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Detection of clinically-relevant *EGFR* variations in *de novo* small cell lung carcinoma by droplet digital PCR

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Abstract

Targeted therapy that utilizes tyrosine kinase inhibitors (TKIs), specific to epidermal growth factor receptors (EGFR) has changed the landscape of treatment of non-small cell lung cancer (NSCLC). The success or failure of this approach depends on presence of certain variations in the tyrosine kinase domain of *EGFR* gene. Generally, patients diagnosed with small cell lung cancer (SCLC) are considered ineligible for TKI therapy owing to the absence of *EGFR* variations. However, there is evidence of these variations being detected in SCLCs, both in *de-novo* and in transformed SCLCs (TKI-treated adenocarcinomas). Despite the presence of clinically-relevant *EGFR* variations in SCLCs, the response to TKIs has been inconsistent. Liquid biopsy is a well-established approach in lung cancer management with proven diagnostic, prognostic and predictive applications. It relies on detection of circulating tumor-derived nucleic acids present in plasma of the patient. In this study, a liquid biopsy approach was utilized to screen 118 consecutive lung cancer patients for four clinically-relevant variations in *EGFR* gene, which included three activating/sensitizing variations (Ex18 G719S, Ex19del E746-A750 and Ex21 L858R) and one acquired/resistance (Ex20 T790M, *de novo*) variation by droplet digital PCR, the most advanced third generation PCR technique. As expected, clinically-relevant *EGFR* variations were found in majority of the non-small cell lung cancer cases. However, among the handful of small cell lung cancer samples screened, sensitizing variations (Ex18 G719S and Ex21 L858R) were seen in almost all of them. Interestingly, Ex20 T790M variation was not detected in any of the cases screened. The results of our study indicate that *EGFR* variations are present in SCLCs and highly sensitive liquid biopsy techniques like ddPCR can be effectively utilized for this purpose of screening *EGFR* variations in such samples.

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Introduction

Small cell lung cancer (SCLC) is a distinct subtype of lung cancer having an aggressive clinical manifestation with a five- year survival rate below 7%. It accounts for approximately 15% of total lung cancer incidences across the globe [1]. SCLC is known to have very short doubling time and often presents with wide spread metastases and endocrine paraneoplastic syndromes. Platinum-based chemotherapy along with radiation remains the mainstay of treatment while, immunotherapy has also been shown to be effective recently [2].

Advent of targeted therapy in the form of tyrosine kinase inhibitors (TKI) has proven to be a boon for the patients with lung cancer. However, this benefit is largely derived by those who have non-small cell lung cancer (NSCLC), most notably, adenocarcinoma of the lung. The driving factor behind the success of TKI therapy in NSCLC is the presence of activating variations in the epidermal growth factor receptor (*EGFR*) gene. Among the frequently observed *EGFR* variations that confer sensitivity to TKIs, small in-frame deletions in exon 19 and point mutations in exon 21 causing a leucine to arginine substitution at codon 858 (L858R) are the "hotspots", comprising about 85-90% of all such *EGFR* variations [3]. The remaining 10% come under the category of uncommon sensitizing variations, of which G719X, a point change at codon 719 that results in substitution of glycine with alanine (G719A), cysteine (G719C) and serine (G719), accounts for approximately 5% of the variations [4]. On the other hand, variations associated with TKI resistance are concentrated in exon 20, with a single nucleotide change at codon 790 resulting in substitution of tyrosine with methionine (T790M) accounting for nearly 50% of all such variations [3].

Initial reports revealing the presence of *EGFR* variations in SCLC came from those small cell lung cancers which were originally adenocarcinomas treated with TKIs. This well-known mechanism of histological transformation resulting in acquired resistance to TKIs is reported to happen in 4-14% of adenocarcinomas treated with EGFR-TKI [5-7]. Such SCLCs were thought to have retained original *EGFR* variation profile of the pre-treatment adenocarcinomas as revealed by the presence of identical variation profile in the same patient before and after transformation [8].

