HUMAN DISEASE-CAUSING PROTEIN HOMOLOGS IN *NAEGLERIA* AMOEBA

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INTRODUCTION

When humans contract Primary Amebic Meningoencephalitis (PAM) from freshwater amoeba *Naegleria fowleri*, death is almost always imminent. From 1962 to 2021, only 2.60% of people in the United States survived the infection, in part because there is no established mechanism to manage or cure PAM. In quest of potential drug targets, we have conducted a BLAST homolog search against human BRCA2 and CFTR genes because both gene products are involved in cell proliferation. The search identified CFTR (cystic fibrosis transmembrane conductance regulator)-like and BRCA2 (breast cancer susceptibility gene 2)-like genes in *N. fowleri* and *Naegleria gruberi*, a nonpathogenic relative. In humans, a mutation in CFTR, the membrane ion channel, results in cystic fibrosis, while a mutation in BRCA2, a member of DNA repair machinery, increases the risk of developing malignant neoplasia such as breast cancer.

Due to safety reasons *N. gruberi* was used for further analyses and biological assays. Protein sequence alignments revealed that human CFTR has 29.53% and 31.35% identities with the CFTR-like protein in *N. gruberi* and *N. fowleri*, respectively, and human BRCA2, on the other hand, has 29.66% and 27.27% identities with *N. gruberi* and *N. fowleri*, respectively. With this level of variation between the proteins in humans and the amoeba, eventual targeting of these gene products may have fewer adverse effects in humans. Importantly, our phosphoproteomics data also show the CFTR-like protein in both *Naegleria* species is phosphorylated at the common serine residue, which is not conserved in human CFTR. Currently, we are investigating the differential expressions of CFTR-like and BRCA2-like gene transcripts in *N. gruberi* by quantitative PCR.

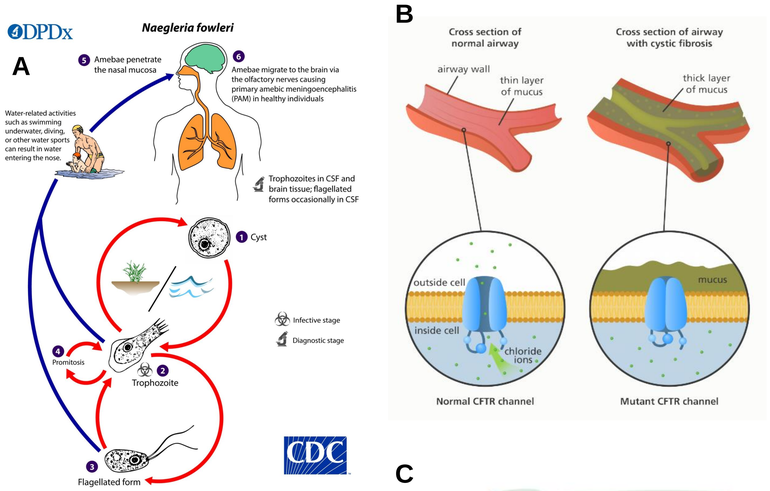

Figure 1. *Naegleria fowleri* Infection Process and Function of Target Genes
(A) This visual depicts the three-stage life cycle of *Naegleria*, as well as trophozoite infection modes and pathways.
(https://www.cdc.gov/parasites/naegleria/pathogen.html)
(B) This model shows the result of the mutated CFTR protein. Chloride ions attract a layer of water which in turn causes cilia hairs to clear out mucus. When the ions are not able to cross the plasma membrane, thick mucus builds up.
(https://www.yourgenome.org/facts/what-is-cystic-fibrosis/)
(C) This diagram represents the general sequence of events that lead to the development of cancer when a tumor suppressor gene is inactivated, as in the case of BRCA2.
(https://www.ck12.org/book/ck-12-biology-advanced-concepts/section/8.25/)

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RESULTS

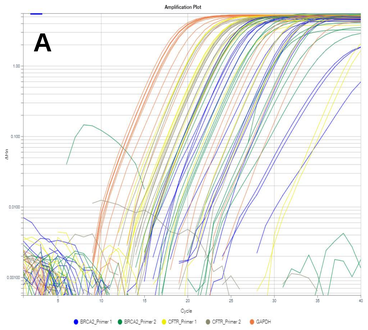

Figure 2. qPCR cDNA Amplification Plot and Melting Curves
(A) This plot shows the real-time amplification of the target gene transcripts with the listed primer sets. (B) BRCA2_Primer 1 Melting Curve (Blue). (C) CFTR_Primer 1 Melting Curve.(Green) (D) CFTR_Primer 2 Melting Curve (Yellow). (E) CFTR_Primer 2 (Gray) and GAPDH (Red) Melting Curves. Each primer set produces unique amplicons that have a specific melting temperature, suggesting non-specific PCR products are present.

