REPORT

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

1. **Name and designation of ICMR-IF :** Dr. Sujit Kumar Bhutia
2. **Address :** Department of Life Science, National Institute of Technology Rourkela, Rourkela-769008, Odisha
3. **Frontline area of research in which training/research was carried out :** Cancer Biology
4. **Name & address of Professor and host institute :**
   Professor Rajesh Agarwal, PhD
   University of Colorado
   Skaggs School of Pharmacy and Pharmaceutical Sciences
   Mail Stop C238
   12850 E. Montview Blvd. V20-2118
   Aurora, CO 80045
5. **Duration of fellowship :** 22nd December 2015 to 3rd April 2016
6. **Highlights of work conducted :**

i) Techniques/expertise acquired:

1. *Abrus* agglutinin induced mitophagy in glioblastoma U87MG cells
2. Abrus agglutinin induced ceramide in glioblastoma U87MG cells
3. *Abrus* agglutinin generated ceramide mediated mitophagy dependent cell death
4. *Abrus* agglutinin found to inhibit epithelial mesechymal transition (EMT) in oral cancer
5. *Abrus* agglutinin demonstrated to modulate ERK expression
6. Study of inhibition of SNAIL by *Abrus* agglutinin
7. *Abrus* agglutinin (AGG) inhibits the expression of $CD44^{+}$ cells in FaDu cells.
8. Inhibition of self-renewal capacity of the orospheres by *Abrus* agglutinin
9. Apoptosis induction with *Abrus* agglutinin in orosphere in FaDu cells.

1. *Abrus* agglutinin induced mitophagy in glioblastoma U87MG cells

We investigated whether *Abrus* agglutinin (AGG) selectively targets and degrades mitochondria following the process of special type of organelle autophagy called mitophagy. Initially we have checked interaction between mitochondria and lysosome and colocalization study was performed. A significant colocalization was noticed in AGG treated U87MG cells, indicating AGG induced mitophagy (Fig.1). Further, we have checked one of the mitochondrial markers by checking expression of TOM20 which is an outer mitochondrial protein. Interestingly, TOM20 expression was decreased with increasing doses of AGG and duration of time of exposure to AGG (Fig.1b). This shows that mitochondrial mass decresed with increasing treatment of AGG as a result of induction of mitophagy.

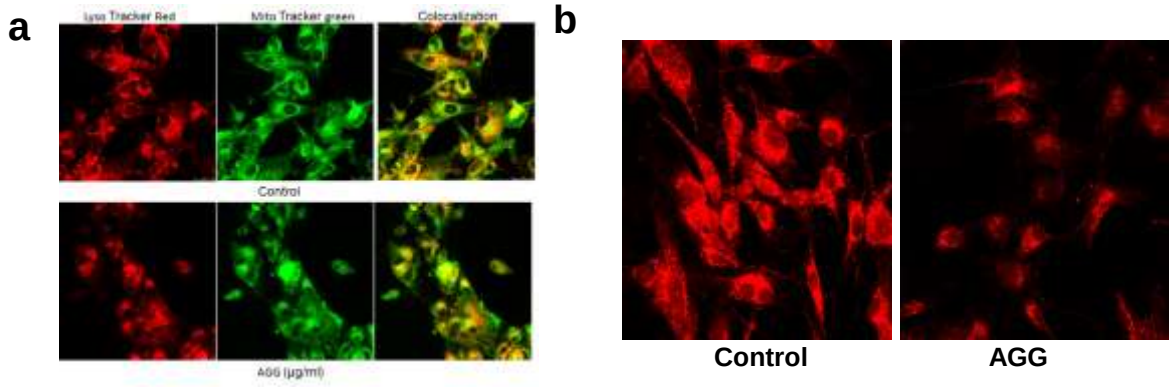

Fig.1: U87MG cells were treated with AGG (10 µg/ml) for 12 h and colocalization of mitochondria and lysosome was analyzed with Mito-Tracker green and Lyso-Tracker red through confocal microscopy (a). Images were taken in confocal microscopy using 630X magnification. U87MG cells were treated with AGG (10µg/ml) for 12 h and the expression of TOM20 was analyzed by fluorescence microscopy (b).

