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Exploring the Impact of Mitochondrial tRNA Mutations on Colorectal Cancer Development and their potential as biomarkers

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Abstract

Aim: Mutations in mitochondrial tRNA (mt-*tRNA*) genes are associated with various diseases including colorectal cancer. The relationship between changes in tRNA genes and cancer is increasingly receiving attention. The focus of this study is to comprehend the possible relationship between mt-tRNA mutations and colorectal cancer incidence and progression.

Study design: The present study we sequenced mitochondrial DNA of 25tumour+25 adjacent normal] samples belonging to Colorectal cancer tissues by Next generation sequencing [NGS] technology. The identified tRNA variants were screened for pathogenicity and structural changes that lead to hampering the function of mt-tRNA protein synthesis**.**

Results: We have identified 10 mt-tRNA mutations across $tRNA^{Ile}$ G4264A, $tRNA^{Ile}$ A4281G, $tRNA^{Ala}$ T5594A, tRNASer$^{[AGC]}$ T12261C, $tRNA^{Leu[CUN]}$ G12300C, $tRNA^{Leu[CUN]}$ A12306C and $tRNA^{Leu[CUN]}$ T12329A. Apart from SNP's, three deletions were identified which are potentially pathogenic: $tRNA^{Ser[AGC]}$A12212del, $tRNA^{Ser[AGC]}$ T12261del, $tRNA^{Leu[CUN]}$ A12328del. Of the identified mutations five of them were novel and were likely pathogenic and reside in extremely conserved positions. Further analysis of copy number suggests the presence of low copy numbers when compared to the matched normal tissues [$p<0.05$]. Hence, it's conceivable these mutations hinder mitochondrial functions, contributing to colorectal carcinogenesis.

Conclusion: Collectively, our data suggest tRNA genes are common sites for pathogenic mutations linked to colorectal cancer, potentially serving as biomarkers for screening and diagnosis.

Keywords: mt-tRNA, Colorectal Cancer, MitoTIP, RNAfold Server, NGS, Pathogenic, mitochondrial dysfunction, mutations, mitochondrial DNA, mitochondrial disease.

Abbreviation: mtDNA-Mitochondrial DNA; nDNA-Nuclear DNA ;mt-tRNA-MitochondrialTransfer RNA.

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Introduction

Colorectal cancer involves unregulated cell division in colon or rectal cells, leading to tumour formation and potentially spreading through adjacent layers(Rawla, Sunkara, and Barsouk 2019). Screening techniques like colonoscopy, sigmoidoscopy, and fecal occult blood tests are crucial for reducing colorectal cancer incidence and mortality. Newly developed genetic testing offers more reliable screening options(Shaukat and Levin 2022; Chung, Ali, and Cash 2022). Cancer evolves through genetic alterations in both nuclear and mitochondrial genes. Mutations in mitochondrial DNA (mtDNA) are ten times more prevalent and detectable than those in nuclear DNA (nDNA) within cancer cells(Lin et al. 2021; Smith, Whitehall, and Greaves 2022; Stenton and Prokisch 2020; Tan et al. 2020). mtDNA mutations affect about 1 in 10,000 individuals, with over 200 pathogenic mutations identified, primarily concentrated within the 22 mitochondrial tRNA (mt-tRNA) genes(Otten et al. 2018).Despite its small size, human mt-tRNA comprises only 4-10% of total RNAs but harbours a significant number of pathogenic mtDNA mutations(Lott et al. 2013). These mutations within mt-tRNAs disrupt transcription and translation processes, leading to dysfunction in the mitochondrial respiratory chain and correlating with various clinical diseases[Figure-1A](Elson et al. 2009).

FIGURE-1

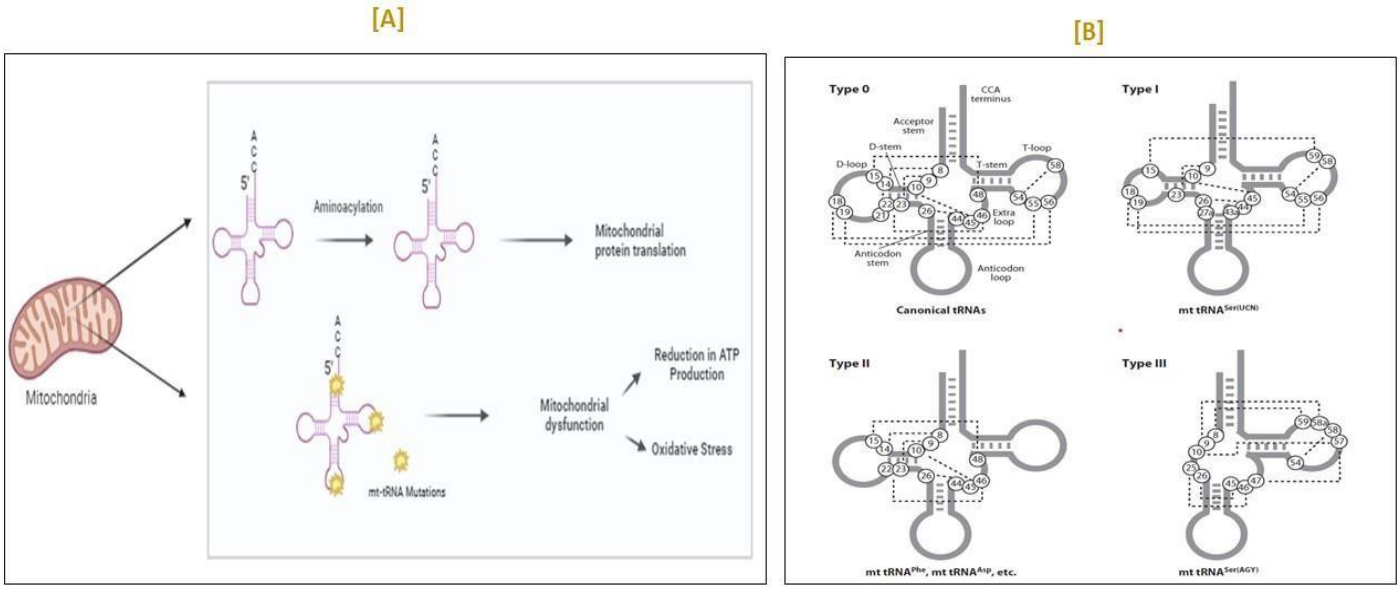

FIGURE 1 : [A] *Mutations in mt-tRNA genes (shown as stars in yellow),reduced mitochondrial protein synthesis, and respiratory chain dysfunction. These mutations lead to metabolic diseases and other disorders* ***[B]*** *Secondary structures of tRNAs within the human mitochondria are illustrated schematically. The standard or canonical tRNA structure is*

denoted as Type 0. Additionally, three distinct types of mitochondrial tRNAs—types I, II, and III—are depicted. Circled numbers denote the nucleotide positions following the tRNAdb numbering system (Juhling F, Morl M, Hartmann RK, Sprinzl M, Stadler PF, Putz J. 2009. tRNAdb 2009: compilation of tRNA sequences and tRNA genes. Nucleic Acids Res. 37:D159–62). Tertiary interactions among nucleobases are highlighted with dotted lines.

