Genetics and Molecular Research

Mitochondrial tRNA mutations may be infrequent in hepatocellular carcinoma patients

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Genet. Mol. Res. 15 (2): gmr.15027665
Received September 17, 2015
Accepted December 22, 2015
Published June 24, 2016
DOI http://dx.doi.org/10.4238/gmr.15027665

ABSTRACT. Mitochondrial DNA mutations have been shown to play important roles in the pathogenesis of hepatocellular carcinoma (HCC). In particular, genes encoding mitochondrial tRNA (mt-tRNA) are hotspots for pathogenic mutations associated with HCC. Recently, an increasing number of studies have reported the involvement of such mutations in this disease. As a result, several mt-tRNA mutations associated with HCC have been described. Some of these are neutral polymorphisms and may not cause mitochondrial dysfunction. Moreover, the molecular mechanisms by which these pathogenic mutations result in HCC remain unclear. To address this problem, we evaluated five mt-tRNA variants ($tRNA^{Val}$ T1659C, $tRNA^{Ala}$ G5650A, $tRNA^{Arg}$ T10463C, $tRNA^{Glu}$ A14679G, and $tRNA^{Pro}$ C15975T) implicated in the clinical manifestation of HCC in humans. We performed evolutionary conservation analysis and used a

bioinformatic tool to predict the secondary structure of the mt-tRNAs carrying these mutations. Using an established pathogenicity scoring system, we classified T10463C and A14679G as neutral polymorphisms, and determined that the T1659C, G5650A, and C15975T variants should be regarded as pathogenic mutations. To the best of our knowledge, this is the first report to establish the pathogenicity of HCC-associated mt-tRNA mutations.

Key words: Hepatocellular carcinoma; mt-tRNA; Mutations; Polymorphisms

INTRODUCTION

Worldwide, liver cancer constitutes the fifth and seventh most common cancer in men (523,000 cases/year, accounting for 7.9% of all cancers) and women (226,000 cases/year, accounting for 6.5% of all cancers), respectively (Ferlay et al., 2010). Hepatocellular carcinoma (HCC) is one of the most common forms of liver cancer and is ranked third in terms of cancer-related mortality. However, the molecular mechanism responsible for HCC remains largely unknown. Several risk factors, including viral hepatitis, cigarette smoking, and alcohol consumption are known to contribute to the development of HCC (Schütte et al., 2009). Moreover, genetic variations, such as hepatitis B virus C1653T, T1753V, and A1762T/G1764A, *UGT1A1*28* $(TA)_7$, and the c.1564A>T mutation in the *MDR1* gene, have been found to be associated with this disease (Wan et al., 2014; Marku et al., 2015; Yang et al., 2015).

However, the mutations identified to date mainly involve nuclear genes. In contrast, the molecular role that mitochondrial (mt) DNA mutations play in HCC remains poorly understood. Human mtDNA is a double-stranded, circular molecule encoding 13 polypeptides and 24 RNA transcripts (2 rRNAs and 22 tRNAs) responsible for the translation of protein-coding sequences (Schon et al., 1997). Three hotspots for HCC-associated mtDNA mutations have been identified: the D-loop (Wheelhouse et al., 2005), the common deletion spanning the protein-coding regions (Yin et al., 2004), and the mt-tRNA genes important for mitochondrial protein synthesis (Abbott et al., 2014).

Variations in mt-tRNA genes have been reported to be associated with a wide range of clinical diseases, including cardiomyopathy, deafness, and Leber's hereditary optic neuropathy. Nevertheless, low genotype-phenotype correlations are very common, for instance, the mt-$tRNA^{Leu(UUR)}$ T3291C mutation (Ding and Leng, 2012) may not alter the secondary tRNA structure and achieves a low rating on the pathogenicity scoring system, besides being poorly conserved (Yarham et al., 2011). To better understand the molecular mechanisms implicating mt-tRNA mutations in HCC, we evaluate here five such HCC-associated sequence variants ($tRNA^{Val}$ T1659C, $tRNA^{Ala}$ G5650A, $tRNA^{Arg}$ T10463C, $tRNA^{Glu}$ A14679G, and $tRNA^{Pro}$ C15975T), and briefly discuss their functional significance.

MATERIAL AND METHODS

Data collection

To identify published articles concerning the association between mt-tRNA mutations

and HCC, we performed a search of PubMed Central and other public databases using a combination of the following key words: "mt-tRNA mutation; hepatocellular carcinoma" or "mitochondrial tRNA variant; hepatocellular carcinoma". Studies were included if they consisted of full-text articles in English describing a case-control investigation. By contrast, articles in which crucial data were not reported in the original paper, or inaccurate reporting was considered to be highly probable were excluded.

