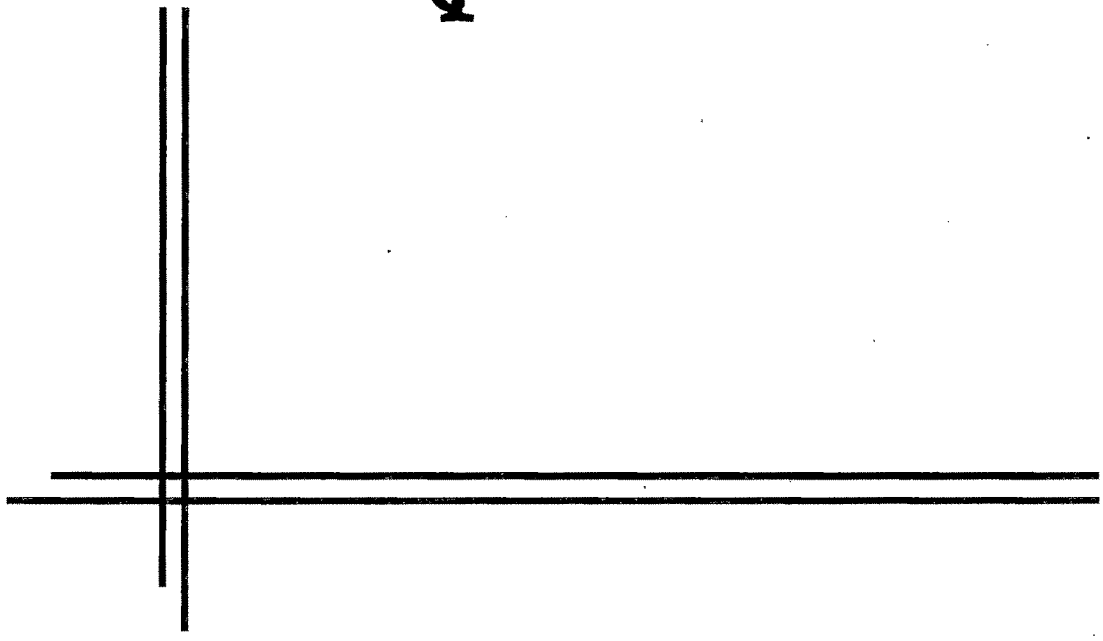
Chapter - 7

Determination of Neuropilin-1 Receptor Expression



7.1. Introduction

Neuropilins (NRP 1 and NRP 2) are membranous receptors capable of binding two disparate ligands, class 3 semaphorins (SEMA 3A) and vascular endothelial growth factors (VEGF-A165), and regulating two diverse systems, neuronal guidance and angiogenesis. NRP1 and NRP2 are expressed by a wide variety of human tumour cell lines and diverse human neoplasms (Bielenberg et al., 2006; Ellis, 2006), and are implicated in mediating effects of VEGF and Semaphorins on the proliferation, survival and migration of cancer cells (Bachelder et al., 2001; Chabbert-de et al., 2006). Table 7.1 summarises the expression of NRPs and other VEGF receptors in a panel of representative carcinoma cells. Over-expression of NRP1 in Dunning rat prostate AT2.1 carcinoma cells increased tumour growth *in vivo* (Miao et al., 2000), while NRP1 knockdown using siRNA inhibited breast carcinoma cell migration (Bachelder et al., 2003), and a peptide targeted to the VEGF binding site of NRP1 induced breast tumour cell apoptosis (Barr et al., 2005). NRP1 is expressed in patient specimens from lung, breast, prostate, pancreatic and colon carcinomas, but not in corresponding normal epithelial tissues (Fukahi et al., 2004). NRP1 has also been found in several other tumours including melanoma (Straume and Akslen, 2003), astrocytoma (Broholm and Laursen, 2004) and neuroblastoma (Fakhari et al., 2002). NRP2 expression was reported in lung cancer (Lantuejoul et al., 2003), neuroblastoma (Fakhari et al., 2002), pancreatic cancer (Cohen et al., 2002), osteosarcoma (Handa et al., 2000) and bladder cancer (Sanchez-Carbayo et al., 2003). It has been suggested that NRP1 is more prevalently expressed in carcinomas (mainly of epithelial origin), whereas NRP2 may be more frequently expressed in non-carcinoma neoplasms such as melanomas, leukaemias and neuroblastomas (Ellis, 2006). However, as Table 7.1 indicates there is no sharp distinction between the types of neoplasms expressing NRPs 1 and 2 (Marcus et al., 2005), and often they are co-expressed. Furthermore, different cell lines derived from the same tumour types, such as glioma (Rieger et al., 2003), may exhibit divergent patterns of NRP1 and NRP2 expression.

