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The investigation of the integrative function of focal adhesion kinase (FAK) in human oesophagel squamous cell carcinoma cell lines

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CHAPTER 5: APPENDIX

5.1 Tissue Culture

DMEM (Dulbecco's Modified Eagle's Medium) Solution

1.37%	DMEM
0.37%	Sodium Bicarbonate
2%	Penicillin (500 U/ml)/Streptomycin (0.5%) solution

Make up to final volume with dH_2O.

Hams F12 Medium Solution

1.07%	Hams F12 medium
0.118%	Sodium Bicarbonate
2%	Penicillin (500 U/ml)/Streptomycin (0.5%) solution

Make up to final volume with dH_2O.

Mix DMEM:Hams F12 in a 3:1 ratio.

Filter-sterilize and store at 4˚C.

Trypsin/EDTA solution

0.02%	EDTA in PBS
0.1%	Trypsin in PBS

Mix EDTA:Trypsin (1:1) to obtain a final concentration of 0.01% EDTA/ 0.05% Trypsin.

Store at 4˚C.

5.2 Phosphate-Buffered Saline (PBS)

136.9 mM	Sodium Chloride
2.68 mM	Potassium Chloride
10.1 mM	Disodium hydrogen orthophosphate
1.75 mM	Potassium dihydrogen orthophosphate

Adjust to pH 7.2-7.3 and make up to final volume with dH_2O.

Autoclave to sterilize.

5.3 Laemmli Lysis Buffer

0.05 M	Tris-HCl, pH 6.8
0.15 M	SDS
10%	Glycerol
5%	β-mercaptoethanol

Adjust pH to 6.8 and make up to final volume with dH_2O.

Store at 4˚C.

For double lysis (Laemmli) buffer, use the same concentrations of solutes but make up to half the volume with dH_2O.

5.4 Phenyl-methyl-sulphonyl fluoride (PMSF) stock solution

0.2 M	PMSF

Make up to final volume with methanol.

5.5 PBS/PMSF/Trazylol solution

0.5%	PMSF stock solution
1%	Trazylol

Make up to final volume in PBS.

5.6 0.5% Triton X-100 Extraction Buffer

0.5%	Triton X-100
50 mM	Tris pH 8.5
150 mM	Sodium chloride
1 mM	Calcium chloride
1 mM	Magnesium chloride
0.01%	Aprotinin
5 µl/ml	PMSF stock solution
10 µl/ml	Trazylol

5.7 0.5% Triton X-100 Extraction Buffer with complete, Mini, EDTA-free protease inhibitor cocktail

10 ml	0.5% Triton X-100 extraction buffer
1 tablet	Complete, Mini, EDTA-free Protease Inhibitor Cocktail (Roche, Germany)

5.8 Protein Estimation

Tri-chloroacetic acid (TCA)

7.5%	TCA

Make up to final volume with dH_2O.

Coomassie Blue

0.26%	Coomassie Brilliant Blue
50%	Methanol

Dissolve, and then add:

10%	Acetic acid

Make up to final volume with dH_2O.

Destain Solution

12%	Glacial Acetic Acid
10%	Methanol

Make up to final volume with dH_2O.

Elution Solution

66%	Methanol
33%	dH_2O
1%	Ammonia

5.9 SDS-PAGE

10% SDS-PAGE

Separating Gel:

375 mM	Separating buffer (Tris-HCl, pH 8.8)
10%	Acrylamide
0.2%	SDS solution (50mg SDS /ml dH_2O)
0.1%	*N'*,*N'*,-Methylenebisacrylamide solution (25mg Bis /ml dH_2O)

Make up to final volume with dH_2O.

Add just before use:

1 mM	Ammonium Persulphate (APS) solution (1.25%)
0.25%	TEMED

Stacking Gel:

125 mM	Stacking buffer (Tris-HCl, pH 6.8)
5%	Acrylamide
0.2%	SDS solution (50mg SDS / ml dH_2O)
0.1%	*N'*,*N'*,-Methylenebisacrylamide solution (25mg Bis/ ml dH_2O)

Make up to final volume with dH_2O.