Subsequently, *de novo* SCLC carrying *EGFR* variations, similar to those seen in NSCLCs, have been reported, mostly from tissue biopsy specimens of the lung [9]. There are very few reports of these variations being detected in plasma by sequencing techniques [10]. However, there are no reports of such variations being detected in plasma by droplet digital PCR (ddPCR) technique, the most advanced third generation ultrasensitive PCR technique. We have previously reported that ddPCR can efficiently detect *EGFR* variations in lung cancer patient-derived plasma [11,12]. Here we are reporting a series of 6 cases of *de novo* SCLC harbouring common as well as rare activating *EGFR* variations in the plasma as detected by droplet digital PCR (ddPCR).

Materials and Methods

Sample collection

One hundred and eighteen consecutive subjects suspected to have lung malignancy based on clinico-radiological features and morphology of the lesion observed during bronchoscopy, were included in the study. Five ml of venous blood was collected in EDTA-coated vacutainers after obtaining informed consent. Plasma was separated within 2 h by centrifugation at 3500 rpm for 10 min. Subjects already under treatment for lung cancer and those who had prior history of malignancy of other organs were not included. Final diagnosis was established by histopathological examination and confirmed by immunohistochemistry (as per WHO classification of lung tumors, 4th ed., 2015) on case to case basis. The study protocol was approved by the Central Ethics Committee of the University.

Cell free DNA (cfDNA) extraction

cfDNA was extracted from plasma using Qia-Amp circulating nucleic acid kit, (Qiagen, Germany) as per the instructions provided by the manufacturer.

Droplet digital PCR assay

ddPCR analysis was done using QX 200 Droplet Digital PCR System (Bio-Rad, USA) as per the protocol for PrimePCR ddPCR Mutation Detection Assays, validated for both FAM and HEX-labelled probes. The assays are designed in such a way that the FAM probe binds to the variant allele whereas the HEX probe binds to the wild type allele. Four variations namely *EGFR* Exon 18 G719S, *EGFR* Exon 19 del E746-A750, *EGFR* Exon 20 T790M, and *EGFR* Exon 21 L858R, were screened using commercially available assays from Bio-Rad. The ddPCR protocol includes four steps. Step 1 is preparation of master mix containing the ready-to-use primer-probe mix with known amount of cfDNA (45-60 ng/sample). Step 2 involves loading the individual samples onto 8-well microfluidic cartridge and mixing with oil to generate the droplets. Step 3 is amplification of individual droplets in a thermocycler and step 4 is measuring the fluorescence of individual droplets in two channels (FAM and HEX) by the droplet reader at the end of the amplification. The results are expressed as number of variant copies / µl of blood. Based on the droplet count, a sample was considered positive for a particular variation when 6 or more FAM positive droplets (variant allele) were detected.

Results

Out of 118 subjects included in the cohort, 110 belonged to NSCLC category, 7 belonged to SCLC category and one subject was classified as undifferentiated carcinoma. ddPCR detected atleast one variant in 79 out of 110 (72%) cases of NSCLC and, in 6 out of 7 (86%) cases of SCLC. One case of SCLC did not show positivity for any of the three variants (*EGFR* Exon 18 G719S, *EGFR* Exon 19 del E746-A750 and *EGFR* Exon 20 T790M) it was screened for. The clinical details including treatment and outcome of these 6 cases of SCLC are presented in Table 1. Histopathological images from two cases are shown as Figures 1 and 2. Depending on the amount of the plasma available, each case underwent screening for a particular number of variations. Of the six cases, only one sample could be analysed for all the four variations. Out of these, SCLC 1, 2 and 5 showed the presence of *EGFR* Exon 21 L858R variation (Figure 3) whereas, SCLC 3, 4 and 6 were positive for *EGFR* Exon 18 G719S variation (Figure 4). The remaining two variations namely, *EGFR* Exon 19 del, EGFR Exon 20 T790M were checked in 3 (SCLC 1, 2 and 5) and 4 (SCLC 2, 3, 4, and 6) samples respectively and none of these samples showed the presence of these two variations. These details have been summarised in Table 2. Being a third generation PCR, ddPCR allows for the calculation of the absolute quantity of the variant DNA (variant load) present in the sample in terms of "number

of copies/µl of the sample". This was estimated for each of the target variation detected. The ratio of FAM positive droplets (variant allele) to HEX droplets (wild type allele) and the fractional abundance (FAM/FAM+HEX) of the variant copies were also estimated for each of the target variation detected. The results are shown in Table 3.