In our present work, we determined the expression of neuropilin-1 in human adenocarcinoma cell line, A549, and mouse melanoma cell line B16F10. This finding will help us in developing neuropilin-1 receptor targeted drug delivery systems (liposomes conjugated with neuropilin-1 antibody; immunoliposomes) for the treatment of cancer or various solid tumors which over express these neuropilin-1 receptors.

Table 7.1. Neuropilins and VEGFRs expression in tumour cells

	NRP1	NRP2	VEGFR2	VEGFR1	VEGFR3
A549 (lung)	++	+	-	-	-
MDA-MB-231 (breast)	+	?	-	++	-
MCF-7 (breast)	+	?	-	++	-
ACHN (kidney)	+	++	-	-	-
SKOV-3 (ovary)	+	+	-	-	-
DU145 (prostate)	++	-	-	-	-
RT112/84 (bladder)	+	?	-	-	-
BxPC-3 (pancreas)	+	?	-	-	-
SK-MEL-5 (melanoma)	+/-	++	-	-	-
MDA-MB-435 (melanoma)	+	++	-	-	-
SK-N-AS (neuroblastoma)	+	-	-	-	ND
SK-N-DZ (neuroblastoma)	+	-	-	-	ND
U87 MG (glioma)	++	+	ND	ND	ND
Astrocytoma(GBM)	+	?	-	-	-
Leukaemia (AML, various)	+	+	-	+/-	+/-
Leukaemia (CML, various)	+	+	+/-	+/-	-

AML, acute myeloid leukaemia cell lines; CML, chronic myeloid leukaemia cell lines; ND, not determined; ?, very low or uncertain expression; +/-, low expression or no expression in other; +, moderate expression; ++, strong expression.

7.2. Materials and Methods

Rabbit anti-neuropilin-1 polyclonal primary antibody was purchased from Santa Cruz Biotechnology, US. Goat anti-rabbit HRP conjugated and FITC conjugated secondary antibodies were purchased from Sigma Aldrich, Mumbai, India. Alexa-568 conjugated goat-anti rabbit secondary antibody was purchased from Invitrogen, USA. Bradford's reagent, Ponceau S solution, Acrylamide and Bis-acrylamide, and DAPI were purchased from Sigma Aldrich, Mumbai. PVDF membrane was purchased from MDI Membrane Technologies LLC, Ambala Cantt, Haryana, India. RIPA Lysis Buffer was prepared as per Abcam protocol handbook. Femto and X-ray films were purchased from Pierce Thermo scientific, US and Kodak, respectively.

Cell Culture

B16F10, a mouse melanoma cell line and A549, lung cancer cell line were purchased from NCCS, Pune, India. The B16F10 and A549 cell lines were maintained in Iscove's Modified Dulbecco's Media (IMDM) and RPMI-1640 (GIBCO) media, respectively. The media was supplemented with 10% heat inactivated foetal bovine serum, FBS (GIBCO) and antibiotics (100U/mL penicillin and 100µg/mL streptomycin). Cultures were maintained at 37 ^{0}C in 5% CO_2 humidified atmosphere.

7.3. Determination of neuropilin-1 expression

7.3.1. Western blotting analysis

Principle:

Heating of cell lysates with sample buffer containing SDS, an anionic surfactant, and dithiothreitol (DTT) (or Tris(2-carboxyethyl)phosphine, TCEP) as reducing agent, reduces protein disulphide bonds to thiol groups and disrupt non-covalent bonds in the proteins, denaturing them, and causing the molecules to lose their native conformation. The proteins migrate through a gel which pore size is determined by the concentration of acrylamide in the gel, under the influence of an electric field. Larger proteins migrate slower than smaller proteins, resulting in a separation of proteins largely based on molecular size. The proteins are then transferred onto a protein-binding membrane (typically nitrocellulose or Polyvinylidene Fluoride, PVDF) under the influence of an electric field. For antibody detection, protein binding sites on the membrane are blocked by incubating with a protein solution such as milk or BSA, and a protein of interest is then specifically bound with an antibody. This primary antibody is then detected with a secondary antibody conjugated to an enzyme, often a peroxidase. A liquid substrate is then added, which in the presence of the peroxidase enzyme emits light in a chemiluminescent reaction, and this light is detected using photographic film. Western blotting is often described as a semi-quantitative technique, with the amount of protein originally present in the lysate proportional to the amount of peroxidase enzyme bound to protein on the membrane, and so the amount of light generated and area and intensity of the band present on the film. The amount of protein in each sample was assessed before loading, to quantify the amount of protein present in the lysates and thus allowing equal loading. Blotting with an antibody to Beta-tubulin that is a housekeeping gene, constitutively being expressed in a cell is used as a loading control.

Preparation of cell lysates:

B16F10 and A549 cells were harvested and then lysed using modified RIPA (Radioimmunoprecipitation assay buffer) buffer (150mM NaCl, 1% Triton X 100, 50mM Tris base, 0.1%SDS, 0.5% sodium deoxycholate, 5mM EDTA, 10mM EGTA, 50mM sodium fluoride, 1mM sodium orthovandate, and protease inhibitor cocktail:1mM phenylmethyl sulfonyl fluoride; 5µg/mL leupeptin; 1µM pepstatin). The cell lysates were

centrifuged (10000rpm for 5minutes), supernatant was collected and stored at -80 ^{0}C until use.

Protein estimation:

Standard 0.5mg/ml Bovine serum albumin (BSA) solution in milliQ water was prepared. 1.25, 2.5, 3.75 and 5µg of BSA from standard solution (2.5µl, 5µl, 7.5µl and 10µl, respectively) were transferred to 96 well plate in triplicate. Volume was made up to 10µl with milliQ water. 10µl water was kept as blank in triplicate. 100µl of Bradford's reagent was added to all sample and blank well and absorbance was taken at 595nm using SoftMax Pro software. The estimation was done in triplicate. Protein concentration was automatically determined by the software, using the BSA standard.

SDS PAGE (Laemmli, 1970):

Gel Casting and Electrophoresis:

Reagents: 30% Acrylamide Mixture (29.2% Acrylamide + 0.8% bisacrylamide) solution was prepared in milliQ water and stored at 4 ^{0}C in amber coloured bottle. 1.5 M Tris HCl, pH 8.8 (resolving buffer) buffer was prepared in milliQ water and pH was adjusted with concentrated HCl. 1M Tris HCl, pH 6.8 (stacking buffer) buffer was prepared in milliQ water and pH was adjusted with concentrated HCl. 10% SDS solution: 10gm of SDS was weighed and added to milliQ water, heated to 70ºC to dissolve SDS completely, and volume was made up to 100mL with milliQ water. 10% APS (Ammonium persulfate) solution in milliQ water: Stored at -20 ^{0}C. N,N,N',N'-Tetramethylethylenediamine (TEMED). Tank Buffer: 196mM Glycine, 50mM Tris HCl and 0.1% SDS. Mini gel dual, assembly, Power Pack and Resolving gel Recipe 30ml volume (for 2 mini gels).

Table 7.2. Composition of resolving gel

Components	8%	10%	15%
H_2O	13.9mL	11.9mL	6.9mL
1.5M Tris-HCl, pH 8.8	7.5mL	7.5mL	7.5mL
10% (w/v) SDS	0.3mL	0.3mL	0.3mL
Acrylamide/Bis-acrylamide (30%/0.8%, w/v)	8mL	10mL	15mL
10% (w/v) ammonium persulfate (APS)	0.3mL	0.3mL	0.3mL
TEMED	0.18mL	0.12mL	0.012mL

APS and TEMED were added at the end.

Depending upon the molecular weight of the protein of interest the gel % was chosen.

Table 7.3. Composition of stacking gel

Stacking gel (5%)	5mL
H_2O	3.4mL
1M Tris-HCl, pH 6.8	0.63mL
10% (w/v) SDS	0.05mL
Acrylamide/Bis-acrylamide (30%/0.8%, w/v)	0.83mL
10% (w/v) ammonium persulfate (APS)	0.05mL
TEMED	0.005ml

APS and TEMED are added at the end.

