Analysis of PIK3CA mutations in tumours from patients with non-small cell lung cancer using pyrosequencing

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Abstract

A subgroup of non-small cell lung cancer (NSCLC) cases harbour mutations in classical oncogenes, which can affect therapy response and prognosis. By therapeutically targeting the corresponding proteins with inhibitory drugs, the clinical outcome for these lung cancer patients may be improved. One of these oncogenes is the phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA) which encodes the catalytic subunit of the phosphatidylinositol 3 kinase (PI3K). PIK3CA is a central regulator in the PI3K/Akt/mTOR pathway, which controls cell growth and apoptosis. Mutations in the PIK3CA gene are considered to up-regulate the kinase activity in tumour cells and through that dysregulate fundamental cellular processes. PI3K inhibitors are currently tested in clinical trials and present a promising therapy option in lung cancer patients. In this study, a pyrosequencing assay for detection of PIK3CA mutations in tumours from patients with NSCLC was established. The three “hot-spot” codons 542, 545 and 1047 of the PIK3CA gene were analysed. The sensitivity of this assay was determined to the presence of 5 % of mutant alleles. In agreement with previous reports, three of the 60 lung cancer cases revealed PIK3CA mutations (5 %). All mutations occurred in exon 9 codon 542 or 545. In line with previous reports, two of the three samples harboured concurrent mutation in the EGFR or KRAS gene. The established pyrosequencing analysis for PI3KCA mutations provides a reliable and cost-effective assay for clinical diagnostics. The determination of the PI3KCA mutation status may help to distinguish patients for treatment targeting the PI3K pathway.

Keywords: PI3K, tumour marker, pseudogene, oncogene, targeted therapy
Introduction

Every year, around 1.59 million people worldwide die from lung cancer, which makes it the most lethal form of cancer.¹ Lung cancer is histologically divided into two groups: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC accounts for more than 80 % of lung cancer cases and can be further classified into three subcategories - adenocarcinoma, squamous-cell carcinoma and large cell carcinoma. The overall survival is poor with only 15 % five years after diagnosis [1, 2]. Surgery is considered to be the only curative treatment, however most lung cancer cases are diagnosed in late stages where only chemotheraphy is the treatment of choice².

Epidermal growth factor receptor (EGFR) and Kirsten rat sarcoma viral oncogene homolog (KRAS) are two genes that may be mutated in NSCLC. Both encoded proteins are included in several pathways within the cell such as STAT-pathway and PI3K/Akt/mTOR pathway, which regulate cell survival and proliferation. Mutations in EGFR and KRAS are known as “driver-mutations” which means that they can induce cancer. Patients with mutated EGFR are generally susceptible for EGFR inhibitors, for example gefitinib and erlotinib, which are proteins that blocks the kinase activity of EGFR. Since the development of these drugs, the clinical outcome regarding patients with mutated EGFR has dramatically improved. KRAS, a protein in the PI3K/Akt/mTOR pathway, is located downstream of EGFR. The EGFR-inhibitory drugs are considered having little effect on patients with mutated KRAS. Unfortunately, to this date, there are no available drugs directly targeting KRAS [3].

Phosphatidylinositol-4, 5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) is a gene that is mutated in various types of cancer including colon-, breast- and lung cancer. PIK3CA encodes for a protein, p110α, which is the catalytic subunit of the lipid kinase enzyme phosphatidylinositol 3-kinase (PI3K) class 1A. PI3K class 1A enzymes are involved in the PI3K/Akt/mTOR pathway and regulate processes such as cell proliferation, differentiation and survival [4]. Previous studies have shown that mutations in PIK3CA could interrupt the regulation of aforementioned processes in the cell, which can lead to tumour growth. Normally, PI3K class 1A phosphorylates the substrate PIP₂ to PIP₃ and the enzymatic antagonist phosphatase and tensin homolog (PTEN) counteracts PI3K by dephosphorylating PIP₃ [5]. The frequency of PIK3CA mutations varies in different types of tumours. In tumours for example in breast- and colon cancer, the mutation frequency can reach 26 %, in ovarian cancer 10 % [6], whilst the frequency for NSCLC is approximately 4 % [7, 8].

More than 80 % of the somatic mutations in the PIK3CA gene occur in three “hot-spots”-regions – codon 542 and 545 in exon 9 and codon 1047 in exon 20 [5, 8]. In this study, these “hot-spots” has been analysed in tumours from patients with NSCLC in order to find possible mutations in the PIK3CA gene.