Discussion

The term "liquid biopsy" refers to the technique of detection of malignancy from blood or any other body fluid and is considered as the non-invasive alternative to the traditional biopsy. It relies on the detection and characterization of tumor derived substances, such as circulating tumor DNA (ctDNA), and circulating tumor cells (CTCs), which are present in the plasma or serum [13,14]. Detection of ctDNA continued to be a difficult task for many decades. This is owing to the fact that cancer-associated variations often evade detection due to their low concentrations relative to the background of wild type DNA in a given sample. However, the recent discovery of the droplet digital PCR (ddPCR) has overcome this difficulty and it is now being widely utilized for non-invasive detection of *EGFR* variations in ctDNA obtained from lung cancer subjects [15-18]. Droplet digital PCR works on the principle of "partitioning effect" where DNA is compartmentalized into water-oil emulsion droplets through microfluidics. These droplets are subsequently amplified individually to provide absolute quantification of the data and expression of values as copies per microlitre of the sample. Zhang *et al*. have reported that ddPCR approach reliably detected as low as 0.1 % variation rates compared to the traditional quantitative PCR methods, which could detect stably up to 1% variation rates [17]. Zhu *et al*., by using *EGFR* variation-positive cell DNA, have opti-

Table 1. Clinical characteristics of small cell lung cancer cases.

Subject code	Age	Gender	Smoking status	Stage at diagnosis	Treatment and outcome
SCLC 1	52	M	S	ED	Lost for follow up after 2 cycles of chemotherapy with cisplatinum and etoposide
SCLC 2	64	M	S	ED	Survived for 1 year after 6 cycles of chemotherapy with cisplatinum and etoposide and radiation therapy
SCLC 3	57	M	NS	ED	Survived for 6 months after 6 cycles of chemotherapy with cisplatinum and etoposide and radiation therapy
SCLC 4	60	M	S	ED	Lost for follow up after 1 cycle of chemotherapy with cisplatinum and etoposide
SCLC 5	58	M	S	ED	Died after 4 cycles of chemotherapy with cisplatinum and etoposide
SCLC 6	58	M	NS	ED	Lost for follow up after 6 cycles of chemotherapy with cisplatinum and etoposide

SCLC, small cell lung cancer; M, male; S, smoker; NS, non-smoker; ED, extensive disease.

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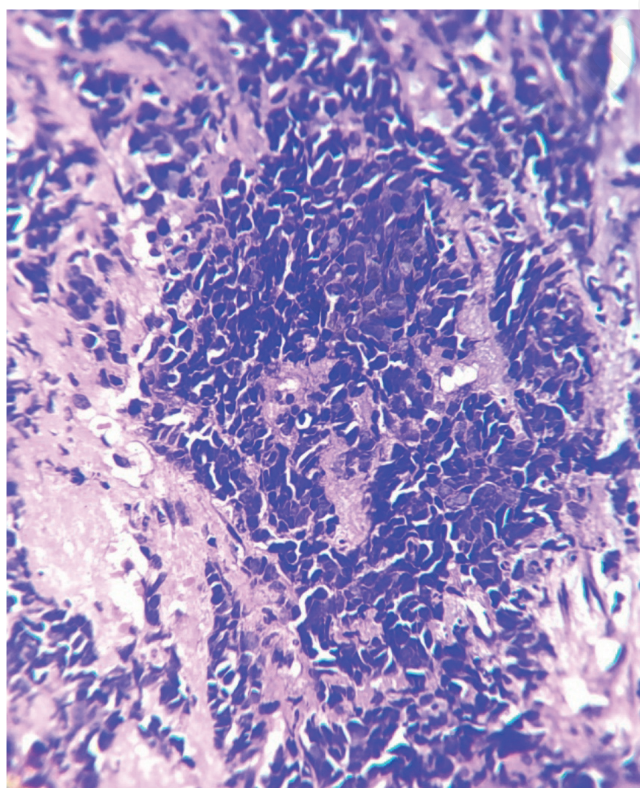

Figure 1. Histopathological image of lung biopsy from SCLC 2 showing features of small cell carcinoma of lung.

