Understanding Cystic Fibrosis Transmembrane Conductance Regulator expression in Heart Failure

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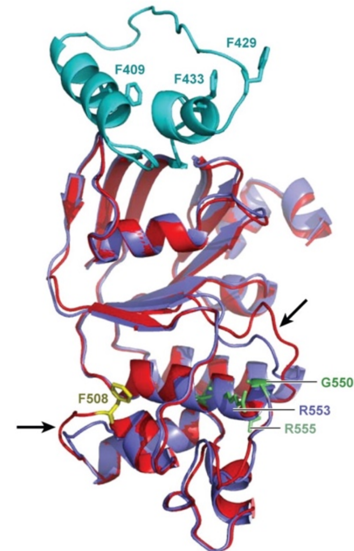
Abstract

Cardiovascular disease, specifically congestive heart failure, is a leading cause of death in the United States. Cystic Fibrosis (CF) is caused by the mutations to the cystic fibrosis transmembrane conductance regulator (CFTR) gene. These mutations result in a defective or absent CFTR protein in the lung epithelial cells. Not a well-known concept is that the CFTR protein is present in more than just the lungs - its presence in the cardiac tissue may be critical for heart function. Preliminary research from the Bowles lab demonstrates that CFTR expression and function are reduced in diseased human cardiac tissue. Also, DNA sequencing suggests a potential cause for this diminishment: a genetic enhancer in the CFTR gene is different in heart failure patients compared to healthy controls. This difference has been seen in a small study of 48 patients. In this independent study, I examined the CFTR gene through PCR and gel electrophoresis analysis, as well as consolidating previous work from the Bowles lab and others in the CFTR field, to provide an in depth look at this region of the gene. The challenges of this PCR study have held back results of amplification data due to complications with the protocol and electrophoresis. Near the end of the study, successful PCR and gel electrophoresis was completed, showing the technique was achievable after trouble-shooting. This technique will be applied to a larger sample set of genomic DNA to be amplified and sequenced. This study may set the stage for using the CFTR enhancer region as a biomarker of heart failure. In addition, it may provide preliminary data to the Bowles lab for the use of CFTR modulation in the treatment of heart failure.

Introduction

The CFTR gene encodes an ATP-binding cassette protein that is in an extensive family of transporters regulated by phosphorylation at specific sites. ATP-binding cassette (ABC) proteins are involved in the transportation of substrates across membranes using the energy derived from ATP, adenosine triphosphate. The protein structure of CFTR is shown in figure 1, reprinted from "CFTR Function and Prospects for Therapy" by John Riordan. A deletion of phenylalanine at F508, located in yellow on the lower left, is a mutation that is often linked with Cystic Fibrosis (CF) (Riordan 2008). This mutation can be heterozygous or homozygous and is present in most CF patients: 70 – 90% of CF patients have at least one deletion at this allele (Bisch et al. 2019). The mutation at F508 causes a misfolding of the protein, which leads to the irregular transport of chloride and sodium ions across the epithelial cell membranes (Riordan 2008). This results in an increased viscosity of airway surface fluid, leading to CF, the buildup of fluid in the lungs and difficulty breathing.



Riordan JR. 2008.
Annu. Rev. Biochem. 77:701–26.

Figure 1. Protein Structure of CFTR. F508 is the site of a deletion of a phenylalanine that leads to the misfolding of this protein. Misfolded or absent CFTR is the main cause of Cystic

When this CFTR gene is mutated, the subsequent protein becomes either mistargeted or dysfunctional. This is thought to play a critical role in the development of cystic fibrosis (Anderson, Sheppard, Berger, & Welsh, 1992). CFTR is also present in a variety of tissues – lung and intestinal epithelia have been heavily investigated, as well as tracheal and intestinal smooth muscle, despite being less studied. CFTR is present in heart tissue, although knowledge on this aspect of the protein has not been researched fully. CFTR has been shown to play a role in myocardial protection (Uramato, Okada, & Okada, 2012). Preliminary studies run by the Bowles lab has shown its presence

may be linked to heart failure, the motivation behind this study. This study will focus on the enhancer region of CFTR, exploring the molecular pathways of CFTR in the context of heart failure. Also, this study will support the foundation to validate CFTR therapeutics, a possible treatment of heart failure.

