumour in lung those were selected to our study.

Sampling

Each patient of 40 who had radiologic evidence of suspected bronchogenic mass were subjected to fiberoptic bronchoscopic biopsy from the accessible mass under either sedation with local anaesthesia or rigid bronchoscopy under general anaesthesia (selected if patient had hypoxemia paO2<80 mmHg or uncontrollable haemoptysis). The biopsied tissue were divided into 2 parts by aid of pathologist; First part for histopathological evaluation using haematoxylin and eosin staining sections to be graded, subtyped and immunohistochemical staining by EGFR monoclonal antibody (Mouse monoclonal antibody, DAKO). Immunoreactivity was regarded as positive if more than 1% of the tumour cells showed cytoplasmic and or membrane reactivities and 2nd part was used for genetic assay;. DNA was extracted (Qiagen,Germany). Tumour tissue from representative 5um thick section cut from formalin fixed and paraffin embeded blocks of each tumour cell contents were above 20%.Real Time PCR. Detection of EGFR mutations in ct DNA extracted from serum samples. Separated serum was stored at −80°C until use. Ct DNA was extracted and purified using a QIAamp Circulating Nucleic Acid Kits (Qiagen, Hilden, Germany) following the manufacturer's protocol and stored at −20°C until use. EGFR mutation status in ct DNA was analyzed using real-time PCR assay . The RT PCR technique is based on a chimeric DNA RNA DNA probe labeled with a fluorescent dye and quencher at each end. The RNA sequence of the probes corresponds to that of the wild-type and point mutations labeled with 5-carboxyfluorescein (FAM) and 6-carboxy-X-rhodamine (ROX), respectively. The primer sequences were as follows: forward primer: 5^0-GCACCACACCTTCTAC AATGA-3^0 and reverse primer: 5^0-TGTCACGCACGATTTCCC-3^0 for a 166-bp amplicon. When mutant molecules are present in the sample and PCR-amplified DNA generates a complete hybrid with the RNA portion of the mutant probe, RNase-H digests the probe at the RNA-DNA heteroduplex into two pieces, leading to a significant increase in fluorescence intensity by separation of the fluorescent dye from the quencher. The intensity of the wild-type probe served as an internal control for the assay. Sample DNA is amplified with a FAM-labeled primer set. PCR products are electrophoresed on a sequencer. When a deletion mutation is present, PCR amplifies the shorter segment of DNA, which creates a new peak in an electropherogram. The 2nd Tissue and serum samples were subjected to beads, emulsion, amplification and magnetics (BEAMing).DNA was extracted and amplified by PCR using primer sequence unique for EGFR TK gene .The amplified PCR product were conjugated with microbeads via streptvidin biotin for highly sensitive and specific purification of mutant DNA sequence via magnetic separation followed by flowcytometry then the concordance between circulating and tissue tumour DNA with mutational analysis were done. Analysis on tumour tissue was used in this study as a gold standard test. Ten healthy human volunteer with no history of and disease were subjected to quantification of free circulatory DNA by real Time PCR to compare between amount of cDNA in healthy and cancer patient.

Results

Table (1): Comparison between ct DNA detected by RT PCR and immunohistochemistry Versus BEAMing in both control and patient groups.

ct- DNA levels(ng/mL)	NSCLC by RT-PCR	NSCLC by BEAMing	Control (10)	p-value
Mean	141.0	159.1	68.7	
Median	118.3	130.0	76	
Std. Deviation	55.3	66.3	13.1	
Range	39.9-234.4	50.1-241.0	40.7-86.3	0.001*

It was found that median & mean value of ctDNA detected By BEAMing technique is significantly higher than ctDNA detected by RT PCR and immunohistochemistry .

*Patient group was significantly higher than in control group.***

Table (2): Relation between circulating tDNA measured by RT PCR and Histopathology of the tumor

Characteristics	No. (40)	Circulating tDNA levels by RT PCR (ng/mL)			
		Range	Mean	Median	P value
Histopathology					0.28
Adenocarcinoma	12	41.8 – 237.3	129.8	115.1	
Squamous cell carcinoma	28	56.1 – 235.3	174.2	124.2	

There is a significant correlation between level of Ct DNA by RT PCR and histopathology of tumour.

