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Research Article

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# Concordant Results of Epidermal Growth Factor Receptor Mutation Detection by Real-Time Polymerase Chain Reaction and Ion Torrent Technology in Non-Small Cell Lung Cancer

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## Abstract

Nowaday screening of non-small cell lung cancer (NSCLC) patients for epidermal growth factor receptor (*EGFR)* activating mutations is carried out in routine diagnostics to select patients who could benefit from *EGFR* inhibitor therapies. We aimed to compare *EGFR* mutation testing by Ion Torrent PGM technology, using AmpliSeq Colon and Lung Cancer panel, with real-time PCR in order to evaluate the accuracy of next generation sequencing (NGS) in detecting clinically relevant *EGFR* mutations in NSCLC.

In total, 368 NSCLC patient samples were tested for *EGFR* by PCR and were also sequenced by Ion Torrent PGM by using AmpliSeq Colon and Lung panel. Samples were formalin-fixed, paraffin-embedded tumor specimens of Finnish NSCLC patients. The mutations studied for comparison were G719X, S768I, T790M, L858R, L861Q, deletions in exon 19 and insertions in exon 20.

Comparison of *EGFR* mutations detectable by both PCR kit and NGS panel, showed a high degree of concordance between the two methods. Out of 368 samples, 31 out of 32 positive by PCR were also positive by NGS, and 336 out of 336 negative by PCR for these mutations were also negative by NGS giving a concordance of 99.7%. Two negative samples by PCR showed insertions in exon 20, which were not detectable by PCR. In one sample NGS failed to detect G719X mutation that had a very weak signal in PCR.

Our study shows that the Ion Torrent PGM technology gives highly comparable results with the golden standard PCR. Thus, this NGS methodology is sensitive and reliable while testing clinically and diagnostically significant *EGFR* mutations in FFPE samples.

**Keywords:** Non-small cell lung cancer; Epidermal growth factor receptor; Mutation; Next generation sequencing; Ion Torrent; PCR

## Introduction

The differences in molecular features of tumors and their predictive role in the response to targeting treatment have become very evident in the last decade. The alterations can be divided into groups based on a type of study they are approached with: protein, RNA and DNA-based methods. The last is used to assess mutations in the genome. In 2004, activating *EGFR* mutations and their role in the response to *EGFR* tyrosine kinase inhibitors (TKI) were described [1-3]. Since then many driver mutations in multiple genes, such as in *ALK*, and *RAS* and *HER* family, have been described, but *EGFR* mutations are still among the most common biomarkers in non-small-cell lung cancer (NSCLC). Currently the US Food and Drug Administration (FDA) has approved three different targeted therapies, afatinib, erlotinib and gefitinib, for advanced NSCLC harboring activating mutations most importantly *EGFR* exon 19 deletions or exon 21 substitution L858R [4].

There are also some other *EGFR* mutations with a clinical interest, such as G719X, L861Q and S768I, which have been associated with a good or partial response to EGFR-TKIs [5,6]. However, the development of resistance to EGFR-TKIs is common in the course of treatment. For instance, the acquired *EGFR* mutation T790M or *MET* amplification can cause the insensitivity to EGFR-TKIs after the preliminary good response [7-9]. Also insertions in exon 20 involving the residues A767, S768, D770, P772 and H773 show lack of response when treated with gefitinib or erlotinib [5,10]. Thus, it would be important to test patients' genetic alterations with the multiplex and sensitive methods.

