Original Article

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Expression of Poly(A)-Specific Ribonuclease in Solid Tumours and Haematopoietic Malignancies

Oncology Section

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ABSTRACT

Introduction: Ribonucleases (RNases) are enzymes involved in degradation of excess messenger Ribonucleic Acid (mRNA) and deadenylation of the poly(A) tail is the most common mechanism involved in mRNA degradation. Among the various RNases involved in deadenylation-dependent mRNA decay, Poly(A)-Specific Ribonuclease (PARN) plays the major role in the deadenylation-mediated mRNA decay. Although PARN is primarily involved in mRNA stability, recent studies suggest other functions of PARN including a role in telomere maintenance, DNA-damage response, and p53 regulation. Altered expression of PARN was also observed in cancers including acute leukaemia, lung squamous cell carcinoma and gastric cancer.

Aim: To determine the levels of PARN transcripts in solid tumours and haematopoietic malignancies.

Materials and Methods: This study was conducted on a cohort obtained from the Department of Oncology at Justice KS Hegde Charitable hospital in Mangaluru, Karnataka, India during January 2017 to June 2017. A total of 26 clinical samples and 14 controls were included. The expression level of PARN in plasma and tissue samples obtained from patients with solid organ tumours and haematopoietic malignancies were analysed by quantitative real-time Polymerase Chain Reaction (PCR) by calculating relative expression using Microsoft excel 2010.

Results: A consistent down regulation of PARN in 88.23% (15 out of 17) of the samples were observed. All the plasma samples (100%, 10 out of 10) obtained from the lung cancer cases, showed down regulation of PARN at statistically significant level, regardless of the subtype of the cancer. In case of haematopoietic malignancies, PARN transcript level was down regulated in five out of seven samples analysed (71.42%), with statistically significant decrease in three of them.

Conclusion: Despite a small sample size, the data presented here show that PARN is down regulated in solid organ cancers, suggesting a tumour-suppressive function of PARN. Screening of a large number of samples would be required to evaluate the true potential of PARN as a novel biomarker in solid organ and haematopoietic cancer.

Keywords: Biomarker, Cancer, Lung cancer, Messenger ribonucleic acid, Nuclease, RNases

INTRODUCTION

Variability in gene expression is a common feature in many types of cancers and messenger Ribonucleic Acid (mRNA) are important in the regulation of gene expression. Excess mRNAs or unused mRNAs are quickly eliminated by various degradation mechanisms. Two types of mRNA degradation pathways exist in a cell, namely the deadenylation-dependent pathway and the endonuclease-dependent pathway [1]. The enzymes involved in mRNA degradation pathways are collectively called RNases, which include the deadenylases, the exonucleases and the endonucleases. The rate-limiting step in mRNA degradation is the initiation of poly(A) tail shortening, the process of deadenylation. Among the three RNases, Poly(A)-Specific Ribonuclease (PARN) is an exoribonuclease that belongs to DnaQ-like superfamily and is highly specific in degrading 3' end located poly(A) tail [2,3]. Besides, poly(A), PARN can degrade poly (U) containing mRNA, albeit less efficiently [4].

Although PARN is primarily involved in degradation of unused or excess mRNA, recent studies have shown that PARN has multiple roles in the cell including a role in telomere maintenance [5], Deoxyribonucleic Acid (DNA) damage response and p53 regulation [6]. Mutations in PARN have been identified in diseases linked to telomere dysfunction including dyskeratosis congenita and idiopathic pulmonary fibrosis [7]. These conditions are usually associated with a high risk of cancer [8,9].

It is conceivable that attenuated degradation of growth-promoting mRNAs or increased turnover of oncosuppressive gene mRNAs can contribute to the initiation of carcinogenesis. Indeed, several RNases are now being considered to have tumour suppressive and/or tumour-promoting roles [1]. Considering that PARN is involved in the first step of mRNA degradation i.e., removal of poly(A) tail, investigating the expression of PARN is of clinical significance in cancer. Indeed, PARN has been shown to be differentially expressed in many types of solid organ cancers and haematopoietic malignancies including gastric tumour, acute lymphoid leukaemia, acute myeloid leukaemia and squamous cell carcinoma [10-12]. Thus, PARN represents as a potential biomarker for cancer.