Sample loading buffer composition:

The cell lysates equivalent to 50µg of total protein were mixed with 3X sample loading buffer (Table 7.4) in a ratio of 2:1 and then samples were boiled for 5min in a boiling water bath to denaturate proteins and then resolved on SDS-PAGE (8%).

Table 7.4. Sample loading buffer composition

Components	3X (10mL)	6X (10mL)
100% glycerol	3mL	6mL
1M Tris pH 6.8	1.5mL	3mL
SDS	6g	12g
2-Mercaptoethanol	300µl	600µl
Bromophenol blue	0.05%	0.05%

Protocol:

The glass plates were swiped with 70% alcohol and assembled. The bottom and the sides of the glass plates were sealed using 1 % molten agarose. When solidified the resolving gel mixture was poured gently leaving some space for the stacking gel. The gel mixture was overlaid with methanol to give a uniform gel front and kept for polymerization for 20-30 minutes. When polymerization was over the methanol and the unpolymerized gel mixture was removed and washed with milliQ water. The stacking gel mixture was poured over the top of polymerized resolving gel and a 1.5mm comb was inserted. Stacking gel was allowed to polymerize, following which the wells were washed with milliQ water and marked. The assembly cathodic and anodic chamber was filled with Tank buffer and the wells were loaded with the denatured sample. Molecular weight marker was also loaded in the well. Electrophoresis was carried out at 25mA for the stacking and then further run was carried out 30mA. Run was stopped when the dye front reached 1mm above the gel end.

Protein Transfer (Towbin et al., 1979):

Reagents: Wet transfer buffer: 25mM Tris, 192mM glycine, 20%v/v methanol, pH 8.3
Poncue S solution: 0.1% Poncue powder in 5% acetic acid.

Protocol:

PVDF membrane was cut according to the size of the gel. The resolved gel and the membrane both were equilibrated with the transfer buffer for 15 minutes. In case of PVDF the membrane was pre-wetted with methanol for 5 minutes before equilibration. Four whatman filter # 3 sheets were cut according to the size of membrane. After equilibration the membrane was kept over the gel and this is sandwiched between the folds of filter paper, two on either side. Care was taken to avoid any bubbles to be trapped. The sandwich was then kept between the electrode plates of the transfer assembly (gel towards the cathode). The transfer assembly was filled with the transfer buffer. Transfer was carried out for 16hrs at constant 10V or for 1 hr at 100V in a cold room. The membrane was stained with Poncue solution till the pink bands appeared. The side containing the bands were marked and then destained by rinsing in milliQ water.

Western Blotting (Timmons and Dunbar, 1990):

Reagents:

TBS (Tris Buffer Saline): 20mM Tris pH 7.5, 150mM NaCl. Autoclaved and kept at 4 ^{0}C.
TBST (Tris Buffer Saline Tween20): To 1 Litre of TBS 1ml of tween-20 is added, stirred well and used.
Blocking Solution: 5% or 7.5% non-fat skimmed milk powder was dissolved in TBST by keeping at 37 ^{0}C for 15 min.
Primary and Secondary antibody dilutions: The antibodies were diluted in 2.5% non-fat skimmed milk in TBST. Primary antibody dilution is 1:500; Secondary antibody dilution is 1:6000.
Femto, Developing cassette, plastic boxes and X-ray films

Protocol:

The membrane was blocked with 5% milk for 2hrs. The blots were then washed gently with TBST for 5 minutes. The blot was taken in a plastic bag, overlaid with rabbit neuropilin-1 polyclonal primary antibody (1:500) and kept in a rotatory shaker for 1hr. Following, the blot was washed with TBST 4 times, 10 minutes each. Blots were then incubated for 1 hr with HRP conjugated goat anti-rabbit secondary antibody (1:6000). The nonspecific binding was removed by 4 TBST washes, 10 minutes each.
The blots were then taken to dark room for visualization of the signal. Femto developing reagent was made as per the company instructions. The blot was kept on a clean glass plate and on the probed side Femto mixture was added and incubated for 5 minutes. After incubation excess reagent was drained on the filter paper and the blot was kept in

the developing cassette in between two transparent plastic sheets. On to the protein side X-ray sheet was kept and exposed for various time range depending upon the signal intensity. The exposed films were developed in the developing machine (Optimax 2010 X-ray film processor, model no. 1160-1-0000). Equal loading was checked by taking β tubulin as a control.