Solbach et al. hypothesize that ABC transporters, similar to CFTR, have altered expression in patients with heart failure (Solbach et al., 2008). The researchers used messenger ribonucleic acid (mRNA) quantification of all known ABC transporters to see if expression is altered; their results show multiple alterations, most relevant to this study that CFTR expression is significantly decreased in patients with heart failure when compared to non-failing controls (Solbach et al., 2008). Watson et al. used these previous findings to initiate a study of CFTR through molecular and genetic methods. Initial histological analysis of myocardium tissues was performed to confirm the results of Solbach et al. showing that mature CFTR protein is expressed less in failing hearts compared to non-failing hearts (Watson et al. 2018). 48 Duke Human Heart Repository (DHHR) participants were submitted for CFTR sequencing by an Ion Torrent assay. DHHR is one of the largest and most well-annotated human heart repositories in the world. Ion Torrent is a next generation sequence panel, comprehensively assessing the DNA sequence across all introns and exons to look for any notable differences. The assay was done by dividing the 48 DHHR participants into three groups: 1) no heart failure, 2) non-ischemic cardiomyopathy (NICM) and 3) ischemic cardiomyopathy (ICM). Ischemia is the restriction or blockage of blood flow to a tissue, and in this case, the heart muscle. Cardiomyopathy is a disease of heart muscle tissue, that often leads to heart failure due to dysfunction in the heart's pumping of blood to the rest of the body. If blockage or partial blockage occurs, as in the case of heart attacks and coronary artery disease, the heart muscle fails or weakens, what is defined as ischemic cardiomyopathy (Felker, Shaw, & O'Connor, 2002). Non-ischemic cardiomyopathy, the other type of heart failure, is not related to coronary artery disease, and is defined as mechanical or electrical dysfunction in the heart muscle that leads to failure (Bluemke,

2010). The study team hypothesized that there is a statistically significant difference in single nucleotide polymorphism data between failing and non-failing controls that will be returned after sequencing.

Results of the Ion Torrent assay showed that there is notable difference between failing and non-failing individuals in the CFTR enhancer region, possibly a source affecting expression differently in these patients (Watson et al. 2018). Figure 2 illustrates that CFTR seems to be less present in the failing myocardium then the non-failing one. This can be seen in the antibody staining of (A) that shows more CFTR expression, compared to the failing myocardium (B), which shows less overall expression. Next, western blots were run on left ventricular tissue samples to examine CFTR expression levels. Figure 3 shows that CFTR protein levels are reduced in both ischemic cardiomyopathy (ICM) and non-ischemic cardiomyopathy (NICM) compared to non-failing controls, similar to the results shown in 1A. Figure 3 also shows a separate western blot to analyze where CFTR is present in heart tissue in general: right ventricle and left ventricle expression of CFTR was consistently higher expression in non-failing patients over ICM and NICM patients. The highest levels of expression were observed in the right atrium of ICM patient samples (Watson et al. 2018). CFTR is also present in the aorta, left atrium, and right atrium, showing its low abundance but large spread across heart tissues.

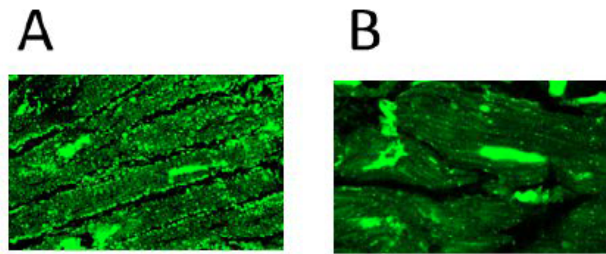


Figure 2. Immunofluorescence of myocardium stained with anti-CFTR antibody from the UNC Cystic Fibrosis Correction Core. A: non-failing, B: failing myocardial tissues. Failing myocardium shows less CFTR expression then non-failing.