Although, a number of previous studies [10-12] have analysed the expression of PARN in cancer, the results were not consistent. This study was aimed at analysing the expression of PARN in plasma and tissue samples obtained from patients with solid tumours and haematopoietic malignancies.

MATERIALS AND METHODS

This study was conducted on a cohort obtained from the Department of Oncology at Justice KS Hegde Charitable hospital in Mangaluru, Karnataka, India during January 2017 to June 2017. The Ethical Clearance (NU/CEC/2016-2017/0079) for the study was obtained from the Central Ethics Committee of Nitte (Deemed to be University).

Inclusion criteria: The sample obtained from subjects aged above 18 years, with confirmed diagnosis of cancer (and only new cases) were included in the study.

Exclusion criteria: Follow-up cancer cases were excluded from the study.

Study Procedure

- A total of 26 clinical samples, including 17 blood and 9 tissues were collected from cancer patients after obtaining informed consent.

- A total of 14 control samples including nine plasma samples from age matched healthy individuals and five normal tissue samples (at least 5 cm away from the edge of the corresponding tumours) of the matched specimen were included in the study.
- Blood samples were collected in Ethylenediaminetetraacetic Acid (EDTA)-coated vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ, and the USA) were immediately brought to the laboratory and plasma was separated by centrifugation at 3500 rpm for 15 min. The tissue samples were immersed in RNAlater, (Thermo Fisher Scientific, and the USA) immediately after collection and were kept at 4°C for overnight to allow complete stabilisation of the Ribonucleic Acid (RNA).

Blood sample: Among the 17 blood samples collected, seven samples were from patients diagnosed with various haematopoietic malignancies including Mucosa Associated Lymphoid Tissue (MALT, n=2), Anaplastic Large Cell Lymphoma (ALCL, n=1), Non Hodgkin's Lymphoma (NHL, n=1), Diffused Large B-cell Lymphoma (DLBCL, n=1), Lymphoid Leukaemia (LL, n=1), and Multiple Myeloma (MM, n=1). The remaining 10 samples were from patients with solid tumours comprising of both Small Cell Lung Cancer (SCLC, n=3), Adenocarcinoma of Lung (ACL, n=2), Squamous Cell Carcinoma of Lung (SCC, n=4) and one non small cell lung cancer (subtype unidentified).

Tissue sample: Out of the nine tissue samples included in the study, four were collected during bronchoscopy of lung, of which three were squamous cell carcinoma and one non small cell lung cancer (subtype unidentified). The other five tumour tissues were collected during surgical intervention of patients with solid organ cancers, which included ovarian cancer (n=1), buccal cancer (n=2), colon cancer (n=1), breast cancer (n=1). The details of samples are shown in [Table/Fig-1]. The histological evaluation of all the samples was done by a pathologist.

Blood/Plasma samples		Tissue samples	
Groups	No. of samples	Groups	No. of samples
Cancer type		**Cancer type**	
Mucosa associated lymphoid tissue	2	Squamous cell carcinoma	3
Anaplastic large cell lymphoma	1	Non small cell lung cancer (Unidentified)	1
Non Hodgkin's lymphoma	1	Ovarian Cancer	1
Diffused large B-cell lymphoma	1	Buccal Cancer	2
Lymphoid Leukaemia	1	Colon Cancer	1
Multiple myeloma	1	Breast Cancer	1
Squamous cell carcinoma of lung	3	**Control**	5
Adenocarcinoma of lung	2		
Squamous cell carcinoma	4		
Non small cell lung cancer (Unidentified)	1		
Control	9		
Total	26		14

[Table/Fig-1]: Details of the clinical samples included in the study.

RNA Isolation and cDNA Synthesis

Total RNA from tissue samples were isolated using a commercial kit (RNeasy Mini kit, Qiagen, Germany) as per the manufacturer's protocol. Total RNA from plasma samples were isolated by Trizol extraction method. The quality and the quantity of the isolated RNA were checked by using a UV spectrophotometer (Eppendorf, Germany) and by performing 3-(N-Morpholino)propane sulfonic acid (MOPS) buffer – Formaldehyde Agarose Gel Electrophoresis (Gel Doc, Bio-Rad, USA). A 1 µg of RNA was reverse transcribed into cDNA using a commercial kit (TaKaRa Prime Script First Strand cDNA Synthesis Kit, Takara, Japan).