Analysis of evolutionary conservation of HCC-associated mt-tRNA mutations

With the aim of elucidating the molecular mechanism through which mt-tRNA mutations might cause HCC, we analyzed the evolutionary conservation of these sequence variants. In brief, we chose 15 vertebrate species and measured the conservation index (CI) for each mt-tRNA mutation. We considered a CI greater than 70% to indicate functional relevance.

Prediction of the minimum free energy (MFE) of mt-tRNAs with and without mutations

The secondary structure of mt-tRNA typically comprises acceptor, D-, anticodon, and T-stems. To establish whether mutations cause thermodynamic changes in mt-tRNA, we used the m-fold program to predict the MFE of such structures with and without these sequence variations (http://unafold.rna.albany.edu/?q=mfold; Mathews and Turner, 2006).

Analysis of mt-tRNA mutation haplogroups

We used PhyloTree (http://www.phylotree.org/tree/main.htm; van Oven and Kayser, 2009) to identify haplogroups associated with the mt-tRNA mutations under investigation.

Assessment of the pathogenic status of each mt-tRNA mutation

We also applied the pathogenicity scoring system originally proposed by Yarham et al. (2011) to each mt-tRNA mutation. According to this classification, an mt-tRNA mutation with a total score greater than 11 points is regarded as "definitely pathogenic", while scores of 7 to 10 points denote "possibly pathogenic" variants, and those less than 6 points signify a "neutral polymorphism".

RESULTS

Study characterization

By searching the literature, we retrieved three studies concerning the association between mt-tRNA mutations and HCC (Wong et al., 2004; Vivekanandan et al., 2010; Yin et al., 2010), from which we identified five sequence variants. These mutations included $tRNA^{Val}$ T1659C (CI = 88.5%), $tRNA^{Ala}$ G5650A (CI = 88.5%), $tRNA^{Arg}$ T10463C (CI = 94.2%), $tRNA^{Glu}$ A14679G (CI = 65.4%), and $tRNA^{Pro}$ C15975T (CI = 94.2%). The features of each study are shown in Table 1.

Table 1. Characterization of mt-tRNA mutations.

Mutation	Location	Reported homoplasmy or heteroplasmy?	MFE (wild type; kcal/mol)	MFE (mutant; kcal/mol)	CI
T1659C	T-loop	Heteroplasmy	-13.88	-12.81	88.5%
G5650A	Acceptor stem	Heteroplasmy	-18.47	-15.13	88.5%
T10463C	Acceptor stem	Homoplasmy	-10.58	-10.86	94.2%
C15975T	T-stem	Heteroplasmy	-19.00	-18.97	94.2%
A14679G	T-stem	Homoplasmic	-20.35	-19.56	65.4%

CI = conservation index.

MFE prediction

We then used the m-fold program to predict the MFE structure of each mt-tRNA with and without the mutations identified. The molecular characterization of each mt-tRNA mutation is given in Table 2. The T10463C, A14679G, and C15975T variants appeared to result in a slight change of entropy when comparing wild-type and mutant sequences. However, this change was significantly larger when considering the T1659C and G5650A mutations, suggesting a potential pathogenic role in carcinogenesis.

Table 2. Pathogenicity scoring system for T10463C and A14679G mutations.

Scoring criteria	T10463C mutation	Score/20	A14679G mutation	Score/20	Classification
More than one independent report	Yes	2	Yes	2	≤6 points: neutral polymorphisms; 7~10 points: possibly pathogenic; 11-13 points (not including evidence from single fiber, steady-state level): probably pathogenic; ≥11 points (including trans-mitochondrial cybrid studies): definitely pathogenic.
Evolutionary conservation of the base pair	Two changes	1	One change	2	
Variant heteroplasmy	No	0	No	0	
Segregation of the mutation with disease	No	0	No	0	
Histochemical evidence of mitochondrial disease	No evidence	0	No evidence	0	
Biochemical defect in complex I, III, or IV	No	0	No	0	
Evidence of mutation segregation with biochemical defect from single-fiber studies	No	0	No	0	
Mutant mt-tRNA steady-state level or evidence of pathogenicity in trans-mitochondrial cybrid studies	No evidence	0	No evidence	0	
Maximum score		3		4	Neutral polymorphisms

Determining haplogroup-specific variants

To distinguish pathogenic mutations from neutral polymorphisms, we used PhyloTree to determine the haplogroup distribution of the mt-tRNA sequence variants under examination. We found T10463C to be a J1c1b1a-specific variant (Figure 1), while T1659C, G5650A, A14679G, and C15975T were not associated with particular haplogroups. This finding indicates that the T10463C variant should be classified as a neutral polymorphism.

