Report

ICMR International Fellowship-2013-14

1. **Name and Designation of ICMR-IF:** Dr. Rana P. Singh,

2. **Address:** 104, Cancer Biology Laboratory, School of Life Sciences, Jawaharlal Nehru University (JNU), New Delhi, India

3. **Frontline area of research in which training/research was carried out:** Proteomics of cancer

4. **Name and address of Professor and Host Institute:** Dr. Yinsheng Wang, Professor at Department of Chemistry, University of California Riverside (UCR), USA

5. **Duration of Fellowship:** 26 Feb 2014 – 25 June 2014

6. **Highlights of work conducted:**

(i) Technique/expertise acquired:

1. Cloning of full length human EGFR in pRK7-flag tag expression vector.

2. Transfection for expression of EGFR-flag in human prostate cancer DU145 cells.

3. Pull-down of EGFR-flag from DU145 cells treated with cisplatin or gamma radiation and silibinin. Pull down of endogenous EGFR using anti-EGFR antibody and dyna beads conjugated with protein A.

4. Trypsin digestion and zip-tip of pull-down for LC-ms/ms analysis.

5. Mascot analysis, identification of post-translational modifications (PTM) of EGFR from chromatogram.

6. Identification of potential interacting proteins with EGFR.

7. Silibinin-cisplatin combination – DU145 cells counting –live/dead

8. Isce-1 plasmid transfection to XL10 strain, amplification and isolation.

9. Study of effect of silibinin on NHEJ (non-homologous end-joining) in response to DNA damage.

10. Study of effect of silibinin on HR (homologous recombination) in response to DNA damage.

11. Site-directed mutagenesis for generating EGFR T693A and EGFR T693D mutant plasmids.

(ii) Research Results, etc.:

1. Cloning of full length human EGFR in pRK7-FLAG-tagged mammalian expression vector.

Human EGFR1 gene is located at chromosome 7p12. It is also denoted as ERBB1 or HER1. Its full length cDNA clone (CDS) has 3630 nucleotides which code for 1210 amino acid peptide. pBABE-EGFR plasmid was a kind gift from Prof. Robert J. Coffey from Vanderbilt University Medical Center, Nasville, TN, USA. This plasmid was inoculated to XL10 competent *E. coli* strain. A colony

PCR was done using 6 different sets of primers. Primers were designed to amplify full length (3630 bp) or N-terminal (2004 bp) or C-terminal (1696 bp); the latter two inserts had overlapping transmembrane region.

PCR products were checked on agarose gel. EGFR-full length insert was identified and purified using Cycle Pure Kit (Omega E.Z.N.A.). EGFR-full length insert (3.6 kb) was digested with pRK7bama (4.7 kb, mammalian expression vector with 3 flag-tags before stop codon) using Sal1-HF and Xba1 over night at 37 degree C. This was followed by Cycle Pure, ligation and transfection to XL10 competent cells followed by overnight incubation for colony growth. pRK7-EGFR-FL plasmid was isolated from the selected colonies and confirmed with restriction digestion (with Sal1-HF and Xba1 or BamH1) followed by agarose gel electrophoresis and also by sequencing (with PR13 primer) using Genomics Core Facility at UCR, CA. Using mini-prep, required amount of pRK7-EGFR-FL plasmid was made for the next step.

2. Transfection for expression of EGFR-FLAG-tag in human prostate cancer DU145 cells and treatment with cisplatin or gamma radiation and/or silibinin.

Advanced (resistant to chemotherapy and radiation treatment) human prostate carcinoma DU145 cells (ATCC, USA) were grown in RPMI1640 medium supplemented with 10% FBS in CO_2 incubator under standard culture condition. At ~60% confluency, cells were transfected with pRK7-EGFR-FLAG-tagged plasmid (1.5 microgram/well in 6-well plate) using lipofectamine-2000 (Invitrogen) and Opti-MEM medium under regular serum condition. After 48 h of transfection, cells were treated with 25 μmol/L of cisplatin or 5 Gy gamma radiation (both are DNA damaging/genotoxic agent and used for cancer treatment) or 100 μmol/L of silibinin (a potential chemo- and radiation-sensitizing agent) or their combination for 3 h. Then, cells were harvested and lysed in Cellytic M- lysis buffer (600 μl/well) and centrifuged at 13000 rpm for 10 min. Cleared lysate was used for the pull-down assay in the next step.