Add just before use:

1 mM	Ammonium Persulphate (APS) solution (1.25%)
0.2%	TEMED

Reservoir Buffer

3.74 mM	SDS
192.5 mM	Glycine
25 mM	Tris-HCl, pH 8.3

Make up to final volume with dH_2O.

Coomassie Blue Stain

0.25%	Coomassie Brilliant Blue powder
50%	Methanol
10%	Glacial Acetic Acid

Make up to final volume with dH_2O.

Destain

10%	Acetic Acid
10%	Methanol

Make up to final volume with dH_2O.

5.10 Western Blot Analysis

Western Blot Transfer Buffer

25 mM	Tris-HCl, pH 8.3
1.41%	Glycine
20%	Methanol

Make up to final volume with dH_2O.

Store at 4˚C.

Tris-Buffered Saline (TBS)

50 mM	Tris-HCl, pH 7.8
147 mM	Sodium Chloride
2 mM	Calcium chloride dihydrate

Make up to final volume with dH_2O.

Blocking buffer (Blotto)

50 mM	Tris-HCl, pH 7.8
2 mM	Calcium chloride
5%	Non-fat milk powder
0.01%	Anti-foam
0.05%	Triton X-100

Make up to final volume with dH_2O.

Developer

6.4 M	Metol
0.6 M	Sodium Sulphite
80 mM	Quinol
0.45 mM	Sodium Carbonate
34 mM	Potassium Bromide

Make up to final volume with dH_2O.

Store at room temperature in the dark.

Fixer

0.8 M	Sodium Thiosulphate
0.2 M	Metabisulphite

Make up to final volume with warm dH_2O.

Store at room temperature in the dark..

5.11 Indirect Immunofluorescence

4% Paraformaldehyde

0.2 M	Sodium dihydrogen orthophosphate
0.6 M	Sodium hydroxide

Mix 166 ml of sodium dihydrogen orthophosphate with 34 ml of sodium hydroxide before adding:

8 g	Paraformaldehyde

Heat to 80 ºC with stirring until solution is clear.

Cool and filter.

pH 7.2

Store at 4ºC.

Elvanol

0.1 M	Tris-HCl pH 8.5
20 %	Polyvinyl alcohol
50 %	Glycerol

Make up to final volume with dH_2O.

5.12 Co-Immunoprecipitation Analysis

Immunoprecipitation Buffer

20 mM	Tris-HCl pH 8.0
0.5%	Nonidet NP-40
0.9%	Sodium chloride

5.13 Determination of protein concentration of whole-cell lysates of the 5 HOSCC cell lines

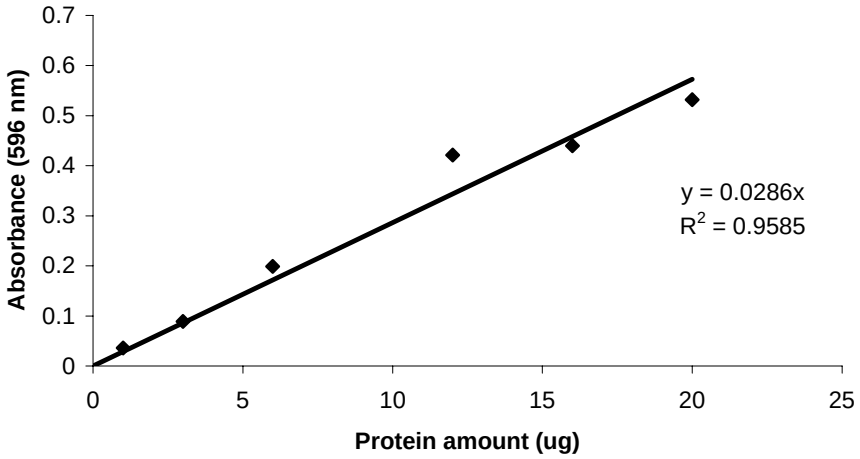


Figure 5.1: Standard curve of absorbance (at 596 nm) versus protein concentration of BSA standards in single lysis buffer.

