POTENTIAL MITOCHONDRIAL–SPECIFIC FUNCTION OF piRNA

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In

Life Sciences with Specialization in Molecular Medicine

By

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CERTIFICATE

I declare that the dissertation entitled "**POTENTIAL MITOCHONDRIAL-SPECIFIC FUNCTION OF piRNA*"*** has been prepared by me under the guidance of Dr. Sandeep Singh, Assistant Professor, and Department of Human Genetics and Molecular Medicine, School of Health Sciences, Central University of Punjab. No part of this project work has formed the basis for the award of any degree or fellowship previously.

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I certify that Shouvik Paul has prepared her research project entitled **"POTENTIAL MITOCHONDRIAL-SPECIFIC FUNCTION OF piRNA"**, for the award of M.Sc. degree of the Central University of Punjab, under my guidance. He has carried out this work at the Department of Human Genetics and Molecular Medicine, School of Health Sciences, Central University of Punjab.

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ABSTRACT

POTENTIAL MITOCHONDRIAL-SPECIFIC FUNCTION OF piRNA.

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Piwi-interacting RNAs (piRNAs) are (26–31 nt) small noncoding RNAs processed from their longer precursor transcripts with the help of Piwi proteins. There are more than 30,000 piRNA genes present in the human genome which now turns out to be emerging player in both homeostasis and diseases. Localization of piRNA and PIWI in the repeat region of the mammalian nuclear genome in germ cells has been reported, although localization and potential functional role of piRNA in the mammalian mitochondrial genome are largely unknown. We have taken 111 piRNA sequences found in the MCF-7 mitochondrial genome, which is obtained by NGS analysis for alignment study. Resulting piRNA have been aligned with DQ112870 North American Homo sapiens mitochondrion genome for studying post-transcriptional roles of piRNA.

Shouvik Paul Dr. Sandeep Singh

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Shouvik Paul

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LIST OF ABBREVIATIONS

S. No	Full Form	Abbreviation
1	Transposable element	TE
2	P-element induced wimpy testis	PIWI
3	PIWI interacting RNA	piRNA
4	Michigan cancer foundation-7	MCF-7
5	Long Interspersed nuclear element	LINE
6	Short interspersed nuclear element	SINE
7	piRNA-induced silencing complexes	piRISCs
8	Aubergine	Aub
9	Argonaute	Ago3
10	Next-generation sequencing	NGS
11	Structure of Y-chromosome locus	SU
12	Piwi,Ago and Zwile	PAZ domain
13	Moloney leukemia virus 10-like protein 1 imported	MOV10L1
14	DEAD-BOX Helicase 4	DDX4
15	Tudor domain containing 12	TDRD12
16	Maelstorm spermatogenic Transposon silencer	MAEL
17	FK506 binding protein	FKBP6
18	Gametocyte specific factor 1	GTSF1
19	Myb-proto oncogenic gene	A-MYB

Chapter I

Introduction

INTRODUCTION:

Our Human genomes encode more than 40,000 genes that control cellular function, regulation, development, and homeostasis. Non-coding RNA (ncRNA) does not encode a protein, but they pose certain roles and functions. Only 3% protein has been translated from mRNA transcripts where rest of noncoding RNA poses important roles in translation, transcription and disease regulation. Small noncoding RNA shows important roles in human genomics (Han Y *et al.,* 2017). Noncoding RNAs includes mi RNA, piRNA, snRNA, rRNA, long ncRNA. Gene expression such as chromatin architecture, transcription, RNA splicing, editing, translation, and turnover can be effectively constrained by this same ncRNAs. They expressed most of our complex characteristics, control disease. These constitute an unexplored world of genetic variation of species. Epigenetic alteration is an orchestrated fashion of controlling gene expression in both disease and homeostasis (Eva-Maria Weick *et al.,* 2014).

Here we talk about piRNA and its role in human disease. PIWI-interacting RNAs (piRNAs) are 24–31 nucleotides long which bind specifically to the PIWI subfamily of Argonaute proteins. piRNA was discovered first in Drosophila melanogaster. piRNA-induced silencing complexes are created by it and repress transposons via transcriptional or posttranscriptional mechanisms. Germline genome integrity is also maintained by piRNA. piRNAs have shown transgenerational inheritance for passing the memory of “self ” and “nonself”. Germline piRNA is involved mainly in the silencing of the selfish genetic element (jumping genes). Regulations, epigenetics, and targets of piRNA have been discussed further. Nevertheless, the potential implication in diagnosis and prognosis roles of piRNA has yet been elucidated (Alan Fu *et. al.,* 2015).

The most abundant expression of PIWI protein has been observed in germline cells where they called as piRISCs and at the 3` terminus of piRNAs have 2`-O-methyl modification sites that processed from single-stranded precursor transcripts. PIWI refers to an individual protein where collectively there is 3 type of Argonaute proteins includes piwi, Aub and Ago3. It is a nuclear protein. piRNAs have no secondary structure motifs. PIWI proteins are mainly expressed in early development and in germ and stem cells, though piRNAs are expressed in many

adult differentiated cells. Because of the processing mechanisms and different types of transposons, piRNA sequences are much more diverse. They often detect in cancers associated with germline or even somatic tissues. (Han Y *et al.,* 2017) The piRNA requires multiple stages of spermatogenesis including de novo DNA methylation, meiosis, and spermiogenesis. The germlines of all metazoa contain electron-dense cytoplasmic structures as nuage. This nuage plays a major role in piRNA maturation and binding of RNA transcript with piwi protein. Mitochondrial protein BmPAPI modulates the length of mature piRNAs (Yuka W *et al.,* 2017).

The complex mechanism of piRNA helps to select and regulate the nonself genes where cytoplasmic factors have been effectively controlling piRNA biogenesis. The function of it cleared currently that piRNAs participate in post-transcriptional activities which are similar to miRNAs by interacting with other Argonaut proteins and silencing mRNAs in the cytoplasm. Robust sequence technology helps to generate many different approaches that helped to answer many questions about piRNAs. Careful analysis of data is highly recommended for this purpose. A piRNA database has been used where 77 million piRNA sequence from 9 organisms have stored. Furthermore, reported piRNA targets also been analyzed. The vast roles and rapidly increasing numbers of piRNA need a specific web platform for piRNA. In RNAcentral, the main database for piRNA is piRNA bank. Outside piRNA bank, RNA quest and piRBase also present. Currently, 32,194 sequence of human is available in piRNA bank.

Presently substantial research has been going on the epigenetic and post-transcriptional effect of piRNA in mammalian cells. In this study, we utilized computational analyses to study the possible presence of Piwi and piRNA transcripts in mitochondria from human normal and cancer cell lines. Next-generation sequencing (NGS) is high-throughput sequencing combines with different modern sequencing technologies (Solexa) sequencing, Roche 454 sequencing, Ion torrent: Proton / PGM sequencing, SOLiD sequencing) have been used to sequencing mcf-7 breast cancer cell mitochondrial genome in our study. These allow us to sequence DNA and RNA much more quickly and cheaply than the previously used Sanger sequencing. They used as such have revolutionized the study of genomics and molecular biology. (Mitochondria-unique genome of NORTH AMERICAN DQ112270 overlapped the piRNA transcripts.) To our

knowledge, this is the first report on the localization of piRNAs and Piwi proteins in mammalian mitochondria where we study adaptability of mutated sequence in mitochondria.