3. Pull-down of EGFR-FLAG as well as endogenous EGFR from DU145 cells.

Cleared lysate was incubated with anti-FLAG-M2 affinity gel/beads (Sigma; 20 μl/sample) over night at 4 degree C on rocker. Lysate was centrifuged at 8000xg for 1 min and beads bound with EGFR-FLAG protein (expressed by the plasmid) was separated from the flow through/supernatant. The flow through was next incubated with anti-EGFR antibody (Cell Signaling Technology) cross-linked with Dyna beads conjugated with protein A (Life Technologies) followed by centrifugation. This step allowed the pull-down of endogenous EGFR expressed in DU145 cells. In both the cases, beads were washed three times with 1x TBS and then subjected to the next process of trypsin digestion.

4. Trypsin digestion and zip-tip desalting of EGFR pull-down.

Washed beads were kept at water bath at 95 degree C for 5 min and then added with DTT and incubated at 37 degree C for 45 min. DTT (10 mM) is a reducing agent that reduces intra – and inter-

molecular bonding through cysteine residues. Then beads were incubated with iodoacetamide (IAA; 25 mM) at 56 degree C for 1 h in dark leading to carboxymethylation. IAA is an irreversible inhibitor of cysteine peptidase and prevents the reformation S-S bonds. Finally, samples were incubated with trypsin (1 μg/sample; sequencing grade) at 37 degree C for 16-18 h.

These trypsin-digested samples were separated from the beads (by centrifugation) and adjusted for pH $\leq$ 4, and subjected to zip-tip desalting procedure. Zip-tips (equivalent to C18 columns) were first washed with a gradient of 100% acetonitrile to 50% acetonitrile+50% H_2O to 100% H_2O, later two containing 0.1% (v/v) formic acid. Samples were passed through these tips ~ 20 times and then eluted in 50% acetonitrile+50% H_2O followed by vacuum drying (Speed Vac, at room temperature). Dried samples were dissolved in 20 μl of ddH_2O and subjected to LC-ms/ms analysis as described below.

5. LC-ms/ms analysis of EGFR pull-down samples.

Desalted peptide samples were automatically injected and separated by online reverse phase liquid chromatography on an EASY nanoLCII and analyzed on an LTQ Orbitrap Velos mass spectrometer equipped with a nanoelectrospray ionization source (Thermo, San Jose, CA). The separation was conducted using a homemade trapping column (150 μm × 50 mm) and a separation column (75 μm × 120 mm), packed with ReproSil-Pur C18-AQ resin (5 μm in particle size, Dr. Maisch HPLC GmbH, Germany). Peptide samples were initially loaded onto the trapping column with a solvent mixture of 0.1% formic acid in CH_3CN/H_2O (2:98, v/v) at a flow rate of 4.0 μL/min. The peptides were then separated using a 105-min linear gradient of 2–40% acetonitrile in 0.1% formic acid and at a flow rate of 300 nL/min. The LTQ-Orbitrap Velos mass spectrometer was operated in the positive-ion mode, and the spray voltage was 1.8 kV. The full-scan mass spectra (m/z 350–2000) were acquired with a resolution of 60,000 at m/z 400 after accumulation to a target value of 500,000. MS/MS experiments were carried out in a data-dependent scan mode where one full scan MS was followed with 20 MS/MS scans. Further, for better quality of MS/MS, the data were also collected in selected-ion monitoring mode. In this mode, the fragmentation of the protonated ions of the unmodified and serine, threonine and tyrosine phosphorylated forms of peptide of EGFR were monitored. All of the MS/MS data were analyzed with Mascot and manually using MS-Product software.

