Designing PCR Primers for Detecting Clinically Actionable Single Nucleotide Variation for Non-Small Cell Lung Cancer

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ABSTRACT: Liquid biopsy has opened up a new era of noninvasive cancer detection, diagnosis, and prognosis using circulating tumor DNA (ctDNA). In this process, ctDNA are amplified due to their small proportion in blood and are sequenced to collect information about cancer. This study focused on designing PCR primers to detect clinically actionable single nucleotide variations (SNVs) in EGFR and BRAF genes specific to non-small cell lung cancer (NSCLC). Identifying the presence of genetic aberrations is useful when choosing the treatments that can work best for the patient with a specific SNV. We evaluated three PCR primer sets for DNA amplification which target EGFR T790M, EGFR L858R, and BRAF V600E, which are SNVs that are clinically actionable for NSCLC. The primers that were designed using this method were eventually used in PCR and sequencing and were verified for their efficacy from the sequencing results. All PCR primer sets that were designed showed high accuracy in amplifying targeted regions when PCR was performed. This study suggests options for effective PCR primer sets and takes a step forward for clinical use of liquid biopsy since the usage of accurate PCR primers increases the probability of successful sequencing and, thus, the reliability of liquid biopsy.

KEYWORDS: Liquid Biopsy; Circulating Tumor DNA; Bioengineering; Polymerase Chain Reaction; Primer Design.

Introduction. Annually, approximately 18.1 million patients are diagnosed with cancer, a disease that leads to a 27% mortality rate.[1] Non-small cell lung cancer (NSCLC) that makes up over 85% of all lung cancer patient diagnoses.[2] Cancer is very difficult to detect in early stages and patients often start treatment too late.[3] The standard for diagnosing and detecting cancer has been invasive tissue biopsies which are done by collecting samples from the actual tumor mass through painful, time consuming, and costly surgical procedures.[4] Additionally, due to the invasiveness of tissue biopsy it is difficult to periodically monitor the status of cancer patients and track treatment responses.

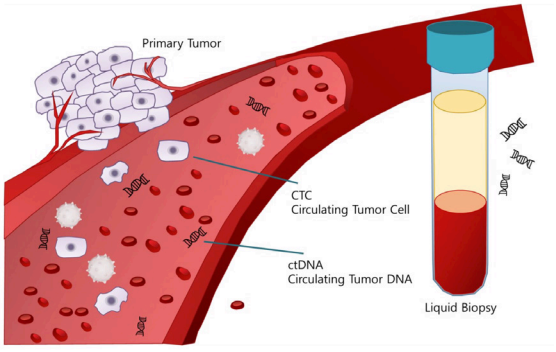

Figure 1. Tumor cells and DNA shed from the primary tumor and circulate around the whole body. Liquid biopsy collects and sequences the ctDNA and gains information about tumor heterogeneity, metastasis, and progression. Unlike conventional tissue biopsies, liquid biopsy is noninvasive, only requiring a vial of blood and providing more accurate data that better reflects the patient's current status.

Liquid biopsy is an emerging noninvasive alternative to detect tumor burdens, identify subclonal sites or transferred metastatic tumors, and track the treatment response through 5-10ml of blood without the need of a biopsy[5] (Figure 1). Liquid biopsy is done by collecting and sequencing the tumor DNA (ctDNA) from the circulating blood that is known to be shed from the tumor cells of the tumor mass. Cell free DNA (cfDNA), or degraded DNA fragments generated during apoptosis and necrosis, are released to the blood plasma and circulate the blood stream. When a growing tumor mass intrudes the blood vessels, the cells and cfDNA from the tumors are disseminated into the blood vessel and circulate.[6] Since the ctDNA contains genetic information of the primary and metastatic tumors of a patient it can be used as a clinical biomarker to monitor, diagnose, and prognose cancer.[5] One can gain information about the originating tumor mass as well as determine the cancer's metastatic progression by looking at the amount of ctDNA in the blood.[6] After obtaining the initial values using real-time quantitative PCR the changing concentration of ctDNA in blood can be significant in clinical settings. The quantity of ctDNA directly reflects the progressing stages of cancer which can be used when monitoring residual cancer after rounds of treatments.[5]

ctDNA is an accessible and accurate clinical biomarker due to its short lifespan, which reflects the patient's status in real-time, and its noninvasiveness, which makes it possible to periodically keep track of cancer patients. The half-life of ctDNA is less than 1.5 hours which ensures the data collected from the ctDNA most accurately reflects the current status of the patient.[6] Accurate reflection of cancer status allows for the development of precision medicine and personalized treat

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ment while avoiding unnecessary treatments that can be costly and toxic to the body. However, it is difficult to accurately detect clinically actionable biomarkers due to the heterogeneity of the tumor.