7.3.2. FACS analysis

Cells were grown up to 60-70% confluency in 35mm tissue culture plate, harvested, washed twice with PBS and then fixed using 1% PFA. Cells in eppendorf tube were incubated with anti-neuropilin-1 rabbit polyclonal antibody (1μg/million cells) for 1hr (redispersed at every 15minutes). Cells were then washed with FACS buffer for 3 times and then incubated with FITC labeled goat anti-rabbit secondary antibody (1:200) for 1hr (redispersed at every 15 minutes). Cells were washed 3 times and finally suspended in FACS buffer for acquisition using FACS Calibur.

7.3.3. Confocal microscopy

Cells were grown on cover slips up to 60-70% confluency. Cells were washed twice with PBS and then fixed using 1% PFA. Coverslips were washed with PBS twice and then were incubated with anti-neuropilin-1 rabbit polyclonal antibody (1:20 dilution in PBS) for 1hr. Cells were then washed with PBS for 3 times and then incubated with Alexa 568 labeled goat anti-rabbit secondary antibody (1:200) for 1hr. Coverslips were washed again with PBS for three times and counterstained with DAPI (4',6-diamidino-2-phenylindole). Cells were washed thrice with PBS for 5 minutes each, mounted using DABCO (1,4-diazabicyclo[2.2.2]octane) and visualized under confocal microscope, Zeiss LSM 510 Meta at 63 X magnification.

7.4. Results and Discussion

Western blot analysis of A549 and B16F10 cell lysates confirms the presence of neuropilin-1 receptor in both the cell lines. We have observed two distinct band at 70kDa and 130kDa with A549 cell line whereas, a single band of 70kDa with B16F10 cell line (Figure 7.1). The semi-quantitative expression of neuropilin-1 receptor was compared with respect to total β tubilin expression in the loaded 50μg of total protein. The tubulin expression varies from cell to cell and therefore we will observe different intensity for equal amount of loaded protein. The β tubilin band intensity was measured using densitometry (semi quantitative method) and compared with neuropilin-1 protein band intensity. The total intensity of both the bands of A549 was compared with intensity of B16F10 band. Our result reveals that the B16F10 cell line (49.34±4.23%) shows more expression of neuropilin-1 protein with respect to β-tubulin content as compared to A549 cell line (35.3±2.38%) (Figure 7.2).

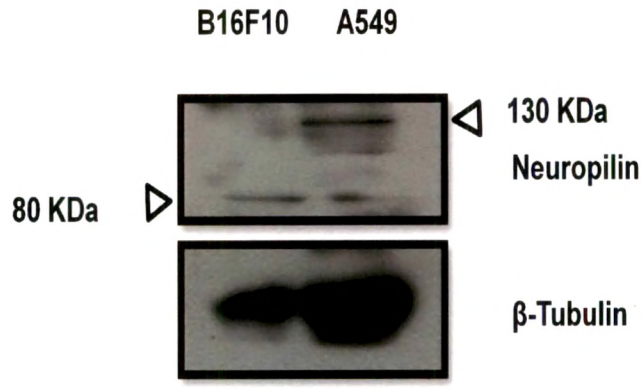


Figure 7.1. Identification of neuropilin-1 receptor of A549 and B16F10 cells by western blotting