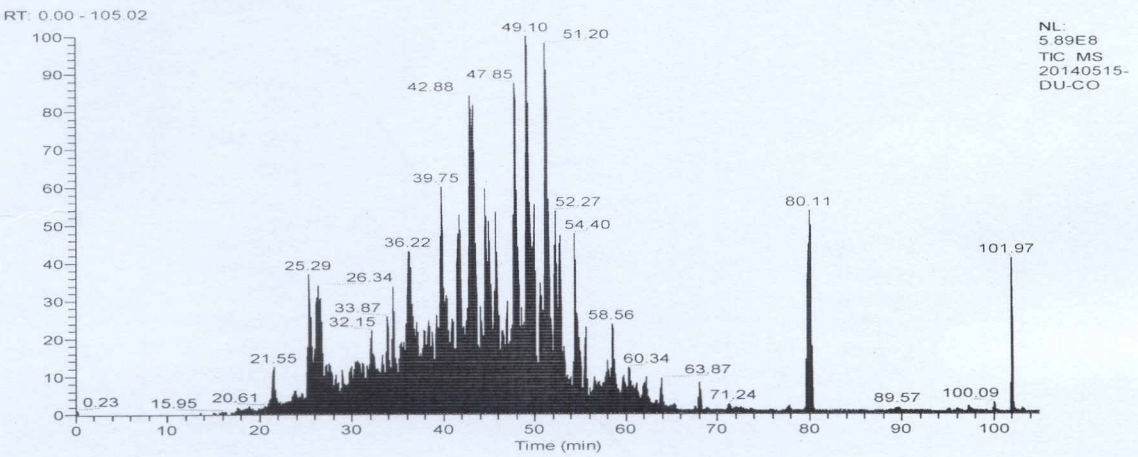

Figure 1. MS scan of EGFR pull-down sample from control DU145 cells. The run time was 105 min showing peaks/abundance for many proteins.

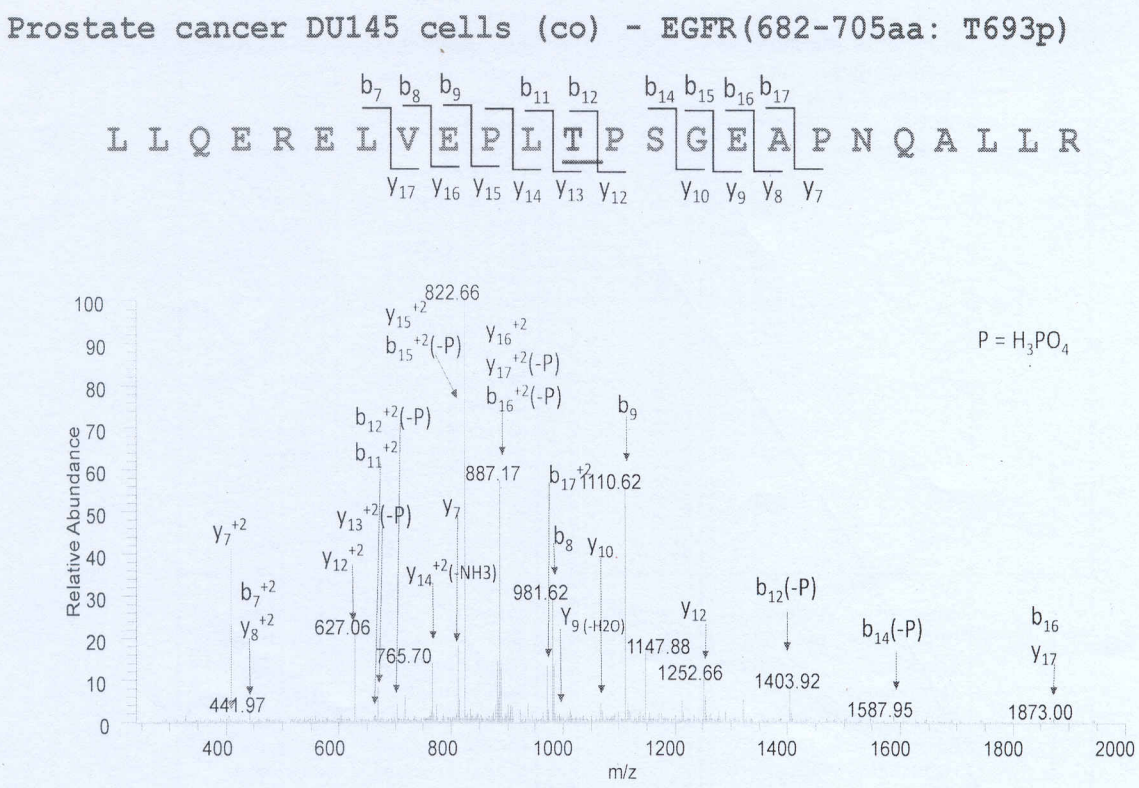

Figure 2. MS/MS analysis of EGFR from DU145 cells showing the showing phosphorylation of threonine at 693 position. We hypothesize that this site may have role in the internalization of the receptor in cytosol or nucleus which needs further studies.