Different tumor cells display distinct features such as cellular morphology and gene mutations.[7] Since cancer displays tumoral genetic heterogeneity, different types and stages of cancers have distinct genetic aberrations that can be used as clinically actionable biomarkers. Single nucleotide variation (SNV) is a type of genetic aberration that occurs when there is a mismatched or missing nucleotide in DNA which leads to codon mismatch and resulting in abnormal activities in cells such as the production of abnormal proteins and severe clinical conditions. For example, epidermal growth factor receptor (EGFR) T790M and EGFR L858R are common single nucleotide mutations that occur in the EGFR gene which develops resistance towards tyrosine kinase inhibitors, a crucial pharmaceutical drug used to treat cancer. B-raf encoding gene (BRAF) V600E is a single nucleotide variation in the BRAF gene which results in oncogenic mutations that cause tumor development. Due to the advancement of sequencing technology, even a single mutated nucleotide can be detected through sequencing in high throughput. To detect the targeted variation, sequencing technologies analyze captured ctDNA. However, ctDNA makes up less than 0.1% of total cfDNAs.[5] Therefore, precise and accurate amplification of ctDNA is crucial. Polymerase chain reaction (PCR) and target-specific primers are required to accurately amplify the mutated region.

In this study, I designed and verified PCR primer sets that amplify the single nucleotide variations clinically actionable for NSCLC patients (Figure 2). After executing PCR on samples using the designed primer sets, the reliability of the primers was verified by finding the mutated region in each sequencing result.

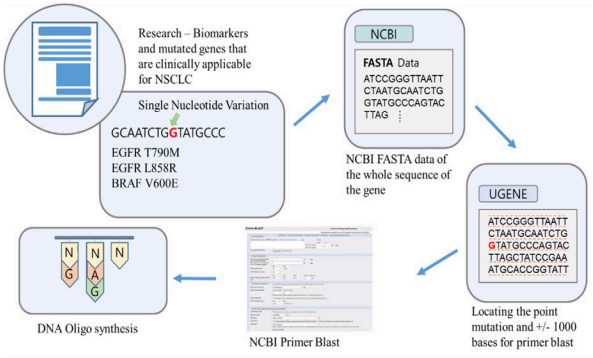

Figure 2. The primer designing and verification process is shown above. Primers are designed through PrimerBlast, an online platform published by NCBI. After choosing the primer sets, primer sequences are sent for oligo synthesis. Using the designed primer, DNA samples, Taq master mix, four samples were amplified. After PCR products were verified for successful amplification through gel electrophoresis the PCR products are purified and sent for Sanger sequencing service. From the sequencing results, the presence of genetic aberrations is verified by comparing the sequence with the original reference sequence of the gene.

Results and Discussion. Designing the primer sets. For the three single nucleotide variations that are clinically actionable for NSCLC, three PCR primer sets (forward/reverse) that target each SNV were designed. The melting temperature, GC%, product length, annealing temperature, and actual sequence is shown in Table 1. All the primer sets are in the appropriate range of Tm and GC% for successful PCR. The measured annealing temperature found through PCR and gel electrophoresis is shown in ranges close to the calculated annealing temperature.

Product Name	Tm(c)	GC%	Expected Product Length	Calculated Annealing Temperature(°C)	Measured Annealing Temperature (°C)	Sequence (5' → 3')
EGFR T790M	F: 60.5 R: 60.5	F: 55 R: 55	355bp	68	68~72	F: GCGTAAACGTCCCTGTGCTA R: CCTTTGCGATCTGCACACAC
EGFR L858R	F: 60.5 R: 62.5	F: 50 R: 60	432bp	68	68~72	F: CTCAGAGCCTGGCATGAACA R: GCTCTGGCTCACACTACCAG
BRAF V600E	F: 60.5 R: 62.5	F: 55 R: 55	433bp	69	68~72	F: AAGAGCCTTTACTGCTCGCC R: CTGATGGGACCCACTCCATC

Table 1. Three different primer sets used in this study

PCR Validation. After designing the PCR primer sets that target EGFR T790M, EGFR L858R, and BRAF V600E, the validity and accuracy of the primer sets were tested (Table 2). The primer suitability for PCR was verified by examining features of the primers such as GC%, melting temperature (T_m), product length, and annealing temperature.