Chapter II
Review of Literature

2. REVIEW OF LITERATURE:

2.1 piRNA and PIWI protein

The piRNA were initially discovered in Drosophila as small RNA transcribed from repetitive elements like retrotransposons, DNA transposon, and SU locus. Since then, it has been found in different organisms. There are thousands of piRNA in different species, although their sequence is not conserved. The piRNA is different from miRNA and siRNA. There 5` end has preferences for uridine and 3` end of piRNA are 2'-o-methylated. By definition, piRNA binds with PIWI protein, which is a subfamily of Argonaut protein. They express specifically in gonads while Ago expresses ubiquitously. Members of AGO protein family binds with various small ncRNAs and involved in RNA induced silencing complex. There are 4 structural domains N-terminal (RNA Duplex unwinding and catalytic activity) MID (incorporates and stabilized 5` ends), PAZ (incorporate 3` ends and determines the length of small RNA) and PIWI domain (RNase H-like fold with conserved aspartate-aspartate-glutamate catalytic motifs which helps to silence there targets through the endonuclease/slicer activity) (Yuka W *et al.,* 2017).

Table 1: Components of piRNA pathway.

Mouse protein	**Knockout phenotype**	**Role in the pirna pathway**	**Fly homolog**
PIWIL 1(MIWI)	Spermiogenic arrest	Primary biogenesis	PIWI
PIWIL2(MILI)	Meiotic arrest	Primary and secondary	PIWI
PIWIL4(MIWI2)	Meiotic arrest	Secondary biogenesis	PIWI
MOV10L1	Meiotic arrest	Primary biogenesis	ARMITAGE
DDX4 (MVH)	Meiotic arrest	Secondary biogenesis	VASA
TDRD12	Meiotic arrest	Secondary biogenesis	YB
MAEL	Meiotic arrest	Primary and secondary	MAELSTROM
FKBP6	Meiotic arrest	Secondary Biogenesis	SHUTDOWN
GTSF1	Meiotic arrest	Transcriptional transposon silencing	GTSF1

A-MYB	Meiotic arrest	Transcription of pirna precursors	

2.2 PiRNA biogenesis

The regulatory function of piRNAs have been accomplished through binding of piRNA with PIWI protein and forming the structural motif of the ribonucleoprotein complex that silenced complimentary sequences. At transcriptional and post-transcriptional level, the main function of this complex is to silence transposable elements that help them to maintain genome integrity. The function of this piRNA complex is highly conserved among species (Eva-Maria Weick *et al.,* 2014).

Germ cells carry the genetic information for future generations; animals have certain mechanisms to protect those lineages from harm. The ping-pong feed-forward amplification mechanism is important for generating sufficient amounts of silencing triggers against highly active elements, and provide a mechanism for sequence diversification by downstream phased piRNA production (Yuka W *et al.,* 2017).

Animals engage in both Post-transcriptional gene silencing and transcriptional gene silencing because of the effects of functional causes of transposons. Indeed, the effect of transposon mobilization has been silenced by PTGS and TGS and cause the destruction of imminent threats (Eva-Maria Weick *et al.,* 2014).

Activated ribonucleotide complex cleaves targeted RNAs at the position of 10 to 11 guide strand. It helps to create 5’ end of RNA that loads into second PIWI protein and helps to create secondary piRNA. It has been observed that uridine have biasness towards 5’ nucleic acid and adenosine has biasness at 10 nucleotide positions. JmjC domain-containing histone demethylation protein 1 (Epe1), chp1 and chp2 create epigenetic activation. After recruitment of HP1a and HMT at nucleus create epigenetic silencing. Carbon catabolite repressed4 negative on TATA-less deadenylation complex associated with Smaug helps in mRNA degradation.

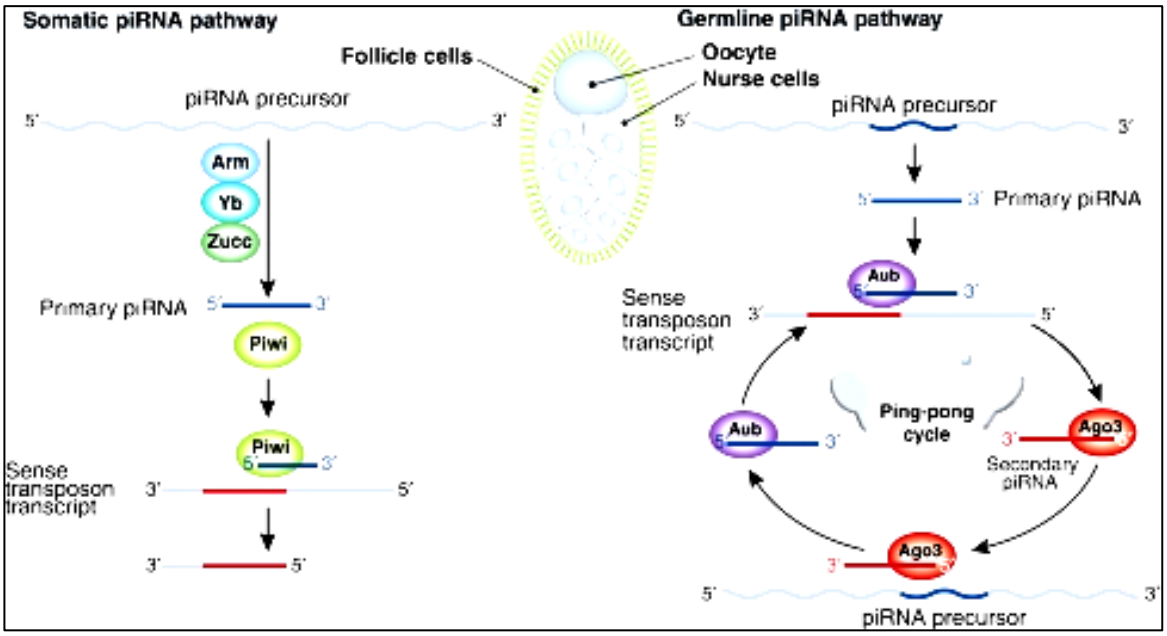

Figure 1. Biogenesis of piRNAs in the germline and in somatic follicle cells. In the germline, piRNAs are generated through an Aub- and Ago3-dependent piRNA amplication cycle, whereas in somatic cells, biogenesis occurs through a Piwi-dependent, Aub- and Ago3-independent pathway**.** (Source: Zamor PD, 2010)