Assessing the pathogenicity of each mt-tRNA mutation

Subsequently, we utilized the updated version of an established pathogenicity scoring system to assess each mt-tRNA mutation. The T10463C and A14679G mutations were assigned total scores of 3 and 4 points, respectively (Table 3), suggesting that they constitute "neutral polymorphisms". However, the T1659C (11 points), G5650A (12 points), and C15975T (12 points) variants were associated with higher scores, implying pathogenicity.

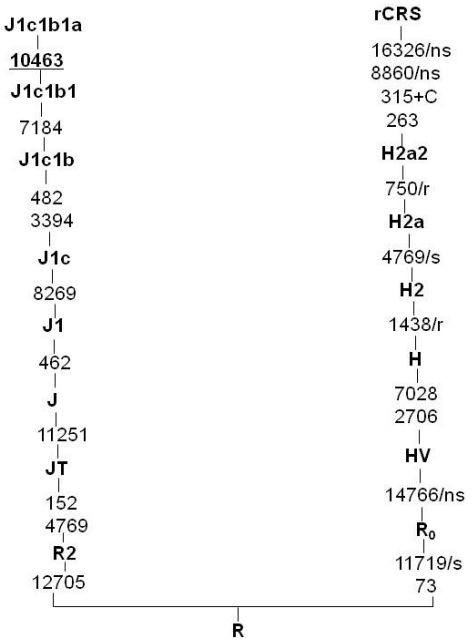

Figure 1. Phylogenetic analysis of the T10463C polymorphism, indicating its place in the human mitochondrial haplogroup, J1c1b1a.

Table 3. Pathogenicity scoring of the T10463C and A14679G variants.

Scoring criterion	T10463C	Score/20	A14679G	Score/20	Classification
More than one independent report	Yes	2	Yes	2	≤6 points: neutral polymorphisms;
Evolutionary conservation of the base pair	Two changes	1	One change	2	7-10 points: possibly pathogenic;
Variant heteroplasmy	No	0	No	0	11-13 points (not including evidence from single fiber or steady-state level
Segregation of the mutation with disease	No	0	No	0	studies): probably pathogenic;
Histochemical evidence of mitochondrial disease	No evidence	0	No evidence	0	≥11 points (including trans-mitochondrial cybrid studies): definitely pathogenic.
Biochemical defect in complex I, III, or IV	No	0	No	0	
Evidence of mutation segregation with biochemical defect from single-fiber studies	No	0	No	0	
Mutant mt-tRNA steady-state level or evidence of pathogenicity in trans-mitochondrial cybrid studies	No evidence	0	No evidence	0	
Maximum score		3		4	Neutral polymorphisms

mt-tRNA = mitochondrial tRNA.

DISCUSSION

Due to its high mutation rate, sequence variations may occur at any of the 73 base-pairs making up the cloverleaf structure of mt-tRNA. It is generally believed that such mutations can alter steady-state levels of functional mt-tRNAs, and consequently result in a failure of mt-tRNA metabolism, including aminoacylation, mitochondrial respiratory chain dysfunction, and increased reactive oxygen species production (Servidei, 2003). Thus, alterations to mt-tRNA genes are commonly regarded as potential causes of diseases such as cardiomyopathy, chronic progressive external ophthalmoplegia, and cancer (Schon et al., 1997). However, there exist a number of mt-tRNA mutations in databases such as MITOMAP and mtDB whose pathogenicity remains to be established, owing to a lack of functional or bioinformatic analysis.