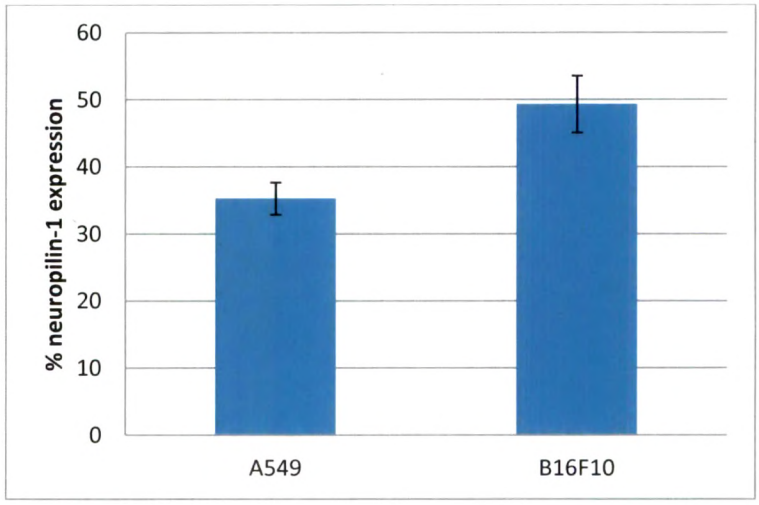


Figure 7.2. The **%** neuropilin-1 protein expression with respect to total β-tubulin content of loaded protein. The B16F10 cells showed significantly high neuropilin-1 expression ($p<0.01$, $p=0.007$) as compared to A549 cells.

The neuropilin-1 expression of A549 and B16F10 cell line was also studied by using FACS technique. The cells were incubated with rabbit polyclonal anti-neuropilin-1 antibody followed by incubation with FITC conjugated anti-rabbit goat secondary antibody. The normal and overlay graphs of secondary control and cell treated with both primary and secondary antibodies are shown in (Figure 7.3). The % relative mean fluorescence intensity (RMFI) of A549 and B16F10 cells are shown in Figure 7.4. The A549 cells showed % RMFI of 52.18±8.23% as compared to B16F10 (MFI was considered 100%) indicating less expression of neuropilin-1 as compared to B16F10.

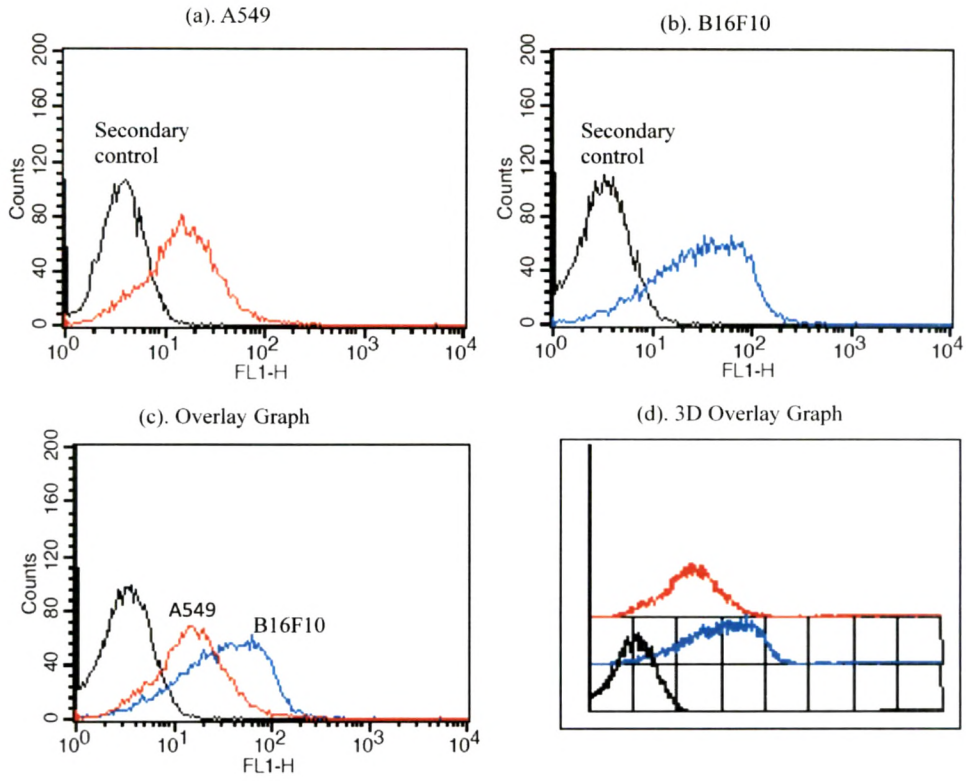


Figure 7.3. FACS analysis of neuropili-1 protein (a). A549 cells (b). B16F10 cells (c). Overlay graph and (d). 3D overlay graph.