5. Data processing and analysis for post-translational modifications (PTM) of EGFR.

Data were processed and searched using Mascot Daemon software (Mascot TM search engine) and the Human IPI data base. 100 ppm precursor ion mass tolerance and 1.0 Da fragment ion tolerance and MUDPIT scoring was applied with a peptide cut-off score of 20 and peptide relevance score of 1. Peptides were filtered based on a false discovery rate cut-off of 1% (strict) and 5% (relaxed). Cysteine S-carbamidomethylation as fixed modification, methionine oxidation as variable modification, and up to two missing cleavages were considered during MASCOT searching. Phosphorylation of EGFR was identified at many amino acids. T693 phosphorylation was present in control which was not detected in silibinin-treated cells, however, this peptide was not detected in cisplatin and combination treatments likely due to low sequence coverage. Likewise, T725 and Y727 were phosphorylated only in cisplatin-treated cells.

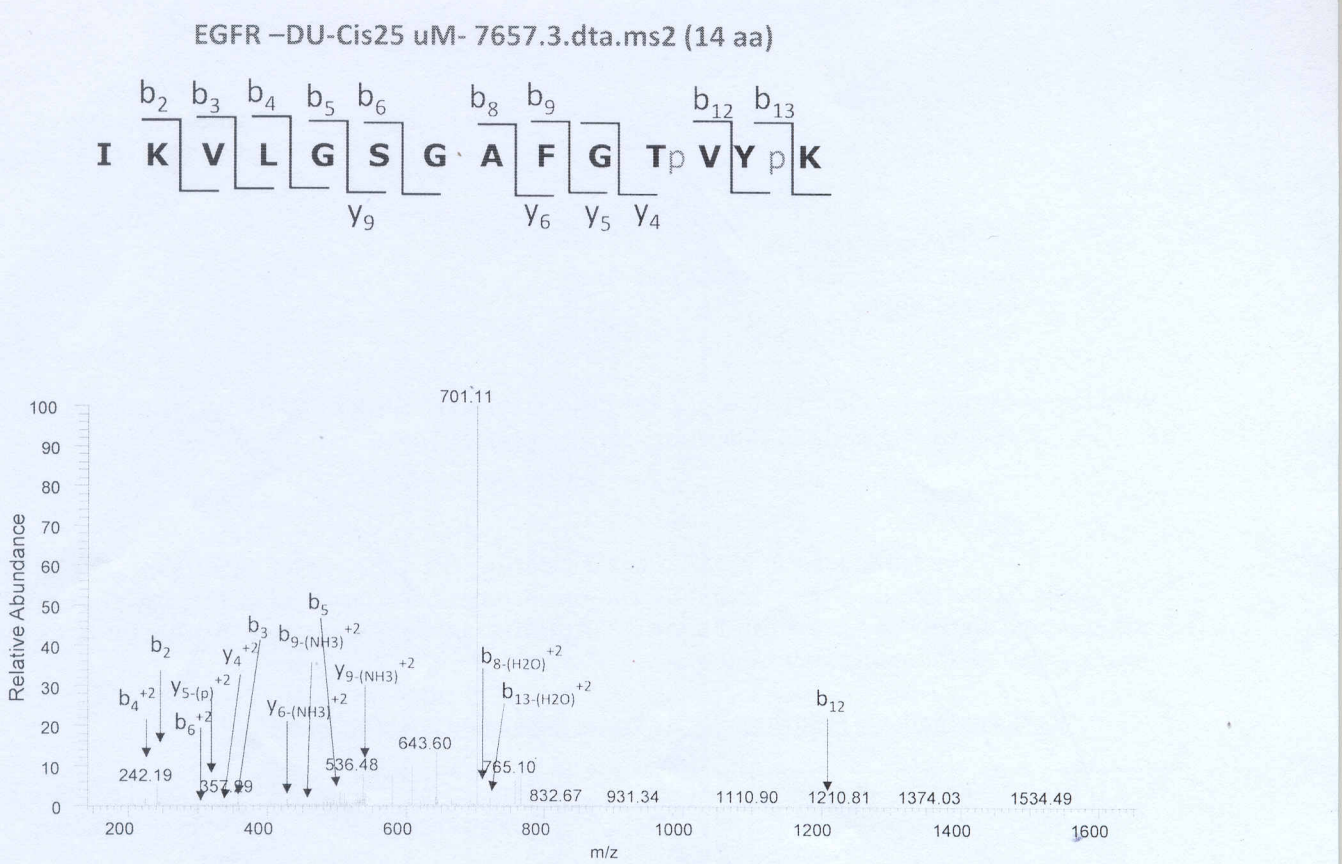

Figure 3. MS/MS analysis of EGFR from cisplatin-treated DU145 cells showing the phosphorylation of threonine-725 and tyrosine-727.

6. Analysis of potential interacting proteins with EGFR. There were peptides representing more than 500 proteins potentially interacting with EGFR. In second and third repeats, these will be narrowed down with better pull-down and with good sequence coverage. There were proteins related to DNA repair such as DNA-PK as well as mesenchymal markers such as vimentin. Further studies are needed explore their bindings with EGFR.