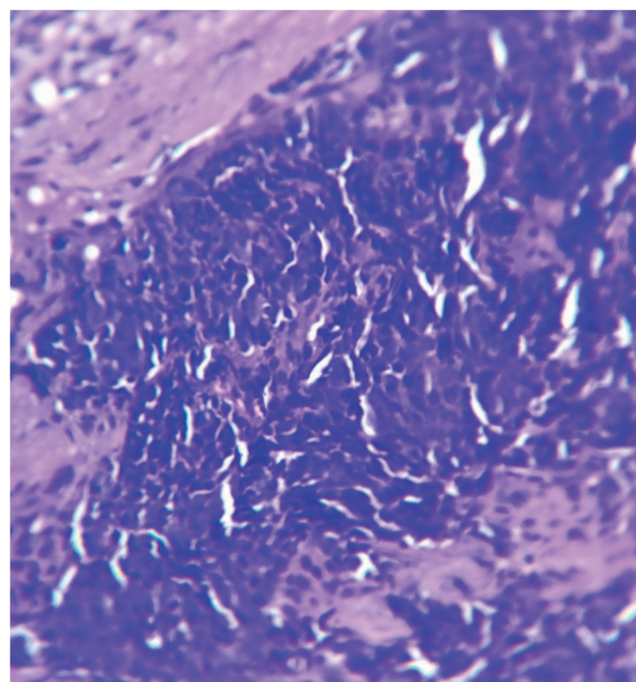

Figure 2. Histopathological image of lung biopsy from SCLC 4 showing features of small cell carcinoma of lung.

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mized the droplet digital PCR assays to reach 0.04% sensitivity [19].

The utility of ddPCR technique is most pronounced in situations where conventional tissue biopsy specimen has low tumor cell burden rendering it inadequate for determining biomarker variation profile by standard molecular methods. As described earlier, favourable response to TKIs depends largely upon the presence of activating *EGFR* variations in the tumor. This technique offers great advantage to the clinicians in real-time monitoring of TKI treatment response

Table 2. Case-wise *EGFR* hotspot variation profile.

Subjectcode	Activating variations			Resistance variation
	G719S	Exon 19E746-A750 del	L858R	T790M
SCLC 1	NS	-	+	NS
SCLC 2	-	-	+	-
SCLC 3	+	NS	NS	-
SCLC 4	+	NS	NS	-
SCLC 5	-	-	+	NS
SCLC 6	+	NS	-	-

SCLC, small cell lung cancer; NS, not screened.