Mutations in PIK3CA are predicted to function as therapy markers in the future. To this date, there are several clinical trials at different stages investigating potential drugs targeting PIK3CA and PI3K, for example NCI trial nr: 01219699, 01708161 and 01613950. These ongoing clinical trials are investigating the effect of the novel drugs and which group of patients that will benefit the most from them. However, the complexity of the PI3K/Akt/mTOR pathway and the interaction with other pathways makes the development of new drugs challenging [9]. Consequently, molecular analyses are an important part regarding the treatment of NSCLC. With up-coming drugs targeting several enzymes in the

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3 Clinical Trials: http://www.clinicaltrials.gov
PI3K/Akt/mTOR pathway, the assortment of molecular analyses must match the future demands.

An obstacle for the molecular analysis of the PIK3CA gene is a pseudogene with 95% homology [10]. In the human genome, there are several thousand of these pseudogenes. They have no apparent function and due to the lack of promoter, most of the pseudogenes are not transcribed like normal genes [11]. To avoid false detection of the pseudogene, an optimized primer design established in a previous study [12] has been applied.

Pyrosequencing is a sequencing-by-synthesis method that exploits a natural phenomenon, which occurs during DNA replication. When DNA polymerase incorporates a nucleotide in the DNA chain and a phosphodiester bond is formed, a release of pyrophosphate from the nucleotide is generated. By adding a range of enzymes and substrates to the reaction, the pyrophosphate is converted into ATP by the enzyme ATP sulfurylase. The ATP then oxidizes the substrate luciferin with the help of the enzyme luciferase. A light signal is emitted and projected as a peak in a pyrogram. The light signal is proportional to the number of nucleotides incorporated, for example two nucleotides in a row will generate a double peak. By adding one nucleotide at a time, the exact sequence will be determined. The enzyme apyrase will clear the reaction from remaining nucleotides before the next nucleotide in the dispensation order is added [13, 14].

Before the pyrosequencing reaction can take place, amplification of DNA is required. To obtain single strand DNA, which is needed for the sequencing, the primers used in the amplification step are biotinylated. The amplified DNA strands with biotin attached are then blended with streptavidin-coated beads. Using the beads, single strand DNA is purified and mixed with amplification primer, before initialising pyrosequencing [15].
The aim of this study was to set up a new pyrosequencing assay for detection of mutations in PIK3CA and screen a cohort of NSCLC patients. Such an assay can be directly applied in clinical diagnostic analysis and distinguish NSCLC patients for targeted therapy.

**Materials and methods**

**Optimization**

Since the pyrosequencing assay for PIK3CA is not currently in the clinical routine, an optimization was needed to evaluate the assay before analysing the NSCLC samples. A previous research project regarding PIK3CA in colon cancer was executed a few years ago. Mutation positive samples from the colon cancer project were used to verify this assay, together with a wild type and a negative control. Primers, PCR conditions and pyrosequencing assays from a previous published study [12] were the basis for the set up of this project. Modifications such as reagents for the PCR reaction, MgCl$_2$ concentration (evaluation of 1.5 mM and 3 mM) and dispensation order for the assay were performed to obtain the best conditions.

After the PCR reaction, the samples were analysed with gel electrophoresis to assure PCR products with correct size before performing pyrosequencing. For the gel electrophoresis, 5 µl of each sample was combined with 1 µl of 6 X Loading Dye (R0611, Thermo Scientific). The samples and GeneRuler Low Range DNA Ladder (Thermo Scientific, Waltham, USA) were applied to a 2 % agarose gel with 1 X TAE-buffer, stained with SYBR® Safe (Invitrogen by Life Technologies, Waltham, USA). In a cast with 1 X TAE-buffer, the gel was run at 150 V for 25 minutes. Then the gel was photographed using Gel Doc™ XR+ System (Biorad, Hercules, CA, USA).
**Samples**

During years 1995 to 2005, NSCLC tumour samples were collected and organized into three different cohorts used for research – a cohort consisting of fresh frozen tissue (n=196), a cohort with formalin-fixed paraffin embedded (FFPE) tissue (n=355) and a cohort consisting of both fresh-frozen and FFPE-tissue (n=189). Mutation analysis of EGFR, KRAS and other oncogenes were performed. In this study, samples from the fresh frozen cohort with DNA that had previously been extracted were analysed. 60 samples were randomly chosen and all samples were used in agreement with Swedish Bio bank Legislation (#2006/235) and approved by the local ethics committee. The molecular analyses used in this study did not cause unnecessary suffering for the patients, who would nevertheless have their tumours removed. The DNA concentrations of the samples were measured spectrophotometrically using NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, USA) and were then diluted with Milli-Q water to a final DNA concentration of 10 ng/µl.

