Annexure-4

Format for Final Report

1. Title of the Project:
"Studies on exosomal proteome and lipidome and their clinical implication in the management of drug resistant and recurrent head and neck squamous cell carcinomas (HNSCC)"

2. Unique ID of the Project (provided by ICMR):
R.F.C No. RCH/NER/4/2016-2017 dated 03/05/2016

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4. Implementing Institution and other collaborating Institutions: **TEZPUR UNIVERSITY**

5. Date of commencement: **17/05/2016**

6. Duration: **Three Years**

7. Date of completion **16/05/2022**

8. Objectives as approved

i) To develop drug resistant sublines of HNSCC cell lines
ii) Isolation and characterization of exosomes derived from drug sensitive and resistant sublines of HNSCC
iii) Proteomics analysis of exosomes derived from drug sensititve and resistant HNSCC

iv) Lipidomics analysis of exosomes derived from drug sensititve and resistant HNSCC

v) Study of role of exosomes derived from drug resistant HNSCC cells in proliferation, migration, invasion and drug resistance of cancer cells.

vi) To study the correlation of proteome and lipidome in drug resistance and their implications in clinical outcomes

9. Deviation made from original objectives if any, while implementing the project and reasons thereof.

As cited in literature hypoxia determines disease aggressiveness and drug response and responsible for poor outcome of several malignancies. Here we have also explored the role of hypoxic exosomes in determining migratory potential and drug response in HNSCC**.**

10. Field/ Experimental work giving full details of summary of methods adopted.

a. Materials Cisplatin (Sigma) was dissolved in 0.9 % NaCl to prepare a 1.66 mM stock . The final test solution contained 0.054 % NaCl. 3-[4,5-Dimethylthiazol-2-yl] 1-2,5-diphenyltetrazolium bromide (MTT) was purchased from HIM**E**DIA

The human head and neck squamous cell carcinoma (HNSCC) cell line Cal 33 was gifted from collaborator's lab, School of Life Sciences, JNU, New Delhi. Cells were cultured and maintained in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotic (penicillin-streptomycin) at 37°C temperature in a humidified atmosphere containing 5% CO_2.

b. Determination of Inhibitory Concentrations:

The inhibitory concentration (IC_{50}) is the optimal concentration at which the survival rate of cells reached the minimum following exposure to an appropriate anticancer drug. Determination of this concentration (IC_{50}) is primordial to the selection of cells for drug resistance. Cells at the logarithmic growth phase were harvested and seeded at a density of 10^5 cells per well in 96-well plates and incubated for proper adherence. Subsequently, the drug-free medium was replaced with fully supplemented fresh medium, containing different concentrations of drugs. The cells were incubated for 72 hours, while maintaining the physiological conditions at 5% CO_2 and 37°C temperature, in a humidified atmosphere. At the end of the treatment period (72 h), 10µl of MTT solution was added to each well. After incubation for 4 h at 37°C, 100µl of solubilizing buffer (DMSO) was added to each well. The viable cells produced a dark blue/purple formazan product, whereas no such staining was formed in the dead cells. The optical densities (OD) of cells treated at different drug concentrations were measured at 570 nm/690 nm using a micro-plate reader and cell viability was calculated as a measure of the optical density (OD) of treated cells relative to the optical density of the untreated controls. Dose-dependent response curve was plotted and the inhibitory concentrations (IC_{50}) of both the drugs (cisplatin) were established.

c. Development of drug resistant cell lines

Cal33 cells were selected for progressive resistance to drug cisplatin. Selection began at a drug dose (dose 1) that was 1000 fold less than concentration at which 50% of parental cells were killed (IC_{50}). The dose was then increased 1.5 fold until maximally tolerated dose is achieved. At each dose cells were maintained for at least 3 generations and an aliquot of cells were stored before each increase in drug dose.

d. Isolation and Characterization of exosomes

Cal33 and FaDu cells were cultured under hypoxia (3-5% oxygen) and the exosomes were isolated after 72h from the culture media by ExoQuick™ method. The isolated exosomes were characterized by DLS for particle size distribution. The exosomes isolated were collected in RNA later for microRNA analysis and lysis buffer for preparation of protein lysate of exosomes for proteome analysis. Further we have co-cultured the exosomes with naïve Cal33 cells and studied their role in cell migration by wound healing method and drug resistance by cell counting.

