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Phenotypic assessment of Cox10 variants and their implications for Leigh Syndrome

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Abstract

Objectives Cox10 is an enzyme required for the activity of cytochrome c oxidase. Humans who lack at least one functional copy of Cox10 have a form of Leigh Syndrome, a genetic disease that is usually fatal in infancy. As more human genomes are sequenced, new alleles are being discovered; whether or not these alleles encode functional proteins remains unclear. Thus, we set out to measure the phenotypes of many human Cox10 variants by expressing them in yeast cells.

Results We successfully expressed the reference sequence and 25 variants of human Cox10 in yeast. We quantitated the ability of these variants to support growth on nonfermentable media and directly measured cytochrome c oxidase activity. 11 of these Cox10 variants supported approximately half or more the cytochrome c oxidase activity compared to the reference sequence. All of the strains containing those 11 variants also grew robustly using a nonfermentable carbon source. Cells expressing the other variants showed low cytochrome c oxidase activity and failed to grow on nonfermentable media.

Keywords *Saccharomyces cerevisiae*, Cox10, Cytochrome c oxidase, Leigh syndrome

Introduction

Leigh Syndrome is a rare genetic condition characterized by progressive neuromuscular defects. This disorder is highly heterogeneous, due in part to the fact that it can be caused by alterations in at least 75 different genes. All of these genetic alterations lead to mitochondrial dysfunction including reduced or eliminated capacity for oxidative phosphorylation [1]. Notably, loss of function in one of these genes, *COX10*, leads to a severe form of Leigh Syndrome that is typically fatal in the first year of life (Table 1). Two patients have been reported to survive longer, albeit with significant pathology [2, 3].

The Cox10 protein is highly conserved in the budding yeast *Saccharomyces cerevisiae*; indeed, expression of the human *COX10* gene in yeast can fully restore function in a strain missing its endogenous *COX10* gene [4]. The Cox10 enzyme is located on the mitochondrial inner membrane and catalyzes the farnesylation of heme [5]. This modified heme group is incorporated as an essential prosthetic group into the cytochrome c oxidase (COX) enzyme, which catalyzes the transfer of electrons from cytochrome c to molecular oxygen.

Eleven different point mutations in the human *COX10* gene have been described in the literature as leading to disease in humans (Table 1). Of course, there are many more alleles in the human population and for the majority

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Table 1 Known pathological variants of human COX10

M1X	Lethal	[6]
T14I/T377I	Pathogenic	[3]
T196K/P225L	Lethal	[7]
N204K	Lethal	[8]
N204D	Lethal	[9]
G288R	Lethal	[10]
D336V/D336G	Lethal	[7]
D336V/R339W	Pathogenic	[2]
P420L	Lethal	[3]

of these we have no direct evidence as to whether or not they encode functional proteins. As of June 17, 2024, the ClinVar database lists 102 known variants of the Cox10 protein, of which nearly three-quarters are of "uncertain significance". We have selected 25 *COX10* alleles for characterization in yeast. This additional knowledge should be useful to clinicians and genetic counselors faced with patients carrying these variants.

Methods

Expression of human COX10 alleles in yeast

A cDNA encoding the reference sequence of human Cox10 was directly synthesized by Eurofins Genomics. To allow for proper expression, this construct included 500 bp upstream and 100 bp downstream of the yeast *COX10* gene. Additionally, three copies myc epitope tag just before the stop codon were included along with *EcoRI* sites to facilitate cloning. This DNA was inserted into the *EcoRI* site of YEplac195 [11] for expression in yeast. The resulting plasmid was either used directly or mutated by whole-plasmid PCR using the Q5 site-directed mutagenesis kit (New England Biolabs) so that it encoded the various alleles. Oligonucleotides used for mutagenesis are listed in Table S1. Plasmids were sequenced to confirm successful mutagenesis. The *COX10* gene of BY4741 strain of *S. cerevisiae* was replaced with the kanMX6 gene [12] using the oligonucleotide primers shown in Table S1. Wildtype and mutant plasmids were transformed [13] into the *cox10Δ* yeast for phenotypic analysis.

Growth assessment

Standard yeast media and growth conditions were used throughout these experiments [13]. Ten-fold serial dilutions of freshly grown yeast were plated onto rich media containing either 2% glucose or 3% glycerol and allowed to grow at 30°C for three days before photographing.