**PCR**

The exons of interest in the PIK3CA gene were amplified with PCR using GeneAmpPCR-system 9700 (Applied Biosystems, Foster City, CA, USA), Platinum® Taq DNA Polymerase Kit (Invitrogen by Life Technologies, Waltham, USA) and the primers presented in Table 1. For each sample, the PCR tube contained 1 X PCR Buffer minus MgCl₂, 1 X Platinum® Taq DNA Polymerase, 1 mM of dNTPs, 0.4 μmol/L of the primers, 3 mM MgCl₂ and 10 ng DNA. PCR conditions were performed as follows: denaturation for 15 minutes at 95°C, then 45 cycles of denaturation for 20 seconds at 95°C, annealing at 60°C for 20 seconds and elongation at 72°C for 20 seconds and subsequently 10 minutes of elongation. Samples were analysed with a negative control (Milli-Q water) and a wild type control MNC (mono nuclear cells from healthy individuals).
Table 1. Amplification primers used in the PCR reaction.

<table>
<thead>
<tr>
<th>Position</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 9 forward primer</td>
<td>5´-Biotin-ATTTCTACAGGCTCTCTCTCT-3´</td>
</tr>
<tr>
<td>Exon 9 reverse primer</td>
<td>5´-CCATTTTAGCAGTTACCTGTGAC-3´</td>
</tr>
<tr>
<td>Exon 20 forward primer</td>
<td>5´-TTCTCTGCTCAGTGAT-3´</td>
</tr>
<tr>
<td>Exon 20 reverse primer</td>
<td>5´-Biotin-TGCTGTATTGTGGAAGATC-3´</td>
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Pyrosequencing

The samples were then analysed for mutations in the PIK3CA gene using the pyrosequencing method. For the pyrosequencing reaction, 18 µl of each PCR sample were used. A mix with streptavidin coated Sepharose-beads (GE Healthcare, Little Chalfont, UK) PyroMark Binding Buffer (Qiagen, Hilden, Germany) and Milli-Q water was prepared and blended with the PCR products on a plate vortex for at least 5 minutes. A mix for each sequencing primer (Table 2) containing 0.3 µmol/L of the primer and PyroMark Annealing Buffer (Qiagen, Hilden, Germany) was distributed onto a PyroMark Q24 Plate (Qiagen, Hilden, Germany). Using the PyroMark Q24 Vacuum Workstation (Qiagen, Hilden, Germany), the PCR-products in the Sepharose mix were denaturated, washed and mixed with the sequencing primers and annealing buffer on the PyroMark Q24 Plate. The plate was then incubated in 2 minutes in 80°C followed by a minimum of 10 minutes in room temperature. Together with the plate, a PyroMark Q24 Cartridge (Qiagen, Hilden, Germany) filled with dNTPs, enzymes and substrate mixture were inserted in the PyroMark Q24 Instrument. Pyrosequencing data was analysed using the software PyroMark Q24 2.0.6. In the case of a mutation, the sample underwent an additional PCR and pyrosequencing analysis to confirm the mutation.

Table 2. Sequencing primers used in the pyrosequencing reaction.

<table>
<thead>
<tr>
<th>Position</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>Codon 542 (exon 9)</td>
<td>5´-TTCTCTGCTCAGTGAT-3´</td>
</tr>
<tr>
<td>Codon 545 (exon 9)</td>
<td>5´-TAGAAATCTTTCTCTG-3´</td>
</tr>
<tr>
<td>Codon 1047 (exon 20)</td>
<td>5´-GAAACAAATGAATGATGC-3´</td>
</tr>
</tbody>
</table>
**Sensitivity**

The limit of detection for this assay was determined using a positive sample from the NSCLC cohort. The tumour cell content of the sample was determined by a pathologist (PM) who analysed the corresponding HE-slide. After determining the tumour cell content, the extracted DNA from the positive sample was then serially diluted 1:2 in six steps with DNA from another patient sample containing non-mutated tumour cells. Pyrosequencing was then performed as previously described.

