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Case Report

Heterogeneous distribution of *EGFR* mutation in NSCLC: Case report

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ABSTRACT

Background: We herein report the case of a patient with advanced lung adenocarcinoma who presented a heterogeneous distribution of *EGFR* mutation.
Case report: A 74-year-old Moroccan male former smoker was diagnosed with advanced lung adenocarcinoma, harboring S768I exon 20 substitution mutation confirmed by Real Time PCR and Pyrosequencing, but not detected by direct sequencing despite 70% of tumor cells. The present report describes a case of minor histologic intratumoral heterogeneity with heterogeneous distribution of *EGFR* mutation.
Conclusion: Both sensitivity and specificity of molecular methods can provide evidence of intratumoral heterogeneity, which may explain the mismatch between the validation of oncology biomarkers and predicting therapeutic response to targeted therapy.

1. Background

Worldwide, lung cancer is the leading cause of cancer death. Over the past decade, the treatment of patients with advanced non-small cell lung cancer (NSCLC) has been revolutionized through use of epidermal growth factor receptor (*EGFR*) tyrosine kinase inhibitors (TKIs) [1]. Recent studies have shown a high intratumor heterogeneity of epidermal growth factor receptor mutations in NSCLC, which facilitates clonal evolution of tumor cells and determines a differential response to TKIs [2].

We herein report the case of a patient with advanced lung adenocarcinoma who presented a heterogeneous distribution of *EGFR* mutation.

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2. Case report

A 74-year-old Moroccan male former smoker was diagnosed with advanced lung adenocarcinoma harboring exon 20 S768I mutation. Microscopically, the cervical lymph node biopsy showed an acinar and papillary adenocarcinoma. Thyroid transcription factor-1 was positive in differentiated cancerous cells, while CK5/6 was negative. The immunohistochemical profile orientates towards a pulmonary primitive origin. Tumoral DNA was extracted from paraffin-embedded tumor tissue. The block with 70% of tumor cells was selected by a pathologist on hematoxylin-, Safran-, and eosin-stained slides. For DNA extraction, 4–7 sections of 5 μm thickness were cut from the selected tumor area. QIAamp DNA FFPE Tissue Kit (Invitrogen) was used for DNA isolation according to the manufacturer's instructions. The first *EGFR* testing was performed using Real Time PCR. The therascreen *EGFR* RGQ PCR Kit is designed to detect In-Vitro the most commonly reported *EGFR* mutations, G719A/S/C in exon 18, 19 deletions in exon 19, T790M, S768I, and 3 insertions in exon 20, and L858R, L861Q in exon 21, using Scorpions® and ARMS® technologies in real-time PCR ('ARMS test', Amplification Refractory Mutation System, Qiagen, Manchester, UK). The fully automated results interpretation revealed *EGFR* exon 20 S768I-positive substitution. To confirm mutations in samples with tumor cells > 25%, we routinely use SANGER sequencing. Mutation screening for exon 20 of the *EGFR* gene was performed using PCR reaction and 2X bidirectional direct sequencing with specific primers that cover the S768I substitution (Table 1) in a 25μl Mix reaction for exon 20. Direct sequencing of purified PCR product was performed using Terminator V3.1 Cycle Sequencing Kit (ABI Prism) and analyzed on Applied Biosystems 3500Dx Genetic Analyzer (Applied Biosystems). The electropherograms are usually reviewed manually to detect all genetic alterations. All mutations detected are confirmed by resequencing of independent PCR products. The patient was diagnosed with S768I-negative adenocarcinoma according to Sanger sequencing. The diagnosis was confirmed through the use of another molecular test; the same extracted DNA used in RT-PCR and Direct sequencing was re-analyzed for the presence or absence of the S768I substitution by Pyrosequencing, using the CE-IVDmarked therascreen *EGFR* Pyro Kit, on the Qiagen PyroMark Q24 device. Pyrosequencing revealed S768I positive sensitizing *EGFR* mutation and suggests that a few tumor cells carry the mutation (Fig. 1).

Finally, the patient was diagnosed with exon 20 S768I-positive lung adenocarcinoma according to Real Time PCR and Pyrosequencing.

3. Discussion

Several studies have reported that anti-*EGFR* agents have extremely high antitumor effects for advanced non-small cell lung carcinoma (NSCLC) patients with a sensitive epidermal growth factor receptor (*EGFR*) gene mutation [3–5], providing clear evidence that TK inhibitors are recommended only for EGFR mutation-positive NSCLC patients.So the detection of *EGFR* mutations is crucial for the selection of NSCLC patients who can benefit from TKI therapy. The present report describes a case of a patient with S768I exon 20 mutation confirmed by Real Time PCR and Pyrosequencing, but not detected by direct sequencing despite 70% of tumor cells. Until now, Direct DNA sequencing testing methods are still considered the standard for the detection of all *EGFR* mutations in NSCLC [6,7]. However, due to its relatively low sensitivity (which requires at least 20%–30% of tumor cells), time consumption, and complexity, another more sensitive easy, and simple methods have been recently developed [7,8]. In our laboratory, the first *EGFR* testing routinely performed is the real-time PCR-based assay that combines the amplified refractory mutation system (ARMS) with Scorpion probes (Scorpion-ARMS), this test is well known to have, a very high sensitivity (1%), eliminate the need for post-PCR confirmation by direct sequencing and has a potential ability to detect on FFPE specimens 29 known *EGFR* mutations, G719A/S/C in exon 18, 19 deletions in exon 19, T790M, S768I and 3 insertions in exon 20, and L858R, L861Q in exon 21. Pyrosequencing is real-time bioluminescence technology, based on direct sequencing-by-synthesis of short stretches of DNA fragments by a novel enzymatic cascade system. The tech-