e. Exosomal Proteome Analysis

The proteins were extracted from the purified exosomes and treated with 100mM DTT at 95ºC for 1hr followed by 250mM IDA at room temperature in dark for 45min. The samples wer then digested with Trypsin and incubated overnight at 37˚C. The peptides were extracted in 0.1% formic acid and incubated at 37˚C for 45 minutes. The solution was then centrifuged at 10000g and the supernatant was collected into a separate tube and vacuum dried and dissolved in 20µl of 0.1% formic acid in water. 10µL injection volume was used on C18 UPLC column for separation of peptides. Liquid chromatography was performed on a ACQUITY UPLC system (Waters, UK). The separation of all samples was performed on ACQUITY UPLC BEH C18 column(Waters,UK)(150mm X 2.1mm X 1.7µm).A gradient elution program was run for the chromatographic separation with mobile phase A (0.1% Formic Acid in WATER), and mobile phase B(0.1% formic Acid in ACETONITRILE) as follows

S.No	Time	Flow	%A	%B	Curve
1	Initial	0.300	98.0	2.0	Initial
2	1.00	0.300	98.0	2.0	6
3	30.00	0.300	50.0	50.0	6
4	32.00	0.300	50.0	50.0	6
5	40.00	0.300	20.0	80.0	6
6	45.00	0.300	20.0	80.0	6
7	50.00	0.300	98.0	2.0	6
8	55.00	0.300	98.0	2.0	6
9	60.00	0.300	98.0	2.0	6

The peptides separated on the column were directed to Waters Synapt G2 Q-TOF instrument for MS and MSMS analysis. A SYNAPT G2 QTOF (Waters, UK) equipped with an electrospray ionization (ESI) source was used for mass spectrometric detection. The raw data was processed by MassLynx 4.1 WATERS. The individual peptides MSMS spectra were matched to the database sequence for protein identification on PLGS software (Protein Lynx Global Server), WATERS. The pathway were identified using UniProt ID with p and q values and the Gene Ontology was done by GOSlim.

The operation parameters were as follows

Experimental Instrument Parameters

- Polarity ES+
- Analyser Resolution Mode
- Capillary (kV) 3.5000
- Source Temperature (°C) 150
- Sampling Cone 45
- Extraction Cone 4.5
- Source Gas Flow (mL/min) 30
- Desolvation Temperature (°C) 350
- Cone Gas Flow (L/Hr) 30
- Desolvation Gas Flow (L/Hr) 80

Acquisition:

Acquisition Time

Start time : 0 min
End Time : 60 min
Source : ES

Acquisition Mode

Polarity : Positive
Analyzer Mode : Resolution

TOF MS:

Da Range

Start : 50Da
End : 1500Da

Scanning Conditions

Scan Time : 0.5 Sec
Data Format : Continuum

Collision Energy:

Function-1 Low Energy

Trap Collision Energy : On – 6V
Transfer Collision Energy : On – 6V

Function-2 High Energy

Ramp Trap Collision Energy : On – 20V to 45V
Ramp Transfer Collision Energy : Off

Cone Voltage:

Cone Voltage: 40V

11. Supported by necessary tables, charts, diagrams and photographs.

1. **Development of drug resistant cell lines**

Cal33 cells were selected for progressive resistance to drug, cisplatin. Selection began at a drug dose (dose 1) that was 1000 fold less than concentration at which 50% of parental cells were killed (IC_{50}). The dose was then increased 1.5 fold until maximally tolerated dose is achieved. At each dose cells were maintained for at least 3 generations and an aliquot of cells were stored before each increase in drug dose. Cal33 cells were exposed to cisplatin through 21 passages over 395 days at concentrations ranging from 0.00779 to 27 µM.