COX assays

Yeast were grown overnight at 30°C with shaking in 15 mL of rich media using 3% raffinose as a carbon source. Raffinose supports growth by cells with and without functional oxidative phosphorylation but does not suppress mitochondrial production like glucose [14]. Cells were washed and lysed in 500 µL cold SH buffer (0.6 M sorbitol, 25 mM HEPES pH 7.4) by vortexing with glass beads for five minutes. Unlysed cells were pelleted by centrifugation at 600 g at 4°C for five minutes, twice. Mitochondria were pelleted by centrifugation at 16,000 g at 4°C for ten minutes and were resuspended in 200 µL of SH buffer [14]. 20 µL of each sample was incubated with 125 µg of reduced cytochrome c in 25 mM potassium phosphate pH 6.2. Absorbance was measured at 550 nm every five seconds for one minute to calculate the rate of cytochrome c oxidation [15]. Reaction rates were normalized to the amount of protein in the lysate as found by the BCA reaction (Pierce). Each sample was measured at least four times.

Results and discussion

We selected 25 human *COX10* alleles whose functions are not clearly understood and expressed them in yeast lacking the endogenous *COX10* to determine how functional the encoded proteins are. This species grows efficiently by anaerobic metabolism and therefore can survive without functional Cox10 protein when provided with a fermentable carbon source like glucose (Fig. 1A, left column). However, carbon sources like glycerol can only be utilized aerobically and thus require a functional Cox10 enzyme. On this fuel source, cells lacking Cox10 do not survive but they thrive if they contain either the yeast or human reference sequence *COX10*. Strains with certain *COX10* alleles, such as S103A or V356M, survive equally as well as those with the reference sequence *COX10*. Other strains, such as those with I127T or Q322P, fail to grow when cultured on glycerol (Fig. 1A, right column), indicating that these protein variants are nonfunctional.

We also directly measured the COX activity in each of these yeast strains. We found that the yeast *COX10* supports slightly more COX activity than the human reference sequence *COX10*, in agreement with a previously published observation [4]. Yeast strains with certain human *COX10* alleles show activities that are close to that provided by the reference sequence while others show very low activities (Fig. 1B).

Unsurprisingly, there is a strong, general correlation between the COX activity measurements and the ability to grow on glycerol. It appears that Cox10 variants that allow approximately 50% of the reference sequence COX activity (see variants S103A, P104L and A328T) grow very efficiently on glycerol and those that allow less than 25% of the reference sequence COX activity (such as I127T and D132Y) fail to grow at all (Fig. 1).

Among the 16 assayed alleles classified as "uncertain significance" by the ClinVar database [16], we find that six (S103A, D152Y, A174T, F209L, C343R, V356M) encode functional proteins. Four alleles are classified as having "conflicting classifications of pathogenicity"; we find that