**Results**

**Optimization**

Before analysis of the NSCLC tumours could begin, optimization of both the PCR and pyrosequencing reactions was needed in order to obtain the best result possible. The results from the PCR reaction when optimizing reagents and MgCl$_2$ concentration, shows slightly stronger bands for the PCR products with 3 mM MgCl$_2$ (Figure 1).

![Figure 1](image1.png)

**Figure 1.** Gel electrophoresis with PCR products containing different MgCl$_2$ concentration. Lane 1 contains a size ladder with base pairs marked to the left. Lane 2 represents exon 9 with 1.5 mM MgCl$_2$, lane 3 exon 20 with 1.5 mM MgCl$_2$ and lane 4 is negative control. Lane 5 represents exon 9 with 3 mM MgCl$_2$, lane 6 exon 20 with 3 mM MgCl$_2$ and lane 7 is negative control.

In the study by Baker *et al* [12], the exon 9 forward primer is biotinylated, later to be selected in the following pyrosequencing pre-treatment step. This means that it is the coding strand
that will anneal with a sequencing primer and DNA-polymerase will start building the sequence of the non-coding strand, which is projected as peaks in the pyrogram. Since the sequencing primers will bind like reverse primers, the codons are sequenced in reverse direction. When interpreting the pyrogram, the codons, which are circled in red, are read from right to left (3´→5´) and translated to the corresponding nucleotides.

During the optimization, programming the pyrosequencing assays in the software PyroMark Q24 2.0.6, was a key event. In order to program the assays, the wild type sequence of the codons must be known. It is programmed under the section “sequence to analyse” i.e. those nucleotides that will be incorporated. When programming the dispensation order, the order in which the nucleotides are added to the reaction, the most common mutations in the specific codon must be taken into account. The Catalogue of Somatic Mutations in Cancer\(^4\) was used in this study to find reports regarding the most common mutations in codon 542, 545 and 1047. Dispensations of nucleotides that may be involved in the mutation, the variable nucleotides, are highlighted in blue in the pyrogram. The software needs a specific amount of peaks incorporated in the wild type sequence, called reference peaks, where a minimum of five peaks is required. When testing the pyrosequencing assays modified in the optimization, it was necessary to localize possible problems in the PCR reaction or the pyrosequencing reaction. Therefore a gel electrophoresis step was added between PCR and pyrosequencing.

**Samples**

Of the 60 samples analysed with pyrosequencing for possible mutation in the PIK3CA gene, three mutations were found (5 %). Figure 2 show the wild type sequence of codon 542 and 545 together with the results of the three mutated samples. One mutation was detected in codon 542 and generated the nucleotide change c.1624G>A which leads to the amino acid

\(^4\) [http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/](http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/)
change p.E542K. Two mutations occurred in codon 545 and generated the nucleotide change c.1633G>A which leads to the amino acid change p.E545K.

Figure 2. Wild type sequences and mutated samples. The x-axis shows the added nucleotides and the y-axis represent the intensity of light. E and S mark the adding of enzymes and substrates to the reaction. The pyrogram is read from right to left with translation to corresponding nucleotides. The red circles highlight the nucleotides included in codon 545 and the blue area underlines the variable nucleotides. A) Wild type sequence GAA of codon 542. B) Mutated sample one from cohort with mutated sequence AAA, nucleotide change c.1624G>A and amino acid change p.E542K. C) Wild type sequence GAG of codon 545. D) Mutated sample two from cohort with mutated sequence AAG, nucleotide change c.1633G>A and amino acid change p.E545K. E) Wild type sequence GAG of codon 545. F) Mutated sample three from cohort with mutated sequence AAG, nucleotide change c.1633G>A and amino acid change p.E545K.
Sensitivity

The tumour cell content of the positive sample in figure 1 F was determined to 50 %. The serial dilution with non-mutated tumour cells generated concentrations of 25 %, 13 %, 6.3 %, 3.1 %, 1.6 % and 0.78 % tumour cells. Figure 3 shows the results of pyrosequencing wild type sequence of codon 545 and samples with the tumour cell content of 50 %, 25 % and 13 %, 6.3 %, 3.1 %, 1.6 % and 0.78 %. The limit of detection for this assay was determined to 6.3 % tumour cells, which is assumed to be the same as 3.1 % mutant alleles. At the concentration 6.3 % tumour cells there was a mutation visible in the pyrogram and measurable in the pyrosequencing software.
Figure 3. Wild type sequence of codon 545 and mutated sample three serially diluted 1:2 with non-mutated tumour cells. The x-axis shows the added nucleotides and the y-axis represent the intensity of light. E and S mark the adding of enzymes and substrates to the reaction. The pyrogram is read from right to left with translation to corresponding nucleotides. The red circles highlight the nucleotides included in codon 545 and the blue area underlines the variable nucleotides. A) show the wild type sequence GAG of codon 545. B) Mutated sample with 50 % tumour cells, mutated sequence AAG. C) Mutated sample with 25 % tumour cells, mutated sequence AAG. D) Mutated sample with 13 % tumour cells, mutated sequence AAG. E) Mutated sample with 6.3 % tumour cells, mutated sequence AAG. F) Mutated sample with 3.1 % tumour cells, mutated sequence AAG. G) Mutated sample with 1.6 % tumour cells, mutated sequence AAG. H) Mutated sample with 0.78 % tumour cells, mutated sequence AAG.
Discussion