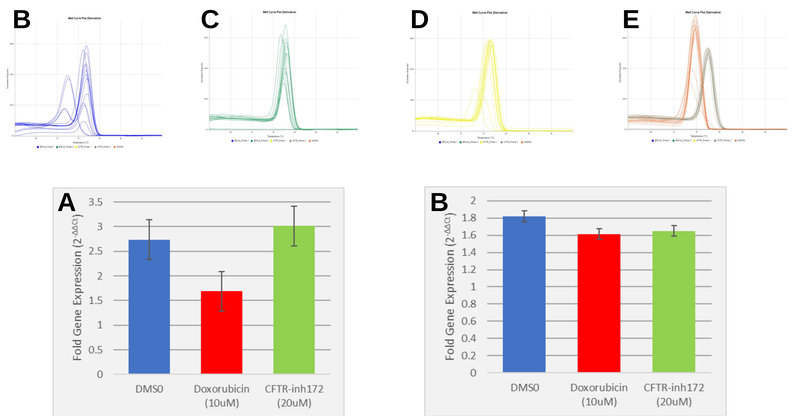

Figure 3. qPCR Fold Gene Expression Experiment Results – Drug Targets
(A) Expression of BRCA2-like gene transcript. (B) & (C) Expression of CFTR-like gene transcript detected by CFTR Primer Set 1 (B) or CFTR Primer Set 2 (C).

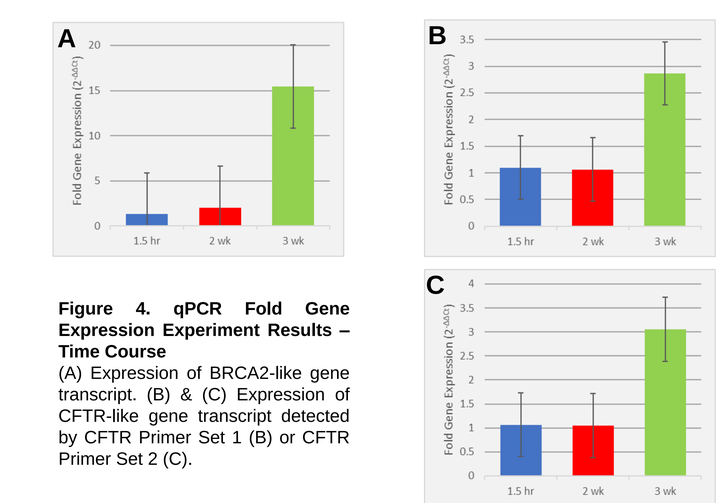

Figure 4. qPCR Fold Gene Expression Experiment Results – Time Course
(A) Expression of BRCA2-like gene transcript. (B) & (C) Expression of CFTR-like gene transcript detected by CFTR Primer Set 1 (B) or CFTR Primer Set 2 (C).

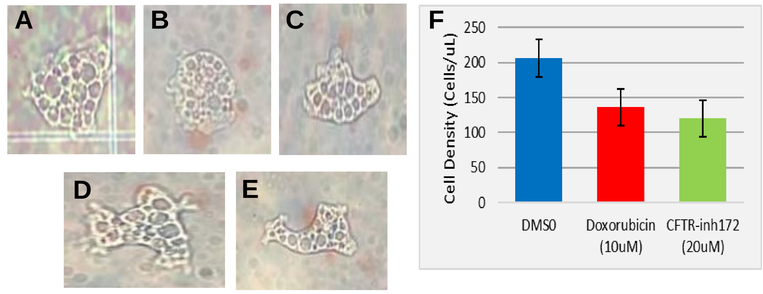

Figure 5. Compound Light Microscope Images of *N. gruberi* and Treated Cell Density
Images captured concurrently with cell density data. (A) Amoeba treated with DMSO solvent after 24 hours. (B) (C) Amoeba treated with 10μM Doxorubicin after 24 hours. (D) (E) Amoeba treated with 20 μM CFTR inh172 after 24 hours. (F) Cell density (Cells/μL) after treatment with DMSO, Doxorubicin, and CFTR-inh172.