A recent study unveiled a new mechanism that connects mt-tRNA modifications to the initiation of a reverse metabolic switch from glycolysis to OXPHOS. This switch promotes tumor cell invasion and metastasis.The mitochondrial genome contains a single tRNA for each of eighteen amino acids, including specific ones for serine ($tRNA^{Ser(AGY)}$ and $tRNA^{Ser(UCN)}$) and leucine ($tRNA^{Leu(UUR)}$ and $tRNA^{Leu(CUN)}$)(Witters et al. 2018). Unlike the typical cloverleaf structure found in tRNAs across different life forms, mammalian mt-tRNAs exhibit unique secondary structures. For instance, mt $tRNA^{Ser(AGY)}$, responsible for AGY codons, lacks the entire D-loop(Tafti et al. 2018). Additionally, other mt-tRNAs, categorized as Type II, lack the conventional D-loop/T-loop interaction, featuring variable size and sequence in D loops and T loops. This structural diversity in mammalian mt-tRNAs serves as an adaptation to evolutionary pressures[Figure-1B](Bohnsack and Sloan 2018; Richter et al. 2021; Karasik et al. 2021).

More than half of the characterized pathology-associated mtDNA mutations are clustered within tRNA genes, with 21 out of 22 tRNA genes implicated in disease-related mutations, except for a very few in $tRNA^{Arg}$ gene. Remarkably, almost half of the identified pathogenic mutations in humans occur in three tRNA genes: $tRNA^{Leu(UUR)}$, $tRNA^{Lys}$, *and* $tRNA^{Ile}$. The $tRNA^{Leu(UUR)}$ gene, with its extensive sequence variability and elevated ratio of pathogenic mutations to neutral variants, possibly owes this to the infrequent use of the UUR codon. Consequently, mutations in $tRNA^{Leu(UUR)}$ may be less detrimental, leading to more frequent identification(Van Haute, Powell, and Minczuk 2017).Despite numerous reported tRNA mutations, only 20% have been definitively proven pathogenic, with 77% categorized as potentially pathogenic in MITOMAP[a Human mitochondrial genome database] and 4% lacking clear annotation(Nakamura, Gojobori, and Ikemura 2000). A study conducted on tumour tissues from colorectal cancer patients revealed an A12308G alteration in human mitochondrial $tRNA^{Leu(CUN)}$**,** suggesting its potential use in cancer screening(Kłos and Dabravolski 2021). The presence of the pathogenic A3243G mutation in reported cases of colon cancer and renal cell carcinoma, along with its absence in normal adjacent tissue, suggests its involvement in the process of carcinogenesis(Lorenc et al. 2003; Sangkhathat et

al. 2005). A study conducted on 80 breast cancer samples revealed 5 possible pathogenic mutations $tRNA^{Val}$ G1606A, $tRNA^{Ile}$ A4300G, $tRNA^{Ser(UCN)}$ T7505C, $tRNA^{Glu}$ A14693G and $tRNA^{Thr}$ G15927A that act as contributors to breast cancer(Ding et al. 2023).

In liver cancer, several mutations have been pinpointed, including T1659C in the $tRNA^{Val}$ gene, G5650A in the $tRNA^{Ala}$ gene, and T10463C in the $tRNA^{Arg}$ gene(Li et al. 2016). A14679G is associated with the $tRNA^{Glu}$ gene, while C15975T is linked to the $tRNA^{Pro}$ gene(Tang et al. 2010). The A12308G mutation within the tRNALeu gene is detected in both colorectal cancer(Mohammed et al. 2015) and oral cancer(Datta et al. 2007). Additionally, alterations such as A7460G in the $tRNA^{Ser}$ gene, G5563A in the $tRNA^{Trp}$ gene, and A12172G in the $tRNA^{His}$ gene have been observed in lung cancer(Wang et al. 2015).Consequently, the mutation pattern in mtDNA serves as a valuable molecular biomarker for cancer, improving the specificity of cancer detection and prediction.

In the present study, we have screened the tumor tissues of colon and rectal tumour and adjacent normal tissues, identified and characterized mt-tRNA variants that can act as biomarkers for detection of colorectal cancer. Present study identified significant mutations in mt-tRNA's $tRNA^{Ile}$, $tRNA^{Ala}$, $tRNA^{Ser[AGC]}$ and $tRNA^{Leu[CUN]}$ including deletions. The mutations were likely pathogenic and mostly disrupted the structure leading to failed tRNA metabolism.

Materials and Methods

Study and sample population:

Study population was composed of 25 Tumour and 25 adjacent non-tumor tissues in an age group of 62±17 years diagnosed with Colon and rectal cancer.The specimens were immediately processed further or frozen at –20 . The study adhered to established protocols and was approved by the Institute of Genetics and Hospital for Genetic Diseases, Hyderabad.

DNA Isolation and PCR amplification of mitochondrial DNA

For DNA extraction, 50 mg of tissue was processed with the Tissue DNA extraction kit [Bioserve India Pvt. Ltd., India]. Whole mitochondrial DNA amplification was done using long-range PCR with TaKaRa LA Taq® DNA polymerase kit [Clontech, South America] with two sets of primers(Gould et al. 2015)[Table:2[A]-.The two ~8.5 kb fragments were amplified using PCR conditions initial denaturation at 94°C for 1 min, followed by 30 cycles of 94°C for 30s, 54°C for 30s, and 72°C for 9 mins, with a final extension at 72°C for 5 mins. Post-

amplification, PCR products were gel purified using the kit from Bioserve India Pvt. Ltd. and quantification through Qubit 2.0 Fluorometer [Thermofisher Scientific, USA.]

Sequencing of Mitochondrial DNA of the tissues using NGS Technology:

Library preparation and sequencing

Library preparation utilized the NEB Next Fast DNA Library Prep Set for Ion Torrent [NEB] following the manufacturer's protocol with steps that include Amplicons fragmentation (200-300bp), end-repair, adapter ligation, amplification, and capture with biotinylated probes. Library profiles were assessed and quantified on the Bioanalyzer 2100 System (Agilent Technologies, Santa Clara, CA, USA). Libraries, pooled in equimolar ratios, were loaded onto the Ion Torrent Chip [318v2] for data generation via the Ion Torrent PGM system [Thermofisher scientific.Pvt.Ltd].

Mitochondrial DNA sequencing analysis

The analysis of sequencing data utilized CLC Genomics Workbench version 20.0.4 [QIAGEN]. Raw data aligned to the Revised Cambridge Reference Sequence (rCRS), applying quality parameters like excluding polyclonal reads, Phred scores >=Q20, three supporting reads per strand for alternate alleles, 500X total sequencing coverage, and a low-frequency variant calling workflow. Variant calling parameters included a minimum coverage of 10, a mismatch of 2, a minimum base quality of 20, a minimum base coverage of 100, and a minimum threshold of 2%. The heteroplasmic analysis covered all identified SNPs, annotation using wANNOVAR tool. Additionally, Mt-tRNA variants underwent further filtration based on MitoTIP information for predicting the pathogenicity of novel variants(Sonney et al. 2017).

Phylogenetic Conservation Analysis.

The conservation index (CI) was computed by comparing human nucleotide variants with those of 14 other vertebrates, defining it as the percentage of species exhibiting the wild-type nucleotide at a given position. The CI≥75% was believed to have functional significance. In the interspecific analysis, 14 vertebrates' mtDNA sequences were utilized, encompassing species such as Homo sapiens, Bos Taurus, Cebus albifrons, Gorilla gorilla, *Hylobates lar*,*Macaca sylvanus*, *Mus musculus*, *Nycticebus coucang*, *Pan paniscus*, *Pan troglodytes*, *Pongo pygmaeus*, *Pongo abelii*, *Papio hamadryas* and *Tarsius bancanus*.