In this study, a sensitive pyrosequencing assay to analyse the most common mutations in codon 542, 545 and 1047 of the PIK3CA gene has been established. This assay was applied when screening 60 NSCLC tumours for possible mutation in the PIK3CA gene and determining the limit of detection.

The MgCl₂ concentration was set to 3mM in the PCR reaction, based on the optimization results of slightly stronger bands in the gel electrophoresis for the PCR products containing 3mM.

By the time the optimization of the PCR step and the pyrosequencing assays were considered completed, analysis of the samples from the NSCLC cohort was initiated.

Three mutations among the 60 analysed NSCLC tumours (5 %) regarding the three “hot-spot” codons in the PIK3CA gene were found. This is consistent with the frequency of 4 % reported in previous studies [2, 6]. All mutations occurred in exon 9: one in codon 542 and two in codon 545. Sample one, harbouring mutated codon 542, generated the nucleotide change c.1624G>A and the amino acid change p.E542K. Both sample with mutated codon 545 (sample two and three) generated the nucleotide change c.1633G>A and the amino acid change p.E545K. Co-mutations were also observed; sample one had a mutated EGFR and sample two harboured a KRAS mutation, which previously had been studied. How KRAS and PIK3CA are interrelated was investigated by Zhao L and Vogt PK [16] along with experiments on the mechanisms causing an up-regulation of the PI3K enzyme by the “hot-spot” codons. Codon 542 and 545 codes for the amino acids glutamine acid, which are a part of the helical domain in the p110α protein and codon 1047 codes for the amino acid histidine,
which is located in the kinase domain. Upon growth factor stimulation and autophosphorylation of receptor tyrosine kinases (RTKs), the regulatory subunit p85, release the p110α subunit and cause an activation of the PI3K enzyme. There is also an interaction between RAS proteins and p110α that can lead to activation. They refer to earlier studies, which have shown that the helical domain mutations interfere with the p85- p110α binding. Mutations in the helical domain make p85 release p110α. By measuring the activation of AKT, the enzyme located downstream of PI3K and truncating a part of the helical domain which binds to p85, they discovered that the helical domain mutations still can cause an activation of PI3K leading to the activation of AKT. In an attempt to inhibit RAS-proteins, they found no activation of AKT. When they tried the same procedure, inhibition of RAS, with mutations in the kinase domain, activation of AKT was detected. When truncating the p110α binding site on p85, they found that mutations in the kinase domain did not cause any activation of AKT. They suggest that mutations in the kinase domain could give an allosteric change of the enzyme leading to activation and this activation is mediated by p85. They conclude that mutations in the helical domain and the kinase domain operate on different pathways which both leads to an up-regulation of the enzyme PI3K.

An early study reported by Kawano O et al [17] shows a result similar to ours. They analysed 235 samples of lung cancer tumours with realtime PCR targeting PIK3CA and found eight mutations (3.4 %) all occurring in exon 9. However, a recent study from Israel presents a dissimilar result. Bar J et al. [18] screened 96 samples of NSCLC tumours from early-discovered cases and detected seven samples with mutation in the PIK3CA gene. Since all of them occurred in exon 20 and specifically in codon 1047, they suggest that the mutational spectrum for PIK3CA differ across the world. In this study they also screened for mutations in other genes such as EGFR, V-raf murine sarcoma viral oncogene homolog B (BRAF) and neuroblastoma RAS (NRAS) and conclude that PIK3CA is the gene most common with co-
mutations. Since driver-mutations generally occur exclusive, this raises some question regarding the status for PIK3CA. It is not determined whether mutations in PIK3CA are considered to be “driver-mutations” when it comes to NSCLC. However, this indicates that PIK3CA might still be a valuable target for therapeutic intervention in NSCLC.

