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Comparison of Standardized Approaches in Detecting *EGFR*, *KRAS* and *BRAF* Mutations

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Abstract

Background: *EGFR* tyrosine-kinase inhibitors have shown efficacy in non-small-cell lung cancer (NSCLC) with specific *EGFR* mutations. The impact of *KRAS* and *BRAF* mutations on therapeutic response remains under evaluation. The study aimed validating the use of mutation detection kits in a routine laboratory.

Methods: The mutation status of the *EGFR, KRAS* and *BRAF* genes, previously determined by Sanger sequencing, was analyzed with two approaches, pyrosequencing (Therascreen® Pyro® kits) and allele specific amplification (Cobas® mutation tests). A set of 70 DNAs from NSCLC tissue samples was selected and harboured 7 *EGFR*, 3 *KRAS* and 4 *BRAF* mutations.

Results: The Cobas® kit missed one *EGFR* and all *BRAF* mutations, and the Therascreen® kit missed one *KRAS* and 2 *BRAF* mutations. The Cobas® kit run in a one-step procedure, while the Therascreen® Pyro® kit included several manual steps, a plate's format change and a final analysis on a separate computer with specific software, allowing access to each experimental result. The Cobas® kit did not give the exact nature in case of mutation. Both kits have thus similar ability to detect mutations.

Conclusions: The Cobas® kit appears suitable for a high-throughput use in a medical laboratory but the synthetic final report presents a limit for full quality of the process. No kit presently integrates flexibility regarding the constant evolution in the set of mutations to be detected.

Keywords: Mutation detection; Accreditation; Targeted drugs

Introduction

Interest in individualizing patient treatment to maximize clinical benefit has become a focus of scientific investigation. During the past decade, *EGFR* tyrosine kinase inhibitor (*EGFR*-TKI) response has been largely studied and the mutation status of the *EGFR* gene has been shown to influence this response. The presence of so-called "activating" *EGFR* mutations, such deletions in exon 19 or L858R mutation in exon 21 as examples, induce *EGFR*-TKI sensitivity, while the acquired mutation T790M in exon 20 lead to secondarily resistance due to a loss of expression of the *PTEN* gene, which is a tumour suppressor gene controlling the PI3K/Akt pathway [1,2]. These binary responses do not appear related to the dosage of *EGFR*-TKI but slightly differ depending of the molecule used (erlotinib or gefitinib). Since 2008, the *EGFR* gene mutation status has been adopted as a prerequisite for *EGFR* TKI based-treatment of non-small-cell lung carcinoma (NSCLC). Somatic mutations activating oncogenes, such the *KRAS* and *BRAF* genes, were generally reported as associated with a lack of response to *EGFR*-TKI. The *EGFR* signaling pathway becomes constitutively active in those tumors, but the mechanism of *EGFR*-TKI resistance remains unclear and there are no sufficient arguments to implement *KRAS* or *BRAF* mutation detection as a consensual tool for therapeutic decision [3,4]. Commercial kits able to establish the *EGFR* mutation status in NSCLC and to predict a specific resistance to *EGFR* TKI progressively emerged and supplant sequencing approaches using the Sanger method. Also the strategy of drug development now moves towards the ability of specifically targeting the RAS pathway [5]. The requirements for the confidence of molecular pathology are high since the results are used to determine the eligibility of patients for treatment using a specific class of drug. Unreliable results might lead to over- or under treatment of patients, as only patients with tumors harboring sensitizing mutations benefit from *EGFR*-TKI. Missing such mutations means a loss of chance for patients who will not receive the drug. On the contrary, missing a resistance mutation will allow a wrong *EGFR*-TKI delivery. Since these drugs are expensive, the availability of safe tests also significantly improves the cost effectiveness of these new treatment modalities [6]. In view of their widespread use in clinical practice, molecular tests need to be both accurate and readily available. Over the world is there a growing trend towards complying with the international standards of ISO15189 for medical laboratories, that is mandatory in France [7]. Contrary to the USA, where in vitro diagnostic product (IVD)

regulation has been developed relating directly to the prescription of drugs, in Europe no regulatory framework exists on which assay(s) are eligible as biomarker of drug response. It is important to be aware that some real-time PCR-based methods do not distinguish between different mutations in the same codon. Likewise, in some kits, the validation studies limit the result to whether a sample is mutant or not. Some screening approaches are not able to detect some mutations such as Val600Lys in the *BRAF* gene for example. As previously reported, mutation spectrum largely differs from a tumor type to another within the same gene, and NSCLC seems to frequently bear complex mutations such in or out of frame indels [8].