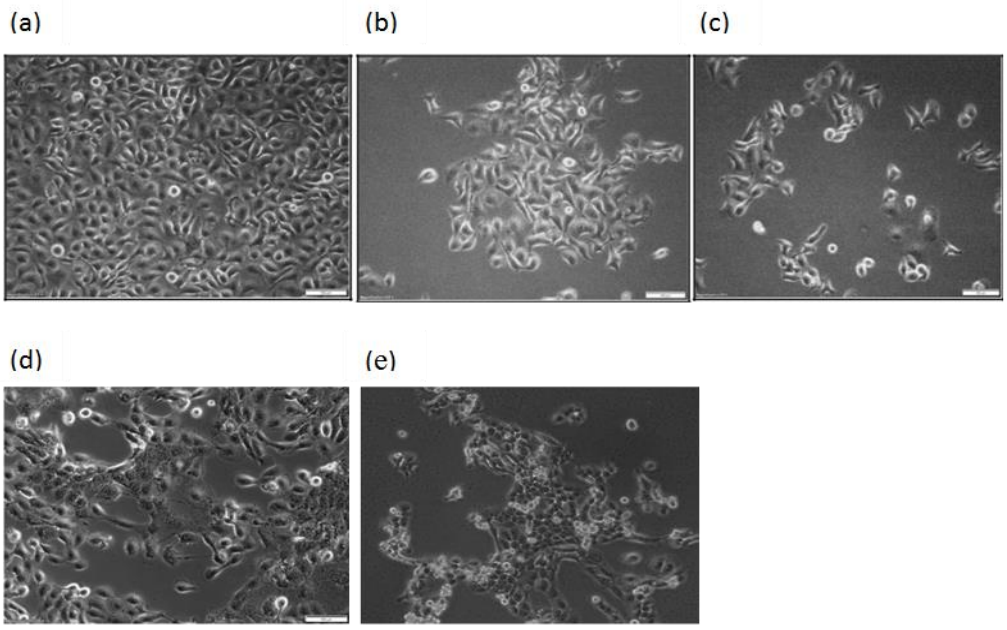


Figure 1. (a) Parental CAL 33 cells; (b) CAL 33 cells at 0.779 µM dose; (c) CAL 33 cells at 2.629 µM dose; (d) CAL 33 cells at 21.65 µM dose; (e) CAL 33 cells at 27 µM dose

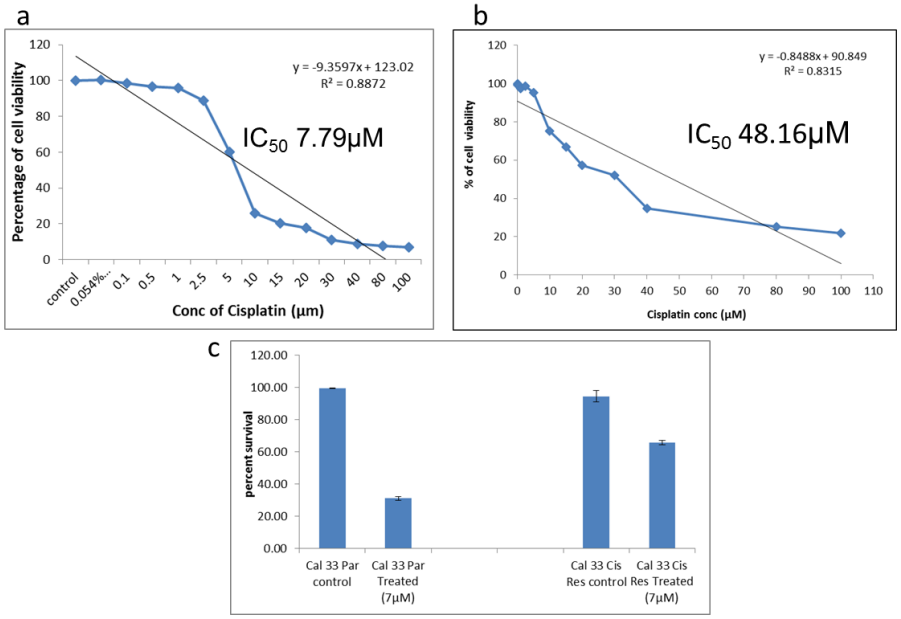
2. Characterization of Cal33 drug resistant subline

a. Cytotoxicity assay

The effect of the cisplatin on the proliferation of sensitive and resistant cell lines were tested in 96-well microtiter plates, using tetrazolium based semi-automated colorimetric (MTT) assay. Cells were plated and exposed to cisplatin. Cell viability was calculated in percent compared to untreated control cells. A minimum of three independent experiments was performed for each of the parental cell lines, sub-lines and final resistant cell lines. The inhibition of cell proliferation and IC_{50} values were determined for each cell line. The resistance index (RI) was determined as the ratio of the IC_{50} of the drug resistant cell line/IC_{50} of sensitive cell line. The resistance indices were evaluated according to the following expression:

$$R = IC_{50} \text{ resistant cell line} / IC_{50} \text{ sensitive cell line}$$

Cal33 CisR subline exhibited RI 6.182 as compared to Cal33 parent cells (Figure 2) suggesting that Cal33 CisR subline is resistant to cisplatin and ready for further study.