In this study, we analyzed the possible role of five HCC-associated mt-tRNA mutations. Of these, T1659C, G5650A, and C15975T have been reported to be associated

with mitochondrial diseases and may be classified as “definitely pathogenic” (Finnilä et al., 2001; Blakely et al., 2004; McFarland et al., 2008; Da Pozzo et al., 2009). At the molecular level, these mutations decrease the stability and aminoacylation ability of the corresponding tRNAs. The $tRNA^{Val}$ T1659C mutation was first described in a child with learning difficulties, hemiplegia, and a movement disorder. This heteroplasmic mutation located on the T-stem of $tRNA^{Val}$ interrupts a conserved Watson-Crick base pair and causes a failure of $tRNA^{Val}$ metabolism. Moreover, the $tRNA^{Ala}$ G5650A mutation has been reported in a patient suffering cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (Finnilä et al., 2001; McFarland et al., 2008). This sequence variation occurs at the site of $tRNA^{Ala}$ recognized by mitochondrial alanyl-tRNA synthetase, which serves a vital function in $tRNA^{Ala}$ aminoacylation, an essential prerequisite for protein translation (Park and Schimmel, 1988). The heteroplasmic C15975T mutation was originally reported in a 56-year-old woman with a history of progressive walking difficulty, and was shown to be absent in healthy controls (Da Pozzo et al., 2009). This mutation introduces a C-A mispair into the T-stem region that may have an effect on tRNA aminoacylation. Functional analysis of this mutation highlighted its pathogenicity, revealing its role in complex I deficiency (Da Pozzo et al., 2009).

The homoplasmic T10463C mutation is located on the acceptor arm of $tRNA^{Arg}$, and belongs to haplogroup J1c1b1a (van Oven and Kayser, 2009; Figure 1). It is interesting to note that mitochondrial haplogroup plays an important role in the development of breast, colorectal, and thyroid cancers (Fang et al., 2010). In addition, a recent report showed that certain mitochondrial haplogroups and somatic mutations are associated with lung cancer in the Han Chinese population (Fang et al., 2015). Since T10463C is a haplogroup-specific variant, it should be regarded as a polymorphism. The A14679G mutation, meanwhile, is located at position 66 in the acceptor stem of $tRNA^{Glu}$. Bioinformatic analysis showed that this mutation causes only a slight MFE change in $tRNA^{Glu}$, and the pathogenicity scoring system employed returned total scores of 3 and 4 points for T10463C and A14679G, respectively (Table 3). From this, it seems clear that these variations should be considered neutral polymorphisms.

Taken together, our results provide direct evidence regarding the pathogenicity of five mt-tRNA mutations associated with HCC. However, our study is limited by a lack of functional analysis, for example, the use of cybrid cells containing these mt-tRNA mutations to examine tRNA stability and protein synthesis defects. We recommend the phylogenetic approach as a useful tool to distinguish neutral single nucleotide polymorphisms from pathogenic mutations. Moreover, we propose that a more careful assessment of mt-tRNA mutations is necessary in cancer studies.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by the Natural Science Foundation of Zhejiang Province (#LY15H030015) and the Wenzhou Bureau of Science and Technology (#Y20140281).

REFERENCES

Abbott JA, Francklyn CS and Robey-Bond SM (2014). Transfer RNA and human disease. *Front. Genet.* 5: 158. PubMed http://dx.doi.org/10.3389/fgene.2014.00158

Blakely EL, Poulton J, Pike M, Wojnarowska F, et al. (2004). Childhood neurological presentation of a novel mitochondrial tRNA(Val) gene mutation. *J. Neurol. Sci.* 225: 99-103. http://dx.doi.org/10.1016/j.jns.2004.07.007

Da Pozzo P, Cardaioli E, Malfatti E, Gallus GN, et al. (2009). A novel mutation in the mitochondrial tRNA(Pro) gene associated with late-onset ataxia, retinitis pigmentosa, deafness, leukoencephalopathy and complex I deficiency. *Eur. J. Hum. Genet.* 17: 1092-1096. http://dx.doi.org/10.1038/ejhg.2009.12

Ding Y and Leng J (2012). Is mitochondrial tRNA $^{Leu(UUR)}$ 3291T>C mutation pathogenic? *Mitochondrial DNA* 23: 323-326. http://dx.doi.org/10.3109/19401736.2012.674119

Fang H, Shen L, Chen T, He J, et al. (2010). Cancer type-specific modulation of mitochondrial haplogroups in breast, colorectal and thyroid cancer. *BMC Cancer* 10: 421. http://dx.doi.org/10.1186/1471-2407-10-421

Fang Y, Yang HY, Shi YH, Cui JH, et al. (2015). Mitochondrial DNA haplogroups and somatic mutations are associated with lung cancer in patients from Southwest China. *Genet. Mol. Res.* 14: 5031-5043. http://dx.doi.org/10.4238/2015.May.12.6

Ferlay J, Shin HR, Bray F, Forman D, et al. (2010). Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int. J. Cancer* 127: 2893-2917. http://dx.doi.org/10.1002/ijc.25516