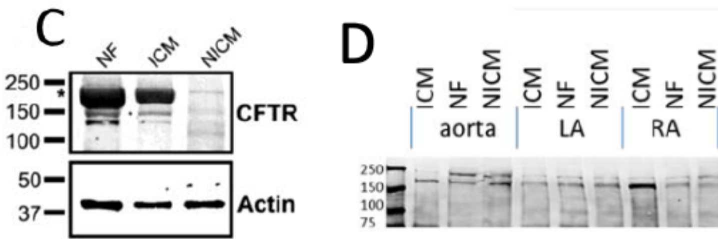


Figure 3. CFTR Protein levels are reduced in heart failure patients. (C) Western blot analysis of ICM and NICM shows reduced CFTR expression compared to non-failing controls. (D) Western blot analysis also shows CFTR is present in the aorta, left atrium, and right atrium.

The Ion Torrent assay performed by Watson et al. also highlighted a possible single nucleotide polymorphism (SNP) candidate in the CFTR enhancer region that may be associated with heart failure. Single nucleotide polymorphisms are variants in single base pairs of DNA sequences when comparing multiple sequences at a time. Here, Watson et al. found 27 SNP variants only in heart failure samples within the 48-sample set. One of these SNPs, labeled below with a blue arrow in figure 4, had only-in-heart-patient hits, meaning that this SNP appeared only in samples of patients with heart failure. Since this SNP is in the enhancer region of the CFTR gene, Watson et al. hypothesized that there may be differential CFTR protein expression between failing and non-failing human hearts (Watson et al. 2018).

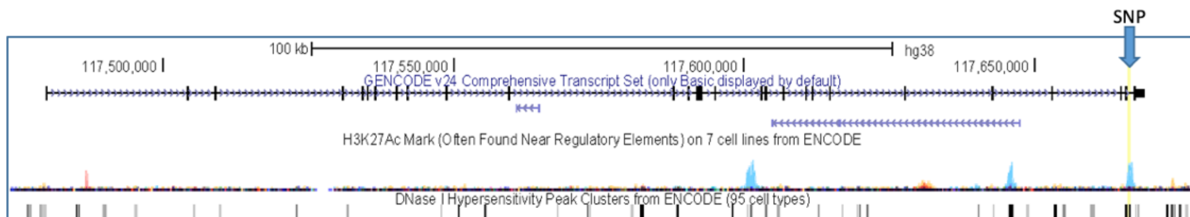


Figure 4. The CFTR enhancer region with a single nucleotide polymorphism that may be putatively linked with heart failure. Using Ion Torrent analysis, Watson and his colleagues obtained genomic sequences from 48 samples of non-failing and failing heart tissue from the Duke Human Heart Repository. 27 SNP variants appeared in their analysis, but one only appeared in heart patient samples alone, shown by the blue arrow.

These preliminary results show that ICM and NICM tissues show decreased CFTR expression, and Watson et al. highlighted a possible SNP candidate in the CFTR enhancer region. My PCR study aims to show if any SNPs in the CFTR enhancer region show any statistically significant differences between failing human heart tissues and non-failing human heart tissues.

Possible results may or may not corroborate the results of previous research showing CFTR to be at low abundance but present throughout the heart. Results may also show that in ICM right atria tissue, CFTR is higher in abundance compared to the corresponding tissue in NICM and non-failing controls. The next step of this investigation is to look at this difference in CFTR enhancer region in a larger sample set to test for statistical significance.

This independent study aims to continue investigating the role of CFTR in the context of heart failure. Specific aims of this study include testing the hypothesis that the differential expression of CFTR between failing and non-failing human hearts first shown by Watson and the Bowles lab is statistically significant in a larger, 750 patient, human heart tissue sample set. Also, this study will examine if the SNP highlighted in Figure 3 is significantly correlated to cardiomyopathies and heart failure in this larger sample set.