Patients and Methods

Involved in somatic mutations detection in our region, PACA-West, the medical laboratory Alphabio evaluated the kits targeted on the *EGFR, KRAS* and *BRAF* mutations in the way of ISO15189 accreditation taking the Sanger sequencing approach as the standard of care. A series of 70 tumor DNAs has been selected from patients affected with NSCLC. All patients signed a written informed consent as a standard procedure for drugs delivery and our institutional review board approved the study. Samples were anonymized. DNAs were extracted from formalin fixed paraffin-embedded tissue specimens. Considering the diversity and heterogeneity of tumor tissue, pathology review and assessment of section quality was first performed to determine the percentage of neoplastic cell in the material to be analyzed. The mutation status on *KRAS* exon 2, *EGFR* exons 18 to 21 and *BRAF* exon 15 had been previously determined by systematic sequencing. They were 7 mutations involving the *EGFR* gene (exons 18 to 21), 3 *KRAS* exon 2 and 4 *BRAF* codon V600. The mutation detection was performed using both Cobas® mutation tests and Therascreen® Pyro® Kits generously provided by Roche Diagnostics (Mannheim, Germany) and Qiagen (Hilden, Germany) respectively.

Results

The kits Cobas® are able to detect 41 mutations at codons 719, 745-759, 768, 769-774, 790, 858 on the *EGFR* gene, 20 mutations at codons 12-13, 61 on the *KRAS* gene, and 4 mutations at codon 600 on the *BRAF* gene. The Therascreen® Pyro® Kits are specified to detect 28 mutations at codons 719, 745-753, 768, 790, 858, 861 of the EGFR gene, 4 and 7 mutations within the same regions in the *KRAS* and *BRAF* genes respectively, and an additional region within the *BRAF* gene including codons 464, 466 and 469. Comparative results are displayed in Table 1. No additional mutation was found in *EGFR, KRAS* or *BRAF*, which had not been detected by sequencing. *KRAS* analysis failed for 5 samples using one or both kits. One *EGFR* point mutation was missed with the kit Cobas®, one *KRAS* and two *BRAF* mutations using the kit Therascreen®. The kit Cobas® missed all *BRAF* mutations.

We then focused on the implementation of the technique. Concentrating on the *EGFR* gene, which mutation status is a prerequisite for therapeutic decision, we took into account the manual and automated steps, and the time of the whole experiments (Table 2). Cobas® analyses one sample for 41 mutations in 3 independent experiments, and Therascreen® 28 mutations in 5 experiments. The Cobas® approach is an automated single step procedure using 3 primer sets in a Cobas Z480 automat that returns final results. The Therascreen® approach is based on a mutation detection using a PyroMark® Q24. Dispatching of the DNA samples is followed by 4 steps, PCR amplification with 4 primer sets, immobilization, washing and dispatching in 24-well plates with 5 primer sets for sequencing then run on the PyroMark® Q24. Rough data have then to be analyzed using the PyroMark® Q24 application v2.0 software on a different computer and data are shown as graphs and ratio of the mutant versus normal profiles at each position screened.

Sample ID	Gene	Sequencing result	Mutation report	
			Cobas®	*Therascreen*®
491448	*EGFR*	c.2156G>C	EX18 G719X	c.2156G>C
491469	*EGFR*	c.2240_2254del	EX19 DEL	c.2240_?del
489432	*EGFR*	c.2573T>G	EX21 L858R	c.2753T>G
452470	*EGFR*	c.2239_2248delinsC	EX19 DEL	c.2239_?del
452978	*EGFR*	c.2573T>G	NM	c.2573T>G
452815	*EGFR*	c.2235_2249del	EX19 DEL	c.2235_?del
475409	*EGFR*	c.2237_2255delinsT	EX19 DEL	c.2236_?del
123146	*KRAS*	NM	na	NM
241138	*KRAS*	NM	na	na
241137	*KRAS*	NM	na	na
025119	*KRAS*	NM	NM	na
304209	*KRAS*	c.38G>A	na	c.38G>A
489482	*KRAS*	c.34_35delinsTT	M	c.35G>C
483271	*KRAS*	c.38_39delinsTT	M	NM