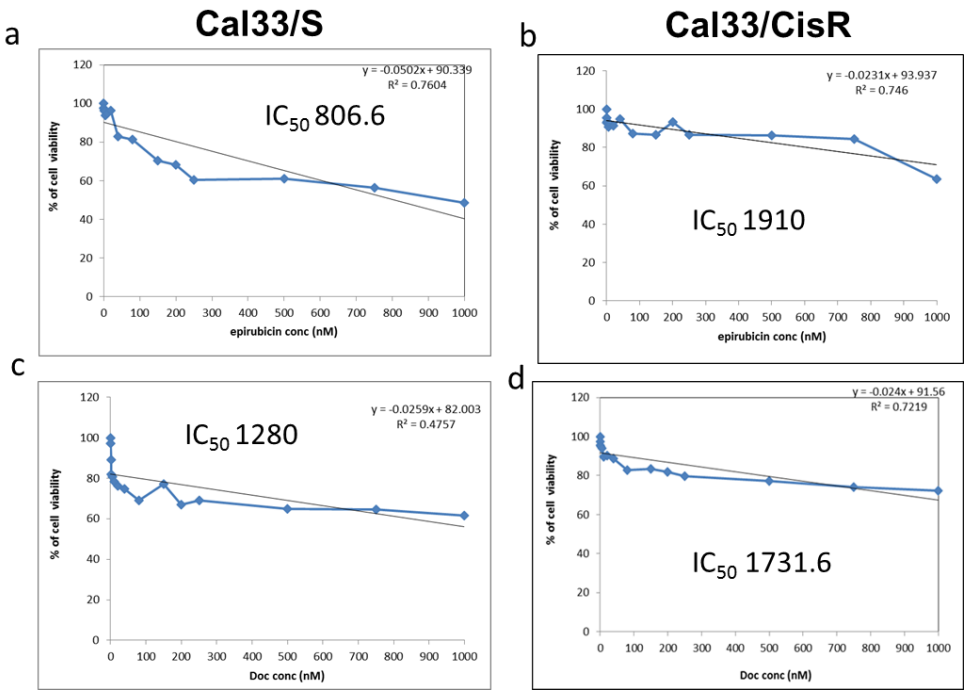
d

Drug	Cells	IC_{50}	Resistance index
Cisplatin	Cal33/S	7.79 µM	1.00
Cisplatin	Cal33/CisR	48.16 µM	6.182

Figure 2 Effects of cisplatin on viability of (a) Cal33 Parent cells; (b) Cal33 CisR; (c) Cal33 CisR subline treated with IC_{50} 7.79 µM; (d) Resistance index of Cal33 CisR subline.

b. Assessment for cross resistance of Cal33 CisR subline

Cross-resistance measurements were performed on the parental and the resistant subline to examine their sensitivity to a wide variety of chemotherapeutic agents. Parental and resistant sublines were seeded into 96-well plates and incubated for proper adherence. Subsequently, the drug-free medium was replaced with fully supplemented fresh medium, containing different concentrations of drugs, docetaxel and epirubicin and the plates were incubated for 72 hr at 37˚C. The suppression of cell proliferation was examined using the MTT assay. The Cal33 CisR subline showed increase in RI=2.367 for epirubicin and 1.352 for docetaxel as compared with parent cells indicating much higher resistance to anthracycline drug epirubicin (Figure 3).



e

Drug	Cells	IC_{50}	Resistance index
Epirubicin	**Cal33/S**	**806.6 nM**	**1.00**
Epirubicin	**Cal33/CisR**	**1910 nM**	**2.367**
Docetaxel	**Cal33/S**	**1280 nM**	**1.00**
Docetaxel	**Cal33/CisR**	**1731.6 nM**	**1.352**

Figure 3 (a) Cal33/S Parent cells and (b) Cal33 CisR treated with Epirubicin; (c) Cal33/S Parent cells and (d) Cal33 CisR treated with Docetaxel; (e) Resistance index of Cal33 CisR subline for epirubicin and docetaxel.

We have developed the cisplatin resistant Cal 33 CisR sublines that exhibited resistance index of 6.18 and cross resistance to epirubicin (RI=2.36) and docetaxel (RI=1.35). As per our defined objectives we were supposed to carry out further work on Cal33 CisR subline subline but unfortunately we lost the cells due to severe contamination in the laboratory.

3. Hypoxia and Drug resistance

Reports have shown that hypoxia in the tumor micro-environment determines disease aggressiveness and drug resistance and is considered as a prognostic indicator of poor outcome in several malignancies. However, the precise mechanisms through which hypoxic condition promotes disease aggressiveness and drug resistance are not well understood.

To study the effect of hypoxia on cell proliferation and morphology human head and neck cancer cell lines –Cal33 and FaDu were exposed to hypoxia (2-5% oxygen) for 72h and cell viability were studied using trypan blue exclusion method (Figure 6). The data showed ~3.10 time more cells in normoxic than hypoxic conditions for both Cal33 and FaDu cells. Lower proliferation rate in hypoxic condition has been accredited to hypoxia-associated deficiencies in other nutrients such as glucose which causes cells to stop or slow their rate of progression.