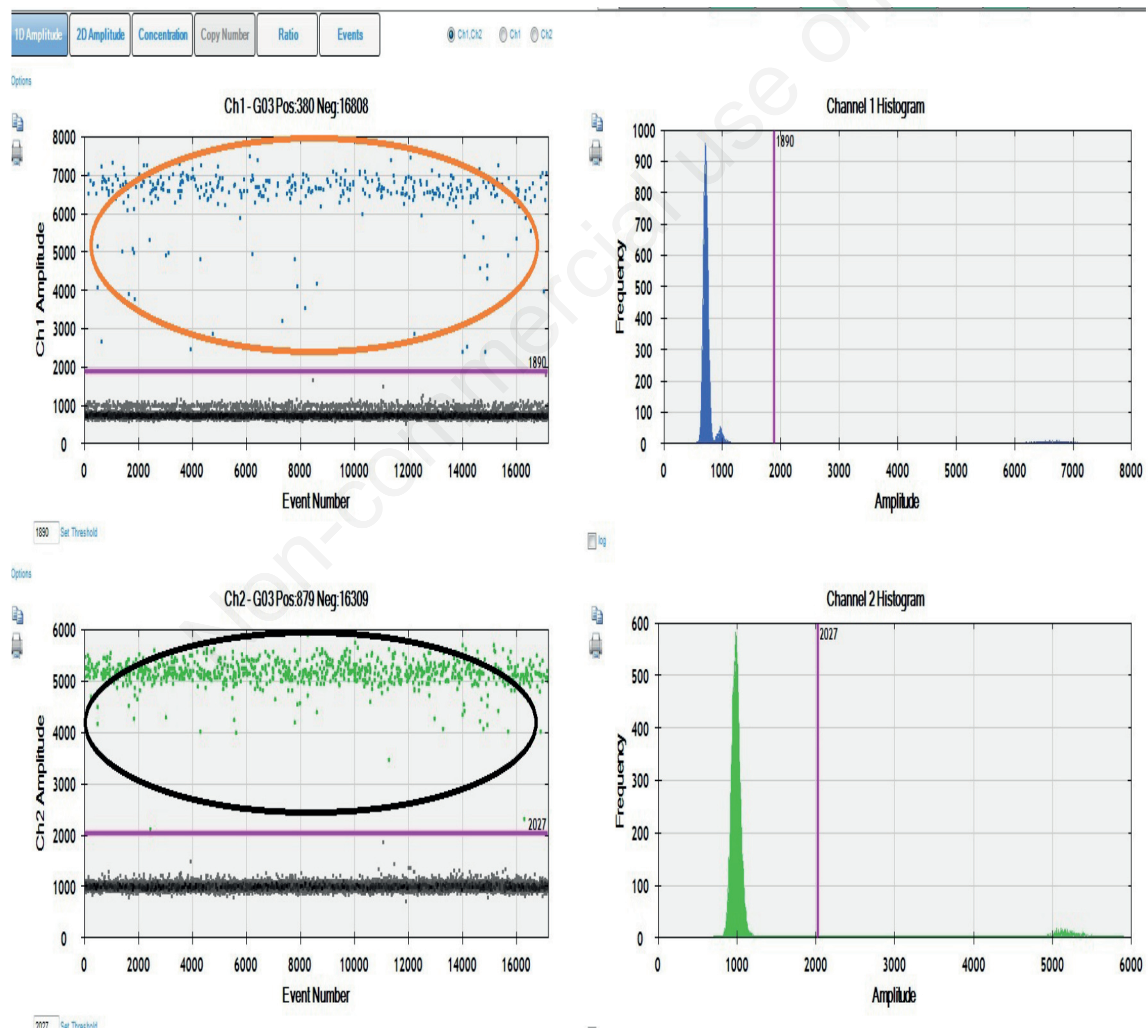

Figure 3. L858R variation assay by ddPCR for SCLC 1. Snapshot of 1D amplitude obtained from the QuantaLife software in QX200 ddPCR platform. The assay included a set of primers and two competitive probes, one labelled with FAM (for L858R variant allele, Channel 1) and another with HEX (for L858R wild type allele, Channel 2). The blue droplets (FAM positive-Orange circle) indicate the presence of mutant copies in the sample. Green droplets (HEX positive-black circle) indicate the wild-type copies. The black droplets below the threshold line (magenta) are the negative droplets having no DNA.

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as well as timely detection of emergence of therapeutic resistance (*EGFR* Exon 20 T790M variation), owing to its non-invasive nature as opposed to the traditional invasive tissue biopsy techniques, which have inherent shortcomings [20].

Incidence of *EGFR* variations in SCLC varies from 1.8% reported from Italian patients [21] to 4.65 % reported from Chinese patients [10]. A Japanese study has reported the incidence to be 4% [22]. Another recent study from a Chinese cohort reported 20% inci-

Table 3. Variant load, ratio (FAM/HEX) and fractional abundance (FAM/FAM+HEX) of variant and wild type droplets in plasma by ddPCR.

Subject code	Target variation	Variant load	Ratio	Fractional abundance (copies/µl)
SCLC 1	L858R	26.3	0.43	29.9
SCLC 2	L858R	2.2	0.13	11.2
SCLC 3	G719S	9.1	0.05	5
SCLC 4	G719S	0.8	0.01	0.8
SCLC 5	L858R	13.9	0.26	20.8
SCLC 6	G719S	20.9	0.10	8.8

SCLC, small cell lung cancer.