Copy number Analysis by RT-PCR

Mitochondrial copy number was assessed using real-time quantitative PCR. For each sample, ND1 specific for mitochondrial DNA and beta-actin specific for nuclear DNA were chosen. Primers for amplification of the ND1 gene (104bp) and Beta-actin gene (105bp) were utilized(Cui et al. 2013)[Table-2B]. PCR cycling conditions were: 50°C for 2 mins, 95°C for 10 mins, followed by 35 cycles of 95°C for 15 secs, 50°C for 30 secs. The melt curve was also generated to ensure single PCR product presence. Each sample was run in triplicates. Ct values for mitochondrial DNA and nuclear DNA were determined for each clinical sample. The average Ct values were calculated, and ΔCt was determined using the equation: ΔCt = (nuclear DNA Ct - mitochondrial DNA Ct). Relative mitochondrial DNA content was calculated using the equation: Relative mitochondrial DNA content = 2 × 2^ΔCt(Furda et al. 2012).

RNA molecular modelling: The structure of mt-tRNA's were predicted using the RNAfold Server program in The Vienna RNA Websuite(Gruber et al. 2008). Apart from the secondary structure, the server predicts the Minimum Free energy [kcal/mol], mountain plots, and ensemble diversity that can help in assessing the effect of structural changes on the function of mt-tRNAs.

Statistical Analysis: The relative mtDNA copy number (mtDNA/b-actin) was determined and patients were divided into subgroups based on the median value of the T/N ratio (mtDNA copy number/b-actin in tumors divided by mtDNA copy number/b-actin in normal controls, multiplied by 100%).). The t-test assessed variance in mtDNA copy number between colon tumors and corresponding non-tumorous tissues (paired t-test) and tumors with different mt-tRNA mutations compared to normal samples without mutations (two-sample t-test) [p-value <0.05].

Results

The colorectal tumours were analysed for mutations across the mitochondrial genome by next-generation sequencing analysis. The clinical and pathological data of the patients involved in the study are detailed in Table 1. The data was analysed on CLC Genomics workbench. Initial data analysis includes quality control of the raw reads to filter out low-quality reads and sequences. The sequencing data, processed through CLC Genomics Workbench, yielded an average depth of ~500X/sample. The mt-tRNA variants were filtered out as they form the main basis of the present study. Mutational screening across 22 mt-tRNA genes led to identifying seven potentially pathogenic variants: $tRNA^{Ile}$ G4264A, $tRNA^{Ile}$ A4281G, $tRNA^{Ala}$ T5594A, $tRNA^{Ser[AGC]}$ T12261C, $tRNA^{Leu[CUN]}$ G12300C, $tRNA^{Leu[CUN]}$ A12306C and $tRNA^{Leu[CUN]}$

T12329A. Apart from SNP's, three deletions were identified which are potentially pathogenic: $tRNA^{Ser[AGC]}$A12212del, $tRNA^{Ser[AGC]}$ T12261del, $tRNA^{Leu[CUN]}$ A12328del. The characterization of all the mutations is listed in Table 3.

Phylogenetic conservation Analysis: Phylogenetic conservation analysis for each mt-tRNA mutation revealed significant conservation indices (CI≥75.0%), suggesting their potential significance in the context of colorectal cancer[Table 3]. The conservation indexes of SNP's ranged between 92-100% whereas the deletions were 100%.

Copy number Analysis:

Mitochondrial copy numbers were evaluated in both tumours and adjacent normal tissues, revealing a relative mitochondrial DNA content of approximately 129±39 for tumours and 88.1±39.9 for adjacent normal tissues. Tumors exhibited a significant reduction in mitochondrial DNA content compared to adjacent normal tissues. Further correlation between mtDNA copy number and clinicopathological factors was conducted using the T/N ratio . Samples were then divided into low and high-copy number groups based on these ratios.Lower mitochondrial content was associated with the tumour stage *(p=0.0353),* with 80% of tumour tissues showing decreased copy numbers compared to 44% in adjacent normal tissues suggesting the role of mtDNA copy number may in colorectal cancer progression [Table-4].

RNA molecular modelling: Using the RNAfold Server at Vienna RNA Websuite, the mt-tRNA's secondary structures were determined. The minimal free energy (MFE) structure of an RNA sequence is its secondary structure characterized by the lowest possible free energy[Table-5]. This configuration is forecasted through a loop-based energy model and the dynamic programming algorithm pioneered by Zuker et al.[38]. Mutation at position G4264A in $tRNA^{Ile}$ resulted in a shift in secondary structure. The Minimum Free Energy (MFE) decreased from -7.5 kcal/mol in the wild type to -4.5 kcal/mol [Figure-2-A,B].

FIGURE-2

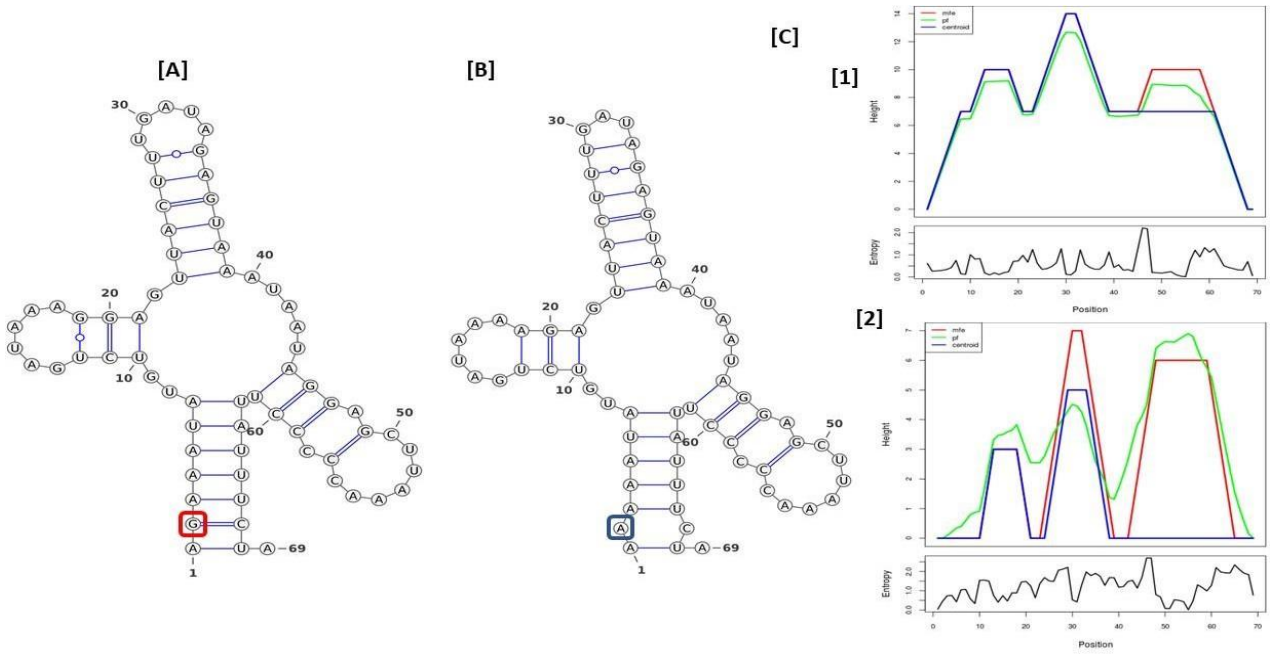

Figure-2:A&B- *Structure of tRNA^{Ile} Wildtype and mutant[G4264A] ;* ***C- i &ii*** *– Mountain plots of Wiltype and Mutant tRNA.*

Similarly, mutation A4281G led to an MFE change to -8.8 kcal/mol [Figure-3-A,B].