2.2.1 piRNA BIOGENESIS IN MAMMALS

During primary processing, the genomic loci that encode piRNAs are initially transcribed as long RNAs, termed piRNA precursors. Pachytene piRNA precursors transcription is initiated by the A-MYB transcription factor. a-myb involved in the positive feedback loop and simultaneously regulate others(Tdrd1, miwi, and mitopld). Another difference between piRNA biogenesis and microRNA/siRNA biogenesis is that computational analysis of regions in the piRNA precursors immediately surrounding pachytene piRNAs shows a lack of stem-loops. Mito PLD/zucchini helps to size the long piRNA transcripts into shorted one that associates with MILI and MIWI. 5′ ends of mature piRNAs have a preference for uridine. MID domain of Ago proteins recognize and the 5′ terminal nucleotide and tend to incorporate miRNAs with a 5′uridine. 5′U biases are conserved. The exact lengths of piRNA intermediates are likely variable. Mg2+-dependent 3′-5′ exonucleolytic activity termed Trimmer activity helps to generate piRNA from primary transcript. TDRKH promotes the 3′ trimming of piRNA intermediates. HEN1 methylated the mature piRNA is 2’-O-methylated at its 3′ end and incorporated into the PAZ domain of the PIWI protein (Hirakata S *et al., 2*014).

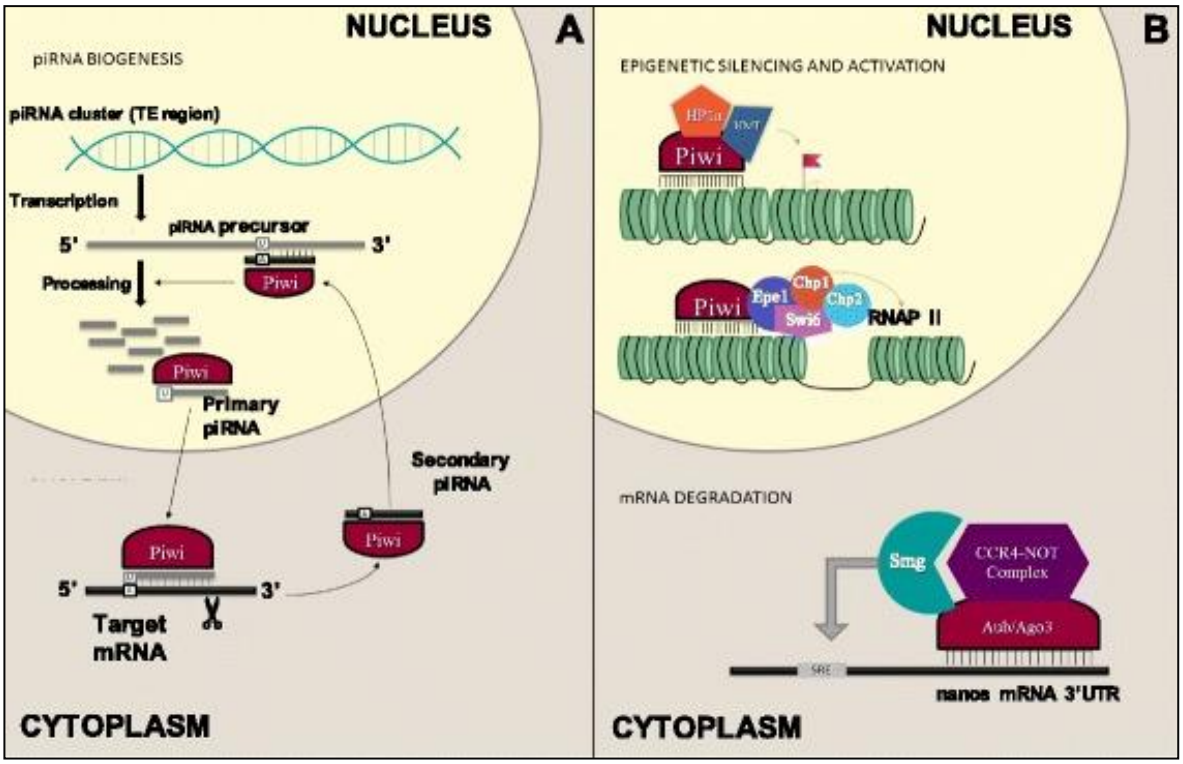

Figure 2: Mechanisms of piRNA-mediated transcriptional silencing. (Source: Weick E, 2014).

2.2.2 Secondary processing (Ping-pong amplification loop)

Primary processing of piRNA generates piRNA transcripts while secondary processing amplifies thing primary piRNA through Ping Pong loop. PIWI proteins cleave their RNA targets between the 10th and 11th nucleotides and generating the 5′ end of the secondary piRNA. Thus, Secondary piRNA intermediates are loaded onto other PIWI proteins and their 3′ ends are also processed by Trimmer activity. Secondary piRNAs, in turn, guide PIWI proteins to RNA targets to slice them and produce primary piRNAs at the same time. In fetal pre-pachytene piRNA, secondary piRNA transcripts observed. In mic, primary piRNAs are loaded onto MILI and secondary piRNAs are loaded onto both MILI and MIWI2 (Olovnikov IA *et al., 2014*). While MIWI2 is involved in the linear generation of secondary piRNAs, MILI forms an intra-amplification loop with itself to amplify piRNAs. Because MILI predominantly associates with fetal pre-pachytene piRNAs that are sensed to transposable elements, it is believed that transcripts of transposable elements are fed into the piRNA pathway to generate the initial pool of primary

piRNAs. Secondary piRNAs are antisense to transposable elements and guide MIWI2 to silence transposable elements in the nucleus (Hirakata S *et al.,*2014).

2.3 piRNA functions beyond transposon silencing; piRNA function in germline tissue

2.3.1 Repression of transposable elements

Functional roles of transposable element in genetic diversity and instability have created pathogenesis by mutational activity and chromosomal rearrangement. (non-LTR), LINE and SINE have shown the important role in certain cancers (Olovnikov IA *et al.,* 2014).

In leukemia, breast, ovarian, and colon cancers, SVA, and Alu have the important role to play as RNA polymerase II binding to TEs compared to non-malignant cell lines. This is the result of an increase in H3K9me3 and HP1 chromatin marks. Recruitment of PIWI, HP1a, and Su(var)3–9 causing overexpression of piRNA and the reduction of RNAPII, and the enrichment of H3K9me2/3.

Indeed, piRNA-mediated epigenetic silencing of TEs in Drosophila has been shown. In Drosophila, PIWI proteins Aubergine (Aub) and Argonaute 3 (Ago3) localize to the cytoplasm and repress transposons at a PT level through the cleavage of transcripts (fig. 2b). The coexistence of both cytoplasmic and nuclear piRNAs supports this theory. Also, it has been reported that Ago3-mediated RNA cleavage helps the production of antisense piRNAs which directs transcriptional silencing by Piwi in Drosophila (Eva-Maria Weick *et al.,* 2014).

piRNA/PIWI-mediated regulation of Transposable Elements has also been well characterized in mammals, mice have three protein. MIWI, MILI, and MIWI2 helps in methylation. In mice, specific piRNA knockdown leads to the derepression of LINE-1and a decrease in piRNA cluster expression has been shown to be associated with increased TE activity (Arrigo *et al.,* 2015).

