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Mitochondrial tRNA$^{Leu(CUN)}$ A12307G variant may not be associated pancreatic cancer

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ABSTRACT. Mitochondrial DNA mutations that lead to mitochondrial dysfunction have long been proposed to play important roles in the development of pancreatic cancer. Of these, alterations to mitochondrial tRNA genes constitute the largest group. Most recently, a variation at position 12307 in the gene encoding tRNA$^{Leu(CUN)}$ has been reported to be associated with this disease. However, the molecular mechanism underlying this relationship remains poorly understood. To assess this association, we evaluated this variant by evolutionary conservation analysis, measurements of allelic frequencies among control subjects, and use of several bioinformatic tools to estimate potential structural and functional alterations. We found this residue to have a high conservation index; however, the presence of the A12307G variation in control subjects revealed by a literature search suggested it to be common in human populations. Moreover, RNAfold results showed that this variant did not alter the secondary structure of tRNA$^{Leu(CUN)}$. Through the application of a pathogenicity scoring system, this variant was determined to be a "neutral polymorphism," with a score of only 4 points based on current data. Thus, the contribution of the

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A12307G variant to pancreatic cancer needs to be addressed in further experimental studies.

Key words: Pancreatic cancer; Mitochondrial DNA; A12307G; Variant; Pathogenic

INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer-related mortality among men and women in the USA, with 43,140 new cases and 36,800 deaths having been reported in 2010 (American Cancer Society, 2010). The five-year survival rate for patients suffering from this cancer is a mere 5%, emphasizing the need for a detailed understanding of the molecular changes underlying this disease, and the application of this knowledge to early diagnosis and therapeutic intervention. It is now generally believed that genetic variants play important roles in pancreatic cancer, for example, polymorphisms in *NAT* 2 and *XRCC4* genes are believed to be associated with this malignancy (Ding and Li, 2015; Liang et al., 2015). Nevertheless, few studies have focused on the relationship between mitochondrial DNA (mtDNA) mutations and pancreatic cancer.

As early as 1956, Warburg (1956) proposed that an important contributing factor to carcinogenesis was the alteration of oxidative phosphorylation (OXPHOS) owing to injured mitochondria. Human mtDNA is a double-stranded, circular DNA molecule of 16.5 kb, including genes encoding electron transport chain proteins (complexes I-IV), ATP synthase (OXPHOS complex V), 2 rRNAs, and 22 tRNAs, and featuring a D-loop region (Wallace and Chalkia, 2013). Mitochondrial dysfunction is a common feature of cancer cells. Somatic mutations of mtDNA have been reported in a variety of cancers, including pancreatic cancer (Jones et al., 2001; Taylor and Turnbull, 2005; Navaglia et al., 2006). These sequence variations, such as intragenic deletions and missense mutations, have been identified in nearly every type of tumor.

Most recently, Lam et al. (2012) investigated sequence variants in the mitochondrial genomes of 286 pancreatic cancer patients and 283 healthy controls using the GeneChip CustomSeq resequencing array. Based on their findings, a homoplasmic A12307G variation in the gene encoding $tRNA^{Leu(CUN)}$ was established as being associated with pancreatic cancer ($P < 0.05$). However, to date, little is known regarding the pathogenic role of the A12307G variant. In this study, we used a phylogenetic approach to determine the deleterious influence of this sequence variation and examined its potential functional effects.

MATERIAL AND METHODS

To reevaluate the dataset, we extracted clinical, genetic, and molecular data from Lam et al. (2012). For comparison, we incorporated previous publications (if any) concerning occurrence of the A12307G variant, to evaluate its frequency and pathogenicity in relation to mitochondrial disease.

To understand the molecular mechanism of this variation in cancer, we first performed a phylogenetic evaluation to calculate the corresponding conservation index (CI), as evolutionary conservation analysis is important in determining the pathogenicity of a candidate tRNA mutation. In addition, A12307G allele frequencies were examined by using

the MITOMASTER program to screen mitochondrial genome sequence variations in the National Center for Biotechnology Information database (www.ncbi.nlm.nih.gov; Brandon et al., 2009).

Effects of the A12307G variant on the secondary structure of tRNA$^{Leu(CUN)}$ were estimated with the RNAfold program, which predicts the minimum free energy (MFE) of a tRNA from its sequence (Gruber et al., 2008). Finally, we applied a pathogenicity scoring system to the A12307G sequence variation. Based on this, a variant is classified as a "polymorphism" if scoring < 6, "possibly pathogenic" with a score between 7 and 10, and "pathogenic" if it scores > 11 (Yarham et al., 2010, 2011).