GC percentage is a percentile that shows how much guanine-cytosine base pairs are contained in the total primer sequence. GC percentage plays a crucial role in PCR because guanine-cytosine pairs have stronger bonds than adenine-thymine pairs. Therefore, amplification is unsuccessful when the GC percentage is not within 40-60%.[8] Too many GC bases in the primer causes the DNA to coil and the primers won't prime well to the DNA if there are not enough GC bases. All the primer sets that were designed for this study were in the appropriate GC percentage of 40%-60%.

The melting temperature of the primers is also an important validator of a primer's effectiveness. A too low T_m will result in loss of specificity while too high T_m will increase the chance of mis-priming. Mis-priming indicates primers priming to unintended regions, primers not priming to the DNA strand, and/or degradation of primers, all of which reduces the specificity and accuracy of PCR. The Tm of the designed primers were in the appropriate range of 56-62 °C.[8]

Table 2. PCR using the designed primer sets was tested on four samples: SKBR3, HL60, Sample 1, and Sample 2. The table below shows the DNA concentration of the amplified product before sequencing and the presence of SNV found from the sequencing result. The initial value of the DNA samples was 1 ng per µl, from which 1 µl was used for all. Samples with known concentration was diluted to 1 ng per µl. From the DNA concentration that was measured after amplification, it was evident that all samples were successfully amplified. There were no SNVs present in any sample. This is an expected result since the samples were not from an NSCLC patient.

Sample name	Primer name	DNA concentration (ng/µl)	Presence of Single Nucleotide Variation
SKBR3	EGFR T790M	11.7	c2369c
	EGFR L858R	7.2	t2573t
	BRAF V600E	7.0	t1799t
HL60	EGFR T790M	5.5	c2369c
	EGFR L858R	7.2	t2573t
	BRAF V600E	4.7	t1799t
Sample 1	EGFR T790M	7.7	c2369c
	EGFR L858R	7.5	t2573t
	BRAF V600E	8.9	t1799t
Sample 2	EGFR T790M	6.2	c2369c
	EGFR L858R	8.1	t2573t
	BRAF V600E	7.3	t1799t

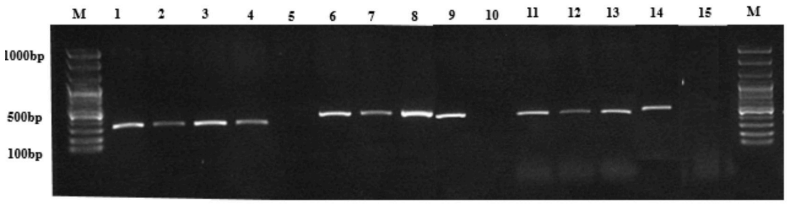

Figure 3. The lanes are the results of amplified products that used primer set for EGFR T790M, EGFRL858R, and BRAF V600E with genomic DNAs from SKBR3, HL60, Sample 1, Sample 2, and nuclease–free water, consecutively..

Figure 4. Presence of SNV was verified in the Sanger sequencing result. (a) The reference SNP was found in NCBI database to locate the single nucleotide variation point in the gene sequence. The sequence marked with a blue box was used to locate the SNV point in the Sanger sequencing result. (b) This is an example of a Sanger sequencing result of the amplified product of Sample 1 amplified using EGFR L858R primer set. Using a part of the sequence found in the reference data, the SNV point marked with a red box was found in the Sanger sequencing result. Since the base was equal to the reference gene, there was no EGFR L858R SNV present in Sample 1.

Verifying the Presence of SNV.

After the samples were amplified using the designed primers, the accuracy of PCR was verified by gel electrophoresis. Using 10µl of samples and 100bp ladder, gel electrophoresis was performed at 160V for 35 minutes. Looking at the gel and comparing with the expected product size of each primer, it is possible to verify whether the amplification was successful. Lanes 1-5 shown in Figure 3 are the results of amplified products that used primer set for EGFR T790M with genomic DNAs from SKBR3, HL60, Sample 1, Sample 2, and nuclease-free water, consecutively. Since all marks are between 300- 400 bp and the expected product size of EGFR T790M primer was 355bp it can be concluded that the amplification was successful. Lanes 6-10 in Figure 3 are the results of amplified products that used primer for EGFR L858R with SKBR3, HL60, Sample 1, Sample 2, and nuclease-free water. Since the expected product size was 432 bp and the band is between 400-500 bp, the amplification was successful. Lastly, lanes 11-14 are the results of the amplified products that used primer for BRAF V600E with SKBR3, HL60, Sample 1, Sample 2, and nuclease-free water. The amplification was successful since the expected product size was 433 bp and the bands are be

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tween 400-500 bp. Lanes 5, 10, and 15 are the negatives that used nuclease-free water instead of samples with amplified DNAs. Since no band was shown, the negative was accurate.