FIGURE-3

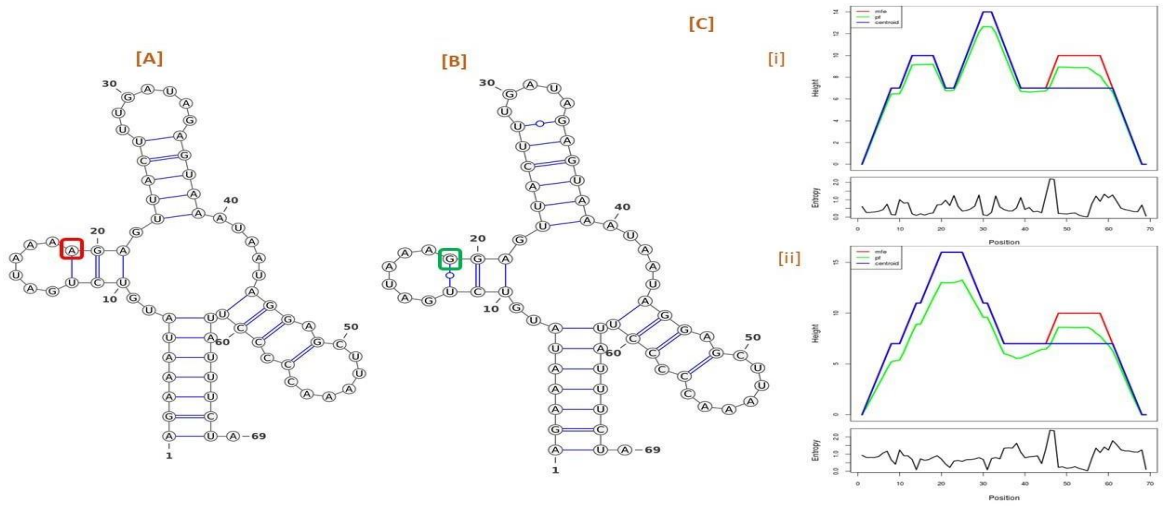

Figure-3: A&B- *Structure of tRNA^{Ile} Wildtype and mutant[A4281G] ;* ***C- i &ii*** *– Mountain plots of Wiltype and Mutant tRNA.*

In *tRNA^{Ala}*, mutation T5594A increased MFE to -4.0 kcal/mol, disrupting the secondary structure from the wild type's -4.9 kcal/mol [Figure-4-A,B].

FIGURE-4

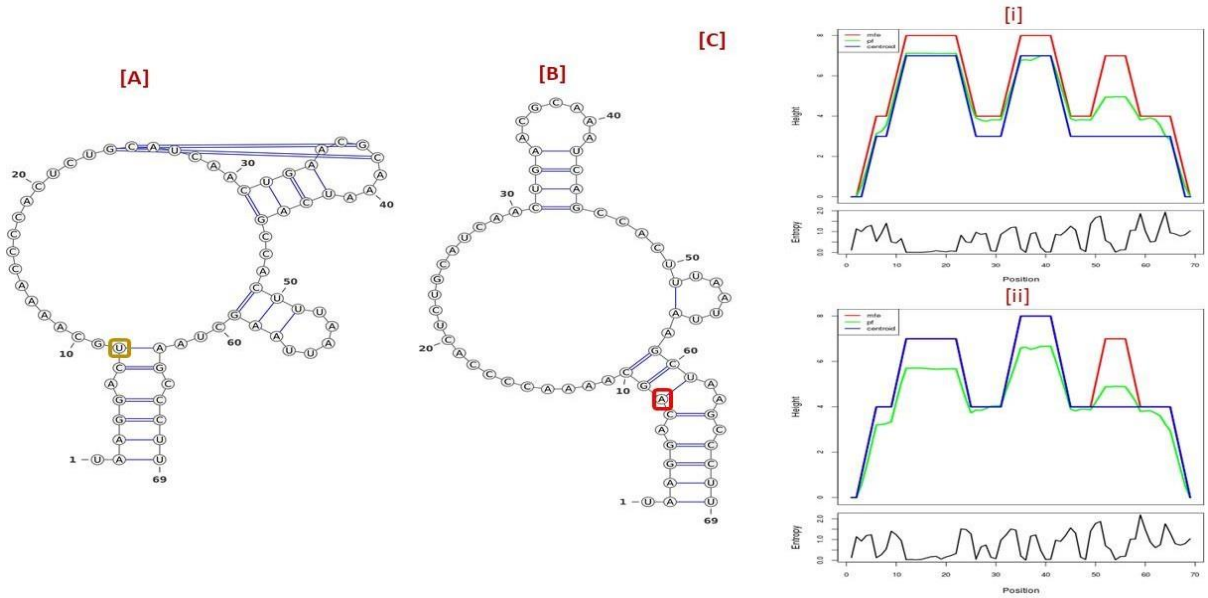

Figure-4: A&B- *Structure of tRNA^{Ala} Wildtype and mutant[T5594A] ;* ***C- i &ii*** *– Mountain plots of Wiltype and Mutant tRNA.*

Mutation T12261C in *tRNA$^{Ser[AGC]}$* increased MFE from -14.9 kcal/mol to -11.4 kcal/mol, impacting the secondary structure [Figure-5-A,B].

FIGURE-5

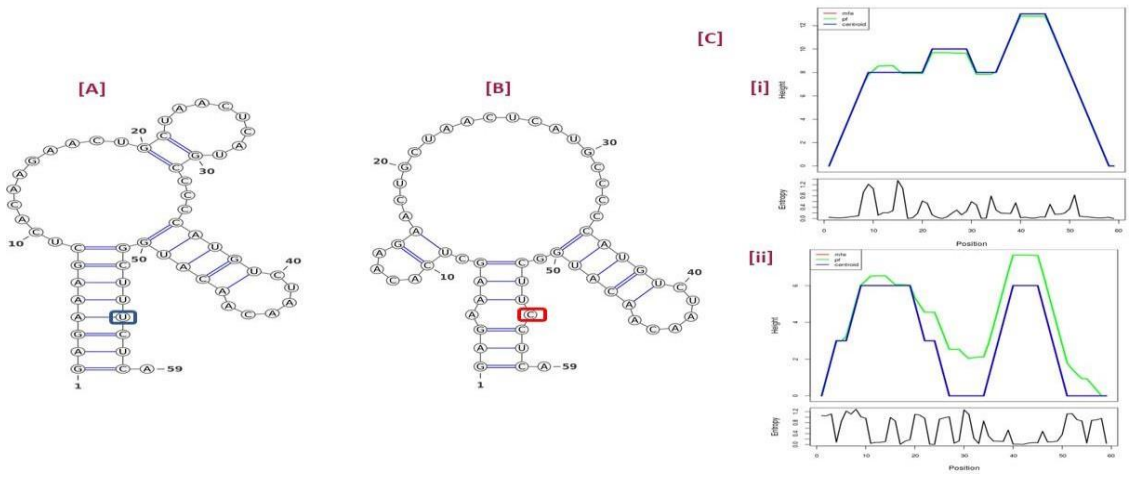

Figure-5: A&B- *Structure of tRNA$^{Ser[AGC]}$ Wildtype and mutant[T12261C] ;* ***C- i &ii*** *– Mountain plots of Wiltype and Mutant tRNA.*

In *tRNA$^{Leu[CUN]}$*, mutations at positions G12300C, A12306C, and T12329A resulted in MFE changes to -14.2 kcal/mol, -13.3 kcal/mol, and -13.9 kcal/mol, respectively. While structural disruption was slight, alterations in bond formation were observed [Figure-6,7,8-A,B].