Another study by Okudela K et al. [19] suggests that PIK3CA mutations and copy number variation may be of equal importance since their research show that these two phenomena occur exclusively and independent from one another. CNV is alterations in the genome, which can lead to gain or loss of genetic material. They also conclude that the mutational frequency is generally low across the world and does not differ, in comparison to the prevalence of KRAS and EGFR. KRAS is considered to be more common in the western world than EGFR, which on the contrary is more common in Asia.

Wang L et al. [20] screened a large cohort of tumours from patients with NSCLC and among 1117 samples, 34 of them (3 %) had a mutation in the PIK3CA gene. Analysis such as amplification, protein expression and mutational status for other oncogenes was also performed. They conclude a frequent coexistence of mutated PIK3CA with EGFR or KRAS and poor survival rate for patient with a mutated PIK3CA and wild type KRAS or EGFR. This indicates that PIK3CA, despite its status regarding driver- or passenger mutations, still has the potential of becoming a target for drugs. Besides the survival rate, it is also important to examine other parameters that could affect investigation of PIK3CA. Some of these parameters are suggested by Chaft JE et al. [21]. Their report included only lung cancer cases with the adenocarcinoma histology and their results showed that PIK3CA mutations coexists with mutations in other oncogenes in many of their cases.

Discussions regarding the variation between different types of cancer and the pathogenesis, therapy response and prognosis of PIK3CA mutations are central in many studies. For instance in colon cancer, no correlation was found between PIK3CA mutation and response to
the EGFR-inhibiting drug cetuximab [22]. In contrast, De Roock W *et al* [23] found a significant correlation between PIK3CA mutation in exon 20 in colon cancer and inferior response to cetuximab. The conflict of these reports implies that there is much to learn about PIK3CA but also that other factors involved in the PI3K pathway, which likely have an influence on therapy response and prognosis, are loss of PTEN and p53 [21].

After the sample analysis, one of the positive NSCLC samples was used to determine the limit of detection. Due to the fact that the DNA concentration should be kept at 10 ng/µl, dilution with non-mutated cells was preferable to water. Using tumour cells from a different sample assured no presence of circulating tumour cells, which otherwise could have infiltrated the normal tissue in the sample harbouring a mutation. This could have the consequences of a higher tumour cell content than expected and would not give the best conditions for analysing the sensitivity of the assay. Determining the tumour cell content is an estimate, which means the true tumour cell content may vary. However, analysing the corresponding HE slide to determine tumour cell content is the current routine in the clinical laboratory. When determining the limit of detection, factors such as copy number variations and aneuploidy were not taken in account [24]. However, for this study it was sufficient to ensure a limit of detection, which agreed with the established sensitivity. In order not to overestimate our assay, the sensitivity was determined to be 10% tumour cells (5% mutant alleles), which also is the accepted limit of detection for pyrosequencing.

When compared to Sanger sequencing, which is the gold standard sequencing method, pyrosequencing has, as previously mentioned, an accepted limit of detection of 5% mutant alleles while Sanger sequencing have approximately 20%. Sanger sequencing also needs a following step consisting of separating the fragments after synthesis while pyrosequencing, as
previously described, synthesises and sequence at the same time. Sanger sequencing can be used for sequencing larger sequences (800 base pairs) whereas pyrosequencing is best suited for shorter fragments (up to 400 base pairs). In this study, pyrosequencing is the ideal method because hot spot point mutations are analysed. Pyrosequencing is both cost-effective and time-efficient, which contributes further to its advantage. However, mutations may be missed in the pyrosequencing analysis due to error when programing the assays. But in the case of analysing a tumour with low tumour cell content, pyrosequencing may find mutations that Sanger sequencing can miss out because of the detection limit, which is higher for Sanger. Depending on the application, both sequencing methods hold strength and weakness [15].

In summary, our results regarding the reported mutation frequency in PIK3CA are consistent with previous studies. However, there are still questions regarding the importance of PIK3CA mutations in NSCLC. Further research investigating for example loss of PTEN and p53, together with mutations and copy number variations of PIK3CA could perhaps give more insight in the molecular field of lung carcinogenesis. Since the mortality rate for patient with NSCLC is very high, there is a need for improved therapy. With this pyrosequencing assay established, patients may be identified for the treatment of PI3K inhibitors.

Acknowledgement

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