**Understanding Interactions of Mutant and Wild Type Cells and their Implications in Chronic Lymphocytic Leukemia Relapse**

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Abstract

**Introduction**

Chronic lymphocytic leukemia (CLL), a slow-developing type of cancer caused by the bone marrow making too many lymphocytes, is the most prevalent type of leukemia in the Western world. Ibrutinib is a CLL treatment that is able to target only cancerous cells by inhibiting the function of a key protein molecule known as Bruton’s Tyrosine Kinase (BTK). By doing so, it blocks signals that stimulate malignant B cells to grow and divide uncontrollably. This causes the division of CLL cancer cells to slow down and sometimes even stops their growth all together. The experiment was conducted to see how the cells of recently relapsed patients operate by determining the sensitivity of Ion Torrenting in the detection of mutant C481S cells.

**Methods**

The experiment utilized DT 40 chicken B cells that were transcribed with BTK wild type and BTK C481S alleles. Eight ratios of BTK mutant (C481S) and BTK wild type (WT) cells were made. DNA was extracted from each of the eight ratios. The final step of the experiment was testing the DNA for the detection of C481S cells through Ion Torrenting, with most ratios being run through 100,000 times.

**Results**

Every ratio of mutant C481S cells had a final frequency, seven of which differed from their original ratio. While the frequencies of the 100:1 and 5,000:1 ratios were both around double what was expected, the most important result was the final frequency of 0.015% for the 10,000:1 ratio. This information showed that Ion Torrenting was a sensitive enough test to detect mutant cells at even the smallest concentration tested.

**Discussion**

The experiment was created as a way to determine the lowest concentration of C481S in a ratio that was detectable through Ion Torrenting. The lowest detectable frequency of C481S (0.015%) was in the 10,000:1 ratio. This result points to the fact that Ion Torrenting is an extremely sensitive test that may even be able to detect smaller concentrations of mutant cells. Overall, these findings are important as the more sensitive that Ion Torrenting can be, the earlier relapse can be identified in a patient. This early detection will enable doctors to switch a patient just entering relapse to a new or alternative treatment therapy.

Introduction

Chronic lymphocytic leukemia (CLL) is the most prevalent type of leukemia in the Western world, with over 15,000 new cases in the US alone in 2012.[[1]](#endnote-1) CLL is a slow-developing type of cancer, most common in older adults, in which the bone marrow makes too many lymphocytes.[[2]](#endnote-2) In patients with CLL, changes in the diseased cells activate oncogenes that promote cell growth and division while deactivating tumor suppressor genes that slow down cell division or cause cells to die at the correct time.[[3]](#endnote-3) Most often, this change is a deletion or loss of part of a chromosome, most commonly of chromosome 13, although chromosomes 11 and 17 can also be affected.[[4]](#endnote-4)

Ibrutinib, developed in the Byrd Lab at the Ohio State University, is a targeted therapy that is the result of years of research dedicated to understanding the differences between cancer cells and normal cells.[[5]](#endnote-5) Since cancer treatment previously focused on killing rapidly dividing cancer cells, it would inadvertently kill normal cells that were also dividing rapidly. Ibrutinib is able to target and affect only the cancerous cells by inhibiting the function of Bruton’s Tyrosine Kinase (BTK), a key protein molecule within the B-cell receptor signaling complex that is vital to the survival of malignant B cells.[[6]](#endnote-6),[[7]](#endnote-7) Ibrutinib blocks signals that stimulate malignant B cells to grow and divide uncontrollably, slowing down and sometimes even stopping the growth of CLL.[[8]](#endnote-8),[[9]](#endnote-9)

Although ibrutinib has been successful at facilitating remissions in CLL patients, it is far from a perfect treatment. At best, ibrutinib-induced remissions last for 3-4 years before mutations within the cells of patients lead to recurrences of the cancer. This trend led to an inquiry into whether a certain type of mutation caused these recurrences or whether several mutations were responsible.

Research into the inquiry was done through experiments designed to determine whether any mutation of an ibrutinib-aided cell had a selective advantage or if they all maintained homeostasis. After an initial experiment showed promising results for only one mutation, another experiment was conducted to detect the concentration of mutant cells after growing in ratios. This was done in an attempt to better understand the environment for relapse.