FIGURE-6

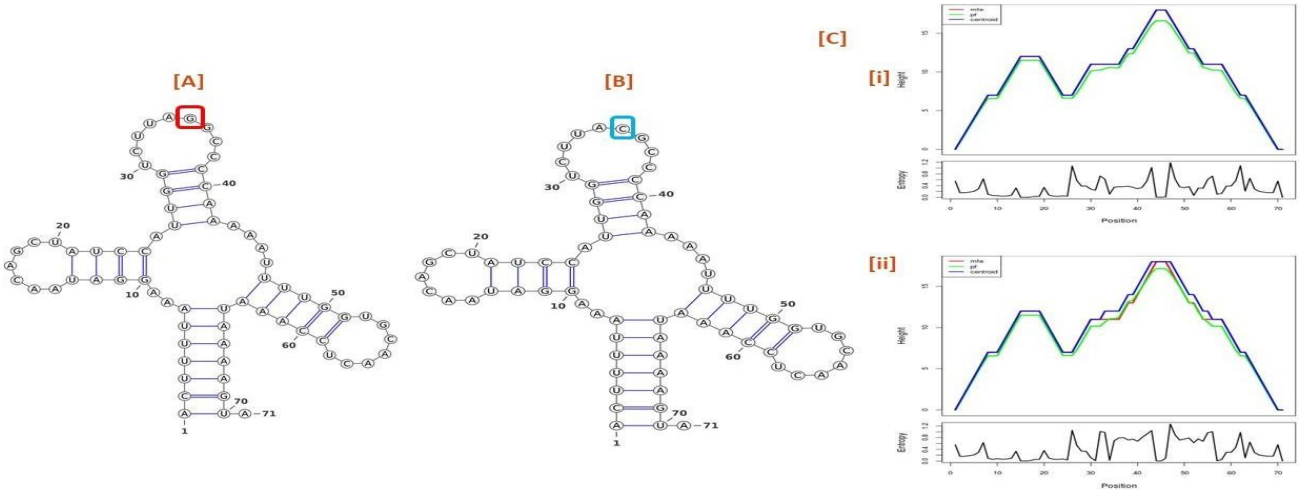

Figure-6: A&B- *Structure of tRNA$^{Leu[CUN]}$ Wildtype and mutant[G12300C] ;* ***C- i &ii*** *– Mountain plots of Wiltype and Mutant tRNA.*

FIGURE-7

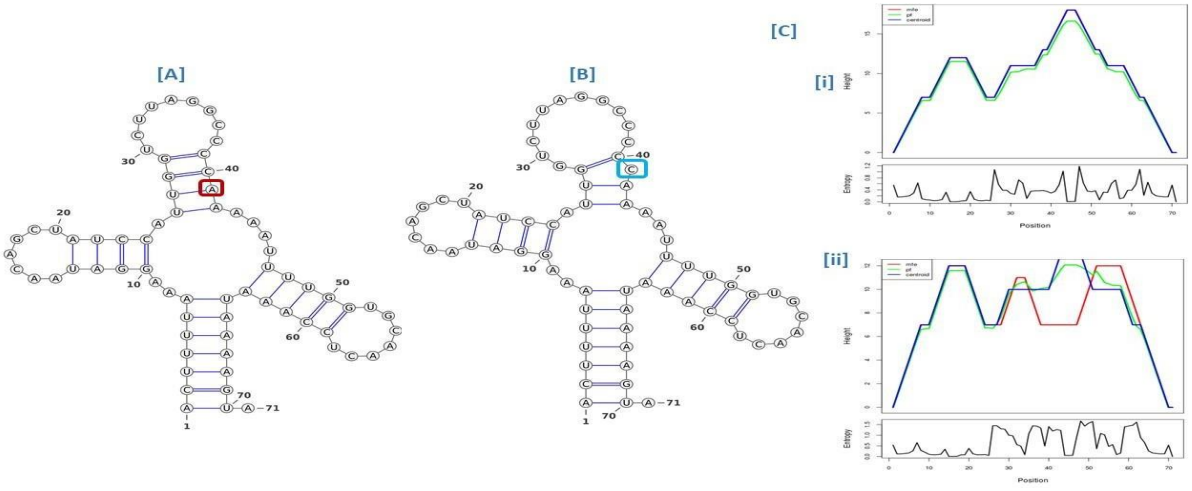

Figure-7: A&B- *Structure of tRNA$^{Leu[CUN]}$ Wildtype and mutant[A12306C] ;* ***C- i &ii*** *– Mountain plots of Wiltype and Mutant tRNA.*

FIGURE-8

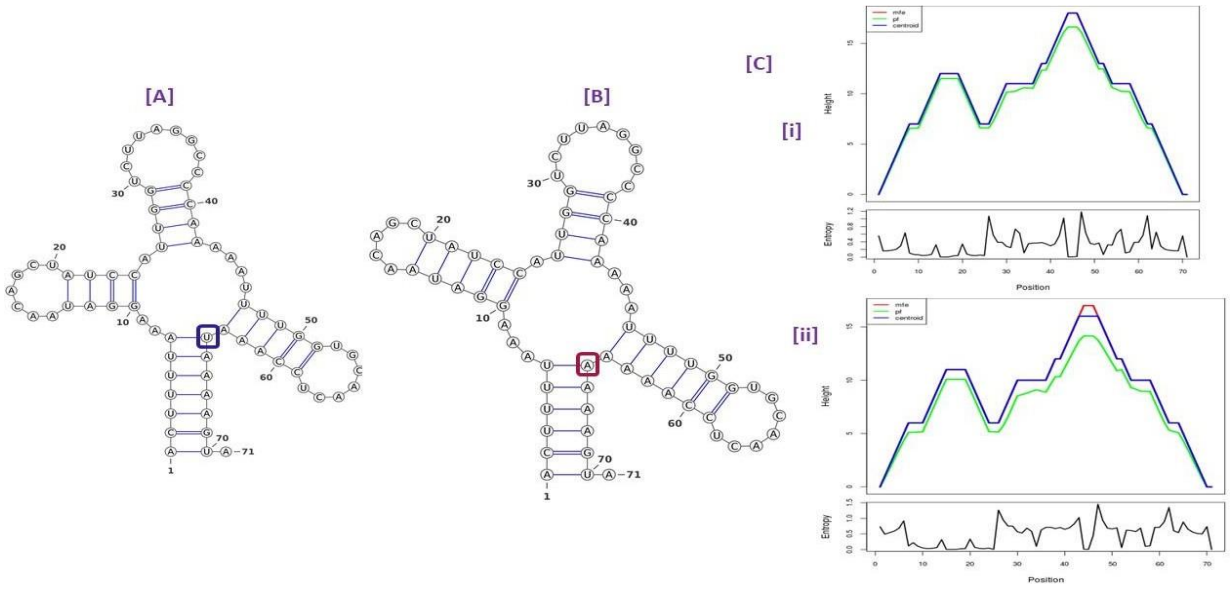

Figure-8: A&B- *Structure of tRNA$^{Leu[CUN]}$ Wildtype and mutant[T12329A] ;* ***C- i &ii*** *– Mountain plots of Wiltype and Mutant tRNA.*

The MFE changes were also observed with deletions in the bases of tRNA structures. Two deletions were observed at positions A12212del and T12261del in *tRNA$^{Ser[AGC]}$* with MFE of normal tRNA being -14.9kcal/mol. Due to the deletion of bases at the positions mentioned above the MFE increased to -10.8kcal/mol and -11.4kcal/mol respectively. The deletion mutations led to disruption in the secondary structure [Figure-9,10-A,B].