2.3.2 Epigenetic activation

Epigenetic activation and piRNA have been extensively linked throughout the human gene expression. They pose certain properties that directly control transcription and euchromatin histone modification. The 3R-TAS1 expression has been positively expressed by piRNA in Drosophila. Knockdown of PIWI shows

opposite effect in the regulatory mechanism of the different part of the genes. (an example is dramatically increased telomere position effect in white eye gene in Drosophila. They are required for this reporter gene as they function as dose-dependent manner. The epigenetic state of 3R-TAS has active highly by this. This piRNA and CSR-1 complex of Argonaute protein in C.elegans extensively recruit histone modifying enzymes that certainly activate epigenetic regulations (Arrigo *et al.,* 2015).

2.3.3 mRNA decay

piRNA not only plays as silencing element by TE repression only. They target mRNA transcripts on late stages of spermatogenesis. Miwi and deadenylase CAF1 having the property of silencing by inducing mRNA deadenylation and degradation (Arrigo *et al.,* 2015).

2.3.4 piRNA expression and functions in somatic tissue

piRNA not only have effective roles in the germline, but also in somatic tissues. In stress response, piRNA might have certain roles as they localized in mitochondrial DNA. Smg-CCR4-NOT-Aub-dependent mechanism of mRNA deadenylation and decay has been observed in Drosophila.

At human bronchial epithelial cells (HBECs), pir-163 has been localized in nucleus and cytoplasm at different stages of cell cycle. Liquid chromatography-mass spectrometry (LC-MS) study implies that it has been interacting with different proteins such as ERM (Ezrin/Radixin/Moesin) family of proteins. They bind active phosphorylated ERM proteins. 3` UTR have been found vastly in piRNA (Eva-Maria Weick *et al.,* 2014).

2.4 Role of piRNA in various diseases

2.4.1 Emerging roles for PIWI proteins in cancer

PIWI proteins are usually expressed in the germline but absent in somatic tissues. Suppressing transposon and maintain genomic integrity is there main characterized role. Although there is evidence that suggested that PIWI proteins are linked to the hallmarks of cancer defined by Weinberg and Hanahan, such as cell proliferation, anti-apoptosis, genomic instability, invasion, and metastasis. This

provides new possibilities for anticancer therapies by targeting of PIWI proteins, which may have fewer side effects due to their potential classification as a CTA (cancer/testis antigen). Also, PIWI functioned as a diagnostic and prognostic marker for many types of cancer, and even to differentiate early- and late-stage cancers (Koduru SV *et al.,* 2017).

2.4.2 PIWIs are involved in promoting cell proliferation

Cell-cycle progression enhances cancer cell growth. It is the reason of cell-cycle promoters serve as good candidates for tumor oncogenes. Inhibition of HIWI suppressed the growth of human gastric cancer cells and induced cell-cycle arrest at the G2/M phase of the cell cycle. The PIWIL2-like (PL2L) protein PL2L60, a splice isoform of PIWIL2 is predominantly expressed in various types of human and mouse tumor cells also found to participate in cell-cycle progression by promoting the G0/1 to S phase transition. The identification of the isoforms of PL2L proteins helps in new insight into the molecular mechanisms of tumorigenesis and a new strategy for cancer diagnostics and anticancer drug development (Han Y *et al.,* 2017).

In breast cancer MDA-MB-231 cells, PIWIL2 activate the STAT3/cyclin D1 pathway, thus promoting cell proliferation. PL2L60 promote tumor cell survival and proliferation in vitro by up-regulating the nuclear expression of NF-κB and increasing STAT3 and Bcl-2 expression.

The theory of cancer stem cells (CSCs) in tumorigenesis accounts for the heterogeneity of tumor tissue by a hierarchy-based model. In human, the HIWI gene was shown to be involved in the maintenance of lung cancer stem cell populations and piwil2 is involved in maintaining the indefinite proliferation potential of Cancer Stem Cells to promote breast tumor growth (Han Y *et al.,* 2017).

2.5 PIWIs are involved in inhibiting cell apoptosis

HIWI silencing in glioma cells helps to increase cell-cycle arrest and promote cell apoptosis. It has also been shown that The expressions of p21, cyclin D1, Bcl-2, and Bax were also significantly altered. In MDA-MB-231 and NIH-3T3 cells, PIWIL2 was acting as an oncogene by activating the STAT3/Bcl-xL signaling

pathway that inhibits apoptosis and to promote proliferation. In HeLa cells, it is verified that PIWIL2 is directly associated with STAT3 protein via its PAZ domain and forms a PIWIL2/STAT3/c-Src triple protein complex. (*Han Y et al., 2017*) This STAT3 protein is further phosphorylated by c-Src and translocates to the nucleus where it binds to the p53 promoter and represses its transcription. This demonstrates that PIWIL2 plays an anti-apoptotic role in tumor cells. In HeLa cells, PIWIL4 was also found to control apoptosis through the p14ARF/p53 pathway without affecting the cell cycle while playing an oncogenic role in cervical cancer. there are also some possibilities that PIWIL4 can directly regulate p14ARF/p53 pathway by inducing H3K9 methylation at the p14ARF locus (Alan Fu *et. al.,*2015).

2.6 PIWIs are involved in facilitating cell migration and invasion

PIWI proteins have been reported to be related to cancer cell migration and invasion. In clinical cases, PIWIL1 expression was found to be associated with the invasion of cervical squamous cell carcinoma (CSCC) and, interestingly, had a statistically significant positive correlation with human papillomavirus 16 (HPV16). In hepatocellular carcinoma (HCC) cell lines, PIWIL1 expression was increased in parallel with the metastatic potential of HCC cell lines, i.e. the expression levels of PIWIL1 in MHCC97L, MHCC97H, and HCCLM3 cells were significantly higher than that in SMMC7721 and HepG2 cells. Even depletion of PIWIL1 caused decreased invasion and metastasis. piwil2 expressed in the cytoplasm, nucleus, or both the cytoplasm and nucleus (C-N) of invasive and metastatic breast cancers, showing a potential relationship with cancer cell migration and invasion (Alan Fu *et. al.,* 2015).

Though the mechanisms underlying the invasion and metastasis of cancer cells are largely unknown, there are proposed some theory often termed the invasion-metastasis cascade. In these steps, epithelial-mesenchymal transition (EMT) is a prominent regulatory program by which transformed epithelial cells can acquire the abilities to invade, to resist apoptosis, and to disseminate. it has been suggested that Some transcription factors, including Slug, Snail, Goose-coid, Twist, and ZEB1, are highly expressed by metastatic cells and have been suggested to play a role in inducing EMT. Though PIWIs influence or are influenced by these transcription factors and/or EMT remains to be investigated. Clearly, cell invasion and metastasis are strongly influenced by the extracellular matrix (ECM). Matrix

metallopeptidase 9 (MMP9), has previously been shown to play a critical role in mediating the tumor microenvironment and potential roles in cancer cell invasion and metastasis. Knockdown of Hiwi inhibits the migration and invasion of glioma cells by reducing the expression of MMP2 and MMP9. PIWIL2 can promote cell migration and invasion by activating the MMP9 in SW620 and SW480 colon cancer cell lines (Ye *et al.,* 2015).