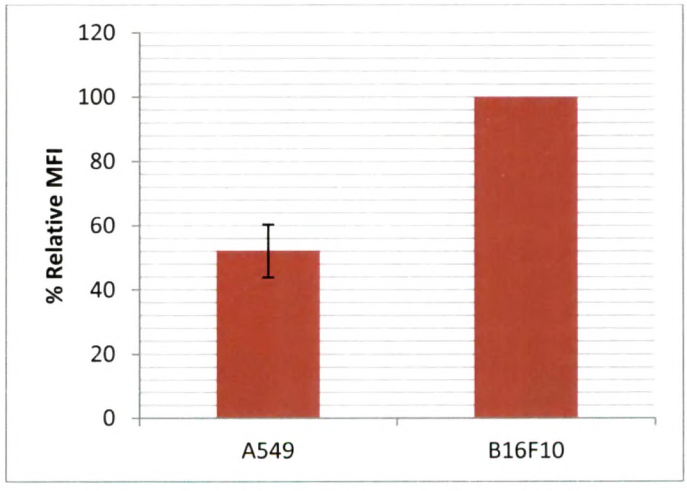


Figure 7.4. The comparison of % relative mean fluorescence intensities of A549 and B16F10 cells. The B16F10 cells showed significantly high neuropilin-1 expression ($p<0.01$, $p=0.001$) as compared to A549 cells.

Neuropilin-1 protein expression in A549 and B16F10 cells was observed using confocal microscope. Cells grown on coverslips were incubated with anti-neuropilin-1 rabbit polyclonal antibody (1:20 dilution in PBS) for 1hr followed by incubation with Alexa-568 labeled goat anti-rabbit secondary antibody (1:200) for 1hr. Cells counterstained with DAPI (4',6-diamidino-2-phenylindole) and analysed using confocal microscope at 63X magnification. The confocal images of A549 and B16F10 cells expressing neuropilin-1 protein are shown in Figure 7.5. Confocal microscopic study clearly confirms the presence of neuropilin-1 receptors in both cell lines tested. The figures clearly depicts neuropilin-1 is a surface, transmembrane and cytoplasmic receptor present in A549 and B16F10 melanoma cells.

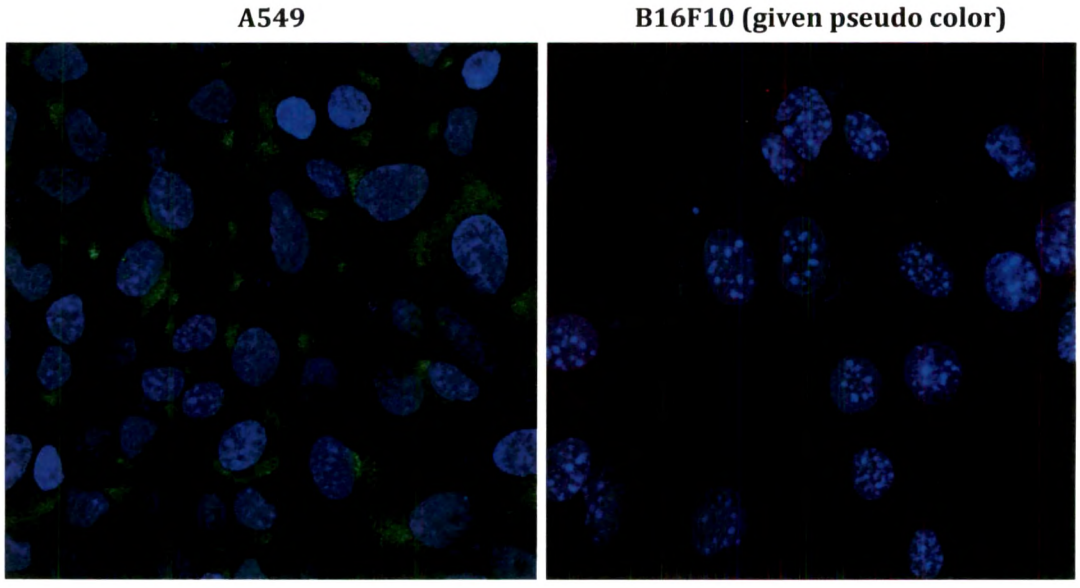


Figure 7.5. Confocal images of neuropilin-1 protein expression in A549 cells and B16F10 melanoma cells

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