2. *Abrus* agglutinin induced ceramide in glioblastoma U87MG cells

The sphingolipid ceramide has been considered as an important second messenger regulates diverse signaling pathways. Besides that ceramide also involved in induction of autophagic and apoptotic cell death. In our present investigation AGG treated U87MG glioblastoma cell induced ceramide in dose and time dependent manner (Fig.2a and b).

3. *Abrus* agglutinin induced ceramide mediated mitophagy dependent cell death

To examine the role of ceramide in AGG induced mitophagy, the expression of TOM-20 was analyzed in presence of ISP-1. The data showed that staining of TOM-20 was decreased with AGG treatment. Interestingly, it showed that the pretreatment of ISP-1 inhibit the decrease of TM-20 staining by AGG indicating AGG induced mitophagy was regulated by ceramide in U87MG cells.

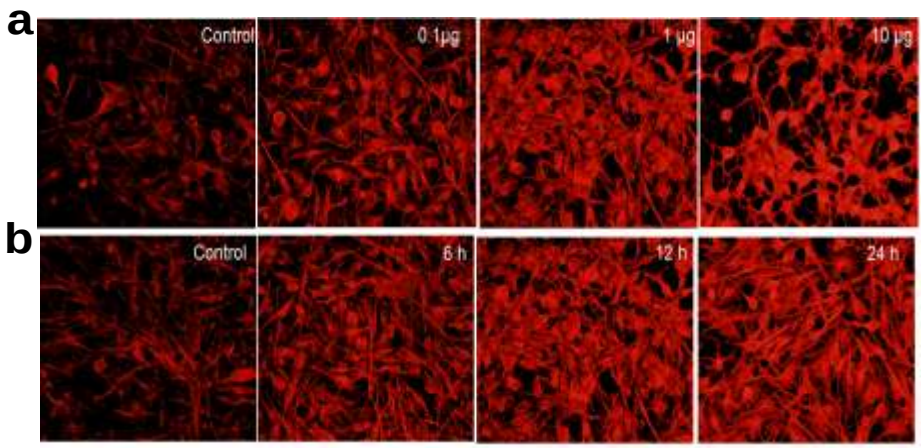

Fig.2. U87MG cells were treated with AGG and the ceramide expression in both dose (0.1, 1.0, and 10 µg/ml) and time (6 h, 12 h, 24 h) dependent manner was quantified by immunofluorescence. Images were taken in fluorescence microscopy using 200X magnification.

4. *Abrus* agglutinin found to inhibit epithelial mesechymal transition (EMT) in oral cancer

Epidermal growth factors play an important role in tumor progression, invasiveness and migration. EGF in cancer cells plays a pivotal role in transformation of epithelial to mesenchymal phenotype subsequently, proving its mettle in cancer cell metastasis and hence supporting stemness. To validate our hypothesis in vitro, FaDu cells were treated with EGF for 30mins. After EGF stimulation, *Abrus* agglutinin was added in dose dependent manner. Results showed pronounced effect of AGG in EGF stimulated FaDu cells by downregulating mesenchymal marker (N-Cadherin) and stemness marker. On other hand the expression of epithelial marker E-cadherin was increased in dose dependent manner in FaDu cells (Fig.3a).

5. *Abrus* agglutinin (AGG) demonstrated to modulate ERK expression

The mitogen-activated protein kinases and their signaling play very important role in cancer progression. We found that AGG inhibited the Ras-dependent extracellular signal-regulated kinase (ERK)1/2 FaDu cells stimulated by the EGF in dose and time dependent manner. Henceforth, we hypothesize MEK/ERK cascade acts as an essential element in EMT in vitro (Fig.3b).