2.7 Functional roles of piRNAs in cancer

piRNA has the vital role in degradation, inhibition of tumor suppressor genes, oncogenes, respectively. Mutagenic retrotransposition and genomic instability have been highly affected by piRNA that's usual role is suppression of genes. DNA hypo/hypermethylation and histone modification are hallmarks of cancer (Ye *et al.,* 2015).

2.8 Altered expression of piRNAs in cancers

There are certain upregulations or downregulations of piRNA observed in cancer. Aberrant expression of the piRNA/PIWI complex and its correlation with clinical features in malignant tissues points out that there are certain roles for piRNAs in cancer. This role is uncertain as the function of piRNA in somatic tissue is still being established.

Table1: Some example of altered expression of piRNA in cancer given bellow.

Cancer Type	**piRNA ID**	**Alteration**	**Detection method**
Breast	piR-4987	upregulated	deep sequencing of matched tumour-normal tissues, validated via PCR
Gastric	piR-823	downregulated	piRNA microarray of cell lines, validated via PCR
Kidney	piR-32051	upregulated	Small RNAseq of tumour tissue, validated via PCR

Liver	piR-Hep1	upregulated	deep sequencing of cell lines, validated in matched tumour-normal tissues via PCR
Lung	piR-L-163	downregulated	small RNAseq of NSCLC cell lines

2.9 piRNA as diagnostic and prognostic marker

It is discovered by Cheng et all that pir-651 is over-expressed by gastric cancer and confirmed it by PCR analysis. They inhibit the G2/M phase of gastric cancer cells in a dose-dependent manner, suggesting its role in potential biomarker in pir-651 for gastric cancer diagnosis. Even it is also reported that it could be upregulated in breast, colon, and lung cancer tissues.

Busch et al. identified 235 upregulated and 369 downregulated piRNA from 109 renal cell carcinoma patient samples. More recently Martinez et all identified that nearly half of expressed piRNA of 358 non-malignant stomach tissue and gastric adenosarcoma sample were over-expressed, includes piR-59056, piR-32105, and piR-58099, which is capable of saving the patient from recurrence. Currently, Krishnan et al discovered 8 piRNA as novel independent prognostic markers in breast cancer (Koduru SV *et al.,* 2017). It was confirmed with cancer genome ATLAS database.

2.9.1 PIWI protein as Diagnostic and Prognostic biomarkers

HIWI expression used as the potential biomarker for diagnosis and prognosis for malignant glioma and Hepatocellular carcinoma. HIWI led to poor outcome in colon adino carcinoma. it was related to poor prognosis in gastric cancer where it leads to less than 5-year survival (Han Y *et al.,* 2017).

Chapter III
Material and Methods

3. METHODOLOGY

Objective

a) To find different mitochondrial targets of piRNA using global alignment tool. (Mitochondrial DNA specific)
b) Validation of alignment studies: The validation of sequences is essential to perform accurate phylogeny and structure/ function analysis.

3.1 BLAST

For finding local similarities between sequences, we used blast. It's a computational biology tool used for comparing nucleotide or protein sequences to sequence databases for finding the highest number of similarity between them, and also analyze statistical significance between them. Here, the mitochondrial sequence derived from MCF7 cell line by NGS analysis were taken (we take 111 piRNA sequence) and mitochondrial genome was aligned to find the highest similarity between them.

3.2 Global Alignment

Global alignment is Needleman-Wunsch algorithm based on dynamic programming. Closely related sequences that are having the same length are very much appropriate for global alignment. Here the alignment study was carried out from beginning till the end of the sequence to find out the best possible alignment. These techniques are used in many different aspects of bioinformatical science. The algorithm explains global sequence alignment for aligning nucleotide or protein sequences. Complete Mitochondrial sequence (North American) is extracted from the mitochondrial database. The extracted sequence was aligned globally with different piRNAs.

3.3 Mitomap

MitoWeb is a platform for distributing mitochondrial information and developing web-based bioinformatics tools for its analysis. The sequences showing high similarity with the query sequences were chosen and their gene location was determined by using mitomap.

3.4 SEQUENCE ALIGNMENT

3.4.1 MUTATION IN PIRNA VS DQ112870 NORTH AMERICAN

Alignment of 2 sequences by changing the observed mutation with wild type

If two sequences in an alignment share a common ancestor, mismatches can be interpreted as point mutations and gaps as indels (that is, insertion or deletion mutations) introduced in one or both lineages in the time since they diverged from one another. In sequence alignments of proteins, the degree of similarity between amino acids occupying a particular position in the sequence can be interpreted as a rough measure of how conserved a particular region or sequence motif is among lineages. The absence of substitutions, or the presence of only very conservative substitutions (that is, the substitution of amino acids whose side chains have similar biochemical properties) in a particular region of the sequence, suggest that this region has structural or functional importance. Although DNA and RNA nucleotide bases are more similar to each other than are amino acids, the conservation of base pairs can indicate a similar functional or structural role.

Chapter IV
Results

4. Results

4.1 GLOBAL ALIGNMENT

Table2: Complete mitochondrial genome (North American, DQ112870) is globally aligned with PIRNA

S. No.	PI RNA	SIMILARITY	ALIGNMENT
1	piR-hsa-12488	**31/31**	Query 2 ----------AGAGTGTAGCTTAACACAAAGCACCCAACTG 32 |||||||||||||||||||||||||| Sbjct 1141 GTACTGGAAAGTGCACTTGGACGAACCAGAGTGTAGCTTAACACAAAGCA CCCAACTTAC 1200
2	piR-hsa-12487	**31/31**	Query 2 ----------AGAGTGTAGCTTAACACAAAGCACCCAACT 31 |||||||||||||||||||||||||| Sbjct 1141 GTACTGGAAAGTGCACTTGGACGAACCAGAGTGTAGCTTAACACAAAGCA CCCAACTTAC 1200
3	piR-hsa-28875	**31/31**	Query 1 GTTTAGACGGGCTCACATCACCCCATAAACA 31 |||||||||||||||||||||||||| Sbjct 181 TGTTTAGACGGGCTCACATCACCCCATAAACAAATAGGTTTGGTCCTAGCC TTTCTATTA 240
4	piR-hsa-27729	**26/26**	Query 1 GCTAAACCTAGCCCCAAACCCACTC 25 |||||||||||||||||||||||||| Sbjct 1201ACTTAGGAGATTTCAACTTAACTTGACCGCTCTGAGCTAAACCTAGCC CCAAACCCACTC 1260 Query 26 C 26 | Sbjct 1261 CACCTTACTACCAGACAACCTTAACCAAACCATTTACCCAAATAAAGTATAG GCGATAGA 1320
5	piR-hsa-27730	**27/27**	Query 1 GCTAAACCTAGCCCCAAACCCACTC 25 |||||||||||||||||||||||| Sbjct 1201 ACTTAGGAGATTTCAACTTAACTTGACCGCTCTGAGCTAAACCTAGCCCCA AACCCACTC 1260 Query 26 CA 27 || Sbjct 1261 CACCTTACTACCAGACAACCTTAACCAAACCATTTACCCAAATAAAGTATAG

