REPORT

Report on participation of the ICMR International Fellow (ICMR-IF) in Training/Research abroad

1. Name and designation of ICMR- IF : Dr Pawan Kumar, Scientist
2. Address : Division of Pathology, ICAR- Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, Pin- 243122 (India)
3. Frontline area of research in which training/research was carried out : Exosome role in carcinogenesis
4. Name & address of Professor and host institute : Dr Gagan Deep, Associate Professor, Department of Cancer Biology, Wake Forest Baptist Medical Centre, Winston Salem, North Carolina, USA
5. Duration of fellowship with exact date : 12 months (05.02.2020 to 28.01.2021)
6. Highlights of work conducted:

(i) Technique/expertise acquired

- Cell culture technique was learned and practically culture the different prostate cancer cell lines (PC3, LnCAP, RC77T, 22Rv1 etc.) in the lab.
- Prostate cancer cell line culturing in the hypoxia CO2 incubator.
- Clonogenic assay and MTT assay to evaluate the effect of the drugs on the cancer cell viability and growth.
- Exosome isolation (ultracentrifugation & ExoQuick method) from the cell culture media and body fluids.
- Exosome characterization (Nanoparticle tracking analysis)
- Western blotting technique
- Confocal microscopy
- Gene silencing in cell by using siRNA
- Real time PCR
- Immunoflourescence and immunohistochemistry
- Mass spectrophotometry
- Statistical analysis on the Graph Pad Prism

ii) **Research results, including any papers, prepared/submitted for publication**

- Exosomes secreted by cancer cell lines under hypoxic and normoxic conditions were characterized. PC3 cells were cultured in RPMI1640 medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin G and 100 μg/ml streptomycin sulfate in the normoxic (37 °C with 21% O2 and 5% CO2) and hypoxic (37°C with1% O2 and 5% CO2) conditions in CO2 incubator and the exosomes were isolated from the conditioned media by ultracentifugation method. Briefly, the cell culture media was first centrifuged at 500 g at 4 °C for 10 minutes and then supernatant was centrifuged again at 10000 g for 30 minutes at 4 °C. Then the supernatant was filtered with 0.22 μm filters (Merck Millipore) and filtrate was concentrated using concentrators. The supernantent was collected and ultracentrifuged at 100,000 g for 120 min (Beckman Coulter). The exosomes were characterized by NTA for total concentration and size (mean). It was observed that PC3 cells growing under the hypoxic conditions produce significantly higher number of exosomes than in normoxic conditions. No significant variation in the sizes of the exosomes secreted in normoxia and hypoxia was observed. Clonogenic and MTT assays were performed to know the colony forming potential and cell viability under treatment with exosome biogenesis inhibitors (DMA & GW4859) and it was overserved that these exosome biogenesis inhibitors lower the cell viability and colony forming potential of the PC3 cells. Confocal microscopy was done to evaluate the expression of CD63 in the cells. Exosome samples were analysed by mass spectrometry to identify the differential protein and RNA expressions.
- Experiment was performed to know the mechanism of paclitaxel (PTX) resistance in the prostate cancer. PTX is a drug used to treat the castration resistance prostate cancers. The PC-3 cells sensitive to PTX (PC3-S) and the PTX resistant PC3 cells (PC3-PTXR) were cultured and assessed for their protein expression and exosome secretion. PC3-PTXR cells were grown in similar media as for PC-3 with addition of 0.2uM PTX concentration in it. It was observed that PC3-PTXR cells secrete comparatively more exosomes than the PC3-S cells, while the size was approximately similar. MTT and clonogenic assay were performed for PC-3 nad PC3-PTXR cells with exosome inhibitors i.e. DMA (5, 10 and 20μg/ml), GW4869 (5, 10 and 20μM), Silymarin (50, 100 and 200μg/ml) and imipramine (5, 10 and 20μg/ml). MTT and clonogenic assay

with the exosome biogenesis inhibitors (DMA, GW4869, sylimarin, imipramine) showed decreased cellular growth and colony formation potential in both PC-3 and PC3-PTXR cells. Animal experiment was done in the mice to evaluate the effect of GW4869 (exosome biogenesis inhibitors) in the PC3-PTXR xenograft and it was observed that GW4869 significantly reduce the tumour volume and weight at 44th day. Western blotting on the cell lysates revealed that CD63 expression was higher in the PC3-PTXR cells than PC3-S. Further, confocal microscopy was done which also showed increased expression of CD63 in PC3-PTXR. Role of CD63 in PTX resistance was evaluated and it was observed by confocal microscopy that PC3-PTXR cells expel PTX out of the cell in small irregular bodies showing high CD63 expression. Silencing of CD63 in the PC3-PTXR cells by using commercially available CD63 siRNA showed decreased cell growth and colony forming potential. Exosomes collected at different time interval after given fluorescent PTX were sent for the mass spectrophotometry to check the level of drug and different proteins.