7. Silibinin sensitizes cisplatin-treated DU145 cells for death. Our laboratory has already found that silibinin enhances radiation-sensitivity of advanced prostate cancer cells. We further, identified here that silibinin strongly sensitized DU145 cells to death. Now, we will be exploring the role of EGFR in this chemosensitizing effect of silibinin in cancer cells.

8. ISce-1 plasmid transfection to XL10 cells, amplification and isolation. I did the ISce-1 plasmid transfection to competent XL10 *E. coli* strain for its amplification and isolation which was used for the experiments related to NHEJ (non-homologous end-joining) and HR (homologous recombination).

9. Study of effect of silibinin on NHEJ in response to DNA damage. U2OS cells stably transfected with EJ5-GFP plasmid were used for the experiment. Following the transfection with ISc -1 (restriction enzyme) and then treatment with silibinin cells. EJ5 has a RE site for ISce-1 in GFP sequence, therefore, only repaired cells can show the fluorescence for GFP. After these treatments, flow cytometric and fluorescence microscopic analyses of cells indicated that silibinin enhances DNA repair by NHEJ mechanism.

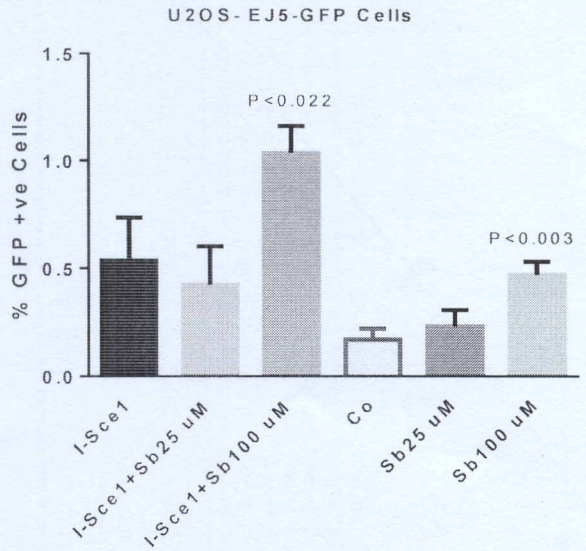

Figure 4. Cells transfected with I-Sce1 plasmid, 3 h later media was replaced with or without Silibinin for 66 h, followed by FACS analysis (Mean±SEM).