FIGURE-9

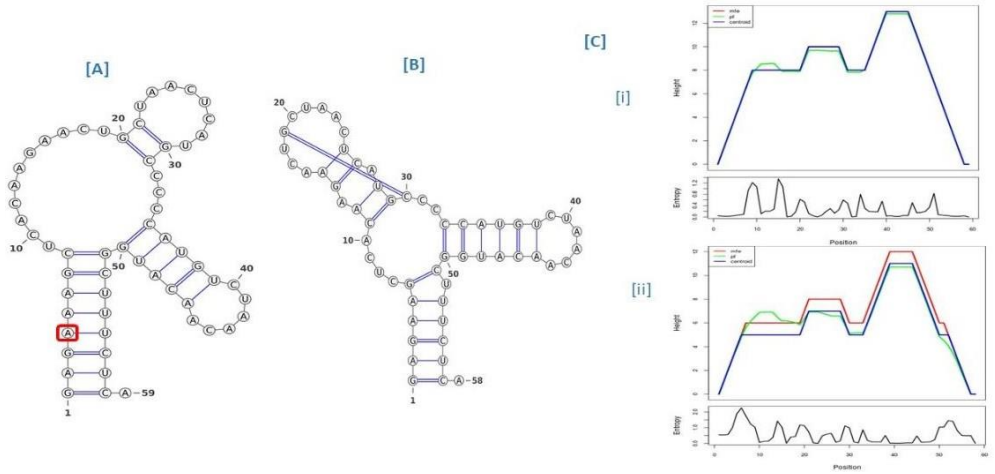

Figure-9: A&B- *Structure of tRNA$^{Ser[AGC]}$ Wildtype and deletion mutant[A12212del] ;* ***C- i &ii*** *– Mountain plots of Wiltype and deletion mutant tRNA.*

FIGURE-10

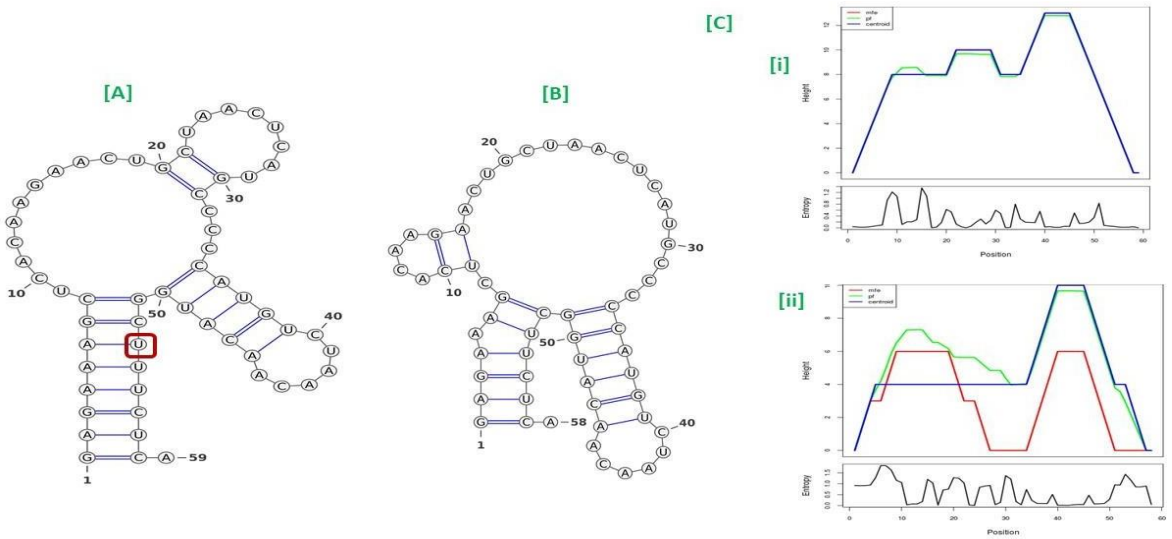

Figure-10: *Structure of tRNA$^{Ser[AGC]}$ Wildtype and deletion mutant[T12261del] ;* ***C- i &ii*** *– Mountain plots of Wiltype and deletion mutant tRNA.*

Another deletion in *tRNA$^{Leu[CUN]}$* at position A12328del led to a slight increase in the MFE from -15.0kcal/mol to -14.8kcal/mol. Though there has been a slightest increase in MFE, there has been disruption in the secondary structure of the tRNA molecule [Figure-11-A,B].

FIGURE-11

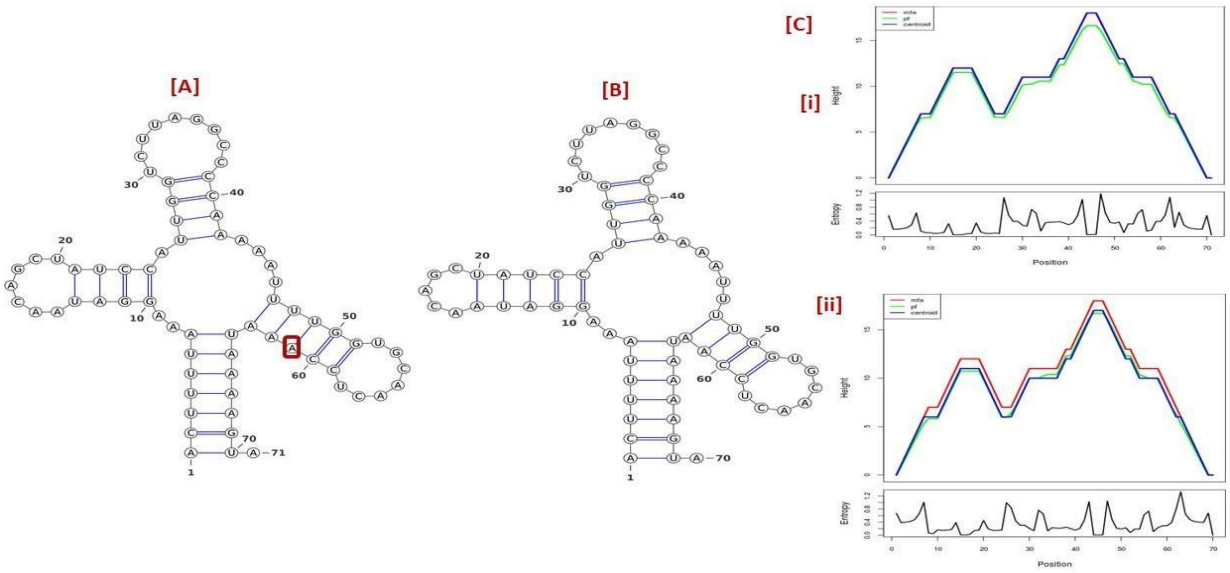

Figure-11: *Structure of tRNA$^{Leu[CUN]}$ Wildtype and deletion mutant[A12328del] ;* ***C- i &ii*** *– Mountain plots of Wiltype and deletion mutant tRNA.*