RESULTS

Diagnostic pitfall of the A12307G variant

Lam et al. (2012) recruited 532 pancreatic cancer patients and 1701 controls to screen common and rare mutations in mitochondrial genomes. However, the selection of sampling method was rather problematic, as the frequency of mtDNA mutations may not be the same in tissues as it is in blood. Thus, cancer tissue specimens should be used, rather than blood samples. From the data, we observed that the A12307G variation was detected in 34 healthy control subjects, with an allele frequency of 13.7%, suggesting that it is common in humans.

MITOMASTER results

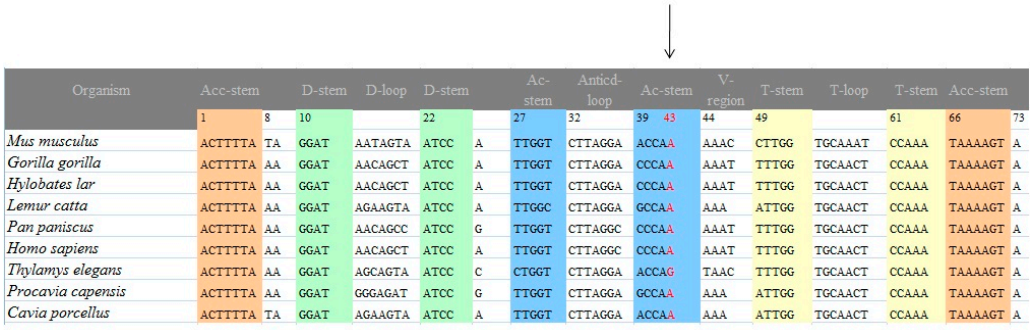
To assess A12307G allelic frequencies, we used the MITOMASTER program to evaluate reported records in GenBank (Brandon et al., 2009). We identified three mtDNA genome sequences containing the A12307G variant in PubMed Central, with accession Nos. JQ706026.1, JQ706032.1, and JQ706045.1 (Table 1). After careful review, we noticed that the complete set of mtDNA sequence variations belonged to human mitochondrial haplogroup U2d (van Oven and Kayser, 2009). Of these variants, some are apparently pathogenic. For example, the *ND2* G5460A mutation is considered to be associated with metabolic disease (Saxena et al., 2006), while the A to G transition at position 8296 in the gene encoding tRNA^{Lys} has been implicated in mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes, and myoclonic epilepsy with ragged-red fibers (Arenas et al., 1999; Sakuta et al., 2002). The common T16189C variant in the D-loop region has been found to play an important role in the development of metabolic syndrome (Palmieri et al., 2011). Moreover, the homoplasmic *CYTB* T15287C mutation has been reported to increase the penetrance and expressivity of aminoglycoside-induced and non-syndromic hearing loss (Janssen et al., 2006). Finally, the A11467G and G12372A mutations identified have been shown to alter brain pH (Rollins et al., 2009). Thus, it appears that while other mutations may play important roles in pancreatic cancer, the A12307G variant does not.

Evolutionary conservation analysis of the A12307G variant

For an insight into the pathogenicity of the A12307G variant, we analyzed the CI of this residue. As shown in Figure 1, this position was highly conserved across different species (CI = 90%).

Table 1. Complete set of mitochondrial DNA sequence variations in patients carrying the A12307G mutation.

JQ706026.1	Variant	JQ706032.1	Variant	JQ706045.1	Variant
D-loop	A73G	D-loop	A73G	D-loop	A73G
	T152C		T152C		T152C
	A188G		T199C		T199C
	T199C		T471C		T471C
	T471C		A16051G		A16051G
	A16051G		T16093C		A16183C
	A16183C		C16148T		T16189C
	T16189C		C16184T		C16234T
	C16234T		T16189C		C16266T
	C16294T		C16234T		C16294T
	T16519C		C16294T		T16519C
12S rRNA	A750G		T16342C	12S rRNA	A750G
	A1438G	12S rRNA	A750G		T1040C
16S rRNA	T1700C		A1438G	16S rRNA	T1700C
	A1811G	16S rRNA	T1700C		A1811G
	A2706G		A1811G		A2706G
ND1	C4025T		A2706G	ND1	C4025T
ND2	A4769G	ND1	C4025T	ND2	A4769G
	G5147A	ND2	A4769G	CO1	G6023A
	G5460A	CO1	C7028T		C7028T
CO1	T6956C	A6/A8	G8557A		A7277G
	C7028T	A6	A8860G	CO2	A7894G
$tRNA^{Lys}$	A8296G		A8938G	A6	A8860G
A6	A8860G		A8982G		A8938G
	A8938G	ND4	A11467G	ND4	A11467G
ND4	A11467G		G11719A		G11719A
	G11719A		A11893G		A11893G
	A11893G	$tRNA^{Leu(CUN)}$	A12307G	$tRNA^{Leu(CUN)}$	A12307G
$tRNA^{Leu(CUN)}$	A12307G	ND5	G12372A	ND5	G12372A
ND5	G12372A		G12651A	CytB	C14766T
	T13789C	CytB	C14766T		A14926G
CytB	C14766T		A14926G		T15287C
	A14926G		A15244G		A15326G
	A15326G		A15326G		
	G15883A				