5.14 Determination of the relative molecular weight of proteins separated by 10% SDS-PAGE

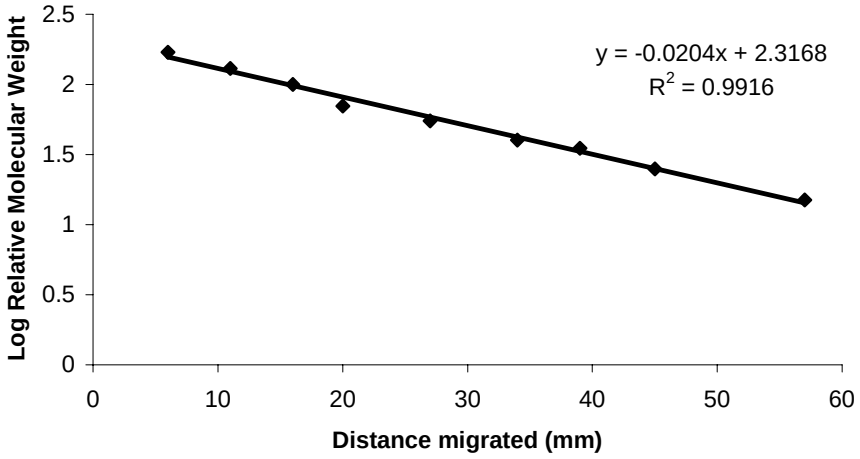


Figure 5.2: Standard curve of relative molecular weight (log) versus distance migrated (mm) by the molecular weight marker.

5.15 Raw densitometric data of western blot showing FAK protein expression levels in whole-cell lysates

Table 5.1: Optical density values obtained by LabWorks™ analysis of polypeptide bands generated by FAK-specific western blot analysis of whole-cell lysates.

CELL LINE	OPTICAL DENSITY VALUES
WHCO1	86.304
WHCO3	27.012
WHCO5	162.31
WHCO6	247.47
SNO	84.962

5.16 Determination of protein concentration of whole-cell lysates of EGF-treated HOSCC cell lines

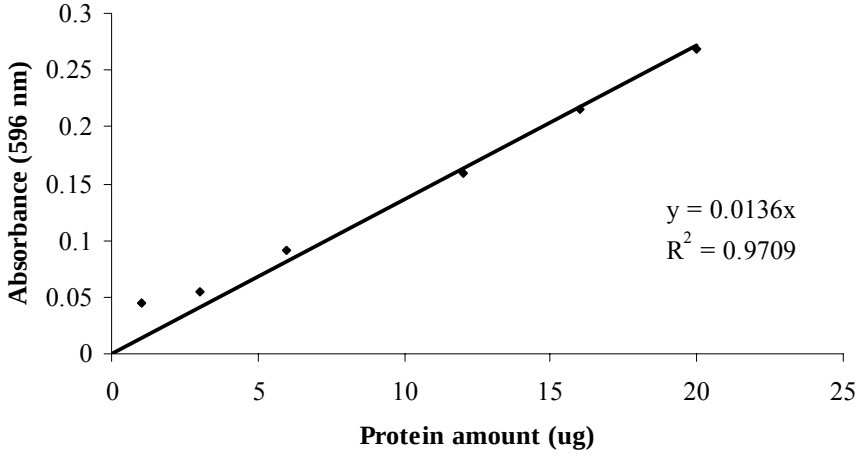


Figure 5.3: Standard curve of absorbance (at 596nm) versus protein concentration of BSA standards in single lysis buffer.

5.17 Raw densitometric data obtained from FAK-specific western blots of EGF-treated whole-cell lysates

Table 5.2: Optical density values obtained using LabWorks™ analysis of the 125 kDa band generated by FAK-specific western blot analysis of whole-cell lysates following EGF treatment of HOSCC cell lines.