Primer Design and Optimisation of PCR Conditions for PARN

The primers for real-time polymerase chain reaction (PCR) were designed using a commercial primer designing tool called Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/). The sequences of the primers are shown in [Table/Fig-2] [11]. The optimisation of the annealing temperature was carried out by gradient PCR in a temperature controlled and automated thermal cycler (Eppendorf, Germany) with temperature ranging from 52°C to 62°C. The optimised temperature was 56°C.

Name	Sequence	Amplicon size	Reference
β-Actin -F	5'-GGACTTCGAGCAAGAGATGG- 3'	235 bp	Maragozidis P et al, [11]
β-Actin -R	5'-AGCACTGTGTTGGCGTACAG-3'		
PARN-F	5'-CTCTGCCTGCGGACTTAAGT-3'	150 bp	Present study
PARN-R	5'-CGCTTTTCCAATTCCGCAAG-3'		

[Table/Fig-2]: Primer sequences for PARN and β-Actin used in the study [11].

Quantitative Real Time PCR

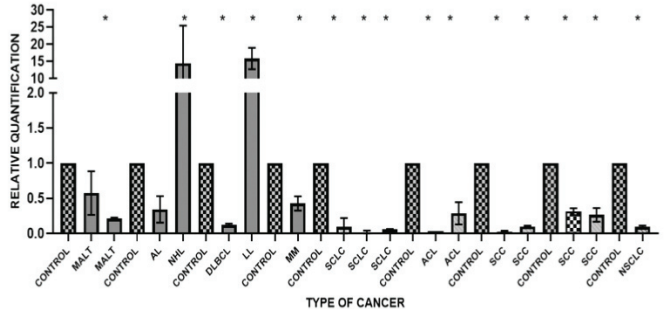
Quantitative Real-Time PCR was performed for expression profiling of PARN in CFX96 Real time System, (Bio-Rad, USA) using SensiFAST SYBR No-ROX Kit (Bioline, UK). The expression levels of PARN in cancer samples were estimated by the ΔΔCq method. The calculated cycle threshold for PARN was normalised against the corresponding ß-actin cycle threshold. The values for cancer samples were expressed as relative quantification ($2^{-\Delta\Delta Cq}$) compared to control samples.

STATISTICAL ANALYSIS

All calculations were performed using Microsoft Excel 2010 and Graph Pad Prism 8.4. Two-tailed unpaired Student's t-test was performed to the obtained $2^{-\Delta\Delta Cq}$ values. Three trials were performed for each set, p-value <0.05 was considered statistically significant.

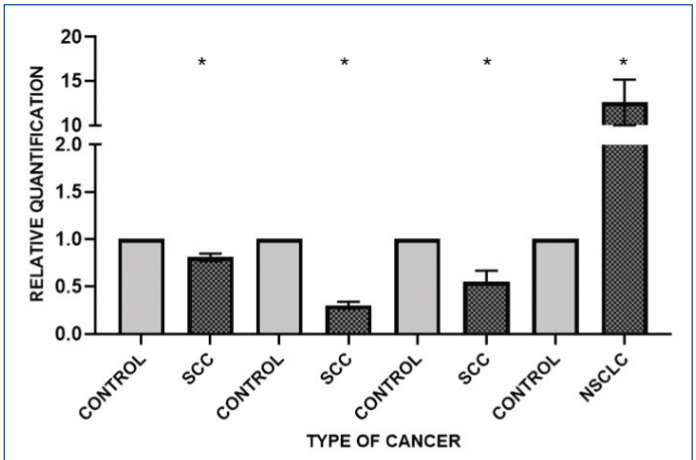
RESULTS

The relative expression of PARN normalised to ß- actin in cancer plasma samples is shown in [Table/Fig-3]. As evident from the results, PARN was significantly down regulated in most of the plasma samples (88.23%, 15 out of 17). All the plasma samples (100 %, 10 out of 10) obtained from the lung cancer cases, showed down regulation of PARN at statistically significant level, regardless of the subtype of the cancer. In case of haematopoietic malignancies, PARN transcript level was down regulated in five out of seven samples analysed (71.42%), with statistically significant decrease in three of them. However, in two samples (NHL and LL) there was a significant increase in PARN expression, suggesting a differential expression of PARN in haematopoietic malignancies. Thus, PARN showed consistent down regulation in lung cancer samples whereas the expression was variable in haematopoietic malignancies.