Table 1
EGFR exon 20 primer sequences used for PCR

Primer name	Primer sequence
***EGFR*-ex-20- F**	5′-ATTCATGCGTCTTCACCTGGAA- 3′
***EGFR*-ex-20-R**	5′-CCCTATCTCCCTTCCCTGATTAC- 3′

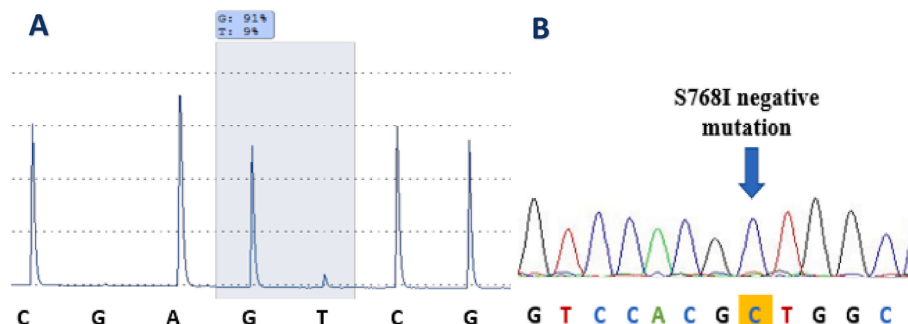

Fig. 1. The electropherogram traces of exon 20 S768I positive mutation by pyrosequencing (A) and confirmed negative by Sanger sequencing (reverse) (B)

nology ensures high accuracy and a sensitive method (5%–10%), which is also fast, simple, robust, and cost-effective [9,10]. The correlation between the percentage of tumor cells and the mutation rates is well addressed. A study by Angulo et al., compared three *EGFR* testing methods, direct sequencing, real-time PCR, and IHC. They concluded that the limit of detection (LOD) of the therascreen test (5%) is much lower than that of direct sequencing; which is reflected in the current case report. The S768I mutation was congruent between the Real Time PCR and Pyrosequencing, but regarding Sanger sequencing, despite 70% of tumor cells, the mutation was not detected. This discordance could be explained by two facts, the different method's LOD and biological sensitivity for analyzing *EGFR* mutations, and by the intratumoral histological and molecular heterogeneity within the same tumor tissue. The negative Sanger sequencing results suggest that only a few tumor cells displayed the S768I mutation.

Many recent studies have revealed that intra- and intertumoral genetic and epigenetic variation affects the cancer signaling pathways [11]. Intratumoral heterogeneity manifests as morphological and molecular variations within the same tumor specimen, caused by genetic instability [2,12]. The tumor microenvironment and the genetic disorders can contribute to genome instability by providing a pool of different mutation rates and types, allowing a genetically distinct subclonal population's evolution driving genetic diversity [13,14]. It is well known that lung adenocarcinoma has a high rate of morphological and molecular heterogeneity, suggesting a differential clinical benefit of anti-*EGFR* TKIs [15]. *EGFR* tyrosine kinase inhibitors have increased response rates, time to progression, and overall survival in *EGFR*-mutated metastatic NSCLCs patients [5]. Sadly, despite their early responsiveness to TKI therapy, almost all patients develop acquired resistance after a median of about 10–13 months. This resistance is explained by a heterogeneity of resistance mutations such as additional *EGFR* mutations (T790M), *PIK3CA* and *BRAF* mutations, *MET* and *ERBB2* amplifications and phenotypic changes including epithelial-mesenchymal transition and non small cell lung cancer to small cell cancer [16].

A minor histologic intratumoral heterogeneity is expressed when a single tumor showed at least 2 different growth patterns in just one histotype [12]. While the major heterogeneity is most often found in adenosquamous carcinoma ADSQ with different histotypes (2 at least) [12]. The present report describes a case of minor histologic intratumoral heterogeneity with heterogeneous distribution of *EGFR* mutation. Prior reports have demonstrated discordant *EGFR* mutation status in distinct sections of the tumor or between the primary and secondary metastatic site [17–20]. A very few cases of heterogeneous *EGFR* distribution are found in the literature. Yatabe et al. study have proved that the heterogeneous distribution of *EGFR* mutation is an extremely rare phenomenon in lung adenocarcinoma [21,22]. The heterogeneous distribution is expressed by the presence of a combination of mutated and wild-type *EGFR* among parts of a single tumor. This event proposes that *EGFR* mutations may acquire just part of the tumor and their remaining *EGFR* wild-type. Independent mechanisms for the partial acquisition of the *EGFR* mutation, mutant allele–specific imbalance (MASI), and *EGFR* amplification heterogeneous distribution might be possible explanations for this rare phenomenon [22]. The intratumor heterogeneity is considered a major obstacle in determining disease diagnosis, cancer progression, prognosis, and successful treatment options [21]. Recent studies have shown that the intratumoral heterogeneity of *EGFR* mutations is associated with the duration of treatment response to *EGFR*-TK: patients with a high rate of *EGFR* mutated allele demonstrate a better clinical response to (TKI) [6]. The challenge for the future is to determine the appropriate management for this substantial minority of cases in which it is hard to predict response to TKI.

4. Conclusion

All three mutation detection methods demonstrated a heterogeneous distribution of *EGFR* mutations in an individual tumor sample, which may not correctly predict response to TKIs. Considering the factors influencing LOD, the sensitivity and specificity of molecular methods can provide evidence of intratumoral heterogeneity, which may explain the mismatch between the validation of oncology biomarkers and predicting therapeutic response to targeted therapy.

Declaration of competing interest

All authors declare that there is no conflict of interest.

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