483336	*BRAF*	c.1798_1799delinsAA	NM	c.1799T>A
484361	*BRAF*	c.1799_1800delinsAA	NM	c.1798_1799delinsAA
462184	*BRAF*	c.1799_1802delinsAAAT	NM	NM
492659	*BRAF*	c.1801A>G	NM	NM
NM: No Mutation; M: Mutation; na: Not Amplified; fails and discordances are highlighted in bold characters. Results obtained using kits are mentioned as reported by the respective software. No mutation was detected in the other 52 samples using all 3 approaches.				

Table 1: Comparative analysis of somatic mutations detected using automated procedures.

Kit	pre-PCR	PCR	post PCR	Detection	Analysis
Cobas	15' (x3)	-	-	1 h 30' (x2)	-
Therascreen	15' (x4)	3 h (x2)	30' (x5)	20' (x10)	10' (x10)
Three and 4 distinct reactions for PCR amplification then 3 and 5 for sequencing were performed using the Cobas® and Therascreen® kits respectively. All steps, as much as possible, are performed in 96-well plates with the exception of detection with Therascreen® that were performed in 24-well plates and the Pyromark® Q24.					

Table 2: *EGFR* analysis on a series of 48 DNA samples

Discussion

In addition to mutation detection using Sanger sequencing, several approaches have been developed with the aim to detect mutations on targeted positions. They are mainly based on allele-specific (AS) amplification, pyro-sequencing, peptide nucleic acid (PNA) clamp PCR, amplification refractory mutation system (ARMS). AS, PNA and ARMS methods use synthetic oligonucleotides to reveal specific mutations. Pyro-sequencing is a less selective approach, as it detect any nucleotide incorporation at a given position. Commercial kits have been further developed, some for diagnostics purpose with a CE IVD label. All these approaches have been exploited to propose mutation detection kits for the *EGFR, KRAS* and *BRAF* genes in routine oncology practice. Kits generally allow detection of the most frequent mutations. Some also detect rare mutations. We select the Cobas® and Therascreen® Pyro® kits because they were theoretically able to detect all mutations found in our samples using Sanger sequencing and run on automats available in the laboratory. Both were CE IVD labeled. The kit Cobas® is based on AS detection, and the kit Therascreen® on pyro-sequencing. On 7 *EGFR*, 3 *KRAS* and 4 *BRAF* mutations, the Cobas® kit missed one *EGFR* and all *BRAF* mutation, and the Therascreen® kit missed one *KRAS* and 2 *BRAF* mutations. Thus the ability to detect mutations in all three genes appeared equivalent for both kits.

The use of Therascreen® kit and PyroMark® Q24 is obviously much more time consuming. This appears the critical limit of the Therascreen® kit for its high-throughput and reproducible use in hospital and medical laboratories. Automation of the whole procedure would be possible, but needs extra robots and software developments thus increasing significantly the cost of the analysis. The use of the Cobas® kit is more comfortable with this respect. Nevertheless, for a given sample, if any of the three experiments fails, the report is not validated. Furthermore the validation limits the result to whether a sample is mutant or not. Such methods are not to be preferred as only the detailed genotype will certify the full quality of the process and avoid any false positive.

To conclude, both kits present positive aspects, the flexibility in interpreting each independent experiment for the Therascreen®, and the simplicity of implementation for the Cobas®. The redhibitory points appear the complexity and duration for the Therascreen® and the lack of transparency for the Cobas®. For this latter, an interesting way would be a 2-steps process giving a synthetic result in case of high confidence data for all distinct experiments, and an access to the result of each experiment separately in case of partial failure, as Therascreen does. Both are limited by the constant evolution of the knowledge about somatic mutations playing a role in drug response [9]. A representative example is the detection of *BRAF* mutations, restricted to V600E with the Cobas® kit.

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