Organism	Acc-stem		D-stem	D-loop	D-stem		Ac-stem	Anticd-loop	Ac-stem		V-region	T-stem	T-loop	T-stem	Acc-stem	
	1	8	10		22		27	32	39	43	44	49		61	66	73
Mus musculus	ACTTTTA	TA	GGAT	AATAGTA	ATCC	A	TTGGT	CTTAGGA	ACCAA		AAAC	CTTGG	TGCAAAT	CCAAA	TAAAAGT	A
Gorilla gorilla	ACTTTTA	AA	GGAT	AACAGCT	ATCC	A	TTGGT	CTTAGGA	CCCAA		AAAT	TTTGG	TGCAACT	CCAAA	TAAAAGT	A
Hylobates lar	ACTTTTA	AA	GGAT	AACAGCT	ATCC	A	TTGGT	CTTAGGA	CCCAA		AAAT	TTTGG	TGCAACT	CCAAA	TAAAAGT	A
Lemur catta	ACTTTTA	AA	GGAT	AGAAGTA	ATCC	A	TTGGC	CTTAGGA	GCCAA		AAA	ATTGG	TGCAACT	CCAAA	TAAAAGT	A
Pan paniscus	ACTTTTA	AA	GGAT	AACAGCC	ATCC	G	TTGGT	CTTAGGC	CCCAA		AAAT	TTTGG	TGCAACT	CCAAA	TAAAAGT	A
Homo sapiens	ACTTTTA	AA	GGAT	AACAGCT	ATCC	A	TTGGT	CTTAGGC	CCCAA		AAAT	TTTGG	TGCAACT	CCAAA	TAAAAGT	A
Thylamys elegans	ACTTTTA	AA	GGAT	AGCAGTA	ATCC	C	CTGGT	CTTAGGA	ACCAG		TAAC	TTTGG	TGCAACT	CCAAA	TAAAAGT	A
Procavia capensis	ACTTTTA	AA	GGAT	GGGAGAT	ATCC	G	TTGGT	CTTAGGA	GCCAA		AAA	ATTGG	TGCAACT	CCAAA	TAAAAGT	A
Cavia porcellus	ACTTTTA	TA	GGAT	AGAAGTA	ATCC	A	TTGGT	CTTAGGA	ACCAA		AAA	ATTGG	TGCAACT	CCAAA	TAAAAGT	A

Figure 1. Alignment of $tRNA^{Leu(CUN)}$ gene sequences from various species. The arrow indicates position 43, site of the A12307G variant.

MFE prediction

We further utilized the RNAfold web server to characterize the potential effect of the A12307G variant on thermodynamic changes to $tRNA^{Leu(CUN)}$ (Gruber et al., 2008). The MFE of the 12307A and 12307G alleles was found to be -16.01 and -15.98 kcal/mol, respectively.

We also analyzed MFE structures generated from wild type and A12307G mutant sequences. As shown in Figure 2, it was quite obvious that this variant did not induce a change in the MFE structure of tRNA$^{Leu(CUN)}$, suggesting that it may not play an important role in pancreatic cancer.

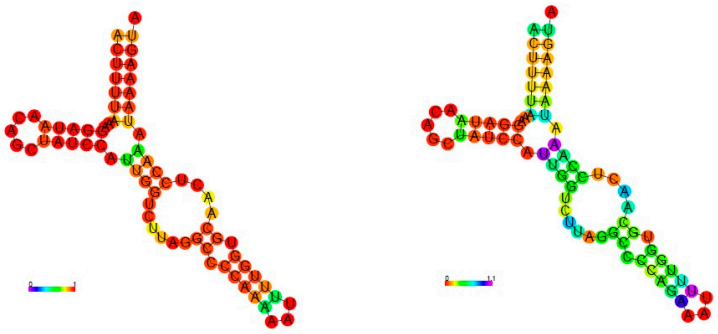


Figure 2. Prediction of the minimum free energy structure of tRNA$^{Leu(CUN)}$ with and without the A12307G variant.

Pathogenicity scoring system

According to a revised pathogenicity scoring system (Yarham et al., 2010, 2011), the A12307G variant was classified as a "neutral polymorphism," with a total score of 4 points (Table 2).

Table 2. Pathogenicity scoring system used to assess the A12307G mutation.