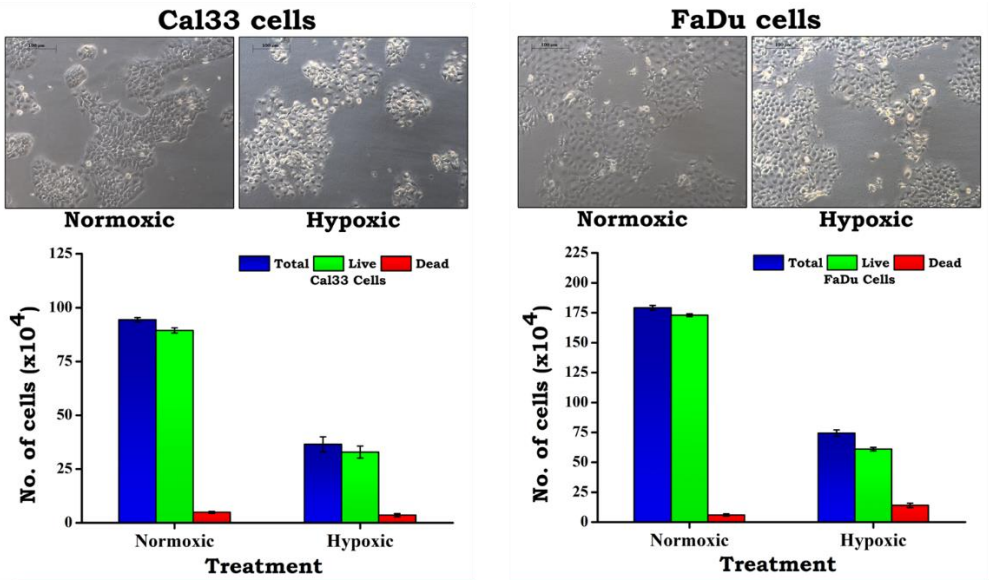


Figure 4 : Cell Viability studies under normoxic and hypoxic conditions on (a) Cal33 cells; (b) FaDu cells.

The morphological changes normoxic and hypoxic Cal33 and FaDu cells were evaluated by microscopy (scale bar- 20□□m). Hypoxia induced changes in structure and morphology, with smaller size, more elongation of the hypoxic structures (Figure 5 and 6) a well-known trait for hypoxic conditioned cells.

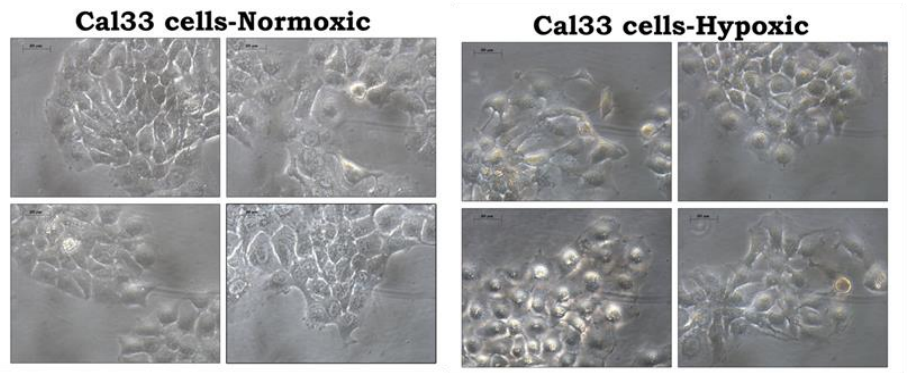


Figure 5 : Microscopic images showing morphological change in Cal33 under normoxic and hypoxic conditions (Scale – 20mm)

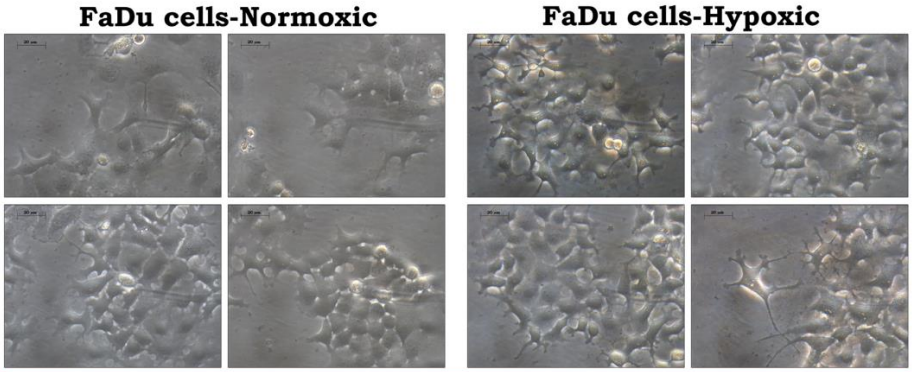


Figure 6 : Microscopic images showing morphological change in FaDu under normoxic and hypoxic conditions (Scale – 20mm)

4. **Hypoxia induced ROS, Autophagy and Lipid accumulation** Hypoxia is reported to increase the intracellular ROS in a variety of cells. The Cal33 and FaDu were exposed to hypoxia (2-5% oxygen) and ROS levels were measured using DCFDA stain by fluorescent microscope. The data showed significant increase in the ROS levels in both head and neck cancer cell lines (Figure 7).