The purified PCR products were then sent for Sanger sequencing for SNV validation (Figure 4). The presence of SNVs was verified by searching one part of reference sequence in the Sanger sequencing result. Verifications were done by searching the reference SNP in the NCBI data base. For example, the reference SNP rs121434569 was used for EGFR T790M. Comparing the reference genome and finding the location of the SNV point (marked with blue box) in the Sanger sequencing result, I was able to determine if the genomic DNA had the clinically actionable genetic aberration. Since the SNV point (marked with red box) is the same as the reference sequence, there is no genetic aberration present.

Likewise, the accuracy of the primers when detecting the targeted SNVs were verified using UGENE by locating both forward and reverse primers in the amplified sequence and determining if the SNV point is in the range between both primers. PCR was performed on DNA samples from SKBR3, HL60, Sample 1, and Sample 2 using the designed primers. Sample 1 and Sample 2 were DNAs extracted from healthy volunteers; for privacy names and details were not provided. All primer sets successfully amplified the targeted region including the SNV point nucleotide. The presence of the genetic aberration was found by searching a part of the gene sequence in front of the SNV from the original sequence at the sequencing result of the amplified PCR products. The sequencing result showed that while no sample had the SNV, every sample had the position of the SNV. It was an expected result since the samples were not from NSCLC patients.

Conclusion. In this study, we designed PCR primer sets for detecting clinically actionable single nucleotide variation in NSCLC and demonstrated their application to four samples. From the results, it was verified that the designed PCR primers were successful in accurate amplification of targeted sections of DNA. Therefore, the three PCR primer sets designed in this research can be used in actual clinical settings, allowing for clinical detection of SNVs in NSCLC patients. Designing effective primer sets and successfully amplifying the ctDNA reduces the errors in PCR and sequencing. Decreasing amplification and sequencing errors is crucial for liquid biopsies to replace conventional invasive biopsies in clinical settings. Higher chances of successful diagnosis will lead to more conventional use of liquid biopsy. However, there are still many improvements in technology needed as well as integration with other platforms and technologies.

Further research should focus on the amplified products being sequenced through next generation sequencing (NGS) and a more sensitive technology can be developed due to the higher sensitivity of NGS than Sanger sequencing. Furthermore, this study did not use cancerous samples thus no genetic aberrations were detected. By using ctDNA from NSCLC patients, the effectiveness and accuracy of the designed primers will be better verified. It will also allow us to research what kind of primers amplify the SNVs with the least errors. Also, different genetic aberrations can be detected and amplified with the use of multiplexed PCR where multiple targeted regions of biomarkers can be amplified using multiple PCR primer sets simultaneously. In order to multiply different SNVs at the same time through high-throughput detection, the melting temperature of the primer sets should be in equal range, as the specificity of primers depend on the melting temperature.

An example of integrating liquid biopsy with other technology is barcode-free NGS error validation technology. This can decrease the sequencing errors that occur when searching for ultra-rare variants such as SNVs that are detected in ctDNAs.[9] This technology allows us to validate if a detection of an ultra-rare variant is a sequencing error by validating the variant through amplification of the sample from the NGS substrate.[9] Since only 1 in 90 detections is a true variant, validating the raw NGS data through the PCR platform can more accurately distinguish true genetic aberrations. Also, for more accurate amplification, Pfu and other types of polymerase can be used instead of Taq polymerase. In this process of PCR validation, the designed primers can be used to amplify the targeted region. Increased accuracy will decrease costs of sequencing processing, thus increasing the accessibility of liquid biopsy in clinical settings.