Materials and Methods

After looking at this preliminary data, as well as the 48 DHHR Ion Torrent Assay performed by Watson et al., I examined the CFTR enhancer region by polymerase chain reaction (PCR) for its association with heart failure. Polymerase Chain Reaction involves repeated heating and cooling of DNA to denature the two strands, separate them, and anneal free-floating nucleotides called deoxynucleotide triphosphates (dNTPs) to essentially replicate the two strands. This process is repeated for 35 cycles to amplify, or copy, the template DNA many times. A larger sample set from the DHHR aimed to provide a better test of statistical significance for differential expression between failing heart tissue and non-failing heart tissue. PCR amplified this region of interest, and gel electrophoresis will show the size of these products. I did this PCR with ICM, NICM, and non-failing control samples stored in the DHHR from 750 heart tissue samples. The products of the PCR will be

sent to a sequencing core at Duke University for Sanger Dideoxy sequencing and will be returned to the study team for analysis. These sequence results will also provide a look at the candidate SNP to see if failing heart tissue does differ significantly from healthy controls. The PCR used CFTR primer pairs flanking the enhancer region of interest, labeled CFTR right and CFTR left. First, the PCR protocol was optimized to get accurate amplification of the CFTR enhancer region.

PCR optimization involved ensuring sufficient amounts of template DNA, quality primers, and proofreading polymerases. DHHR template DNA is of high purity, used in multiple other genotyping studies and targeted sequencing. Also, I optimized my PCR protocol by altering the temperatures of each step to amplify the most amount of DNA possible. Initial temperatures were from the Taq PCR kit manual kit from New England Biolabs (NEB): initial denaturation at 95°C for 30 seconds, denaturation at 95°C for 15-30 seconds, annealing at 49°C for 1 minute, extension at 68°C for 1 minute, and final extension at 72°C for 5 minutes. These steps were repeated after extension for 30 cycles. 49°C was chosen for the annealing temperature since annealing is usually done around 5°C below the melting temperature of the primer (for CFTR right, this temperature is 54.2°C). Also, I used Taq polymerase from New England Biolabs initially in PCR optimization to allow for alterations in temperature; the more valuable Q5 proofreading polymerase from NEB will be used afterwards, and also on the 750-patient sample. Q5's polymerase ability to correct for base pair annealing errors is important in the amplification this study is attempting, since Taq's error will not allow for accurate amplification of the CFTR enhancer region.

Initial PCR concentrations were calculated using the manual given by NEB with the Taq PCR kit and altered slightly to account for DNA concentrations. The volumes and final concentrations are given in this table below for a 50 μL reaction:

PCR component	Volume	Final Concentration
Taq Buffer (10X)	5 µL	1X
Deoxynucleotide solution (10mM)	1 µL	200 µM
CFTR primers (100 µM)	1 µL (each)	0.2 µM
DNA template (10 ng/µL)	1 µL	1 ng/ 50 µL
Taq DNA Polymerase	0.25 µL	1.25 units/ 50 µL PCR
Nuclease-free water	40.75 µL	Bring reaction to final volume

Table 1. Initial concentrations used in the PCR reaction of this study. These concentrations were chosen from the Taq PCR kit provided by New England Biolabs, as they recommended. These were not the final concentrations after optimization.

These PCR concentrations and temperatures changed after optimization, and the new temperatures and concentrations are listed in the results below. These components will be placed in a 0.5 mL PCR tube on ice in a master mix. They were placed in separate reaction tubes and run through a MJ Research PTC-200 Thermal Cycler with the cycling conditions described above.

PCR products will be purified and quantified from these DHHR genomic DNA templates and will be submitted to the DNA sequencing core for Sanger Dideoxy sequencing. I intended to look at different options for sequencing that the Bowles lab had previously used, like Sanger Dideoxy and Ion Torrent, but the project did not progress far enough to sequencing examine these different routes for sequence analysis. Fundamentally, Sanger Dideoxy sequencing was planned after amplification, which digests DNA into fragments with labeled nucleotides. The fragments are separated by length by capillary gel electrophoresis, and then each label nucleotide is excited by a laser. The light emitted matches each base in the sequence with the correct nucleotide, giving the overall sequence (Sanger, Nicklen, & Coulson, 1977). Afterwards, the data of this sequencing will be returned to the study

team, allowing for analysis of any correlations between SNP data and patient information in the context of heart failure. Also, this sequencing analysis will show any statistically significant SNPs in the CFTR enhancer region of interest with the increased sample set.