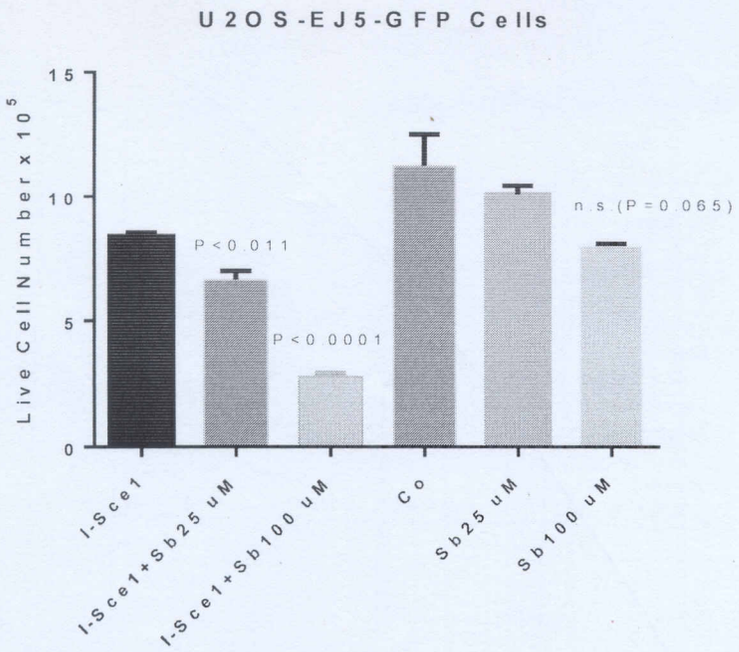

Figure 5. In the similar treatment as in Figure 4, live cell number decreased in I-Sce1 + silibinin treatments.

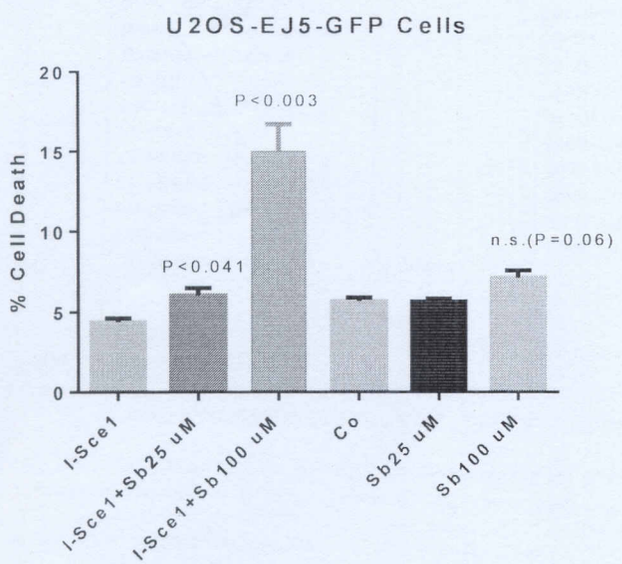

Figure 6. In the similar treatment as in Figure 4, the number of dead cells increased in I-Sce1 + silibinin treatments.

10. Study of effect of silibinin on HR in response to DNA damage. U2OS cells stably transfected with DR-GFP plasmid having RE site for ISce-1 in GFP sequence were used for the experiment. In this case, homologous recombination is required for GFP expression after ISce-1 transfection. Flow analysis of cells showed that silibinin enhances DNA repair by HR mechanism.

11. Site-directed mutagenesis for generating EGFR T693A and EGFR T693D mutant plasmids. I also learnt how to make mutant and mimetic plasmids using RT-PCR. Primers were designed to generate EGFR T693A and EGFR T693D mutant plasmids. This will be helpful in defining the functional role of a particular PTM in EGFR.

Out-reach activities

1. **Invited Seminar** at Department of Chemical Sciences (Graduate Toxicology Programme), University of California Riverside (UCR), CA, USA (12 March 2014). Title: "Chemoprevention of photocarcinogenesis by small molecules from plants"
2. American Association of Cancer Research, Annual Meeting, San Diego, CA, USA (5-9 April 2014). **Poster presentation,** title: "Silibinin radiosensitizes prostate cancer cells by enhancing radiation-induced cell death and inhibiting nuclear EGFR-mediated DNA repair"
3. **Invited Seminar** at Department of Pharmaceutical Sciences, University of Colorado Denver, CO, USA (12 May 2014). Title: "A novel role of silibinin in enhancing radiotherapeutic response in prostate cancer"

(iii) Proposed Utilization of the experience in India:

The techniques learnt and preliminary work done in the area of proteomics and DNA damage/repair will be helpful in moving ahead with the proposed objectives in the research proposal at JNU, New Delhi. This visit to UCR, CA has now established a research collaboration with my laboratory.

21/7/2014

Signature of ICMR-IF

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