Scoring criteria	A12307G mutation	Score	Classification
More than one independent report	Yes	2	≤ 6 points: neutral polymorphism;
Evolutionary conservation of the base pair	Highly conserved	2	7-10 points: possibly pathogenic;
Variant heteroplasmy	No	0	11-13 points: (not including single-fiber, steady-state level
Segregation of the mutation with disease	No	0	evidence): probably pathogenic;
Histochemical evidence of mitochondrial disease	No evidence	0	≥ 11 points (including trans-mitochondrial cybrid studies):
Biochemical defect in complex I, III, or IV	No	0	definitely pathogenic
Evidence of mutation segregation with biochemical defect from single-fiber studies	No	0	
Mutant mt-tRNA steady-state level or evidence of pathogenicity in trans-mitochondrial cybrid studies	No	0	
Total score		4	Neutral polymorphism

mt = Mitochondrial.

DISCUSSION

Cancer is considered to comprise a heterogeneous set of divergent molecular and cellular features, and increasing evidence suggests that mitochondria play a key role in carcinogenesis (Chatterjee et al., 2006; Hu et al., 2015). Mutations in mtDNA may lead to mitochondrial dysfunction and the metabolic reprogramming of malignant cells, leading to

the modulation of cellular processes involved in the initiation and progression of cancer. Somatic mtDNA mutations have been identified in many human tumors and are common in pancreatic cancer (Jones et al., 2001; Navaglia et al., 2006). Some of these mutations are localized in OXPHOS-complex genes and the D-loop region, while others occur in mt-tRNA genes. Mutations in these latter have an impact on secondary and tertiary tRNA structure, and may cause translational and transcriptional defects, inducing mitochondrial respiratory chain dysfunction.

mt-tRNA point mutations have been associated with a diverse range of clinical phenotypes, including epilepsy, deafness, diabetes, cardiomyopathy, and encephalopathy (Florentz et al., 2003; Yarham et al., 2010). To date, over 200 mt-tRNA mutations have been linked to mitochondrial disease, but sufficient evidence to justify a classification of "definitely pathogenic" exists for less than half of these (Yarham et al., 2011), demonstrating the difficulties faced when characterizing potentially pathogenic variants. Distinguishing mt-tRNA polymorphisms from mutations is important, since failure to do so will inevitably lead to poor diagnostic and genetic advice.

In this study, we reanalyzed the possible role of the $tRNA^{Leu(CUN)}$ A12307G variant in pancreatic cancer through the application of bioinformatics and phylogenetic conservation analysis. We notice that the homoplasmic A12307G variant is localized at position 43 in the anticodon stem of $tRNA^{Leu(CUN)}$ (Figure 3). It is generally believed that each cell contains hundreds to thousands of mtDNA copies. Once a pathogenic mutation occurs, a population of this pathogenic mtDNA coexists with wild-type mtDNA, a condition known as heteroplasmy. When the frequency of pathogenic mtDNA in a cell increases and exceeds a threshold, a clinical phenotype arises. This observation highlights the importance of heteroplasmic mtDNA mutations in clinical diseases, and suggests that the homoplasmic A12307G variant may not be involved in the pathogenesis of pancreatic cancer.

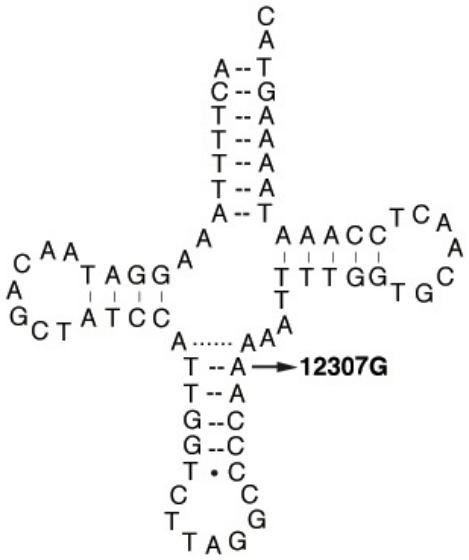


Figure 3. Secondary structure of $tRNA^{Leu(CUN)}$. The arrow indicates the A12307G variant.

To assess the potential deleterious role of this variant, we applied the following criteria: a pathogenic mutation should be 1) present in < 1% of healthy controls; 2) affect a residue with a CI > 75%, as proposed by Ruiz-Pesini and Wallace (2006); 3) potentially induce structural and functional alterations; and 4) score highly on the pathogenicity scoring system. We found that the A12307G variant was present in healthy controls at a frequency of 13.7%, and RNAfold results showed that it did not alter the MFE structure of tRNA$^{Leu(CUN)}$. In addition, this mutation was assigned only 4 points by the pathogenicity scoring system employed, being categorized as a "neutral polymorphism" based on current data. Thus, the role of the A12307G variant in pancreatic cancer needs to be addressed in further experimental investigations.

Conflicts of interest

The authors declare no conflict of interest.

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