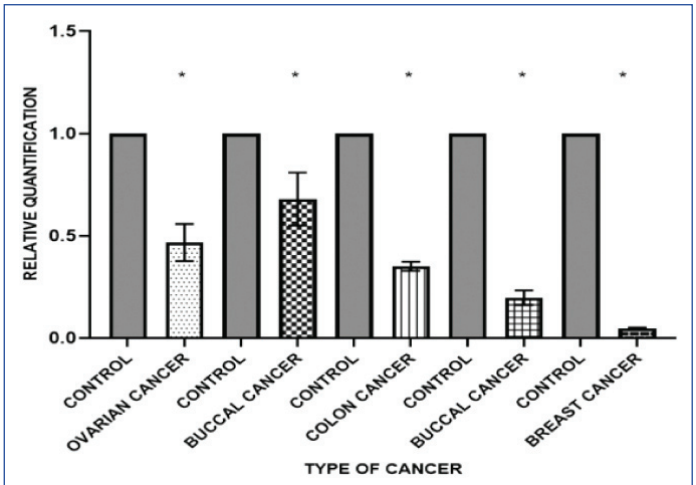
[Table/Fig-3]: Relative Expression of PARN mRNA in cancer plasma samples normalised to ß-actin. mRNA levels from plasma samples were quantified by qRT-PCR considering ß-actin as a house keeping gene. Each histogram represents expression of PARN in plasma samples of the respective cancer subtypes.
*p-value <0.05 was considered as statistically significant

The data obtained from the tissue samples were highly consistent with the results obtained for plasma samples of solid organ cancers with three out of four samples showing down regulation of PARN mRNA expression [Table/Fig-4]. Among these, the squamous cell carcinoma samples showed significant down regulation of PARN mRNA while the non small cell lung cancer type (subtype unidentified) showed increased expression of PARN transcript level. Interestingly, the PARN level in plasma for this sample showed down regulation, suggesting a differential expression. However, the results obtained for adeno and squamous type were highly consistent in both tissue and plasma samples.



[Table/Fig-4]: Relative Expression of PARN mRNA in cancer tissue samples normalised to ß- actin. The cancer sample type is mentioned below each histogram. *p-value <0.05 was considered as statistically significant

In order to further confirm if the results obtained from the tissue samples of lung cancer were consistent with other types of solid organ cancers, authors checked the PARN mRNA level in five other samples comprising ovarian, breast, colon and buccal cancer tissues. The results were highly consistent with that obtained for lung cancer cases, with all the five samples showing down regulation of PARN mRNA [Table/Fig-5]. The p-values obtained after applying t-test to the obtained $2^{-\Delta\Delta Cq}$ values to each of the sample are given in the [Table/Fig-6]. The expression levels of PARN shown in the figures are the relative quantification normalised to internal control with the levels in control samples calculated as 1.0 as per the $^{2\wedge\text{-ddCt}}$ method.



[Table/Fig-5]: Relative Expression of PARN mRNA in cancer tissue samples normalised to ß-actin. The cancer sample type is mentioned below each histogram. *p-value<0.05 was considered as statistically significant

Cancer type	Relative expression	p-value
Plasma sample		
MALT lymphoma	0.576009	0.305973
MALT lymphoma	0.212254	0.012394
Anaplastic large cell lymphoma	0.3343099	0.07316
Non Hodgkin's lymphoma	14.45303	0.347288
Diffuse large B cell lymphoma	0.1205339	0.000512
Lymphoid leukaemia	15.85932	0.042086
Multiple myeloma	0.426258	0.042086
Small cell lung carcinoma	0.096539	0.000617
Small cell lung carcinoma	0.019763	$5.6X10^{-5}$
Small cell lung carcinoma	0.058608	0.000127
Adenocarcinoma of lung	0.030655	$2.99X10^{-5}$
Adenocarcinoma of lung	0.28685	0.04359
Squamous cell carcinoma of lung	0.027916	$6.25X10^{-5}$
Squamous cell carcinoma of lung	0.097414	0.000227
Squamous cell carcinoma of lung	0.309128	0.005034
Squamous cell carcinoma of lung	0.264586	0.016586
Non small cell lung carcinoma	0.094576	0.000404
Tissue Samples		
Squamous cell carcinoma of lung	0.8115	0.03814
Squamous cell carcinoma of lung	0.3033	0.002513
Squamous cell carcinoma of lung	0.5527	0.061870
Non small cell lung carcinoma	12.5931	0.04638
Ovarian cancer	0.4679	0.0276
Buccal cancer	0.6800	0.1311
Colon cancer	0.3516	0.001199
Buccal cancer	0.1980	0.00200
Breast cancer	0.0464	$0.53X10^{-5}$