Results

Challenges arose in the initial runs of PCR on this genomic DNA; little to no product was present when analyzed through gel electrophoresis. Despite assessment of temperatures and techniques with PCR runs with standard plasmid and genomic DNA, the gels with the genomic DNA from still showed very opaque bands. Multiple practice PCR runs were performed successfully with plasmid DNA with different temperatures and concentrations of template DNA, since plasmid DNA concentrations are higher than genomic DNA concentrations when doing PCR. Most of the study I trouble-shot my PCR protocol to find the best temperatures for denaturation, annealing, and extension. I found a different set of temperatures then the initial runs worked best for product, along with the advice of Chunbo Wang, Ph.D., also in the Bowles lab. We also changed the concentrations of PCR components, displayed below.

Changing the protocol of the PCR improved the yield of the amplified product. We found the best temperatures for higher product were an initial denaturation at 94°C for 5 minutes, denaturation at 94°C for 40 seconds, annealing at 56°C for 1 minute, extension at 72°C for 20 seconds, and final extension at 72°C for 7 minutes. Q5 as the polymerase was also used instead of Taq. Taq was not successful in any of the previous PCR runs, and Q5's proofreading capabilities may have provided the difference in yield when our final PCR runs and gel electrophoresis results were successful. Both Taq and Q5 are polymerases that have similar properties in PCR, so it is not understood why Taq was

not successful – Q5 in this technique is proven to work, however. The concentrations of the protocol were altered with the addition of Q5 for a 25 μL reaction:

PCR component	**Volume**	**Final Concentration**
Q5 mix (dNTPs, Buffer, & Q5 Polymerase)	12.5 μL	1X
CFTR primers (100 μM)	0.5 μL (each)	0.5 μM
DNA template (10 ng/μL)	2 μL	20 ng/ 25 μL
Nuclease-free water	9.5 μL	Bring reaction to final volume of 25 μL

Table 2. Final concentrations used in successful PCR. These concentration values are taken from Q5 PCR kit protocol provided by NEB.

These volumes are chosen as instructed by the NEB protocol for the Q5 master mix, and provided the best results for the PCR reaction. The study team and I concluded that 2 μL of template DNA worked for the PCR reaction, different from the 1 μL recommended by the PCR kit.

Chunbo Wang, Ph.D. did successfully run PCR on 6 samples from the 48-sample set, shown in Figure 5. This result from the gel electrophoresis shows sharp bands of DNA around 300 base pairs in length in comparison to a Quick-Load Purple 1 kilobase DNA ladder in the first lane. Clearly this PCR technique is achievable and can be replicated. Chunbo Wang, Ph.D. and the Bowles team

will continue to apply this PCR protocol to more samples in the 48-sample set to ensure the success of amplification and replicate the results of the Ion Torrent Assay conducted by Watson and his colleagues. Next, the project will expand to the 750-sample set of human heart tissue stored in the DHHR, and amplified product will be sequenced and returned to the study team. The results of this study show that PCR can be optimized for this genomic DNA, and can successfully amplify the enhancer region for sequence analysis.

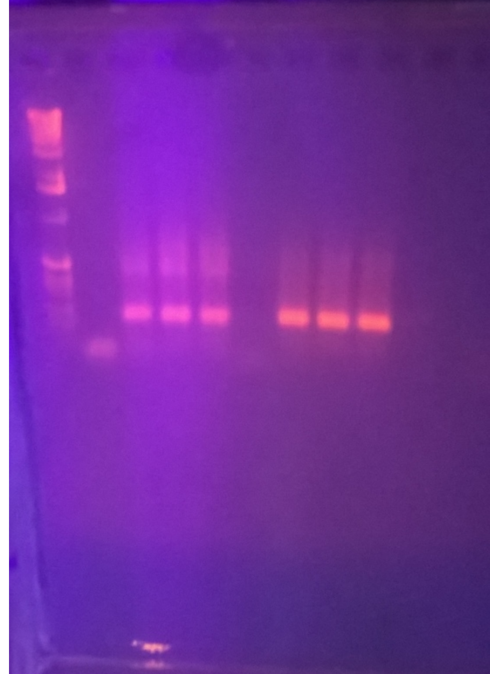


Figure 5. Successful PCR run and gel electrophoresis result on 6 samples from the 48 sample set of human heart tissue from the Ion Torrent assay. The gel was analyzed through UV spectroscopy. The amplified product presents at around 300 bp.