6. Study of inhibition of SNAIL by *Abrus* agglutinin

Snail is a zinc-finger transcription factor with its master mind of EMT in several tumor progressions. There are 3 family members of Snail; Snail1, Snail2/ Slug, and Snail3. We are interested in studying Snail1 or Snail as it is more commonly known. It binds to E-box consensus sequence of their target genes. It suppresses the transcription of epithelial genes, down-regulating E-cadherin expression, a cell adhesion molecule which leads to decreased cell adhesion to the neighboring cells and enhances transcription of mesenchymal genes with increased propensity for cell migration, invasion, tumorigenicity. Our molecule AGG effectively inhibited the snail upregulation in FaDu cells (Fig.3c).

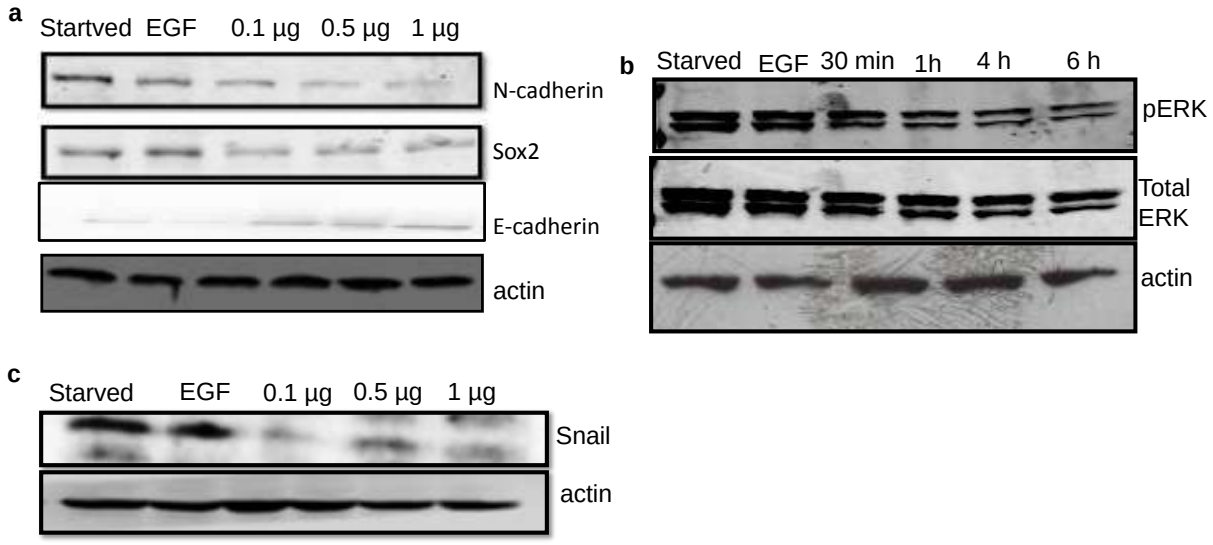

Fig.3: FaDu cells were starved for 16-18 h and stimulated with EGF for an hour with AGG treatment for 24 h and expression different EMT protein was analyzed by Western blot (a). ERK expression was evaluated in time dependent manner by Western Blot (b). Expression of snail in EGF stimulated FaDu cells were demonstrated by Western blot (c).

7. *Abrus* agglutinin (AGG) inhibited the expression of CD44 $^{+}$ cells in FaDu cells

The CD44 is the most commonly studied cancer stem cell marker in oral cancer. The CD44 is a transmembrane receptor which binds with Hyaluronic acid (HA) and signals many cancer related events like proliferation, invasion and metastasis. The CD44 is also associated with apoptosis resistance, drug resistance, and stemness. In this study we investigated the role of AGG in expression of CD44 cells in FaDu cells. The FaDu cells were treated different concentration of AGG and the flow cytometry data showed that AGG was able to decrease the percentage of

$CD44^{+}$cell and increase percentage of $CD44^{-}$cells in dose dependent manner. Further FaDu cells were incubated with the low dose AGG (0.1µg/ml) for different time periods. The flow cytometry analysis showed increase in the $CD44^{-}$ population time, indicating AGG inhibited the proliferation of cancer initiating/stem cells in FaDu cells.