The mountain plot illustrates RNA secondary structure, where height corresponds to position. Plateaus represent loops, with hairpin loops as peaks, while slopes indicate helices. The X-axis denotes nucleotide position, and the Y-axis shows the number of base pairs enclosing each nucleotide. RNAfold visualizations use color-coded lines: Red for the most stable predicted Minimum Free Energy (MFE) structure, Green for pair probabilities indicating base pair likelihood, and Blue for the centroid structure, reflecting the ensemble of folded states. The plots representing *tRNA^{Ile}* at positions 4264 and 4281 displayed changes attributed to mutations. Specifically, the mutated structure showed a higher Minimum Free Energy (MFE) plot at position 4264 [Figure-2[C]], and alterations in plateaus and slopes in the plot at position 4281 [Figure-3[C]]. Concerning the mutation at position 5594, the wildtype protein exhibited the highest MFE peak, which was disrupted by the mutation, indicating increased potential for various conformations as the coloured lines no longer aligned [Figure-4[C]]. For the mutational position 12261 in *tRNA^{Ser}*, the plot pattern demonstrated alterations in plateau and slope heights, along with a decrease in plot height compared to the wild-type structure [Figure:5[C]]. Mountain plots were scrutinized for mutations at positions 12300, 12306, and 12329 in tRNA-Leucine [CUN]. Position 12300 displayed minimal deviation from the wild type, whereas at position 12306, slight modifications hinted at regions favouring an irreversibly stable structure. Conversely, position 12329 exhibited no significant alterations in the mountain plot [Figure-6[C], 7[C],8[C]]. Additionally, plots were generated for deletions at these positions. In tRNA-Serine [AGC], at position 12212, the prominence of the MFE structure was indicated by a higher red line [Figure-9[C]]. Mutation at position 12261 disrupted tRNA's secondary structure, evident in plateau and slope dynamics [Figure-10[C]], while deletion at position 12328 showed no notable changes except for the increased presence of the MFE structure [Figure-11[C]]. The Ensemble diversities of the wildtype tRNA and mutated structure were compared to get insights into the potential flexibility upon a mutation or deletion. The values of all the structures with mutated/deleted bases [Table-5] show higher diversity when compared to the original mt-tRNA structure.

Discussion

Colorectal cancer (CRC), involving cancer of the colon-rectum, poses a substantial health challenge, ranking as the third most frequently diagnosed and second deadliest cancer worldwide(Zuker and Stiegler 1981)[https://www.who.int/news-room/fact-sheets/detail/cancer]. In this study we employed the advanced technology of Next generation sequencing for screening the mutations across mt-tRNAs of colorectal cancer tumour and adjacent normal tissues .As a result, seven mt-tRNA mutations and three significant deletions were detected. The structural changes conferred by these mutations were studied using RNAfold Server. Also, MitoTIP tool predicted the pathogenicity of identified variants.

The mutation G4264A located in acceptor arm of $tRNA^{Ile}$ disrupted the base pairing of 2G-67C that is highly conserved. The variant can be predicted to be likely pathogenic based on the MitoTIP scores. Structural disruption can be evidenced by an increase in the MFE upon mutation. The variant has been classified as a VUS mutation [rs1603219385](Alston et al. 2021; Yarham et al. 2010; Hossain et al. 2022). Possibly the mutation can disrupt the binding of amino acids to the tRNA molecule during the process of aminoacylation that can lead to impaired translation of mitochondrial genes, affecting the synthesis of proteins critical for oxidative phosphorylation and energy production.

Another mutation detection in $tRNA^{Ile}$ is A4281G(Wong et al. 2020; Rajkumar et al. 2005). This heteroplasmic transition mutation was located in the D-stem of the tRNA disrupts the evolutionarily highly conserved base pair and is likely to have functional importance. Also, structural disruption is observed with also decrease in the MFE [-8.8 kcal/mol]. This is further corroborated by earlier findings: in one instance, a T > C transition at nucleotide position 4274, which disrupts the same base-pair in the DHU stem leading to diseased states. Investigations into the molecular mechanism underlying the m.4274T > C mutation's impact on mitochondrial protein synthesis have revealed a decreased efficiency in the post-transcriptional modification of the mutant $tRNA^{Ile}$. Specifically, this substitution hinders the efficiency of aminoacylation(Emmanuele et al. 2011; Chinnery et al. 1997; Borthwick et al. 2006; Levinger, Giegé, and Florentz 2003).The T5594A mutation has been identified in the acceptor arm of $tRNA^{Ala}$. This mutation position is highly conserved and likely a pathogenic mutation. The MFE for the structure upon acquiring mutation was found to be increased [-4.0kcal/mol] in comparison to the wild-type structure. The phylogenetic analysis of this mutation and mtDNAs from the other 10 vertebrates revealed that the nucleotide T at the conventional position 5594 was extremely evolutionarily conserved. The occurrence of this mutation across the samples in

the study indicated a significant role in pathogenicity and hypothesized to cause an impaired translation of mitochondrial genes during oxidative phosphorylation. The T12261C mutation in $tRNA^{Ser[AGC]}$ disrupts the well-conserved bonding between 4A-55C, indicating a potentially pathogenic mutation supported by an increase in the Minimum Free Energy (MFE) (-11.4 kcal/mol). Located in the aminoacyl acceptor stem, this mutation has structural and functional implications for mt-tRNA. Analysis of single muscle fibers revealed a significant correlation between the presence of the m.12261T>C mutant load and COX deficiency. Applying the recently updated canonical pathogenicity scoring system confirmed its classification as 'pathogenic,' scoring 13 out of a potential 20 points(Kelley, Steinberg, and Schimmel 2001). Three substitutions were observed in mt-tRNA $tRNA^{Leu[CUN]}$ - G12300C, A12306C, and T12329A. All three mutations are observed in highly conserved bases. The G12300C mutation did show a slight increase in the MFE when compared to the wild-type structure without major structural disruption . Mutation A12306C disrupts the highly conserved 27T-41A upon acquiring mutation. An increase in the MFE has been observed. Both mutations were observed in the anticodon arm of the tRNA structure. These mutations are predicted to be possibly pathogenic as they potentially affect the ability of the tRNA to fold properly and bind to its cognate amino acid or the mRNA codon. The T12329A mutation is located in the acceptor arm and a structural disruption has been observed leading to an increase in the MFE. Mutations in the acceptor arm impair aminoacylation, reducing functional mt-tRNAs. This disrupts mitochondrial protein synthesis, leading to fewer adequately charged tRNAs for translation. Consequently, there's a deficit in crucial proteins vital for oxidative phosphorylation, impeding cellular energy production(Karasik et al. 2021).

Three significant deletions were observed in the tRNA i.e., two in $tRNA^{Ser[AGC]}$ and one in $tRNA^{Leu[CUN]}$ respectively. Both the mutations of $tRNA^{Ser[AGC]}$ A12212del and T12261del were present on the acceptor arm of the tRNA at highly conserved positions. These deletions affect the conserved bonding and may lead to structural disruptions that increase the MFE of the structure. Based on the MitoTIP predictions both the deletions are likely pathogenic. The deletion in the TΨC arm of $tRNA^{Leu[CUN]}$ A12328del led to a slight increase in the MFE of the structure but not at a significant level. The mutations on TΨC arm are generally considered more tolerant to mutations nevertheless but sometimes affect the translation efficiency. This exciting study proposes that blocking mt-tRNA writers or mitochondrial protein synthesis could offer a promising therapeutic strategy to hinder the spread of tumor cells from primary tumors.