Discussion

The outcomes of this PCR study further advance the project's goal of understanding the CFTR enhancer region's role in cardiovascular function. Although the aim of statistical analysis of the candidate SNP mentioned above is not finished, the Bowles lab will continue this research and obtain sequence data to answer this aim. Thus, interpretation and implications of the results of this study will occur once sequencing analysis will be finished.

Future directions with this study could be to use different methods of PCR, like real-time PCR. Also called quantitative PCR, real-time PCR is a method to quantitatively assess the size of the product by monitoring with a fluorescent dye. By labeling the products, we can measure the accumulation of a fluorescent signal for precise quantification of the amount of PCR product, eliminating the need for gel-electrophoresis. This method of amplification may be useful since this

study involves large amounts of PCR runs on 750 patient samples, and running gel electrophoresis on each sample may prove time-consuming. The Bowles lab may use TaqMan real-time PCR as an option for this study in the future.

For sequencing, methods other than just Sanger Dideoxy sequencing may help analysis after PCR amplification. Ion Torrent assay is a next-generation sequencing method that uses semiconductors and high-throughput methodology to rapidly construct sequences of DNA from as little as 10 ng of template DNA. Watson and his colleagues used this method for the preliminary research before this study (Watson et al. 2018). This system is done by identifying genetic alterations, single nucleotide polymorphisms, insertions, deletions, copy number variations and large genomic rearrangements (Ion Torrent). Ion AmpliSeq can target sequences for greater specificity, focusing on a single gene of interest when sequencing. This specificity is helpful for our study, because we focus solely on the enhancer region of CFTR in this study. One issue we have considered is Ion Torrent's cost – the assay is fairly expensive and may not be within the grant's budget in the future of this study.

If CFTR does show differential expression in failing and non-failing heart tissue, this may prove a useful diagnostic tool, even a possible treatment for heart failure by modulation of CFTR. A preliminary model system has been established using human cardiac tissue from the DHHR to examine the properties of CFTR in the heart and its susceptibility to modulation by pharmacological methods (Bowles 2018).

One pharmacological method is CFTR modulators; this study could validate and support the use of these as therapeutics for heart failure. There are multiple modulators that have been approved to be used in the clinic. Ivacaftor (developed as VX-770, tradename Kalydeco) has been shown to reduce developed tension of the heart (Figure 6). This was done using a "tissue bath system" and exposed to VX-770, a CFTR potentiator. Figure 6 illustrates that there is a 16.4% decrease in average peak height (2 μM, n=7). NICM tissues show the most reduction, although only two tissues were examined, so these results are not conclusive. This PCR study could further show that CFTR therapeutics are worth investigating as an effective treatment for heart failure.

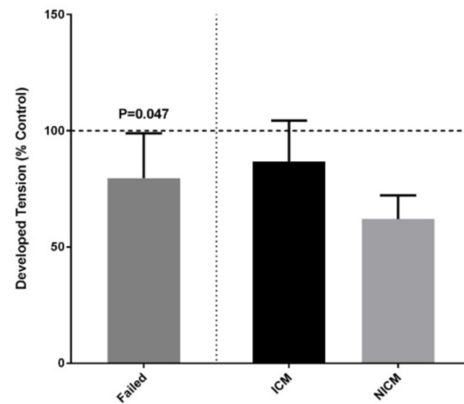


Figure 6. Heart tissue treated with VX-770, a CFTR potentiator. This result shows a 16.4% decrease in developed tension in these tissues.

In conclusion, this study shows decreased CFTR expression in failing heart tissue compared to non-failing heart tissue by histological evaluation as well as western blot analysis, and preliminary results from an assay of 48 sample set of human heart tissue from the Duke Human Heart Repository show a differential expression between failing tissue and non-failing tissue. This differential expression may be due to a candidate SNP in the enhancer region of CFTR. Future work will carry out this investigation of the enhancer region to a sample set of 750 participants, also tissue samples from the DHHR, to see if any statistically significant differences appear in expression. PCR products will be purified, quantified, and sent for Sanger sequencing. Results of this study may show that CFTR therapeutics is a valid path to the treatment of heart failure.

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