MATERIALS AND METHODS

Cell culture *N. gruberi* (strain NEG-M, ATCC#30224) was cultured and maintained in petri dish at room temperature in media that consists of 2% Bacto™ casitone, 10% FBS, 1% yeast extract, and penicillin-streptomycin (50U/ml)
Pharmacological studies Doxorubicin (MedChemExpress, HY-15142) at 10 μM or CFTR inh-172 (MedChemExpress, HY-16671) at 20 μM was introduced to $0.5x10^3$ *N. gruberi* cells, with DMSO solvent serving as a control. Cell numbers were counted using a hemocytometer after 24 hours.
RNA Purification & cDNA Synthesis *N. gruberi* cells were collected and washed with Chalkley's solution (0.006% $CaCl_2$, 0.1% NaCl, and 0.004% KCl) once. The cells were lysed and stored in TRIzol (ThermoFisher Scientific) at -80ºC until further use. PureLink RNA Mini Kit (ThermoFisher Scientific) was used to isolate and purify total RNA by following the manufacturer's instructions. After centrifugation of cell lysates in TRIzol, 450 μL of the aqueous phase was added with ethanol to a fresh RNase-free Eppendorf tube. RNA was then bound, washed, and eluted in 30μl H_2O. Transcriptor High Fidelity cDNA Synthesis Kit (Roche. Cat. No. 05-081-955-001) was used to synthesize cDNA by following the manufacturer's instructions. Primer annealing was done by incubating total RNA and primers (random hexamer and anchored-oligo($dT)_{18}$) at 65ºC for 10 min and followed by immediate chilling on ice. Reaction buffer, RNase inhibitor, dNTPs, DTT, and Reverse Transciptase were added to the primer annealed RNA. Reverse transcription was then conducted at 50ºC for 20 min, followed by denaturation of the enzyme at 85ºC for 5 min. cDNAs were stored at -80ºC until use.
qPCR SYBR Green qPCR Master Mix (Intact Genomics) and 5μl of cDNA were used for qPCR reaction in QuantStudio 5 system (ThermoFisher Scientific) with 40 cycles of 95ºC (15 sec) and 60ºC (30 sec), using the below primer sets, followed by a melting curve step:

CFTR Primer Set 1 (forward): GGTCTGGTTGGTTCTGGTAAA
CFTR Primer Set 1 (reverse): CCAGGCTTGTTGAGGAACATA
CFTR Primer Set 2 (forward): TTGGTGGGAGCTGTAGTTATTC
CFTR Primer Set 2 (reverse): GACAATGACAGACCAGCCATA
BRCA2 Primer Set 1 (forward): GTATCCGCCTCGAATAGAACTAAC
BRCA2 Primer Set 1 (reverse): CTTCAGTTGGTGGTGGTCTATC
BRCA2 Primer Set 2 (forward): CCACCACCAACTGAAGGATT
BRCA2 Primer Set 2 (reverse): CTGTTGCAGGTGGACGATTA

Cell imaging Olympus CH compound microscope was used to capture the images of *N. gruberi* cells treated with DMSO, Doxorubicin, or CFTR-inh172.

CONCLUSIONS

- CFTR-like and BRCA2-like genes were identified in *Naegleria* genomes, and their gene expressions were confirmed.
- Generated qPCR primers which yielded specific amplification of target gene transcripts
- Cell Density data indicated Doxorubicin and CFTR-inh172 negatively affected *N. gruberi* growth at these concentrations.
- The Time Course study displayed elevated gene expression in BRCA2-like and CFTR-like genes at Week 3.

FUTURE DIRECTIONS

- Identify housekeeping gene qPCR primers that will not be affected by a time course.
- Investigate if BRCA2 pathway is conserved in *Naegleria*.
- Determine if Rad-51 is present in *Naegleria*.
- Identify more potential drug targets based on protein homology.
- Determine localization of Doxorubicin; verify if this drug targets the nucleus.
- Determine if CFTR-inh172 binds to CFTR-like protein; investigate receptor activity.