			GCGATAGA 1320
6	piR-hsa-27731	**31/31**	Query 1 GCTAAACCTAGCCCCAAACCCACTC 25 |||||||||||||||||||||| Sbjct 1201 ACTTAGGAGATTTCAACTTAACTTGACCGCTCTGAGCTAAACCTAGCCCCA AACCCACTC 1260 Query 26 CACCCT 31 |||| | Sbjct 1261 CACCTTACTACCAGACAACCTTAACCAAACCATTTACCCAAATAAAGTATAG GCGATAGA 1320
7	piR-hsa-15023	**31/31**	Query 1 TGCCCCCATGTCTAACAACATG 22 ||||||||||||||||||| Sbjct 11761 CTTATTTACCGAGAAAGCTCACAAGAACTGCTAACTCATGCCCCCATGTCT AACAACATG 11820 Query 23 GCTTTCTCA 31 |||||||| Sbjct 11821 GCTTTCTCAACTTTTAAAGGATAACAGCTATCCATTGGTCTTAGGCCCCAA AAATTTTGG 11880
8	piR-hsa-26492	**29/29**	Query 1 GACATCCCGATGGT 14 |||||||||||||| Sbjct 2521 AGAGTCCATATCAACAATAGGGTTTACGACCTCGATGTTGGATCAGGACAT CCCGATGGT 2580 Query 15 GCAGCCGCTATTAAA 29 |||||||||||||| Sbjct 2581 GCAGCCGCTATTAAAGGTTCGTTTGTTCAACGATTAAAGTCCTACGTGATC TGAGTTCAG 2640
9	piR-hsa-26681	**28/28**	Query 1 GAGAAAGCTCACAAGAACTGCTAACTCA---------- 28 |||||||||||||||||||||||| Sbjct 11761 CTTATTTACCGAGAAAGCTCACAAGAACTGCTAACTCATGCCCCCATGTCT AACAACATG 11820
10	piR-hsa-26684	**29/29**	Query 1 GAGAAAGCTCACAAGAACTGCTAACTCAT 29 |||||||||||||||||||||||||| Sbjct 11761 CTTATTTACCGAGAAAGCTCACAAGAACTGCTAACTCATGCCCCCATGTCT AACAACATG 11820
11	piR-hsa-26685	**28/28**	Query 1 GAGAAAGCTCACAAGAACTGCTAACTCAT 29 |||||||||||||||||||||||||| Sbjct 11761 CTTATTTACCGAGAAAGCTCACAAGAACTGCTAACTCATGCCCCCATGTCT AACAACATG 11820
12	piR-hsa-26686	**27/27**	Query 1 GAGAAAGCTCACAAGAACTGCTAACTCATG 30 ||||||||||||||||||||||||||| Sbjct 11761 CTTATTTACCGAGAAAGCTCACAAGAACTGCTAACTCATGCCCCCATGTCT AACAACATG 11820
13	piR-hsa-21622	**31/31**	Query 1 TGTGAATCTGACAACAGAGGCTTACGACCC 30 ||||||||||||||||||||||||||| Sbjct 11701 TGTAAATATAGTTTAACCAAAACATCAGATTGTGAATCTGACAACAGAGGCT TACGACCC 11760 Query 31 C 31 | Sbjct 11761

			CTTATTTACCGAGAAAGCTCACAAGAACTGCTAACTCATGCCCCCATGTCT AACAACATG 11820
14	piR-hsa-9491	**32/32**	Query 1 TGAATCTGACAACAGAGGCTTACGACCC 28 |||||||||||||||||||||||||| Sbjct 11701 TGTAAATATAGTTTAACCAAAACATCAGATTGTGAATCTGACAACAGAGGCT TACGACCC 11760 Query 29 CTTA 32 |||| Sbjct 11761 CTTATTTACCGAGAAAGCTCACAAGAACTGCTAACTCATGCCCCCATGTCT AACAACATG 11820

4.2 Mitomap and BLASTN Result

Table 3: piRNA and Its Target associated genes

PiRNA	**TARGETS**
piR-hsa-12488	MT-RNR1
piR-hsa-12487	MT-RNR1
piR-hsa-27729	MT-RNR1
piR-hsa-27730	MT-RNR1
piR-hsa-27731	MT-RNR1
piR-hsa-15023	MT-ND4
piR-hsa-26492	MT-RNR2
piR-hsa-26681	MT-ND4
piR-hsa-26684	MT-ND4
piR-hsa-2668	MT-ND4
piR-hsa-26686	MT-ND4
piR-hsa-21622	MT-ND4
piR-hsa-9491	MT-ND4

4.3 Upregulation and downregulation in piRNA levels in cancer through references from Literature study.

1. PIR-has-12487 and pir-hsa-12488 are deregulated in renal cell carcinoma.

2. PIR-has-27729 and pir-hsa-27730 are deregulated in colorectal cancer tissue sample (Srinivas V Koduru *et al*., 2017).

4.4 Resulting PiRNA have been aligned with DQ112870 north American homo sapiens mitochondrion:

Observed that **piR-hsa-12488** and **pir-hsa-12487** on DQ112870 (MT-RNR1) aligned with each other although there is mutated nucleotide sequence present. Changing those sequences with Wild-type does not show any alignment. It has the certain role in deaf associated diseases. On DQ112870 alignment with both **piR-hsa-27729** and **pir-hsa-27730** individually, the resulted mutated nucleotide sequence has been reported in pancreatic cancer.