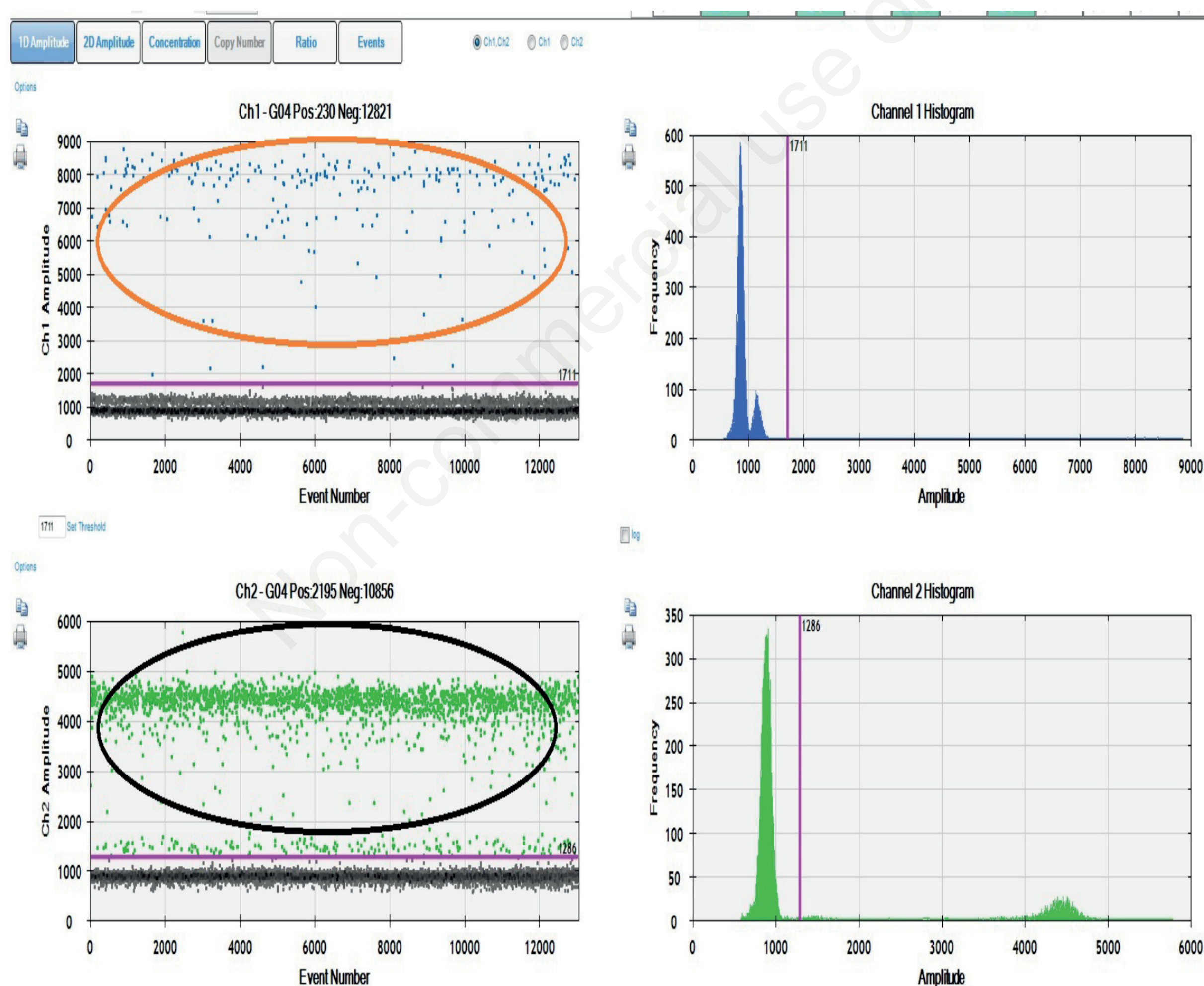

Figure 4. G719S variation assay by ddPCR for SCLC 6. Snapshot of 1D amplitude obtained from the QuantaLife software in QX200 ddPCR platform. The assay included a set of primers and two competitive probes, one labelled with FAM (for G719S variant allele, Channel 1) and another with HEX (for G719S wild type allele, Channel 2). The blue droplets (FAM positive-Orange circle) indicate the presence of mutant copies in the sample. Green droplets (HEX positive-back circle) indicate the wild-type copies. The black droplets below the threshold line (magenta) are the negative droplets having no DNA.

