Circulating Tumor DNA as a Biomarker for Head and Neck Squamous Cell Carcinoma

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Abstract

Introduction

Background. Detection and quantification of cell-free circulating tumor DNA (ctDNA) in plasma of cancer patients provides the possibility for noninvasive detection and monitoring of tumors. In patients with head and neck squamous cell carcinoma (HNSCC), potential uses of ctDNA analysis include detection of residual disease and tumor recurrence following surgery.\textsuperscript{1,3}

Methods. We evaluated samples from 3 HNSCC patients. Mutations in tumor DNA were identified by targeted sequencing and visualized in the Integrating Genomics Viewer. Key mutations were selected for each tumor sample, and PCR primers were designed and tested for use with a sequencing technology called SiMSen-qseq, which generates barcoded next-generation sequencing libraries that help identify and remove polymerase errors in sequencing data, allowing for detection of mutant alleles at a frequency <0.1%:\textsuperscript{2} SiMSen-qseq was used to verify presence of mutations in the tumor DNA. Cell-free DNA was then isolated from patient plasma and SiMSen-qseq was applied to detect and quantify ctDNA. Results were compared with clinical staging and treatment.

Results and Conclusion. Four to five mutations in each tumor were selected for SiMSen-qseq. Primers were designed and tested for all three patients. SiMSen-qseq analysis of the plasma DNA is in process for one patient. Plasma samples were available at the time of surgery, after surgery and shortly after documented tumor recurrence. SiMSen-qseq results are pending; however, we hope to detect ctDNA at the time of surgery and hypothesize that we may also detect ctDNA prior to clinical documentation of relapse. This would indicate the significance of ctDNA in monitoring HNSCC in patients after surgery.

Methods

Approach

Barcoding Enables Identification of True Mutations vs Polymerase errors

Figure 5: Diagram of SiMSen-qseq protocol after design of hairpin primers - SiMSen-qseq is a Next-Generation sequencing technology that creates barcoded libraries to identify rare variant detection in DNA samples. The barcodes, when run through PCR, help with this identification of mutations, as SiMSen-qseq removes such polymerase errors in sequencing data. Hairpins protect these barcodes, and prevent them from mispriming during PCR. As a result, very small frequencies of mutant alleles (<0.05%) can be identified using this technology.

Figure 6: Target Primer test result examples - the first result is an amplification plot (example of CSMD2_106 shown above, Fig. 9). In this plot, the first three curves represent three different genomic DNA concentrations at which the primer was tested and run through qPCR. Then, the melt curve (example of SACS, Fig. 8 shown left, Fig. 8) is used to make sure the no-template control (NTC) contains no nonspecific product that was accidentally amplified.

Figure 7: Primer design spreadsheet - After selecting a few mutations from each tumor sample, we designed target primers for each of those mutations and documented their sequence, amplicon and window sizes, G-C content, melt temperatures, and predicted melt temperatures. The table above shows all of this data. Green represents successful designing and testing, and red represents either unsuccessful designing or failure during qPCR testing.

Figure 8 & 9: qPCR Target Primer test result examples - the first result is an amplification plot (example of CSMD2, c06 shown above, Fig. 9). In this plot, the first three curves represent three different genomic DNA concentrations at which the primer was tested and run through qPCR. Then, the melt curve (example of SACS, Fig. 8 shown left, Fig. 8) is used to make sure the no-template control (NTC) contains no nonspecific product that was accidentally amplified.

Figure 10: Round 2 qPCR plots of hairpin primers - The red-orange line represents DNA being amplified with the TP53 primer, whereas the blue lines shows a no-template control (NTC), which lacks DNA but still has the primer. The amplification plot shows the increase in DNA in the PCR reaction as each denatured, annealed and extended cycle progresses. The melt curve shows product that melts at a certain temperature; since the DNA curve is shifted to the right of the NTC curve, we can safely conclude that the DNA sample contains specific product.

Figure 11: Example of a positive SiMSen-qseq result - The results for this project are still pending. The expected result is similar to the one shown; the presence of a mutation on the tumor DNA is marked by a rise in mutant allele frequency at a specific base pair, in this case 12345678. If there is a similar increase in frequency at the same location in the plasma DNA, that same mutation can be assumed to exist in the plasma DNA.

Discussion

Of the twelve target primers which were designed and tested in this project, eleven of them tested well under real-time PCR. CBX4-N3_3971 consistently resulted in a nonspecific product for the cTaq, and has been redesigned (SiMSen-qseq).

As for the fragment analyses of the first patient’s plasma, the peak in the middle of the graph represents the amount of PCR product found. Although two of these three plasma samples contained relatively low amount of DNA, we hope to see results when the DNA is run through SiMSen-qseq.

For the first patient, we have three plasma samples: one taken at the time of surgery for removal of the tumor, shortly after surgery, and after recurrence of HNSCC. SiMSen-qseq should show positive results for the first sample, but we hope to also see positive results for the second sample. This would indicate the clinical significance of ctDNA as a cancer biomarker, as it was found approximately one month before clinical documentation of the relapse.

At the beginning of this program, I came in as a rising high school senior interested in molecular biology, but with little hands-on experience in professional scientific labs. The first three weeks were a wash, with me constantly forgetting to Vortex the plates before running the plates. After the wash, I was directed more properly, or not changing pipette tips between various additions of solutions into tubes, which would lead to contamination and, eventually, inconclusive results. Now, I am able to (almost) consistently pipet accurately and cleanly, and am more conscious of each procedure, to make sure that no important detail is left out of my attention. Without the developing of those skills, I would not be able to do this project successfully, as it demands accuracy in testing each primer and transferring any solutions between each step.

References


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Figure 12: Patient plasma fragment analysis - We used a previously designed target primer (TP53) and used it in a PCR with the plasma-isolated DNA for the first patient (left), all diagrams labeled “plasma.” The HN01 tumor fragment analysis is used as a positive control when the plasma is run through SiMSen-qseq.