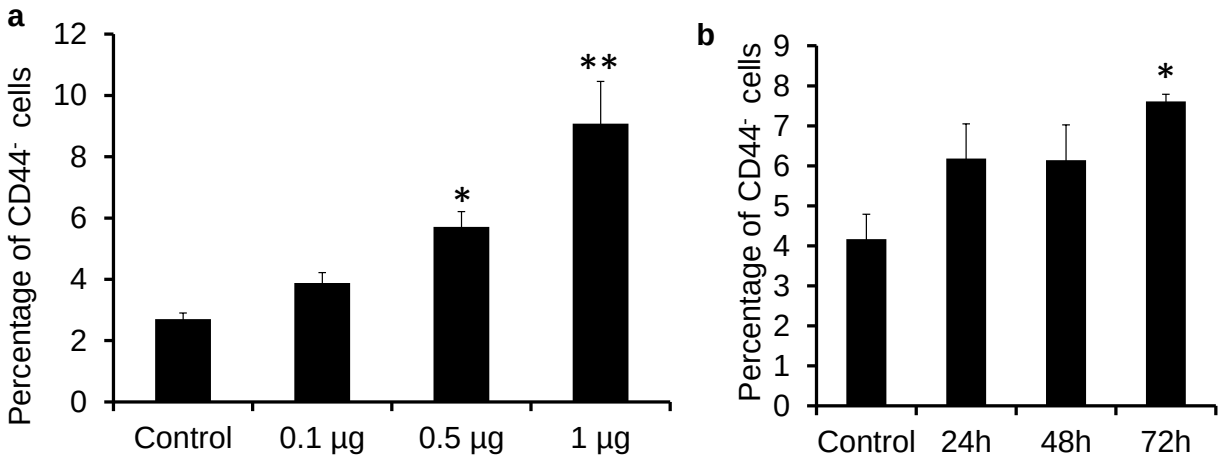

Fig.4: FaDu cells were treated with different doses of AGG and after 24h, CD44 expression was evaluated through flow cytometry (a). FaDu cells were incubated with the lowest dose of AGG (100 ng) for 24 h, 48 h and 72 h to evaluate the CD44 expression by flow cytometry (b).

8. *Abrus* agglutinin inhibited the self-renewal capacity of the FaDu derived orospheres

Oral cancer-initiating/stem cells are enriched in nonadherent spherical clusters of cells, termed orospheres, and these cells are capable of yielding secondary spheres and differentiating along multiple lineages. To examine whether AGG could suppress the formation of spheres *in vitro*, we treated FaDu cells with for 24 h and then cultured these cells in nonadherent conditions to determine orosphere formation for 2 weeks. Compared to control, AGG inhibited in a dose-dependent manner not only the number of orospheres but also the size of the spheres, indicating a reduced self-renewal capacity of these initiating/stem cells. Further, we determined the effect of AGG on Wnt signaling in cancer-initiating/stem cells by analyzing the levels of protein expression in this signaling pathway and AGG treated orospheres resulted in a significant dose-dependent downregulation of β-catenin in FaDu derived orospheres.

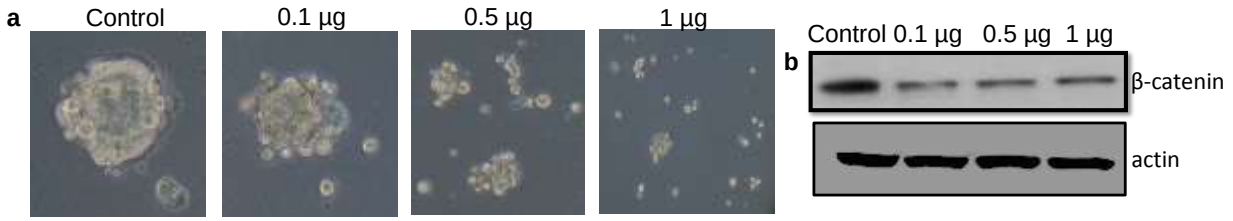

Fig.5: Orospheres were grown in the ultra-low adherent plate in a serum free media supplemented with bFGF, hEGF and nitrogen supplement. The images were taken by bright field microscopy at 400X. The FaDu-derived orosphere were treated with AGG for 24 h and expression of β-catenin was analyzed by Western blot.