.. observed mutation

.. mutational hotspot

.. reported mutation

Locus	Disease	Allele	RNA	Homoplasmy	Heteroplasmy	Status	MitoTIP †	GB Sequences (# FL / # CR)*	Reference
MT-RNR1	Possibly DEAF-associated	T1180G	12S rRNA	+	-	Reported	N/A	0	references
MT-RNR1	DEAF-associated	C1192A	12S rRNA	+	-	Reported	N/A	6 (6/0)	references
MT-RNR1	DEAF-associated	C1192T	12S rRNA	+	-	Reported	N/A	9 (9/0)	references

piR-hsa-12488 on DQ112870 (MT-RNR1) 1192C> DEAF-associated (probability of mutation)

Query 1 CAGAGTGTAGCTTAACACAAAGCACCCAACT 31

||||||||||||||||||||||||||||||||

Sbjct 1167 CAGAGTGTAGCTTAACACAAAGCACCCAACT 1197

piR-hsa-124887 on DQ112870 (MT-RNR1) 1192C> DEAF-associated (probability of mutation

)Query 1 CAGAGTGTAGCTTAACACAAAGCACCCAACT 31

||||||||||||||||||||||||||||||

Sbjct 1167 CAGAGTGTAGCTTAACACAAAGCACCCAACT 1197

MITOMAP: mtDNA Somatic Variants

Nucleotide Position	Locus	Nucleotide Change	Homoplasmy	Heteroplasmy	Cell or Tissue Type	MitoTIP †	GB Sequences (# FL / # CR)*	Referen
1243	MT-RNR1	T-C	+	-	pancreatic cancer cell line	N/A	662 (662/0)	referen

piR-hsa-27729 on DQ112870 (MT-RNR1) pancreatic cancer cell line 1243 >c

Query 1 GCTAAACCTAGCCCCAAACCCACTCC 26

||||||||||||||||||||||||||

Sbjct 1236 GCTAAACCTAGCCCCAAACCCACTCC 1261

piR-hsa-27730 on DQ112870 (MT-RNR1) pancreatic cancer cell line 1243 >c

Query 1 GCTAAACCTAGCCCCAAACCCACTCCA 27

||||||||||||||||||||||||||||

Sbjct 1236 GCTAAACCTAGCCCCAAACCCACTCCA 1262

piR-hsa-27731 on DQ112870 (MT-RNR1) pancreatic cancer cell line 1243 >c

Query 1 GCTAAACCTAGCCCCAAACCCACTCCACC 29

||||||||||||||||||||||||||||||

Sbjct 1236 GCTAAACCTAGCCCCAAACCCACTCCACC 1264

piR-hsa-26492 on DQ112870 (MT-RNR2) no reported mutation observed

Query 1 GACATCCCGATGGTGCAGCCGCTATTAAA 29

||||||||||||||||||||||||||||||

Sbjct 2567 GACATCCCGATGGTGCAGCCGCTATTAAA 2595

piR-hsa-15023 on DQ112870 (MT-ND4) no reported mutation observed

Query 1 TGCCCCCATGTCTAACAACATGGCTTTCTCA 31

||||||||||||||||||||||||||||||||

Sbjct 11799 TGCCCCCATGTCTAACAACATGGCTTTCTCA 11829

piR-hsa-26492 on DQ112870 (MT-RNR2) no reported mutation observed

Query 1 GACATCCCGATGGTGCAGCCGCTATTAAA 29

||||||||||||||||||||||||||||||

Sbjct 2567 GACATCCCGATGGTGCAGCCGCTATTAAA 2595

MITOMAP: Reported Mitochondrial DNA Base Substitution Diseases: Coding and Control Region Variants

Nucleotide Position	Locus	Nucleotide Change	Reported Disease	Homoplasmy	Heteroplasmy	Amino Acid Change	GB Sequences (# FL / # CR)*	References
11777	MT-ND4	C-A	Leigh Disease	-	+	R-S	0	references
11778	MT-ND4	G-A	LHON / Progressive Dystonia	+	+	R-H	118 (118/0)	references

MITOMAP: mtDNA Somatic Variants

Nucleotide Position	Locus	Nucleotide Change	Homoplasmy	Heteroplasmy	Cell or Tissue Type	MitoTIP †	GB Sequences (# FL / # CR)*	References
11781	MT-ND4	T-C	+	-	pancreatic cancer cell line, MNGIE fibroblasts	N/A	0	references
11794	MT-ND4	T-C	-	+	oral cancer	N/A	21 (21/0)	references

piR-hsa-26681on DQ112870 (MT-nd4) no reported mutation observed

Query 1 GAGAAAGCTCACAAGAACTGCTAACTCA 28

||||||||||||||||||||||||||||

Sbjct 11771 GAGAAAGCTCACAAGAACTGCTAACTCA 11798

piR-hsa-26684 on DQ112870 (MT-nd4) no reported mutation observed

Query 1 GAGAAAGCTCACAAGAACTGCTAACTCAT 29

|||||||||||||||||||||||||||||

Sbjct 11771 GAGAAAGCTCACAAGAACTGCTAACTCAT 11799

piR-hsa-26685 on DQ112870 (MT-nd4) no reported mutation observed

Query 1 GAGAAAGCTCACAAGAACTGCTAACTCATG 30

||||||||||||||||||||||||||||||

Sbjct 11771 GAGAAAGCTCACAAGAACTGCTAACTCATG 11800

piR-hsa-26686 on DQ112870 (MT-nd4) no reported mutation observed

Query 1 GAGAAAGCTCACAAGAACTGCTAACTCATGC 31

||||||||||||||||||||||||||||||

Sbjct 11771 GAGAAAGCTCACAAGAACTGCTAACTCATGC 11801

piR-hsa-21622on DQ112870 (MT-nd4) no reported mutation observed

Query 1 TGTGAATCTGACAACAGAGGCTTACGACCCC 31

||||||||||||||||||||||||||||||

Sbjct 11731 TGTGAATCTGACAACAGAGGCTTACGACCCC 11761

piR-hsa-9491 on DQ112870 (MT-nd4) no reported mutation observed

Query 1 TGAATCTGACAACAGAGGCTTACGACCCCTTA 32

||||||||||||||||||||||||||||||||

Sbjct 11733 TGAATCTGACAACAGAGGCTTACGACCCTTA 11764

4.5 Mutation in PIRNA Vs DQ112870 NORTH AMERICAN

4.5.1 Alignment of 2 sequences; change the observed mutation with wild type.

OBSERVED MUTATION

If two sequences in an alignment share a common ancestor, mismatches can be interpreted as point mutations and gaps as indels (that is, insertion or deletion mutations) introduced in one or both lineages in the time since they diverged from one another. In sequence alignments of proteins, the degree of similarity between amino acids occupying a particular position in the sequence can be interpreted as a rough measure of how conserved a particular region or sequence motif is among lineages. The absence of substitutions, or the presence of only very conservative substitutions (that is, the substitution of amino acids whose side chains have

similar biochemical properties) in a particular region of the sequence, suggest that this region has structural or functional importance. Although DNA and RNA nucleotide bases are more similar to each other than are amino acids, the conservation of base pairs can indicate a similar functional or structural role.