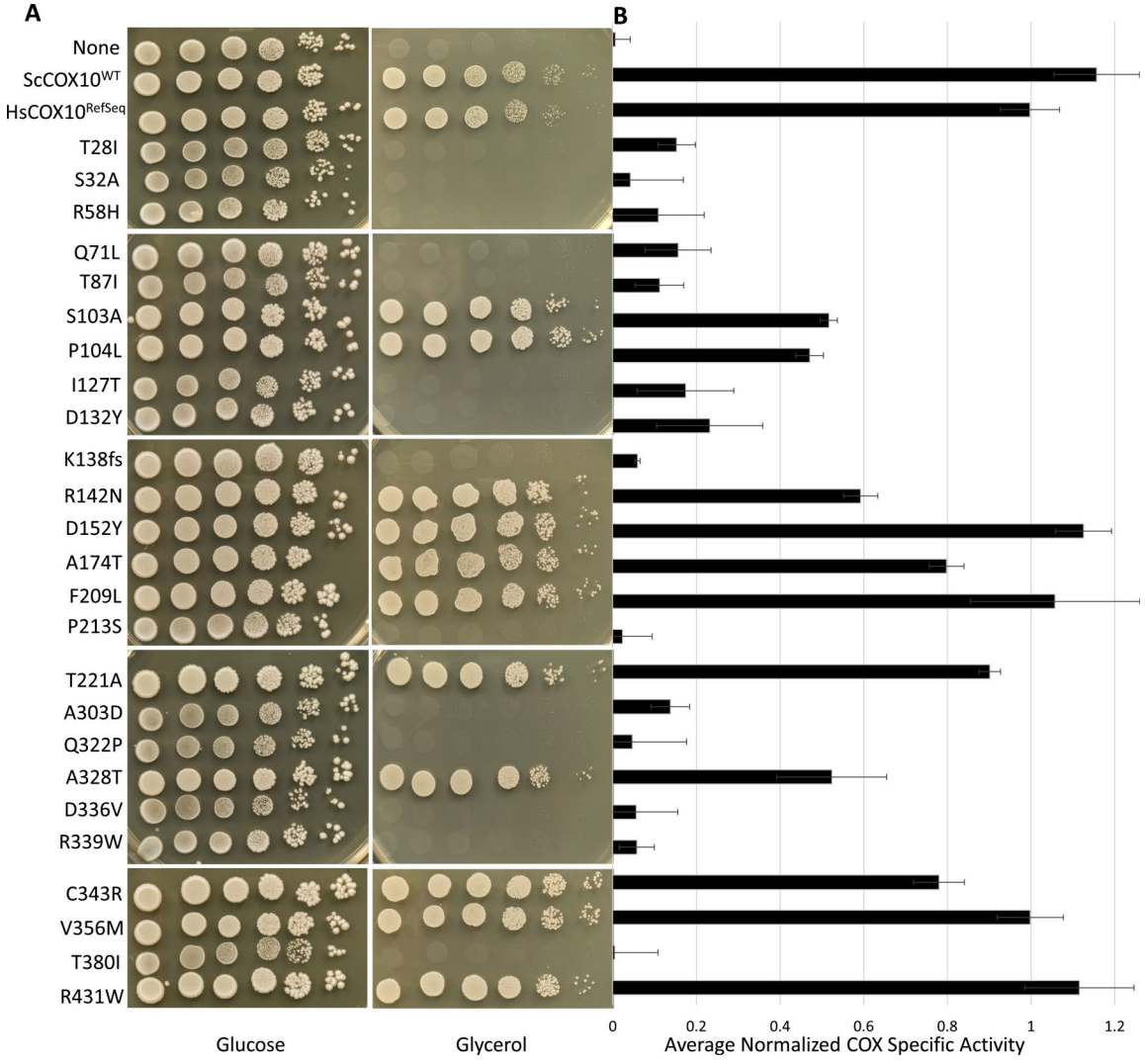

Fig. 1 Activity of human *COX10* variants. (**A**) Ten-fold serial dilutions of yeast cells carrying the indicated *COX10* variant or an empty vector were plated on rich media with either glucose or glycerol as the carbon source. (**B**) The same strains were lysed and COX activity was measured. The resulting specific activities were normalized to the sample containing the human *COX10* reference sequence. Error bars show one standard deviation

three of these alleles (P104L, A328T and R431W) encode functional proteins and one (T87I) does not. T221A is classified as "likely pathogenic" and T28I is classified as "benign/likely benign"; our data disagree with these two predictions. We believe that our in vivo measurements of the activity of these alleles will provide important, actionable information for clinicians and genetic counselors who may come across patients with these alleles in their practices.

The D336V and R339W alleles were selected for a slightly different reason. Pitceathly et al. have reported that a human with these two alleles survived to adulthood and that both alleles encode nonfunctional proteins [2]. We thought that using more sensitive enzyme assays might reveal partial activity from one or both proteins but found that both are fully nonfunctional (Fig. 1). Recently, a second patient with unusual *COX10* alleles has been described who survived past infancy [3]. This patient is homozygous for two alleles, T14I and T377I. The second variant is highly similar to the T380I variant that we found to be nonfunctional in that both alterations are located in the sixth transmembrane domain [17], strongly suggesting that the patient's Cox10 protein will be nonfunctional. Thus, it remains unclear how either patient has survived when most people with nonfunctional Cox10 variants die in infancy (Table 1). Perhaps specific alleles of other genes found in these patients can partially suppress the loss-of-function in *COX10*. Identifying these genes could potentially point to interventions to treat Leigh Syndrome patients and is an area that our laboratory is pursuing.

Limitations

Despite the strong evolutionary conservation in oxidative phosphorylation mechanisms, it is possible that these variants will have a different level of activity in humans compared to yeast cells.

Abbreviations

COX Cytochrome c oxidase

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13104-024-06879-5.

Supplementary Material 1

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Author contributions

SDJ designed the study and supervised the work. TSV, EBL, AM, KM, RD, NEO and SDJ performed the experiments and analyzed the resulting data. All authors read and approved the final manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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