PCR-based mutation testing kits and Sanger sequencing have become as golden standard methods for diagnostic purposes. Next generation sequencing (NGS) methods are fast, economical, sensitive and multiplexable, and they are slowly replacing the traditional methods. However, before implementation of NGS in diagnostic settings, it is important to thoroughly test and compare results to those with standard routine diagnostic methods. Some previous studies have shown the Ion Torrent PGM system to be accurate in mutation analysis by using the Ion AmpliSeq Colon and Lung cancer Panel and/or the Ion AmpliSeq Colon and Lung Cancer Research Panel V2 compared with Sanger sequencing [11,12], the Ion AmpliSeq™ Cancer Panel compared with direct sequencing of peptide nucleic acid-locked nucleic acid polymerase chain reaction (PNA-LNA PCR) product [13] and custom panel of cancer genes compared with Sanger sequencing [14], although these have been performed on smaller sample sizes. In this study, we

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compared the *EGFR* mutation statuses of 368 Finnish NSCLC tumor specimens detected by the real-time PCR method and the targeted amplicon-based Ion Torrent NGS by using the Colon and Lung Cancer Panel performed on DNA extracted from formalin-fixed, paraffin-embedded (FFPE) tumor material in order to evaluate the accuracy of NGS in detecting clinically important *EGFR* mutations.

## Material and Methods

### Patients

In total, we collected retrospectively 566 FFPE tumor specimens obtained via either diagnosis or surgical procedures, of NSCLC patients treated at the Hospital District of Helsinki and Uusimaa (HUS), Finland, during 2006–2014. Of those, tumor DNA from 368 NSCLC specimens were studied for *EGFR* mutations by both the real-time PCR and the amplicon–based NGS using the Colon and Lung Cancer Panel with the Ion Torrent PGM sequencing technology. Tumor content and histological type of NSCLC were confirmed by pathologist. Tumor content ranged from 5 to 90%, in 90% of the samples, the content was at least 20%. Patient characteristics are presented in the table 1.

### DNA Extraction and Mutation Detection

DNA was isolated from FFPE sections using the QIAamp DNA Mini kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's instructions, with small modifications [15]. DNA concentration was assessed using the Qubit® 2.0 Fluorometer (Thermo Fisher Scientific).

### PCR Analysis

In our previous study, the tumor DNA samples of over 500 NSCLC patients were tested for *EGFR* mutations by using the Therascreen EGFR PCR Kit (Qiagen®, Manchester, UK) according to the manufacturer's protocol, on the ABI7500 platform or the cobas® EGFR Mutation Test (Roche Molecular Systems, South Branchburg, USA) according to the manufacturer's protocol, on the cobas® z480 platform [16]. Of those, 368 samples were studied also by Ion Torrent and in this study we compare the results.

| Feature | N (%) |
|---|---|
| **Histology** | |
| ADC | 265 (72) |
| SCC | 58 (16) |
| LCC | 28 (8) |
| ADSQ | 11 (3) |
| Other NSCLC | 6 (2) |
| **Gender** | |
| Male | 211 (57) |
| **Median age, years** | 66 |
| **Smoking[a]** | |
| Never-smoker | 31 (8) |
| Light ex-smoker | 29 (8) |
| Medium ex-smoker | 133 (36) |
| Current smoker | 170 (46) |
| NA | 5 (1) |

Abbreviations: ADC: Adenocarcinoma; LCC: Large Cell Carcinoma; SCC: Squamous Cell Carcinoma; ADSQ: Adenosquamous Carcinoma; NSCLC: Non-Small Cell Lung Cancer

[a]light ex-smoker: smoking <20 years, cessation >10 years ago; medium ex-smoker: smoking >20 years, ceased; and current smoker: current smokers, smoking >20 years

**Table 1:** The characteristics of the patients included into this study.

### Ion Torrent AmpliSeq Sequencing

Ten nanogram of DNA were used to prepare the barcoded libraries with the Ion AmpliSeq™ Library kit 2.0 (Thermo Fisher Scientific). The Colon and Lung Cancer panel covered 504 mutational hotspots and targeted regions (totaling 14.6 kb) in 22 genes, including *EGFR*. Template preparation and enrichment was performed with the Ion OneTouch™ 2 System (Thermo Fisher Scientific). Finally, sequencing was carried out using Ion 316™ chips on the Ion Personal Genome Machine System (PGM™, Thermo Fisher Scientific) and with the Ion PGM™ Sequencing 200 kit v2 (Thermo Fisher Scientific).