piR-hsa-27729 on DQ112870 (MT-RNR1) pancreatic cancer cell line 1243 >c

Query 1 GCTAAACTTAGCCCCAAACCCACTCC 26 **25/26**

Sbjct 1236 GCTAAACCTAGCCCCAAACCCACTCC 1261

Query 1 GCTAAACCTAGCCCCAAACTCACTCC 26 **25/26**

Sbjct 1236 GCTAAACCTAGCCCCAAACCCACTCC 1261

piR-hsa-27730 on DQ112870 (MT-RNR1) pancreatic cancer cell line 1243 >c

Query 1 GCTAAACTTAGCCCCAAACCCACTCCA 27 **26/27**

Sbjct 1236 GCTAAACCTAGCCCCAAACCCACTCCA 1262

Query 1 GCTAAACCTAGCCCCAAACTCACTCCA 26 **26/26**

Sbjct 1236 GCTAAACCTAGCCCCAAACCCACTCCA 1261

piR-hsa-26492 on DQ112870 (MT-RNR2) no reported mutation observed

Query 1 GACATCCCGACGGTGCAGCCGCTATTAAA 29 **28/29**

Sbjct 2567 GACATCCCGATGGTGCAGCCGCTATTAAA 2595

Query 1 GACATCCCGATGGTACAGCCGCTATTAAA 29 **28/29**

Sbjct 2567 GACATCCCGATGGTGCAGCCGCTATTAAA 2595

piR-hsa-15023 on DQ112870 (MT-ND4) no reported mutation observed

Query 3 CCCCCATGTCTAACAACATGGCTTTCTCA 31

||||||||||||||||||||||||||||| **29/29**

Sbjct 11801 CCCCCATGTCTAACAACATGGCTTTCTCA 11829

piR-hsa-26429on DQ112870 (MT-RNR2) no reported mutation observed

Query 1 GACATCCCGACGGTGCAGCCGCTATTAAA 29

|||||||||| |||||||||||||||||| **28/29**

Sbjct 2567 GACATCCCGATGGTGCAGCCGCTATTAAA 2595

Query 1 GACATCCCGATGGTACAGCCGCTATTAAA 29

|||||||||||||| |||||||||||||| **28/29**

Sbjct 2567 GACATCCCGATGGTGCAGCCGCTATTAAA 2595

piR-hsa-26681on DQ112870 (MT-nd4) no reported mutation observed

Query 1 GAGAAAGCCCACAAGAACTGCTAACTCA 28

|||||||| |||||||||||||||||| **27/28**

Sbjct 11771 GAGAAAGCTCACAAGAACTGCTAACTCA 11798

Query 1 GAGAAAGCTCACAAAAACTGCTAACTCA 28

|||||||||||||| |||||||||||| **27/28**

Sbjct 11771 GAGAAAGCTCACAAGAACTGCTAACTCA 11798

piR-hsa-26684 on DQ112870 (MT-nd4) no reported mutation observed

Query 1 GAGAAAGCCCACAAGAACTGCTAACTCAT 28

|||||||| |||||||||||||||||| **28/29**

Sbjct 11771 GAGAAAGCTCACAAGAACTGCTAACTCAT 11798

Query 1 GAGAAAGCTCACAAAAACTGCTAACTCAT 28

|||||||||||||| |||||||||||| **28/29**

Sbjct 11771 GAGAAAGCTCACAAGAACTGCTAACTCAT 11798

piR-hsa-26685 on DQ112870 (MT-nd4) no reported mutation observed

Query 1 GAGAAAGCCCACAAGAACTGCTAACTCATG 28

||||||||| |||||||||||||||||||| **29/30**

Sbjct 11771 GAGAAAGCTCACAAGAACTGCTAACTCATG 11798

Query 1 GAGAAAGCTCACAAAAACTGCTAACTCATG 28

|||||||||||||| |||||||||||| **29/30**

Sbjct 11771 GAGAAAGCTCACAAGAACTGCTAACTCATG 11798

Query 1 GAGAAAGCTCACAAGAACTGCTAACTCAT 29

|||||||||||||||||||||||||||| **29/29**

Sbjct 11771 GAGAAAGCTCACAAGAACTGCTAACTCAT 11799

piR-hsa-26686 on DQ112870 (MT-nd4) no reported mutation observed

Query 1 GAGAAAGCCCACAAGAACTGCTAACTCATGC 28

||||||||| |||||||||||||||||||| **30/31**

Sbjct 11771 GAGAAAGCTCACAAGAACTGCTAACTCATGC 11798

Query 1 GAGAAAGCTCACAAAAACTGCTAACTCATGC 28

|||||||||||||| |||||||||||| **30/31**

Sbjct 11771 GAGAAAGCTCACAAGAACTGCTAACTCATGC 11798

Query 1 GAGAAAGCTCACAAGAACTGCTAACTCAT 29

|||||||||||||||||||||||||||| **29/29**

Sbjct 11771 GAGAAAGCTCACAAGAACTGCTAACTCAT 11799

piR-hsa-21622on DQ112870 (MT-nd4) no reported mutation observed

Query 3 TGAATCTGACAACAGAGGCTTACGACCCC 31

|||||||||||||||||||||||||||| 29/29

Sbjct 11733 TGAATCTGACAACAGAGGCTTACGACCCC 11761

piR-hsa-9491 on DQ112870 (MT-nd4) no reported mutation observed

Query 3 AATCTGACAACAGAGGCTTACGACCCCTTA 32

|||||||||||||||||||||||||||||| 30/30

Sbjct 11735 AATCTGACAACAGAGGCTTACGACCCCTTA 11764

Chapter V
Discussion

5. DISCUSSION

It is highly aligned with mutated sequence rather than wild one. So, It may have the potential role in disease. It might be acting against mutated DNA. It has been shown that neither mitochondrial DNA, nor wild-type type is aligned properly with sequence, but rather it was mutated sequence of pi-RNA that aligned perfectly in 4 cases. In mitochondria, normal as well as mutant genome copy express perfectly. its indicate that piRNA might be binding with mutant RNA originating from mutant genome regulating activities of genomic integrity.

5.1 CONCLUSION

It has been shown that some wild-type mitochondrial DNA was not aligned properly with the piRNA sequence, but mutated sequences are aligned perfectly in 4 cases. It is also observed that certain mutations in mitochondrial genome having better alignment, indicating that mutated mitochondrial genome might be regulated by piRNA at the Post-transcriptional level. In mitochondria, normal as well as mutated genome copy express similarly. It indicates that piRNA might be binding with mutant RNA (originating from mutant genome) regulating activities of genomic integrity.

5.2 FUTURE PPECTIVES

Quantitative real-time polymerase chain reaction analysis by knockdown/mimic of selected piRNA could determine the validation of piRNA. We can identify and validate multiple novel piRNAs associated with tumorigenesis and histological differentiation and also these piRNAs could be further exploited for the development of useful biomarkers in diagnosis, prognosis, and treatment of various cancer types.

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Student Approval Form

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Degree	M.Sc. Life Sciences with specialization in Molecular Medicine
University	Central University of Punjab
Guide	Dr. Sandeep Singh
Project Title	Potential Mitochondrial-specific function of piRNA
Year of Award	2018

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Declaration

I declare that all the changes suggested by the VC nominee examiner in the Research Project entitles “Potential Mitochondrial-specific function of piRNA” submitted by me for the award of degree of Master’s in Science in Life Sciences with specialization in Molecular Medicine in the Department of Human Genetics and Molecular Medicine has been incorporated in the Research Project.

(Shouvik Paul)

Department of Human Genetics and Molecular Medicine

School of Health Sciences

Central University of Punjab

Date:

(Dr. Sandeep Singh)

Department of Human Genetics and Molecular Medicine

School of Health Sciences

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Date:

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