Table (3): Relation between circulating tDNA measured by RT PCR and stage of the tumor

Stage	**No.**	**Range**	**Mean**	**Median**	**P value**
III	**24**	**40.3-237.6**	**121.3**	**106.6**	**0.001***
IV	**16**	**115.7-235.8**	**175.1**	**194.5**	

The level of ct DNA increased in NSCLC staged IV more than staged III by histopathological evaluation.

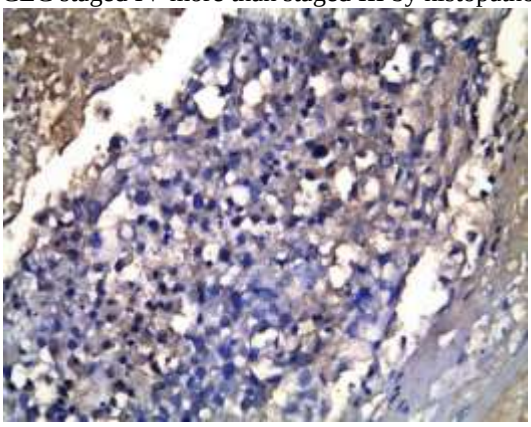

Fig 1: Squamous cell carcinoma showed positive EGFR expression [x400]

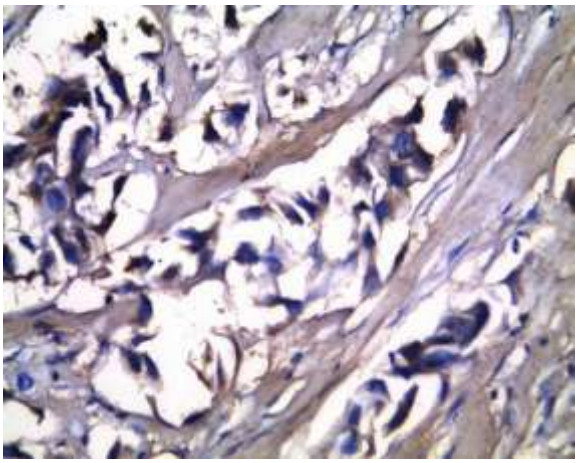

Fig 2: Lung adenocarcinoma showed positive EGFR expression [x400]

Discussion

Lung cancer is one of the most common causes of death worldwide. Bronchoscopy for diagnosis of lung cancer is an invasive measure. The development of a new non invasive method for diagnosis of EGFR mutated lung cancer is a challenge. Blood sample for EGFR mutation assessment is promising especially tumor samples are inaccessible. The aim of the present study was to assess the correlation between blood content of ct DNA of patients with metastatic NSCLC with histopathological state of tumour and to assess immunohistochemistry and the BEAMing technique in detection of EGFR mutation compared to RT PCR. Histopathology for 40 patients with advanced NSCLC & Blood samples,

DNA extraction, RT PCR, immunohistochemistry and BEAMing of EGFR on both peripheral ct DNA and tumour cells were done. This study was in accordance with Knneth etal., 2007 who found that the concordance between BEAMing and RT-PCR in

detecting EGFR mutation was 90% (8). Our result correlated with Detterbeck et al, 2009 who found that the highest sum of sensitivity 90%, specificity 86%, positive predictive value 90% and negative predictive value 90 %(9). Daniel SW et al .2016 found that the accordance between biopsy and Ct DNA was 61% while mutation was detected in 35%of patients of 2ry EGFR TK resistance (10). Our study was in difference with Kimura et al 2006& 2007 and Moran 2007, who found that detection rates for EGFR mutation in blood (Plasma & serum) of ct DNA was 31% (11&12&13). This may be due to difference in samples: plasma or serum and differences in DNA extraction techniques and mutation may be due to difference in patient clinical stages.

Conclusion

Plasma ct DNA is a promising alternative to biopsy for EGFR testing. Detectable mutation in blood was associated with more advanced disease and poorer prognosis. Afatinib improved outcomes in EGFR mutation-positive patients regardless of blood mutation status.

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