Finnilä S, Tuisku S, Herva R and Majamaa K (2001). A novel mitochondrial DNA mutation and a mutation in the Notch3 gene in a patient with myopathy and CADASIL. *J. Mol. Med.* 79: 641-647. http://dx.doi.org/10.1007/s001090100268

Marku E, Maltese PE, Koni M, Capodicasa N, et al. (2015). Polymorphism of *UGT1A1*28* $(TA)_7$ and liver damage in hepatitis B virus-positive patients in Albania. *Genet. Mol. Res.* 14: 5221-5228. http://dx.doi.org/10.4238/2015.May.18.13

Mathews DH and Turner DH (2006). Prediction of RNA secondary structure by free energy minimization. *Curr. Opin. Struct. Biol.* 16: 270-278. http://dx.doi.org/10.1016/j.sbi.2006.05.010

McFarland R, Swalwell H, Blakely EL, He L, et al. (2008). The m.5650G>A mitochondrial $tRNA^{Ala}$ mutation is pathogenic and causes a phenotype of pure myopathy. *Neuromuscul. Disord.* 18: 63-67. http://dx.doi.org/10.1016/j.nmd.2007.07.007

Park SJ and Schimmel P (1988). Evidence for interaction of an aminoacyl transfer RNA synthetase with a region important for the identity of its cognate transfer RNA. *J. Biol. Chem.* 263: 16527-16530.

Schon EA, Bonilla E and DiMauro S (1997). Mitochondrial DNA mutations and pathogenesis. *J. Bioenerg. Biomembr.* 29: 131-149. http://dx.doi.org/10.1023/A:1022685929755

Schütte K, Bornschein J and Malfertheiner P (2009). Hepatocellular carcinoma--epidemiological trends and risk factors. *Dig. Dis.* 27: 80-92. http://dx.doi.org/10.1159/000218339

Servidei S (2003). Mitochondrial encephalomyopathies:gene mutation. *Neuromuscul. Disord.* 13: 848-853.

van Oven M and Kayser M (2009). Updated comprehensive phylogenetic tree of global human mitochondrial DNA variation. *Hum. Mutat.* 30: E386-E394. http://dx.doi.org/10.1002/humu.20921

Vivekanandan P, Daniel H, Yeh MM and Torbenson M (2010). Mitochondrial mutations in hepatocellular carcinomas and fibrolamellar carcinomas. *Mod. Pathol.* 23: 790-798. http://dx.doi.org/10.1038/modpathol.2010.51

Wan YY, Wang XW, Hui HX and Wan L (2014). Association between the c.1564A>T genetic polymorphism of the *MDR1* gene and hepatocellular carcinoma in Chinese population. *Genet. Mol. Res.* 13: 6820-6826. http://dx.doi.org/10.4238/2014.August.29.3

Wheelhouse NM, Lai PB, Wigmore SJ, Ross JA, et al. (2005). Mitochondrial D-loop mutations and deletion profiles of cancerous and noncancerous liver tissue in hepatitis B virus-infected liver. *Br. J. Cancer* 92: 1268-1272. http://dx.doi.org/10.1038/sj.bjc.6602496

Wong LJ, Tan DJ, Bai RK, Yeh KT, et al. (2004). Molecular alterations in mitochondrial DNA of hepatocellular carcinomas: is there a correlation with clinicopathological profile? *J. Med. Genet.* 41: e65. http://dx.doi.org/10.1136/jmg.2003.013532

Yang Y, Sun JW, Zhao LG, Bray F, et al. (2015). Quantitative evaluation of hepatitis B virus mutations and hepatocellular carcinoma risk: a meta-analysis of prospective studies. *Chin. J. Cancer Res.* 27: 497-508.

Yarham JW, Al-Dosary M, Blakely EL, Alston CL, et al. (2011). A comparative analysis approach to determining the pathogenicity of mitochondrial tRNA mutations. *Hum. Mutat.* 32: 1319-1325. http://dx.doi.org/10.1002/humu.21575

Yin PH, Lee HC, Chau GY, Wu YT, et al. (2004). Alteration of the copy number and deletion of mitochondrial DNA in human hepatocellular carcinoma. *Br. J. Cancer* 90: 2390-2396.

Yin PH, Wu CC, Lin JC, Chi CW, et al. (2010). Somatic mutations of mitochondrial genome in hepatocellular carcinoma. *Mitochondrion* 10: 174-182. http://dx.doi.org/10.1016/j.mito.2009.12.147