Alignment to the hg19 human reference genome and variant calling was performed by the Torrent Suite Software v.4.0.2 (Thermo Fisher Scientific) by the default threshold settings. For quality scores, coverage and strand biases those were a quality score of minimum of 6, relative read quality of minimum of 6.5, coverage of minimum of 6 for SNP/COSMIC variant and 15 for indel, and strand bias maximum of 95% for SNP/COSMIC variant and 90% for indel. Alignments were visually checked with the Integrative Genomics Viewer (IGV) (v.2.3.34, Broad Institute) [17].

## Results & Discussion

We compared the *EGFR* mutation detection in 368 NSCLC FFPE tumor samples between the real-time PCR method and the amplicon-based Ion Torrent PGM sequencing technology (Tables 2 and 3). The mutations were detected in 9% (34/368) of the patients. The observed mutation status pattern was highly comparable with the results from other studies as illustrated in our previous study by the standard PCR [16]. We detected a very high concordance between the results by the two methods used. Comparing only those mutations detectable by PCR in 368 samples, 31 out of 32 positive by PCR were also positive by NGS, and 336 out of 336 negative by PCR for these mutations were also negative by NGS giving a concordance of 99.7%. When taking the PCR results as true positive, the number of *EGFR* positive by NGS, were 31 (true positive = 31; false positive = 0) and negative 337 (true negative = 336; false negative = 1). The sensitivity and specificity of detecting clinically common mutations L858R, deletions in exon 19, insertions in exon 20, G719X, S768I by the Ion AmpliSeq Colon and Lung Cancer Panel as following: 96.9% and 100%, respectively.

Other studies carried on relatively smaller sample size have shown a good concordance between PCR and NGS mutation testing [15,18,19]. Our results from a larger cohort clearly indicate that clinically important *EGFR* mutations can be detected with high accuracy by amplicon based NGS and is suitable in diagnostics. Similarly, recent comparisons between Sanger sequencing and NGS have shown similar results, giving greater sensitivity to NGS [11, 12, 15, 18-20]. Studies comparing the Ion

| Mutation | Detected by PCR | Detected by NGS |
|---|---|---|
| L858R | 15 | 15 |
| Deletion in exon 19 | 12 | 12 |
| G719X | 4 | 3 |
| G719X & S768I | 1 | 1 |
| Insertion in exon 20 | 0 | 2[a] |
| Other COSMIC mutation[a] | 0 | 12 |
| No mutations | 336 | 322 |
| Total | 368 | 368 |

[a]Not detectable by Therascreen EGFR mutation testing.