CELL LINE	EGF EXPOSURE DURATION (hours)			
	0	**0.5**	**3**	**12**
WHCO1	437.77	339.48	400.35	339.15
WHCO3	129.22	106.64	178.27	195.08
WHCO5	184.39	197.34	261.97	104.11
WHCO6	619.85	591.8	695.14	487.5
SNO	123.19	297.71	355.85	267.5

5.18 Determination of protein concentration of Triton X-100 membrane extracts of HOSCC cell lines

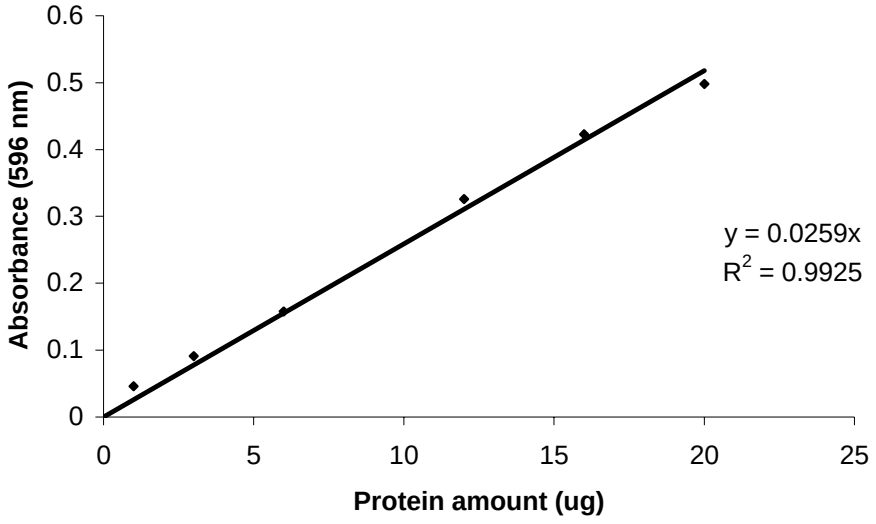


Figure 5.4: Standard curve of absorbance (at 596 nm) versus protein concentration of BSA standards in Triton X-100 membrane extraction buffer.

5.19 Raw densitometric data of western blot showing the 85 kDa and 125 kDa FAK variants in Triton X-100 membrane extracts of HOSCC cells

Table 5.3: Optical density values obtained using LabWorks™ analysis of FAK-specific western blotting of Triton X-100 membrane extracts of the HOSCC cell lines.

CELL LINE	FAK ISOFORMS	
	125 kDa	**85 kDa**
WHCO1	122.01	181.42
WHCO3	89.65	184.94
WHCO5	238.08	285.84
WHCO6	296.46	504.02
SNO	146.21	293.39

5.20 Determination of protein concentration of Triton X-100 membrane extraction of SNO with and without a protease inhibitor cocktail (Roche)

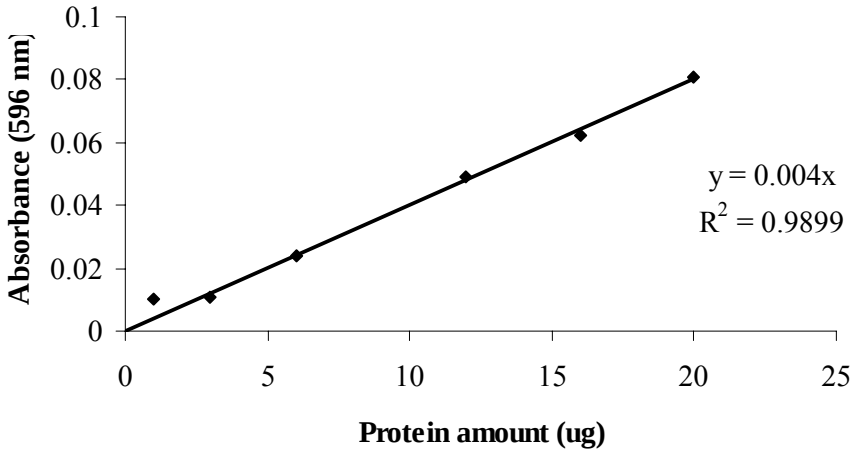


Figure 5.5: Standard curve of absorbance (at 596 nm) versus protein concentration of BSA standards in Triton X-100 membrane extraction buffer.