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dence, but it is not clear whether the cases were *de novo* SCLC or transformed SCLC [23]. In contrast, in this first communication from India, we report the presence of at least one hot spot *EGFR* variations *de novo* in 86% of the SCLCs screened. Though the small sample size (n=6) could be a factor contributing to such high percentage, we strongly believe that the effective incidence rate of *EGFR* variations in SCLCs in India maybe higher than available reports. In fact, in our unpublished data, we noticed a staggering 72% incidence of *EGFR* variations (presence of at least one hotspot variation) among the cases with NSCLC of the same cohort, which in itself is a higher number compared to the literature published so far. There have been two comprehensive studies on *EGFR* variations in Indian lung cancer patients, one in 2011 where the incidence was found to be 51.8% (24) and another in 2013, where it was estimated to be around 35% (25). These studies used ARMS-PCR and probe-based real time PCR respectively as techniques for variation detection and there was no mention on the lung cancer type of the samples used. Although our data indicates a much higher rate of incidence, we are of the opinion that the higher sensitivity (variation detection capability) of ddPCR is the main factor behind higher incidence noted in our cohort, across NSCLC and SCLC cases. Indeed, we have reported earlier about the presence of *EGFR* variations even in non-malignant lung pathologies [26].

A recent review of 59 reported cases of SCLC with EGFR variations by Seigele *et al*. [27] revealed a greater than 3:1 female: male predominance and a marked preponderance of never smokers over smokers, unlike the general findings in SCLC, where smokers predominate. Also, EGFR Exon 19 del and EGFR Exon 21 L858R were the most encountered variations. These are considered as common activating variations in *EGFR* gene. Another review of 67 cases by Marcoux et al. [7] also reported nearly similar findings. Notably, both these studies had included NSCLC-transformed SCLCs as well as *de novo* SCLCs in different proportions. In contrast, all 6 patients from our cohort were *de novo* SCLC and male smokers. The EGFR Exon 18 G719S and EGFR Exon 21 L858R were detected in 3 cases each and, EGFR Exon 19 del was not detected. It is to be noted that, EGFR Exon 18 G719S is a relatively uncommon activating variation [3]. Hence, it can be inferred that the cases in this cohort harboured common and uncommon variations in equal proportions.

Most of the reported cases of EGFR variations in SCLC have utilized biopsy specimen of the lung tissue for molecular analysis. There are very few reports where "liquid biopsy" approach was utilized to detect these variations. Although plasma-based approaches, followed by either quantitative estimation or by utilizing Next Generation Sequencing (NGS) were employed to check for EGFR variations in SCLC [10,28], so far there has been no report on use of ddPCR in detection of EGFR variations in SCLC and ours happens to be the first such report, to the best of our knowledge.

As far as treatment of SCLC harbouring EGFR variations with TKIs is considered, the results so far have been mixed. While some researchers have found favourable response [9,29-31], others have reported poor response to TKI [10,28,32]. The mechanisms accounting for lack of response include lack of phenotypic expression of EGFR variation as confirmed by immunohistochemistry [32]. In our cohort, we could do immunohistochemistry to check for EGFR protein expression in only one of the six patients, and it was found to be negative. Since overexpression of EGFR is usually associated with oncogenic transformation of EGFR gene, due to certain mutations, this may indicate that the EGFR variant detected by ddPCR did not lead to phenotypic expression of the EGFR at the protein level. Other reported mechanisms implicated in TKI-resistance include the presence of variations in the EGFR downstream signalling genes like KRAS, BRAF, PIK3CA and PTEN [5,28,33].

Conclusions

Traditionally, TKI therapy is considered a better alternative to conventional chemotherapy in NSCLC and it is generally accepted that EGFR variations, particularly those that are clinically relevant, are not present in SCLC. However, recent reports have strongly indicated that SCLCs also harbour these variations. Although it is rare, screening for EGFR variations in SCLC is worth exploring given the fact that the presence of such variations opens up the possibility of offering TKI therapy to these patients. Here we show the presence of clinically-relevant EGFR variations in a handful of SCLC cases that were encountered in our cohort. However, none of them were considered for a TKI therapy as the decision on the therapeutic regimen by the oncologist was purely based on the histological classification. It is not possible to predict whether these six cases would have responded to TKI, if they were considered for the same. However, the fact that despite a small number, we still found the presence of these clinically-relevant EGFR variations in these samples using an ultrasensitive technique highlights the need for screening SCLCs also for EGFR variations. In our cohort, there was an overwhelmingly large percentage of NSCLC cases, as expected for lung cancer. Thus, our observation on SCLCs could be an over representation. However, the results of our study strongly show that EGFR variations are present in SCLCs and highly sensitive liquid biopsy techniques like ddPCR can be effectively utilized for this purpose of screening EGFR variations in such samples.

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