Brief report on BRNS funded PROJECT

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Sanction Number: 35/14/02/2020/35025

Mode of Execution: Research Project

Date of Start: 30-07-2020

Date of Completion: 31-03-2023

Total Amount Sanctioned (in Lakhs): 56.516

Amount Received (in Lakhs with date): Rs.31.804; 30-07-2020

Institutes involved in case of MoU/CRP: NA

Category:

Title: Modulating post translational modification of the oncogenic signaling kinase p21-activated kinase-1 (PAK1) to circumvent radio resistance in cancer cells

Name of PI & Affiliation: Dr.Ganesh Venkatraman Professor, Department of Human Genetics, Sri Ramachandra Institute of Higher Education and Research, Porur, Chennai-600116

Name of CI & Affiliation: Dr S Gouthaman, Professor Professor, Department of Surgical Oncology, Sri Ramachandra Institute of Higher Education and Research, Porur, Chennai- 600116

Name of PC & Affiliation: Dr.Archana Mukherjee, Head, RadiopharmaceuticalsDivisionBARC, DAE

Name of major Equipments procured and their cost : Spectramax M5; 20,00,000

Present working status of the Equipment: **Under working order:** Working

Number of other users & their affiliation and % use by others: 30%

Details of the High cost consumables used:

PAK1, pPAk1 – Affinity purified Antibodies

Cell Culture Media, FBS, etc

Mass Spectrometry Reagents

Patent with brief description: None

Number of Journal Publications with impact factor (attach list as Annex- I): Under preparation

Number of symposia presentations:

Number of staff trained under this project: 1

List of Objectives as mentioned in original proposal :

The objective of the project is to identify novel phosphorylation sites in PAK1 upon exposure to IR

(List accomplishments/ short falls against each of the objectives)

Objectives	Accomplishments
To identify different phophorylation sites (known and unknown) that are modulated by ionizing radiation. • To achieve this objective, we will screen a panel of human cancer cell lines for PAK1 phosphorylation using *in vitro* techniques. • We will be selecting representative cell lines from different cancer types, for which radiotherapy is a standard care and we will be using clinically relevant doses. Upon IR exposure, whole cell lysates will be collected and PAK1 phosphorylation status will be determined. • Workout the optimal dose of IR and time required to induce Pak1 activity	We have Screened a panel of Oral Cancer cell lines for expression of PAK1 by Immunoblotting. We have performed Immunofluorescence using Confocal microscopy to identify sub-cellular localization of Pak1 in Oral cancer cells We have collected Oral cancer tissues samples from head & neck cancer patients for determining PAK1 expression & activation We have standardized IHC on tissue sections We are standardizing the IR dose exposure for the cells to activate Pak1 We are finalizing the protocols for Mass Spectrometry identification of Phosphorylation sites and have performed trial experiments to confirm phosphorylation detection.

CELL LINES

Cell lines namely T4074, SCC131, SCC172, SCC745, SCC969, RPMI 2650, FBM, MDA MB 231 were maintained in appropriate growth media with supplements.

IMMUNOBLOTTING

Protein lysates were prepared in Radioimmunoassay precipitation buffer (RIPA) with protease and phosphatase inhibitor. The lysates were then resolved by 8% sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose membrane, followed by blocking with 5% skimmed milk for 1 hour and then probing with specific antibody (PAK1 - CST, USA and pPAK1 Thr 212 - Sigma, USA).

Fig 1. **Immunoblot for expression of PAK1 in Oral Squamous Cell Carcinoma Cell lines**

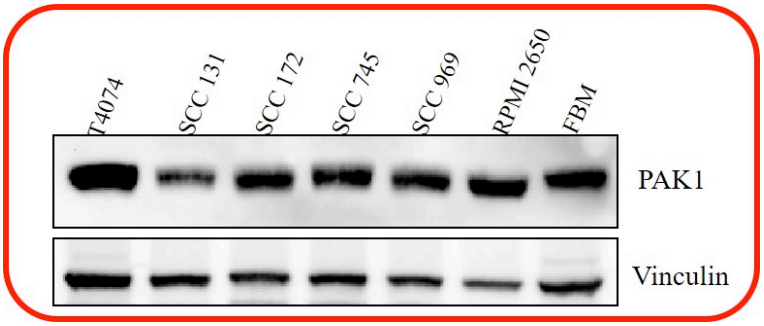
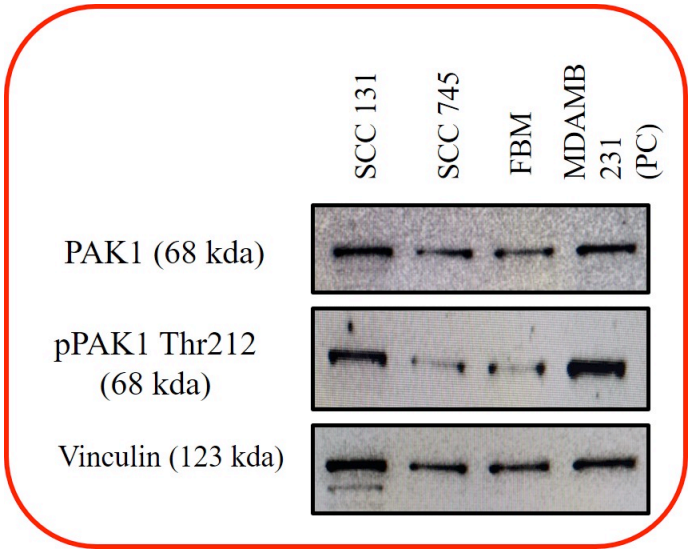