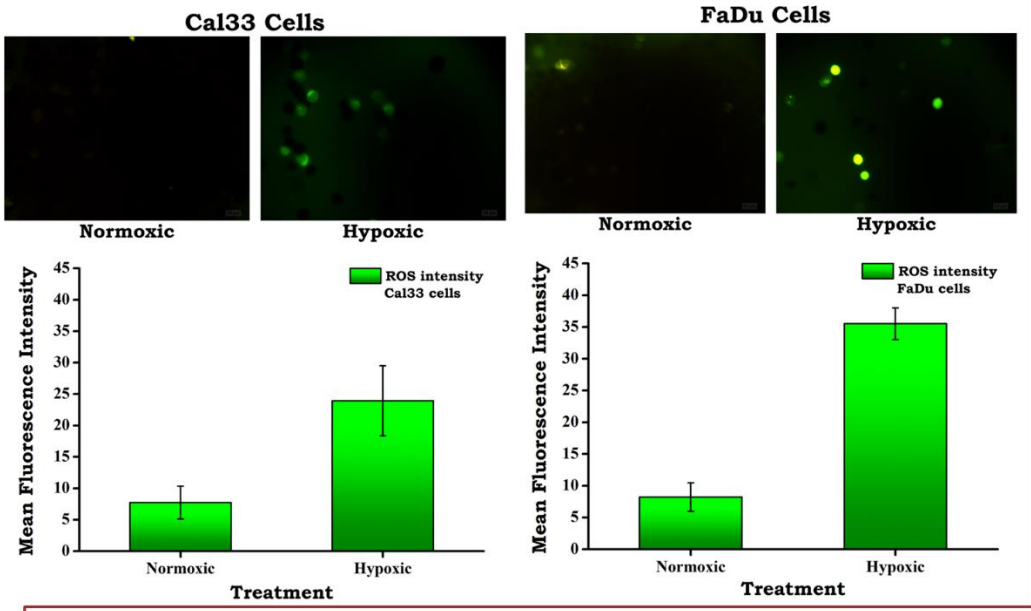


Figure 7: ROS evaluation using Fluorescent microscopy -DCFDA staining of (a) Cal33 cells, (b) FaDu cells under normoxic and hypoxic conditions (Scale – 20mm). Images were processed by ImageJ and the MFI was evaluated indicating ROS intensity.

Kasper et al. hypothesized that autophagy, a lysosomal degradation pathway, may be involved in reducing ROS during periodic hypoxia through removal of ROS producing species. To study autophagy Cal33 and FaDu cells were exposed to hypoxic condition and were stained with Acridine Orange (AO), imaged using fluorescent microscope (100X and 400X). The microscopic images were processed using ImageJ software and the number of acid vesicles (AVOs) and the degree of acidity (DOA) was analyzed. The data showed increase in the AVOs and DOA under hypoxia in both Cal33 and FaDu cells (Figure 8 and 9). He et al. (2012) demonstrated hypoxia induced autophagy or increased autophagy activity is associated with increased radio-resistance of tumor cells.