- In another study, the 40 human prostate cancer tissues sections were processed for immunohistochemistry to determine the expression of HIF-1 alpha and CA9. The tissue section comprised of 20 from patients (African American-10, Caucasian American-10) with non-recurrence of prostate cancer and 20 from the patients (African American-10, Caucasian American-10) with prostate cancer recurrence. It was observed that the expression of both HIF-1 alpha and CA9 was comparatively higher in the prostate cancer tissues of the patients where tumour did not recur than the patients with recurrence of the prostate cancer.
- CPT1a knockdown or overexpression experiment: 22Rv1 cells were transfected to knockdown and overexpressing the CPT1A. The area of necrosis in the CPTKD was comparatively less than the corresponding vector control. In CPT overexpression group the area of necrosis was comparatively high than the corresponding control. Further, expression of Ki67, cyclin D1, CD31, VEGF and LYVE1 was analysed in the tissue section. No significant difference in the expression of proliferation marker Ki-67 in CPT1A knockdown tumor tissues compared to vector control. Was observed. There was a slight decrease in Cyclin D1 cells in the knockdown group compared to the vector control, however, this was not statistically significant. Similarly, no differences in

microvessel density (CD31+ vessels) between the CPT1A knockdown and vector tumors was seen. Significant decrease in the percentage of strongly stained cells (+3) in the CPT1A knockdown group compared to the vector was observed. LYVE-1 microvessel density (LYVE-1+) was slightly decreased in CPT1A knockdown tumors compared to the vector group. Similarly, expression of these biomarkers was analysed in overexpression group. It was observed that no significant change in Ki-67 and CD31 expression was seen in CPT1 overexpressing tumors but a slight but statistically significant decrease in cyclin-D1 expression was observed. Statistically significant decrease in the percentage of strongly stained VEGF and VEGF-D in the CPT1A overexpressing tumors compared to vector controls.

Publications:

- Ashish Kumar, Susy Kim , Yixin Su , Mitu Sharma , **Pawan Kumar,** Sangeeta Singh , Jingyun Lee, Cristina M. Furdui, Ravi Singh, Fang-Chi Hsu, Jeongchul Kim, Christopher T. Whitlow, Michael A. Nader, Gagan Deep. (2021). Brain cell-derived exosomes in plasma serve as neurodegeneration biomarkers in male cynomolgus monkeys self-administrating oxycodone. eBiomedicine, 63:103192. https://doi.org/10.1016/j.ebiom.2020.103192
- Leslimar Rios-Colon, **Pawan Kumar,** Susy Kim, Mitu Sharma, Yixin Su, Ashish Kumar, Sangeeta Singh, Molishree Joshi; Isabel Schlaepfer, Deepak Kumar, and Gagan Deep (2021). Role of palmitoyltransferase 1 in regulating growth of prostate cancer cells under hypoxic conditions. Cancer letters (Submitted).
- **Pawan Kumar,** Mitu sharma, Susy Kim, Yixin Su, Ashish Kumar, Sangeeta Singh, Gagan Deep (2021). CD63 mediates the paclitaxel resistance through extracellular vesicles in prostate cancer cells. (Under preparation)

iii) **Proposed utilization of the experience in India:** The experience provided by this training definitely improved my technical, scientific and analytical skills. The exposure of handling and growing the cell line in the lab has made me confident in growing the cells at my lab. This experience will be utilized me in designing the projects to isolate the animal disease viruses in the cell lines at my lab. Virus isolation will further be utilized for the pathogenesis studies of the associated disease and also it will help in

development of the new diagnostics. The techniques learned will help in performing the experiments and generating the reliable and concrete data, which can prove the hypothesis of my projects.

In animal / veterinary research, the role of exosome in the disease initiation and progression, and development of diagnostic has not been explored much. So, the exposure of the exosome isolation and characterization will be utilized to develop project in which the role of the exosome in the pathogenesis of the animal diseases and neoplastic conditions will be explored. This will help in establishing the lab, where exosomes work can be done and collaborations with the researchers interested in exosomes research can be made. The use of the exosomes inhibitors in understanding the exosomes role in carcinogenesis during my study will help me in studying the pathways or mechanism of the different diseases processes by utilizing the specific inhibitors or blockers for the effector molecules or receptors.

Place: Bareilly

Dated: 11/03/2021

Signature of ICMR-IF

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