The experiment, created as a way to understand how the cells of recently relapsed patients operate, was conducted as a simulation of the process. It started with DT 40 chicken B cells that were similar to the B cells where CLL originates in humans. These cells had either BTK wild type or BTK C481S alleles transcribed into them in an attempt to determine how easily the mutant C481S cells could be detected. The experiment involved the maintenance of eight different cell ratios to see if there was a point at which only BTK WT could be detected. The cells were split 1:20 with media, two to three times a week, until there were enough BTK WT cells to make every ratio. After all ratios were made, one million cells from each of the eight ratios were used in DNA extraction. Lastly, the DNA from each ratio was run through Ion Torrent deep sequencing to determine the sensitivity of detecting the mutant C481S cells in each ratio.

Methods

Throughout the project, there were two experiments. The first experiment was done to see if there was a certain type of mutation that had a selective advantage over another. The set-up and testing were almost exactly the same as its successor, with the exception of the cells that were used. However, the results were inconclusive and it was difficult to gain any information from all but one mutation, BTK C481S.

The second experiment was based on using ratios of the mutant BTK C481S to BTK WT to simulate the environment before, during, and after a relapse in CLL by using DT 40 chicken B cells. The first step to creating the desired cells was binding the desired plasmid (BTK WT or BTK C481S) into a virus. Next, the DT 40 chicken B cell was infected with the virus in order to transcribe human BTK. After that, the virus was killed. Lastly, puromycin was inserted to keep plasmids in cells.

Media was made in order give the cells an environment to grow enough in number to match the desired ratios of mutant to wild type cells. The media was a 15% Fetal Bovine Serum (FBS) solution that was used two to three times weekly to make 1:20 ratios of cells (1 mL cells: 19 mL of media). The process started with 840 mL of RPMI media. 150 mL of FBS and 10 mL of p/s/g were added to create the desired solution. The solution was then filter sterilized and the final volume was 1,000 mL (1 L).

The concentrations of cells also had to be counted. Small samples of both BTK WT and BTK C481S were placed in Isoflow sheath fluid in a vial in an effort to count and save 1E6 cells. 10 mL of sheath fluid was put into a clean counting vial. 10 uL of the BTK C481S cell line was taken and put into the 10 mL of sheath fluid. The cell line was counted three times and the concentrations were averaged. The volume of fluid that contained 1E5 cells was then calculated to be 3.61 uL using this information. The process was repeated for the BTK WT cell line.

Next ratios had to be made for cell pellets. There was a C481S constant, a 10 WT: 1 C481S ratio, a 50 WT: 1 C481S ratio, a 100 WT: 1 C481S ratio, a 500 WT: 1 C481S ratio, a 1,000 WT: 1 C481S ratio, a 5,000 WT: 1 C481S ratio, and a 10,000 WT: 1 C481S ratio. The volume of liquid needed for 1E5 cells of BTK C481S was then used as the 1 in every ratio while the volumes of liquid needed for 1E6 (24.1 uL), 5E6 (102.2 uL), 1E7 (204.5 uL), 5E7 (1.022 mL), 1E8 (2.045 mL), 5E8 (10.22 mL), and 5E9 (20.45 mL) of BTK WT were used for the 10, 50, 100, 500, 1,000, 5,000, and 10,000 in their own respective ratios. The cell lines for both BTK WT and BTK C481S were split two to three times a week until there were enough BTK WT cells to make all of the ratios.

DNA extraction followed this process as dNeasy spin columns were used for 1E7 patient cells of each ratio. The cells were thawed in a 37°C water bath for 5 min. The cell pellets containing each ratio was transferred from a cryovial to a labeled 15 mL conical tube and filled to the top with PBS. Each of the eight combinations was spun at 1,000 rpm for 5 min. The supernatant was decanted and the cell button was suspended in ~400 uL PBS. 40 uL proteinase K was added to each 15 mL tube containing cells. Then the 400 uL buffer AL (lysis buffer) was added to each tube. The tubes were then mixed through pulse vortexing for 15 seconds. Each tube was incubated at 56°C for 10 minutes. 400 uL Etoh (96-100%) was then added to each sample. Again, these were mixed by pulse vortexing for 15 seconds.

The contents of each tube were pipetted into new dNeasy spin columns. Each was centrifuged at ~13,000 rpm for one minute. Next the dNeasy spin columns were placed in new 2 mL collection tubes. 500 uL of buffer AW1 was then added. The tubes were centrifuged again at ~13,000 rpm for one minute. The dNeasy spin columns were once again put into new 2 mL collection tubes. 500 uL of buffer AW2 was added. Once more, the tubes were centrifuged at ~13,000 rpm for one minute and the dNeasy spin columns were put into new 2 mL collection tubes.