5.21 Determination of protein concentration of Triton X-100 membrane extracts of EGF-treated HOSCC cell lines

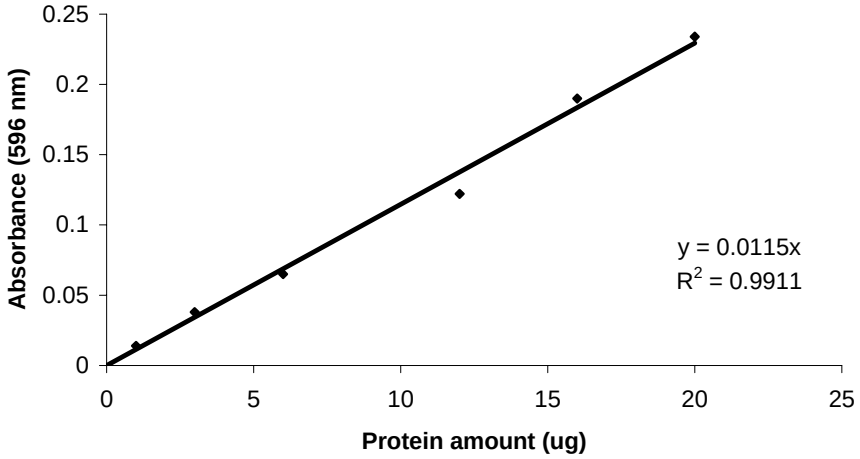


Figure 5.6: Standard curve of absorbance (at 596 nm) versus protein concentration of BSA standards in Triton X-100 membrane extraction buffer.

5.22 Raw densitometric data obtained from western blots of Triton X-100 membrane extracts of EGF-treated HOSCC cells

Table 5.4: Optical density values obtained using LabWorks analysis of 125 kDa bands generated by FAK-specific western blot analysis of membrane extracts following EGF treatment of HOSCC cell lines.

CELL LINE	EGF EXPOSURE DURATION (hours)			
	0	**0.5**	**3**	**12**
WHCO1	30.25	133.50	151.58	37.87
WHCO3	166.38	156.18	462.59	130.94
WHCO5	202.60	220.89	227.83	181.33
WHCO6	253.13	139.73	167.54	49.48
SNO	1037.70	817.23	769.50	744.74

Table 5.5: Optical density values obtained using LabWorks™ analysis of 85 kDa bands generated by FAK-specific western blot analysis of membrane extracts following EGF treatment of HOSCC cell lines.

CELL LINE	EGF EXPOSURE DURATION (hours)			
	0	**0.5**	**3**	**12**
WHCO1	92.65	610.14	813.48	298.56
WHCO3	325.45	273.72	268.31	152.45
WHCO5	197.86	244.64	226.26	198.28
WHCO6	360.94	304.32	272.37	161.54
SNO	1003.50	645.98	440.16	552.92

5.23 Raw densitometric data obtained from western blots of EGF-treated membrane extracts immunoprecipitated with anti-phosphotyrosine antibody and blotted with FAK

Table 5.6: Optical density values obtained using LabWorks™ analysis of the 85 kDa bands generated by FAK-specific western blot analysis after EGF treatment of HOSCC cell lines and immunoprecipitation with an anti-phosphotyrosine antibody.

CELL LINE	EGF EXPOSURE DURATION (hours)			
	0	**0.5**	**3**	**12**
WHCO1	252.71	273.96	330.76	287.29
WHCO3	109.58	55.87	149.22	124.05
WHCO5	377.75	470.83	175.46	153.08
WHCO6	610.27	110.87	328.80	130.25
SNO	29.69	825.03	629.30	31.61