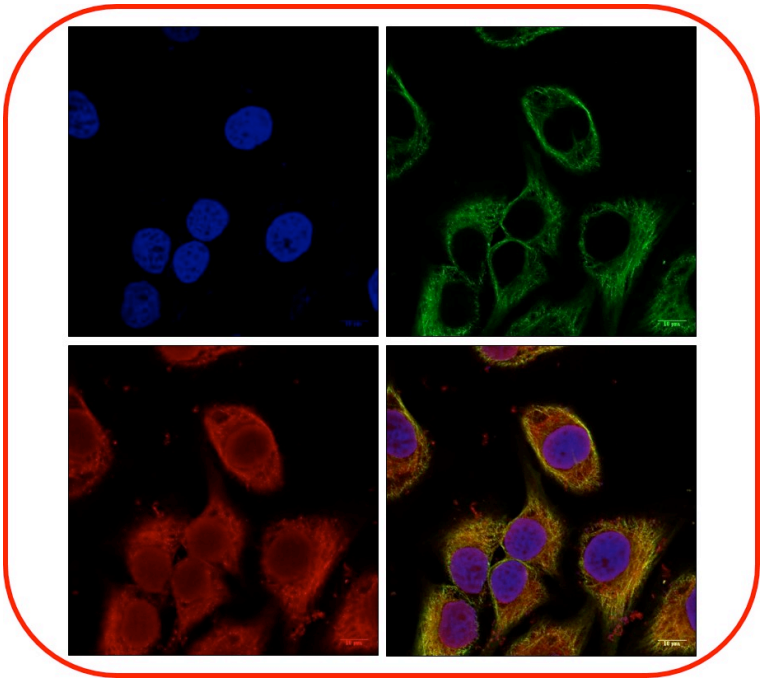
Fig 2. **Immunoblot for expression of pPAK1 in Oral Squamous Cell Carcinoma Cell lines**



IMMUNOFLUORESCENCE

Cells were plated onto glass coverslips in culture dishes and allowed to attach overnight. After semi-confluency, cells were fixed for 20 min in 1 % paraformaldehyde (PFM). They were permeabilized using 0.1 % Triton X 100 for 3–5 min. A series of PBS rinse thereafter. The cells were labelled with the primary antibody cocktail (PAK1 – CST, USA, Beta Tubulin – Santa Cruz, USA) for one hour in the moist chamber. The sections were given 1X PBS washes and detected with the secondary antibody Alexa Fluor 546 (Life Technologies, Carlsbad, CA) conjugated with goat anti-rabbit IgG and Alexa Fluor 488 (Life Technologies, Carlsbad, CA), conjugated with goat anti-mouse IgG. The nuclei were counterstained with DAPI and mounted using Vectashield Anti-Fade mountant. Confocal images were captured by sequential scanning under appropriate fluorescent filters using Zeiss LSM 880 System and Zeiss Zen version 3.2 software.

Fig 3. **Immunofluorescence for expression of PAK1 in Oral Squamous Cell Carcinoma Cell line SCC-131**



Blue – Nucleus stained with DAPI
Green – Beta Tubulin stained with Alexa Flour 488
Red – PAK1 stained with Alexa Flour 546
Merged image

IMMUNOHISTOCHEMISTRY

5µm thick Formalin Fixed Paraffin Embedded (FFPE) oral squamous cell carcinoma and matched normal tissue sections were obtained from the Department of Pathology, SRIHER. IHC using PAK1 antibody (CST, USA) was performed. A standard laboratory procedure was used. Briefly, the slides were dewaxed in graded xylenes and consecutively hydrated in graded alcohols. Antigen retrieval was carried out with heated high pH TRIS-EDTA buffer method followed by peroxide blocking, TRIS buffer washes, and overnight incubation in moist chamber with the respective primary antibody. The sections were given TRIS buffer washes and incubated with the universal kit for secondary antibody (Bio SB), counter-stained, and mounted using DPX mountant. The air dried sections were observed under Leica DM 2000 LED Light Microscope and IHC images were captured using Microscope software platform Leica Application Suite.