Conclusion

In summary, this research emphasizes mitochondrial studies' potential in identifying high-risk individuals for cancer and developing early detection methods using bioinformatics and phylogenetic based approach. Next-Generation sequencing allows precise detection of novel mutations, illuminating their role in tumorigenesis. Further exploration of mitochondrial tRNAs influence on tumour development is warranted. Pathogenic mt-tRNA mutations like $tRNA^{Ile}$ G4264A, A4281G, and $tRNA^{Ser[AGC]}$ T12261C, may contribute to colorectal cancer through mitochondrial dysfunction, though confirmation is needed. This study enhances existing data by considering factors such as structural disruption and copy number. Novel mutations like $tRNA^{Ala}$ T5594A and $tRNA^{Leu[CUN]}$ G12300C, A12306C, T12329A, require further investigation. Despite some mutations showing minimal impact, they could still serve as valuable biomarkers. Larger studies are needed to validate these findings due to the study's limited sample size.

Declaration of Interest

The authors report no conflicts of interest.

Authors Contributions

The authors are grateful to MNJ Institute of Oncology for providing the samples for the study. Ramya Gadicherla in study design, sample processing, data analysis, and drafting of manuscript. Dr. Niraj Rai for providing sources for Next Generation Sequencing. Dr. Rajath Othayoth in Data analysis. Dr. Srinivasulu Kamma in study design, and monitoring of work progress, helped in drafting the manuscript and Corresponding author. All authors read and approved the final manuscript.

Ethical Approval and Informed consent

The study was approved and conducted under the surveillance of the Institute of Genetics and Hospital for Genetic Diseases, Hyderabad and Department of Genetics, University college of Science, Osmania University , Hyderabad. All the participants provided consent in the format suggested by the authorities[Ethical approval certificate enclosed as separate attachment document].

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1) **Table 1: Demographic and Clinicopathological data of the samples used in the study**

Clinical Feature	Stratification	Number of people[%]
Age[45-80]	<60 years	14[56%]
	>60 years	11[44%]
Gender	Male	13[52%]
	Female	12[48%]
Tumour location	Sigmoid Colon	6[24%]
	Ascending Colon	7[28%]
	Rectum	12[48%]
Stage[TNM Classification]	T1	4[16%]
	T2	7[28%]
	T3	9[36%]
	T4	5[20%]

2) **Table-2:**

A] Primers for MT DNA amplification. The two sets of primers amplify ~ 8.5kb region each of the Human mitochondrial genome.
[Ref: Gould MP, Bosworth , *etal.*,doi: 10.1371/journal.pone.0139253.]

Amplicon-1 Primers	**F**-5'- AAATCTTACCCCGCCTGTTT-3' **R**-5'-AATTAGGCTGTGGGTGGTTG-3'
Amplicon-2 Primers	**F**-5'-GCCATACTAGTCTTTGCCGC-3' **R**-5'-GGCAGGTCAATTTCACTGGT-3'

B] Primers specific for amplification of ND1 region of 104bp and Beta-Actin region of 105bp

ND1 Primers	**F**-'5'-TAATGCTTACCGAACGAA-3' **R**-'5'-TTATGGCGTCAGCGAAGG-3'
Beta-Actin	**F**-5'-GCAAAGTTCCCAAGCACA -3' **R**-5'- AAGCAAGCAGCGGAGCAG -3'

3) **Table 3:** Clinicopathological parameters for assessing the copy number in 25 samples of tumour and normal of Colorectal Cancer.

Clinicopathological parameters	Group	mtDNA/b-actin mtDNA copy numbers (T/N ratio)	Mt copy number		P-value
			Low	High	
Age	<60	51.9±14.1	11	3	0.220
	>60	81.7±11	10	1	
Stage					
	T1	65.65±10.15	3	2	0.0353*
	T2	73.1±19.6	5	4	
	T3	62.5±24.6	5	4	
	T4	69.5±14	7	4	

4) Table:4-

A] List of mt-tRNA mutations identified in the study

S.No	tRNA	Sequence Alteration	Location	Watson-Crick basepairing	Conservation Index[%]	Homoplasmy/ Heteroplasmy	Pathogenicity
1	$tRNA^{Ile}$	G4264A	acceptor arm	G-A	100	Heteroplasmy	likely pathogenic
2	$tRNA^{Ile}$	A4281G	DHU ARM	A-G	100	Heteroplasmy	likely pathogenic
3	$tRNA^{Ala}$	T5594A	acceptor arm	T-A	100	Homoplasmy	likely pathogenic
4	$tRNA^{Ser[AGC]}$	T12261C	acceptor arm	T-C	100	Heteroplasmy	possibly pathogenic
5	$tRNA^{Leu[CUN]}$	G12300C	anticodon arm	G-C	100	Heteroplasmy	possibly pathogenic
6	$tRNA^{Leu[CUN]}$	A12306C	anticodon arm	A-C	92.8	Heteroplasmy	possibly pathogenic
7	$tRNA^{Leu[CUN]}$	T12329A	acceptor arm	T-A	100	Homoplasmy	likely pathogenic
8	$tRNA^{Ser[AGC]}$	A12212del	ACCEPTOR ARM	NA	100	NA	possibly pathogenic
9	$tRNA^{Ser[AGC]}$	T12261del	ACCEPTOR ARM	NA	100	NA	likely pathogenic
10	$tRNA^{Leu[CUN]}$	A12328del	TΨC arm	NA	100	NA	likely pathogenic

B] Mutational occurrence across Tumor and Near Normal tissues

Mutation	Number of samples observed[Normal]n=25	Number of samples observed[Tumor] n=25	*P-value[<0.05]*
G4264A	4[16%]	11[44%]	0.029
A4281G	2[8%]	11[44%]	0.0155
T5594A	3[12%]	11[44%]	0.012
T12261C	5[30%]	13[52%]	0.022
G12300C	3[12%]	8[32%]	0.091
A12306C	4[16%]	12[48%]	0.11
T12329A	8[32%]	14[56%]	0.099
A12212del	3[12%]	13[52%]	0.015
T12261del	5[30%]	17[68%]	0.061
A12328del	4[16%]	15[60%]	0.013

5) Table-5 : Summary of analysis of mt-tRNA mutations using RNAfold Webserver

tRNA's	WILD TYPE MFE[kcal/mol]	**Ensemble Diversity	MUTATION	*MFE[kcal/mol]	Ensemble Diversity	Structural Disruption
$tRNA^{Ile}$	-7.5	8.05	G4264A	-4.5	19.68	Yes
			A4281G	-8.8	13.57	Yes
$tRNA^{Ala}$	-4.9	9.86	T5594A	-4.0	11.03	Yes
$tRNA^{Ser[AGC]}$	-14.9	3.11	T12261C	-11.4	8.42	Yes
$tRNA^{Leu[CUN]}$	-15.0	4.48	G12300C	-14.20	6.49	No
			A12306C	-13.3	11.68	Disruption in bonds
			T12329A	-13.9	8.22	No
$tRNA^{Ser[AGC]}$	-14.9	3.11	A12212del	-10.8	7.82	yes
			T12261del	-11.4	9.4	yes
$tRNA^{Leu[CUN]}$	-15.0	4.48	A12328del	-14.8	5.18	yes

*MFE: Minimum Free Energy. MFE refers to the predicted **most stable secondary structure** for an RNA molecule

**Ensemble diversity: This refers to the variation or heterogeneity within the possible secondary structures of an RNA molecule. A value higher in comparison to the wildtype structure suggests a highly homogenous ensemble with the MFE structure being dominant.