Another way of integrating this study with other technology is using the designed primers to amplify circulating tumor cells (CTCs). Although sequencing results of ctDNA provide valuable information about a tumor, analysis of CTCs cells can provide a different genomic profile of the cancer. Single CTCs can be captured from whole blood using an optomechanically-transferrable chip and laser-induced isolation of microstructure on optomechanically-transferrable-chip sequencing (LIMO seq) which allows each cell to be sequenced in single cell level.[10] Captured single CTCs are whole genome amplified and go through NGS to provide a detailed analysis of the heterogeneous genome profile of the cancer. However, whole genome amplification has a limitation in lower accuracy due to amplification artifacts which can result in errors when looking for ultra-rare variants such as SNV. By replacing whole genome amplification with PCR, the designed primers can be used for more accurate amplification of DNAs or single CTCs where only clinically valuable targeted regions will be amplified. Additionally, by performing multiplex PCR with multiple designed primer sets, it is possible to obtain more clinical information with higher accuracy than the sequencing results of whole genome amplified CTCs.

With integration with other technologies, liquid biopsy has potential to become the future standard of accurate and noninvasive cancer diagnosis, prognosis, and monitoring. Designing clinically actionable primer sets as described herein will be the first step in high-performance liquid biopsy.

For P. digitatum treated with Stereocaulon sp., the best performance was obtained with the ethanol extract (ET 50), followed by chloroform (CL 50) and isopropanol (IS 50). The 20 μL ethanol (ET 20) treatment was also very effective as it had a 9.0 mm halo diameter. This shows that P. digitatum is especially sensitive to lichen compounds dissolved in ethanol.

In the experiment with Cladonia sp. extracts, the best performance was observed with the isopropanol extract (IS 50),

followed by ethanol (ET 50), chloroform (CL 50) and ethyl acetate (AE 50), as shown in Figure 5.

Aqueous extracts (AD 20 and 50) and hexanes (HE 20 and 50) had low performances in all experiments.

Conclusion. The lichens of the genera Stereocaulon and Cladonia used in this project present substances that inhibiting the growth of S. cerevisiae and P. digitatum.

Extracts of Stereocaulon sp. had a better performance than the Cladonia sp. Extracts because they generated larger-diameter halos under the same cultivation conditions for both fungi.

Greater inhibition was observed in the treatments using a higher extract dose (50 µL) which shows the inhibitory effect is dependent on the extract concentration.

The solvents used have no inhibitory effect on fungal growth as demonstrated in the control treatments.

In all the experiments that utilized solid culture medium, the extracts with the greatest inhibition of fungal growth were those of ethanol, chloroform, and isopropanol while the hexane and aqueous extracts had the lowest performance. The ethyl acetate and acetone extracts had varied effects.

The lichen extracts of Stereocaulon sp. and Cladonia sp. have the potential to formulate phytosanitary products or drugs for controlling pathogenic fungi. For this, it will be necessary to determine the components of each extract.

Further research must be conducted to test these extracts on other fungal and bacteria species of agricultural interest as well as to determine the minimum dose necessary to inhibit microorganism growth and study the biochemical profile of each extract.

Methods. The research was carried from March to August 2018 in the teaching laboratory at the Complejo Educacional Chimbarongo in Chimbarongo, Chile.

Stereocaulon sp. was collected in the Manquemapu locality of Purranque, Chile and the Cladonia sp. was collected in a sclerophyllous forest located in El Sauce. The lichen material was dried for two weeks then crushed in a food processor.

The solvents used to make the extracts were distilled water, absolute ethyl alcohol, isopropyl alcohol, ethyl acetate, acetone, chloroform, and hexane. To make the extracts, 80 ml of each solvent was mixed with 20 g of lichen material and macerated for a week at 6 ˚C in hermetic glass jars then sieved and filtered.

The fungi were cultivated on Potato-Dextrose-Agar medium in 90 mm plastic Petri discs. Inoculation was carried out by applying 1 mL of physiological saline solution containing approximately 5,000 colony-forming units (CFU) of Saccharomyces cerevisiae and 3,800 CFU of P. digitatum onto the agar medium.

Mycological susceptibility tests were performed using the disk diffusion method in solid media using 6 mm diameter filter paper discs. In each Petri dish, 3 discs with 20 µL and 3 discs with 50 µL were placed. The Petri dishes were placed in a digital incubator at 25 ˚C for 72 hours.

Four experiments were carried out with 28 treatments. Doses of 0 (C = control), 20 and 50 µL of each of the seven types of extract were evaluated. The control treatment consisted of disks with 50 µL of each solvent. The growth inhibition halos were measured with a ruler for the data analysis.

A unifactorial analysis of variance with a level of significance of p = 0.05 was performed. The means were separated by the Tukey test when there was a significant difference at p = 0.05.

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