The tubes were then centrifuged at ~13,000 rpm for three minutes to dry the membrane. The flow-through and collection tubes were discarded. Each dNeasy spin column was then placed into a clean 1.4-2 mL centrifuge tube. 200 uL buffer AE (elution buffer) was then pipetted directly onto the membrane and allowed to sit for five minutes. The tubes were centrifuged at 13,000 rpm for one minute to have the DNA in elution. Lastly, the nanodrop was used to check the purity and concentration of samples.

The final step to the experiment was DNA testing as determined by ion torrenting. One ratio of cells was read nearly 150,000 times, while others were read over 300 times, to ensure the validity of data gathered.

Results



Table 1: Most pertinent information from raw data reorganized into more concise draft

The table above highlights the most important parts of the raw data: chromosomes, gene IDs, allele names, ratios of cells, reference genes, variant genes, frequencies, and coverages. The chromosome section shows which chromosome of DNA the testing occurred in. All of the testing was done in the X chromosome that had been transcribed into the chicken cell as it is a perfect model for human reaction since all humans have an X chromosome.

The gene ID highlights which gene is being tested. This gene is always BTK as it is the signaling pathway for the majority of B-cell cancers including CLL. The allele name is also consistent throughout as the mutant allele used for testing in every ratio was C481S.

The WT:Mutant ratios were a measure of how many cells of both BTK WT and BTK C481S were in each ratio. The ratios started with a C481S constant then a 10:1 BTK WT:BTK C481S ratio. It proceeded to go up by a factor of two or five from that point onward.

Reference genes were the ones in sequence that were considered to be the wild-type. Every reference gene within the DNA code was cytosine (C). Conversely, the variant genes were guanine (G) since the cells had transcribed themselves into complementary pairs by the time the DNA was tested.

Frequency is the percentage of C481S cells relative to the total number of cells. For this reason, it mattered when the final frequencies of C481S differed from the original ratios. The first ratio, the C481S constant, should have a frequency of 100% and does. The 10:1 ratio has a final frequency of 8.1%, which is considerably lower than the 10% it started with. The 50:1 ratio’s final frequency should be 2% but it is slightly lower at 1.7%. Surprisingly, the 100:1 ratio has a frequency that is almost double the 1% it started with at 1.8%. The 500:1 ratio should have a frequency at 0.2% but is slightly higher at 2.4%. The 1,000:1 ratio’s frequency should be 0.1% but is smaller at 0.07%. The frequency of the 5,000:1 ratio is 0.04%, exactly double its expected frequency of 0.02%. Lastly, the frequency of the 10,000:1 ratio should be 0.01% and is actually 0.015%. The fact that every ratio had a final frequency shows that Ion Torrenting is a sensitive enough testing procedure that it can detect a single mutant cell even when it is among 10,000 normal cells.

Original coverage essentially represents how many times the same part of the DNA code was tested. The 500:1 ratio was tested 148,161 times, the most of all, while the 10:1 ratio was tested the least at 307 times. The other ratios tended to be tested around 50,000-100,000 times.

Discussion

The experiment was designed as a simulation of what happens in both healthy and diseased bodies based on ratios of wild type and mutant cells. The DT 40 chicken B cells used in the simulation share many genetic similarities to human cells. Specifically, the DNA readings that came from the X chromosome that was transcribed into the chicken cell are an exact replica of what they would be in a human X chromosome. For this reason, both the BTK WT and C481S cells are believed to have acted just as they would in a CLL patient.

The simulation results provide key new information with respect to relapse, providing insight about the behavior of CLL before, during, and after taking ibrutinib. Before taking ibrutinib, the new knowledge can inform which environment (ratio of mutated cells to wild type cells) is conducive to relapse. During the treatment, knowing how wild type and mutant cells interact may help doctors predict when a relapse could occur.

Overall, the experiment was conducted to determine the lowest concentration of C481S in a ratio that still had enough mutant cells to be detected through Ion Torrenting. Since every ratio had a final frequency greater than 0%, the lowest detectable frequency of C481S is in the 10,000:1 WT:C481S ratio, which had a frequency of 0.015%. This is a threshold of extreme sensitivity as it means that 1.5 mutant (i.e. cancerous) cells can be read among 10,000 normal cells. The sensitivity at which Ion Torrenting can detect mutant C481S cells is critical as it enables earlier detection of relapses in patients. This early detection will enable doctors to switch a patient just entering relapse to a new or alternative treatment therapy.

The detection of mutant cell in a 10,000:1 ratio is a positive step forward. However, its frequency of 0.015% indicates that there may be even larger ratios of normal cells to mutant cells where the mutants can still be detected. Thus, these larger ratios must be tested to see if Ion Torrenting deep sequencing has an even greater sensitivity when it comes to DNA testing.

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