ANALYSIS OF IHC SECTIONS

The slides were examined by an experienced pathologist and scored for the intensity of staining as follows,

Q = P * I

Q - QUICK SCORE

P - POSITIVE CELLS

I - INTENSITY OF STAINING

MAXIMUM SCORE IS 300

Fig 4. **Immunohistochemistry for expression of PAK1 in Oral Squamous Cell Carcinoma tissue samples (mag:x200)**

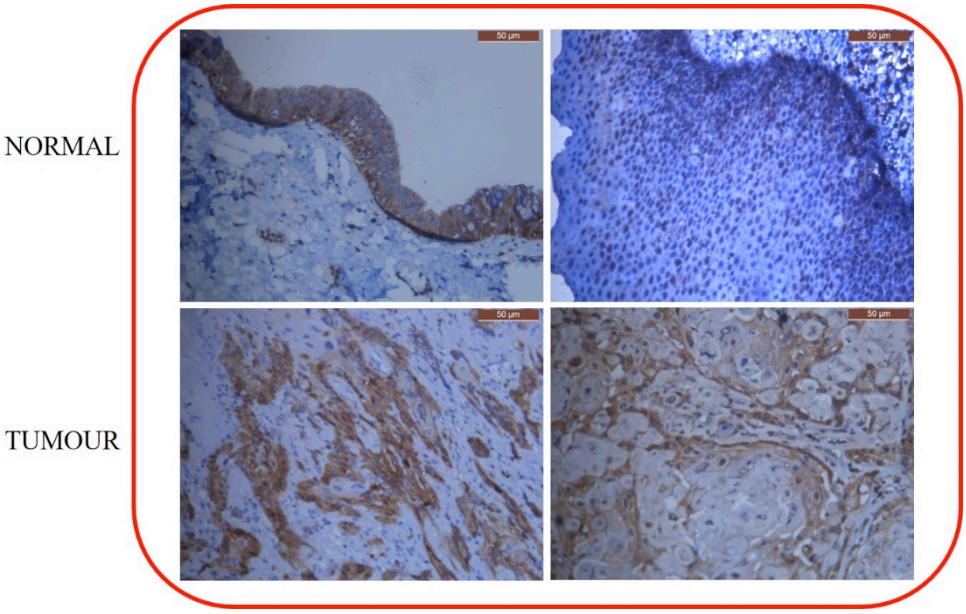


Table 1. **Q - Score for expression of PAK1 in Oral Squamous Cell Carcinoma tissue samples with matched normal**

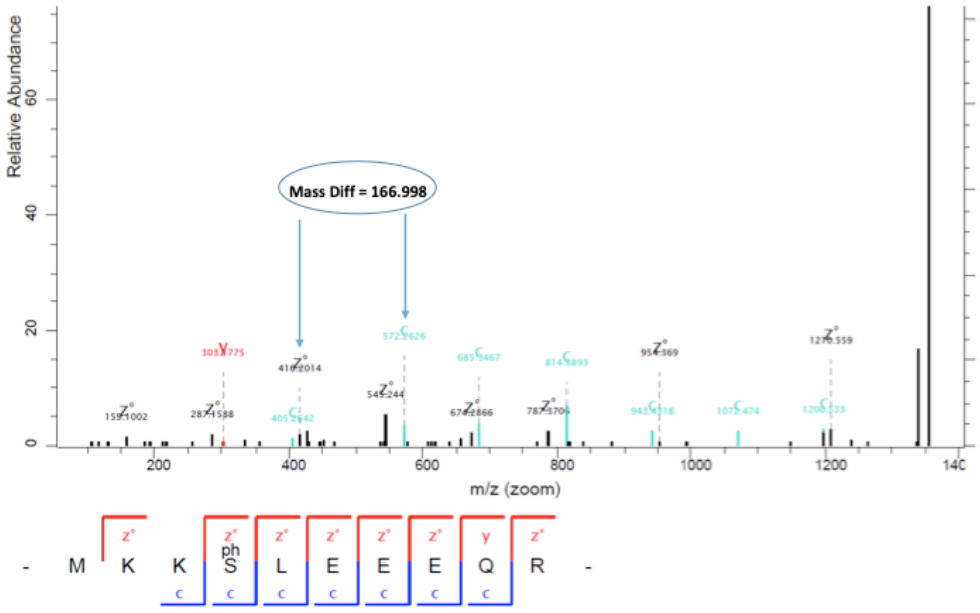
S.NO.	PAK1 Q SCORE (Normal)	Localization	Pak1 Q SCORE (Tumor)	Localization
1	30	N+/C+	80	C +
2	160	C+	270	N+/C +
3	270	C+	160	C +
4	210	N+	240	N+/C +
5	120	N+/C+	240	N+/C +

PAK1 Mean Q Score (Normal): 158
PAK1 Mean Q Score (Tumor): 198
Legends:
N- Nucleus
C – Cytoplasm

Mass Spectrometry analysis of protein lysates from PAK1 expressing & knock out cells.