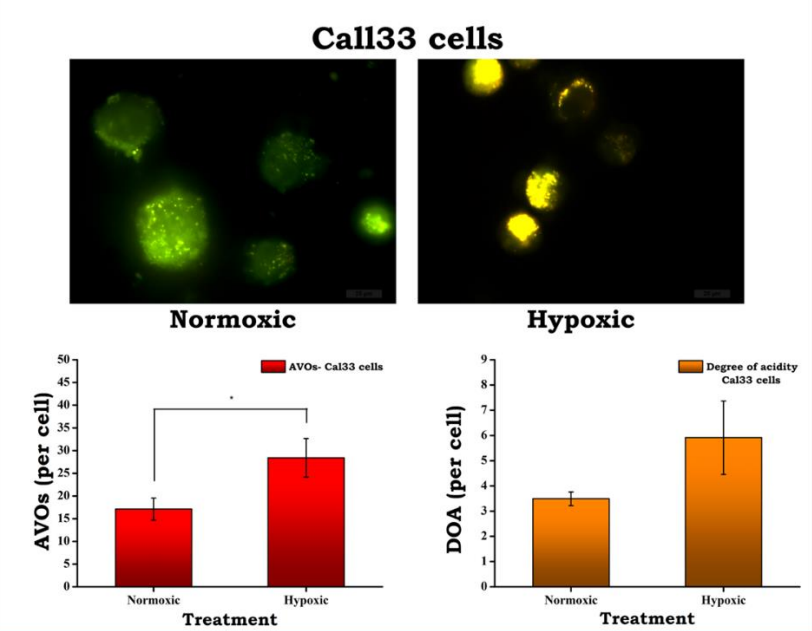


Figure 8: Autophagical study using fluorescent microscopy–AO staining of (a) Cal33 cells under normoxic and hypoxic conditions (Scale – 20mm). Images were processed by ImageJ and the AVOs, DOA was evaluated. (*=0.01<p<0.05)

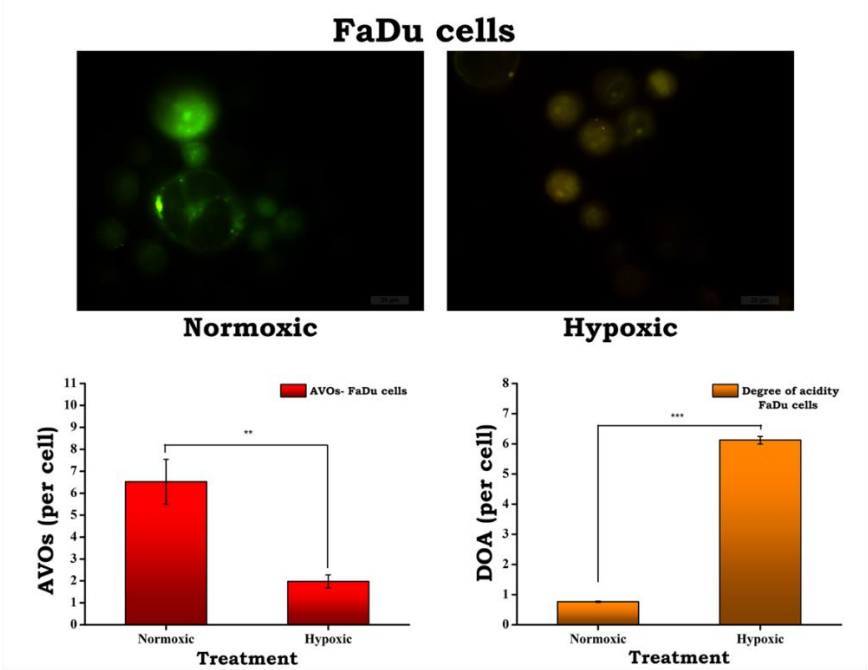


Figure 9: Autophagical study using fluorescent microscopy–AO staining of (a) Cal33 cells under normoxic and hypoxic conditions (Scale – 20mm). Images were processed by ImageJ and the AVOs, DOA was evaluated. (**=0.001<p<0.01, ***=p<0.001)

The hypoxia-dependent regulation of lipid metabolism is not well found in literature. In our study we evaluated the presence of lipid droplet (LD) accumulation within Cal33 cells (under normoxic and hypoxic conditions) using Oil Red O staining. The LDs could be seen interspersed between the clear vacuoles as depicted in the microscopic images. We observed increase in lipid accumulation expressed as absorbance (OD) per one lakh cells 1 lakh cells (Figure 10).