**Table 2:** The comparison of EGFR mutation result between PCR and AmpliSeq colon and lung panel.

| S. No | Patient | Mutation by PCR | Mutation by NGS | Mutant read frequency, % (NGS) | Tumor content, % |
|---|---|---|---|---|---|
| 1 | 359 | L858R | L858R | 14 | 20 |
| 2 | 66 | L858R | L858R | 37 | 55 |
| 3 | 505 | L858R | L858R | 6 | 20 |
| 4 | 546 | L858R | L858R | 74 | 45 |
| 5 | 203 | L858R | L858R | 25 | 40 |
| 6 | 217 | L858R | L858R | 5 | 35 |
| 7 | 157 | L858R | L858R | 10 | 40 |
| 8 | 160 | L858R | L858R | 30 | 55 |
| 9 | 105 | L858R | L858R | 21 | 35 |
| 10 | 27 | L858R | L858R | 16 | 30 |
| 11 | 560 | L858R | L858R | 11 | 30 |
| 12 | 554 | L858R | L858R | 3 | 10 |
| 13 | 486 | L858R | L858R | 44 | 60 |
| 14 | 671 | L858R | L858R | 17 | 70 |
| 15 | 603 | L858R | L858R | 3 | 10 |
| 16 | 233 | Del Ex19 | Del Ex19 | 60 | 35 |
| 17 | 199 | Del Ex19 | Del Ex19 | 34 | 50 |
| 18 | 508 | Del Ex19 | Del Ex19 | 31 | 65 |
| 19 | 215 | Del Ex19 | Del Ex19 [a] | 10 | 30 |
| 20 | 52 | Del Ex19 | Del Ex19 | 10 | 35 |
| 21 | 211 | Del Ex19 | Del Ex19 | 55 | 70 |
| 22 | 70 | Del Ex19 | Del Ex19 | 33 | 45 |
| 23 | 631 | Del Ex19 | Del Ex19[b] | 28 | 55 |
| 24 | 653 | Del Ex19 | Del Ex19 | 12 | 15 |
| 25 | 587 | Del Ex19 | Del Ex19 | 26 | 60 |
| 26 | 646 | Del Ex19 | Del Ex19 | 54 | 70 |
| 27 | 632 | Del Ex19 | Del Ex19 | 18 | 10 |
| 28 | 239 | G719X | G719X | 42 | 55 |
| 29 | 107 | G719X | no mutation[c] | 0 | 70 |
| 30 | 234 | G719X | G719X | 37 | 50 |
| 31 | 688 | G719X | G719A | 37 | 30 |
| 32 | 672 | G719X; S768I | G719A; S768I | 12; 11 | 45 |
| 33 | 166 | No mutation[d] | D770_N771insSVD | 24 | 75 |
| 34 | 580 | No mutation[d] | D770_N771insSVD | 23 | 30 |
| 35 | 513 | No mutation | S720F; A767V | 3; 6 | 60 |
| 36 | 467 | No mutation | V774M | 8 | 45 |
| 37 | 350 | No mutation | S784F | 3 | 40 |
| 38 | 236 | No mutation | A864T | 2 | 70 |
| 39 | 34 | No mutation | A864T | 3 | 60 |
| 40 | 311 | No mutation | D770N | 3 | 15 |
| 41 | 521 | No mutation | P741L | 3 | 25 |
| 42 | 534 | No mutation | V765M | 2 | 70 |
| 43 | 344 | No mutation | A871T | 4 | 90 |
| 44 | 244 | No mutation | A871T; G874S | 4; 4 | 50 |
| 45 | 674 | No mutation | R776H | 3 | 40 |

[a]Missed by Ion Torrent Software, but seen clearly by visualization in 9.9% of the reads.
[b]Novel 2bp deletion followed by 10bp deletion.
[c]Detected by Therascreen, but is uncertain with weak signal.
[d]Not detectable by Therascreen EGFR mutation testing kit.

**Table 3:** The patients with EGFR mutations detected by either method.

Torrent technology are also in concordance with this study, suggesting it to be eligible method in diagnostics [11,12,19].

The comparison of the two methods showed discrepant results in two samples. One patient with *EGFR* deletion by PCR, was missed by the Ion Torrent software (probably filtered out by the variant calling setting), although it was clearly seen in 10% of reads with IGV. Another patient with G719X mutation in PCR was clearly negative for this mutation by NGS even though the read depth at this location was 2204x. Since, this sample had a very low concentration in PCR (Ct 38.97 and dCt 11.70) and was flagged as doubtful; it is not certain whether it is false positive in PCR or false negative in NGS.