PAK1 knockout cells were washed with 1X PBS twice and cell lysate was collected with RIPA buffer containing both protease and phosphatase inhibitors. Samples were sonicated at 30% amplitude for five cycles with 10sec ON/OFF. Lysates were quantified and 2mg of the protein lysates were used for GST-PAK1 pull-down assay. Briefly, the samples were precleared with GST beads to remove the noise signals that might be caused by binding of certain proteins to the GST. The samples were then incubated in rotation at 4^{o}C with GST tagged active PAK1 enzymes. This was followed by incubation of samples with GST beads over-night at 4^{o}C with rotation for pull-down. The next day, the beads were washed extensively with TED buffer. The GST beads conjugated with the active PAK1 enzymes now contain the interacting partners of the PAK1. Kinase reaction (with 10mM $MgCl_2$ and 2mM $MnCl_2$) was prepared and the beads were incubated at 30^{O}C for 3 hours with or without ATP. Further, the samples were prepared for mass spec analysis by denaturation, reduction, alkylation, precipitation. Tryptic digestion was carried overnight at 37^{o}C. Digested samples were used for phospho-enrichment with TiO_2 beads and finally were desalted and dried using speedvac. Dried samples were re-suspended in 0.1% formic acid. Samples were injected (2.5µL) in triplicates and mass spec run was performed using Thermo Nano-LC/MS/MS Orbitrap Elite – ETD mode. Raw files obtained from the Mass-spectrometry instrument were used for analysis using MaxQuant 1.6.3.3 software. Phospho (ST) input was given to identify phospho-peptides.

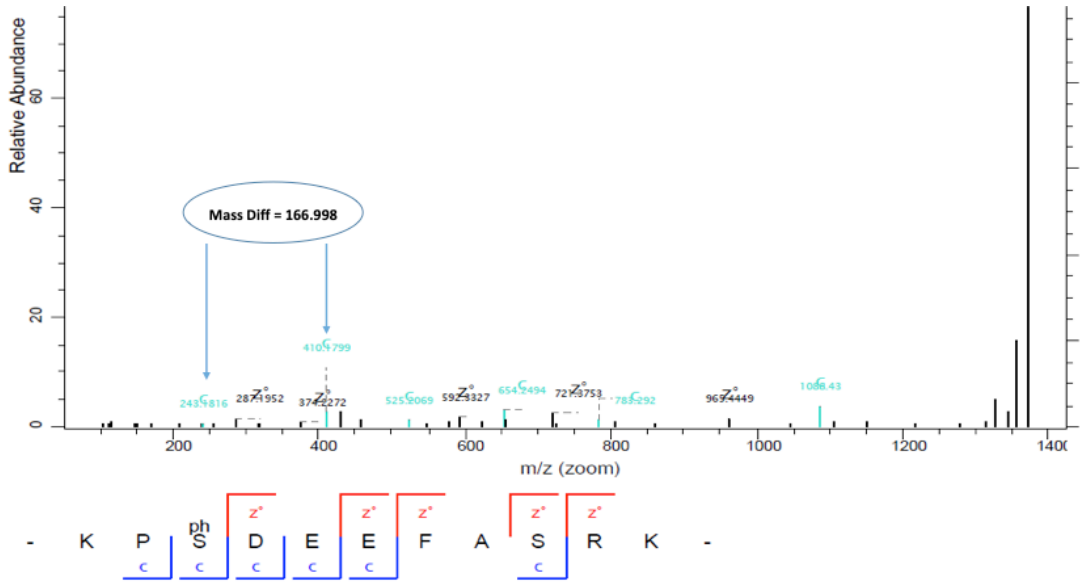
Figure 5. Mass Spectrometry spectrum of Phosphorylated sites in cell lysates.



Identified Peptide Sequence: DEVQELRQDNKKMKKSLEEEQRARKDLEKLV

Phosphorylation site: 689

Figure 6. Mass Spectrometry spectrum of Phosphorylated sites in cell lysates.



Identified Peptide Sequence: TMKKLLPKRKPERKPSDEEFASRKSTAALEE

Phosphorylation site: 644