ANNEXURE-I

Preparation of Lowry C reagent for protein estimation

a) Copper sulphate in 1% sodium potassium tartarate (1% w/v) 0.5gm of copper sulphate was dissolved in 1% sodium potassium tartarate (Prepared by dissolving 1gm of sodium potassium tartarate in 100ml of distilled water).

b) Sodium carbonate in 0.1M sodium hydroxide (2% w/v)- 2gms of sodium carbonate was dissolved in 100ml of 0.1M sodium hydroxide.

2ml of solution (a) was mixed with 100ml of solution (b) just before use.

WESTERN PROTOCOL

After cutting the stored tumor sample in several pieces collected in eppendorf microfuge and frozen in liquid nitrogen. These samples were stored at -80°C for later study.

A. Preparation of cell lysates- The samples stored at -80°C wee removed just before the experiment from the deep freezer. Lysis buffer 100-200ul was added to each tube and the tissue was mixed with pipette tips on ice for 10 min and centrifuge at 14,000 rpm in an eppendorf microfuge for 10-20 min at 4°C. The supernatant was transferred to a new tube and the pellet was discarded. The protein concentration was measured with Bradford assay (Bio-Rad). Proteins were mixed with equal amount of 2x sample buffer (Loading buffer), boiled for 5 min and centrifuge to bring down condensation prior to loading gel.

B. Polyacrylamide gel (14.5 cm x 16.5 cm)

1. Agarose plug: 1% agarose was dissolved in 1x resolving gel buffer
2. Resolving gel: 24 ml of a 9% gel (5.4 ml 40% acrylamide/bisacrylamide 29:1, 3 ml 8x Resolving gel buffer, 15.6 ml water, 12 µl TEMED and 60 µl 20% ammonium persulfate (APS))
3. Stacking gel: 8 ml (1 ml 40% acrylamide/bisacrylamide 29:1, 2 ml 4x Stacking gel buffer, 5 ml water, 8 µl TEMED, 21.6 µl 20% ammonium persulfate

C. Preparation of gel- The glass plates and spacers (1.5 mm thick) were assembled and an agarose plug was poured (1-2 mm). Running gel was added about 1cm below the wells of the comb (~20 ml) and sealed with 1 ml water-saturated butanol. After setting of gel, butanol was removed and gel was rinsed with deionized water. The stacking gel was added (~5 ml) and the comb was inserted immediately. The gel was set in gel ring and immersed in buffer after setting of stacking gel.

D. Running the gel- The protein sample was loaded into the wells along with marker and run with constant current (35-37mA with voltage set at > 300 V) using the Bio-Rad mini-gel transfer system (Bio-Rad Laboratories, Cambridge, MA). Usual running time was about 2.5 hr. The glass plates were removed from the electrophoresis apparatus and placed on a paper towel. Using a spatula, pry the plates were parted and the orientation of the gel was marked by cutting a corner from the bottom of the gel. The gel was removed from the electrophoresis apparatus and incubated in western transfer buffer for approximately 10 min to remove detergent.

E. Preparation of membrane- A piece of Immobilion-P-transfer membrane (Millipore Immobion-P #IPVH 000 10) was cut and wet for about 30 min in methanol on a rocker at room temp. Methanol was then replaced with 1x blotting buffer until ready to use.

F. Membrane transfer- The sponges, filter papers were soaked in 1x blotting buffer and "sandwich" for Bio-Rad's Transblot. (Sponge-filter paper-gel - membrane-filter paper–sponge). The blot was transferred for 1 hr at 1 amp at 4°C. After completion, the membrane was blocked overnight in blocking buffer.

G. Antibodies and detection- The membrane was incubated with primary antibody diluted in blocking buffer for 60 min at room temp and washed with 3 x 10 min with 0.05% tween 20 in PBS. The membrane was further incubated with secondary antibody conjugated to horseradish peroxidase (HRPO; 1:1000-1:10000 dilution) for 60 minutes at RT. After five washes with

TBST, the membrane was developed for 5 minutes using western blot chemiluminescent substrate (Super signal West Femto maximum sensitivity substrate, Pierce). The blot was again washed 3 x 10 min with 0.05% tween 20 in PBS. X-ray film was exposed to the blots for appropriate time periods to visualize the chemiluminescence signal corresponding to the specific antibody-antigen reaction. The film was fixed and developed with Hi-Fix liquid fixer and X-ray film developer (Indo Chem) respectively.

Buffers for Westerns

Lysis buffer: (0.15 M NaCl, 5 mM EDTA, pH 8, 1% Triton X100, 10 mM Tris-Cl, pH 7.4). Just before using add: 1:1000 5 M DTT, 1:1000 100 mM PMSF in isopropanol, 1:1000 5 M aminocaproic acid.

2x sample buffer (Loading buffer): 130 mM Tris-Cl, pH8.0, 20% (v/v) Glycerol, 4.6% (w/v) SDS, 0.02% Bromophenol blue, 2% DTT (DTT should be added just before the buffer is used, from 1M stock)

8x Resolving gel buffer: (100 ml) 0.8 g SDS (add last), 36.3 g Trizma base (= 3 M), Adjust pH to 8.8 with concentrated HCl

4x Stacking gel buffer: (100 ml) 0.4 g SDS (add last), 6.05 g Trizma base (= 0.5 M), Adjust pH to 6.8

10x Running buffer: 1 L 30.3 g Trizma base (= 0.25 M), 144 g Glycine (= 1.92 M), 10 g SDS (= 1%)--add last Do not adjust the pH.

10x Blotting buffer: 1 L 30.3 g Trizma base (= 0.25 M), 144 g Glycine (= 1.92 M), pH should be 8.3; To make 2 L of 1x Blotting buffer: 400 ml Methanol, 200 ml 10x Blotting, buffer, 1400 ml water

Blocking buffer: 0.5 L 3% Bovine serum albumin (Fraction V), Make up in PBS and sterile filter.

Then add 0.05% Tween 20. Keep at 4°C to prevent bacterial contamination.

Stripping buffer: 0.5 L (sterile filter solution and keep at 4°C) 0.2 M Glycine, pH 2.5, 0.05% Tween 20.