[Table/Fig-6]: Relative expression and p-values of samples analysed in this study. p-value <0.05 was considered as statistically significant

Taken together, these results suggest that PARN is consistently downregulated in solid organ tumours whereas in haematopoietic malignancies, it showed variable expression with elevated levels in some samples and decreased levels in some.

DISCUSSION

Traditionally, PARN is known for its role in mRNA stability. It plays a significant role in the rate-limiting step in mRNA decay pathway, i.e., removal of poly(A) tail at the 3' end of mature mRNA. Several exciting findings in recent years have revealed that PARN plays multiple roles in a cell apart from its main role, i.e., de-adenylation. Besides its role in TP53 regulation [6] and stability of noncoding RNAs [13-15], PARN is also involved in telomere maintenance [16]. Recently, PARN has been implicated in several genetic conditions involving bone marrow failures and telomere dysfunction [17-20]. These conditions are usually associated with a high risk of cancer development.

Given the fact that PARN is involved in the regulation of TP53, an important tumour suppressor gene including [6,21], examining the expression level of PARN in cancer samples becomes very relevant. Indeed, Maragozidis P et al., 2012, 2015 showed altered PARN expression particularly in acute lymphoid and myeloid leukaemias and squamous cell carcinoma of lung. [11,12]. In this study, the aim was to examine the expression of PARN mRNA in cancer samples, particularly in lung cancer and lymphomas and to evaluate its role as a potential biomarker. The results, although preliminary, showed that PARN expression was down regulated in majority of the samples analysed in this study. While the solid organ tumours showed a consistent down regulation in both plasma and tissue samples, the results obtained from lymphoma and leukaemia cases were variable. The present study data on haematopoietic samples corroborate previous studies that reported altered expression of PARN in acute leukaemias [11,12]. However, with regards to the solid organ cancer data, the present study results are in contrast with previous reports that showed either differential expression of PARN in squamous cell lung cancer [12] or overexpression in gastric cancer [10]. Although preliminary, the present study data indicated

that PARN was down regulated in lung cancer cases, including the major subtypes, the non small cell and small cell. Similar results were obtained in other types of solid organ cancer analysed, suggesting that PARN is downregulated in solid organ cancers.

Limitation(s)

Despite a small sample size, the results were consistent for all the major organ cancer analysed in the study. However, a large number of samples representing a wider repertoire of cancers and their subtypes must be analysed for investigating the actual role of PARN as a potential biomarker. Since, the histological subtype directly influences the treatment option in lung cancer it would be worth examining the exact role of PARN in lung tumourigenesis. Due to the small sample size analysed, it is not possible to generalise the present study data for all types of cancer.

CONCLUSION(S)

Considering the tumour suppressive role of PARN, the present study results are in expected lines as the majority of the samples, particularly the lung cancer cases, showed decreased expression, which is usually the case for a tumour suppressor gene. The data presented here show that PARN is down regulated in solid organ cancers, suggesting a tumour-suppressive function of PARN. Future studies should be directed towards understanding the precise role of PARN in tumourigenesis with focus on specific subtypes of an organ tumour and by identifying the target mRNAs of PARN through a combination of clinical and functional studies using integrated animal model systems. Also, screening of a large number of samples would be required to evaluate the true potential of PARN as a novel biomarker in solid organ and haematopoietic cancer.

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Author contribution: NB and DPN conducted the laboratory procedures, did literature review and prepared the first draft. SA carried out sample preparation. RV and VS collected samples and acquired clinical data. AC designed the study, analysed the results and prepared and edited the final version of the manuscript. All authors reviewed the manuscript and approved the final version. Both NB and DPN have the same contribution and equal credit should be given for the same.

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