One important parameter of considerable significance in detecting mutations in tumor samples by NGS is deciding appropriate cut-off for mutation allele frequency, so as it maximizes sensitivity and minimizes

false positive rate. Since the proportion of tumor cells in tumor samples can vary greatly among different samples, it can greatly affect the mutant allele proportion. We therefore also analyzed the tumor content of sample, as estimated histologically, in relation to percent of mutant allele among total reads. Overall, the percentage of mutant reads ranged between 3 and 75% (average 28%) for all *EGFR* mutation. In this study, we could successfully detect *EGFR* mutations in samples with tumor content of 10% that had mutant reads percentage ranging between 3 and 18. At a cut-off of 3% for reads with mutant allele, we could detect all clinically relevant mutations without any false positives. This suggests NGS to be a sensitive method and can detect mutant alleles as low as 3% of the reads without false positives. Previous study has reported the limit of detection 1.3% or less for the Ion Torrent AmpliSeq panel [20]. Percentage of mutant reads was positively co-related to tumor percentage, though not very strongly (Pearson's correlation R=0.5). At least five samples had mutant reads more than 50% that could indicate a probable amplification of the mutant *EGFR* allele.

In addition to *EGFR* mutations that could be detected by both PCR and NGS, 12 patients had other COSMIC mutations detected by NGS, but not seen by PCR as they are not included in the PCR panel. This is a great advantage of NGS to detect all kind of mutations and distinguish them. By NGS, for instance, exon 19 deletions and exon 20 insertions are detectable in more detail, as PCR cannot distinguish them. By NGS we were able to identify two samples with exon 20 insertions (p.D770_N771insSVD) which were not detected by PCR, because it is not included in the PCR panel (Figure 1). Mutant reads were around 20% in both these cases by NGS. Similarly, we detected novel 2bp deletion in patient 631, which is not described previously. Although, this deletion was detected by PCR it did not identify it (since it only detects deletions without characterizing them). Moreover, we could clearly identify different variations of exon 19 deletion with NGS (Figure 2).

Another strength of the amplicon based Ion Torrent NGS is the sufficiency of small amount of starting material (ten nanogram of DNA), and its applicability to FFPE specimens. Moreover, Ion Torrent NGS workflow is user-friendly and fast.

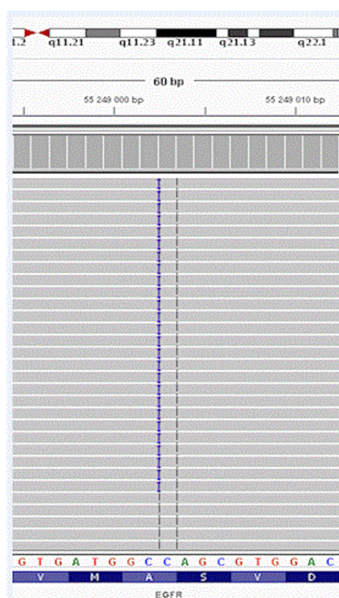


**Figure 1:** IGV visualization of insertion P.D770_N771insSVD detected by NGS, but not by PCR.

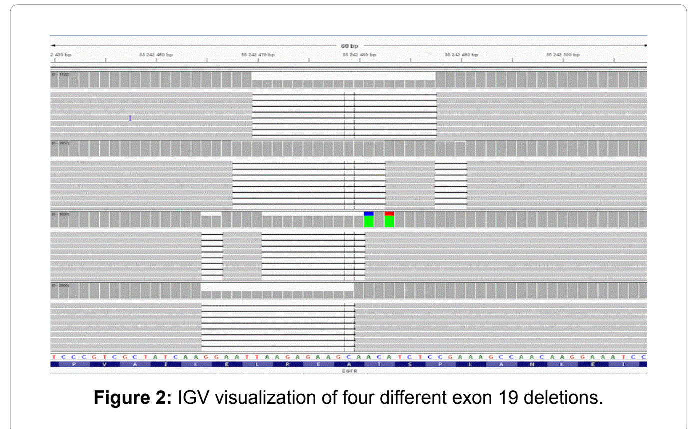

**Figure 2:** IGV visualization of four different exon 19 deletions.

## Conclusion

As conclusion, this study shows that the Ion Torrent technology gives highly comparable results with the golden standard PCR. Thus, this NGS methodology is sensitive and reliable while testing clinically and diagnostically important *EGFR* mutations from FFPE samples.

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