9. Apoptosis induction with *Abrus* agglutinin in orosphere in FaDu cells

Initially, FaDu derived orospheres were treated with different concentration of AGG and cell viability was determined by trypan blue exclusion method. Our data showed that AGG dose dependently decreased the cell viability of cancer stem cells derived from FaDu cells. Further, Annexin V staining performed 24 h after AGG treatment demonstrated a strong dose-dependent induction of apoptosis in cancer-initiating/stem cells. Dose-dependent apoptosis induction by AGG was further confirmed after AGG treatment by increased cleavage of PARP and caspases, downregulation of the antiapoptotic protein Bcl-2 and upregulation of the proapoptotic protein Bax in orosphere from FaDu cells.

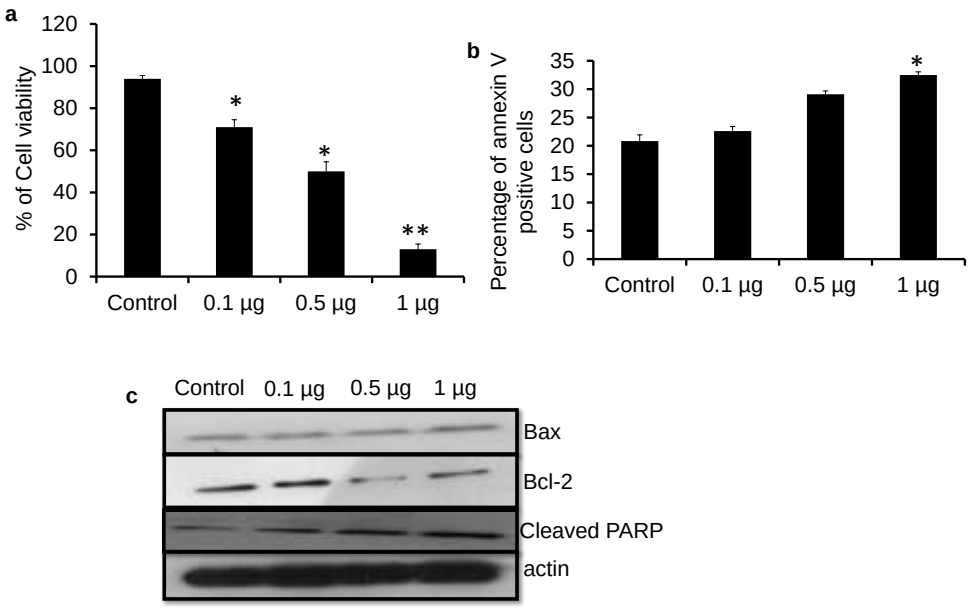

Fig.6. The FaDu derived orospheres were treated with different doses of AGG, and the cell viability was calculated by trypan blue exclusion method. The orospheres were treated in a dose dependent manner, stained with FITC-Annexin V to study apoptosis by flow cytometry. The orospheres were treated with AGG for 24 h and expression of apoptotic related proteins was analyzed by Western blot.

ii) Proposed utilization of the experience in India:

On returning to India, the knowledge and the technical expertise obtained during the ICMR fellowship will be utilized to identify and develop natural chemopreventive molecules for cancer treatment and prevention. Research projects will also focus on monitoring anticancer activity of natural molecules available in india for development of cancr therapeutics. Additionally, this fellowship will also enable the establishment of international collaboration between the National Institute of Technology, Rourkela the Department of Pharmaceutical Sciences University of Colorado Cancer Center. This collaborative effort will seek grant funding from relevant funding agencies, both in the United States and in India. Moreover, after the successful findings of molecular mechanisms of AGG, we can try it for the clinical trials.

Signature of ICMR-IF

ICMR Sanction No. INDO/FRC/452(Y-64)/2015-16-IHD

Dated 15th December 2015