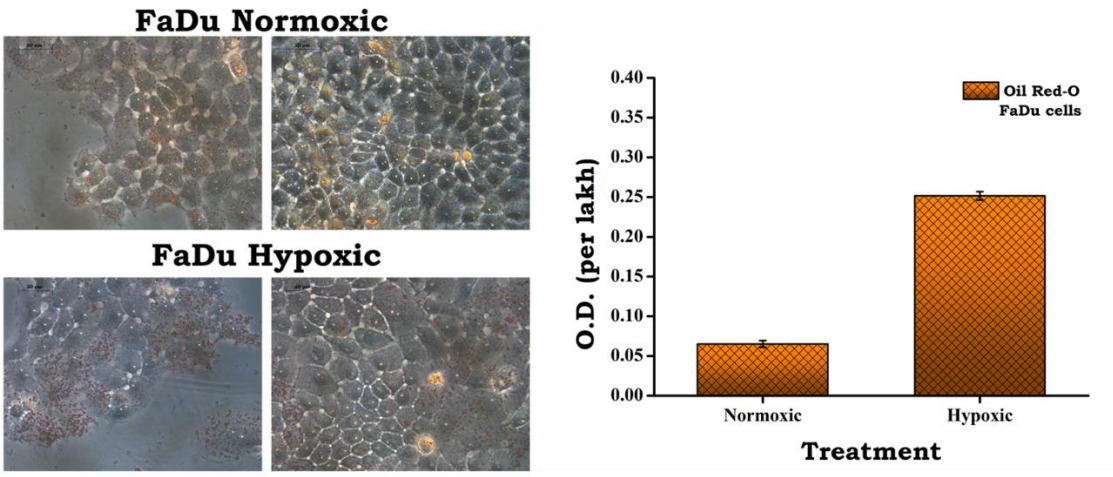


Figure 10 : Lipogenic studies using OIL –RED O method: FaDu cells exposed to normoxic and hypoxic conditions (Scale – 20mm)($p<0.001$)

5. Hypoxia and Apoptosis

Hypoxia is commonly found in solid tumours of various origins. Selection by hypoxia renders tumour cells resistant to hypoxia induced apoptosis. These cells with a reduced apoptotic potential may also explain the resistance of many solid tumours to cancer treatment. A better understanding of the regulation of apoptosis by hypoxia in solid tumours may not provide novel insight into tumour behavior but also in developing effective therapeutic strategies.

Here we tried to understand the role hypoxia in inducing apoptosis in Cal33 cells. The Cal33 cells were treated under hypoxic (2-5% O_2) conditions for 72h and thereafter cell were stained with AO/EtBr and imaged by fluorescent microscope. There was increase in the levels of necrotic cells but not much changes were observed in the levels of apoptotic cells (Figure 10).

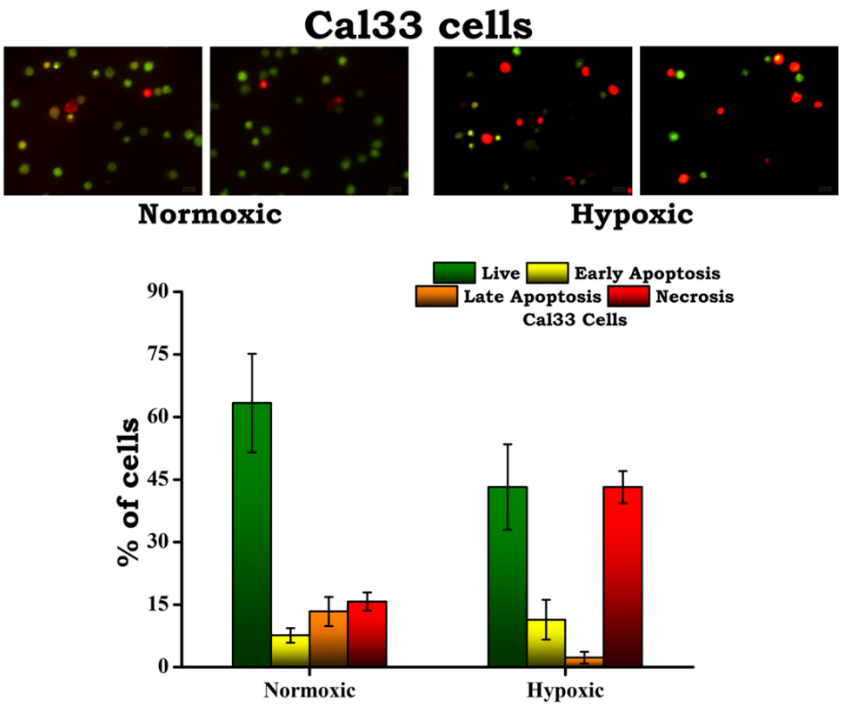


Figure 11: Apoptosis using AO/EtBr method: Cal33 cells exposed to normoxic and hypoxic conditions (Scale – 20mm)

6. **Isolation and characterization of exosomes**

   The exosomes were isolated from Cal33 and FaDu cell lines cultured under hypoxic conditions for 72 h. The size distribution of the exosomes were evaluated by Dynamic Light Scattering (DLS) method. The DLS analysis showed the exosomes size range from 50-1000 nm (Figure 12). The DLS analysis showed the exosomes size range from 50-1200 nm. The large size of particles might be due to aggregation of exosomes

7. **Exosomes and cell migration and drug resistance**

   Hypoxia is reported to induce migratory potential in the cells. Here we have examined the migration of Cal 33 cells treated with exosomes isolated in response to hypoxia. For this cells were seeded in 6-well plates and grown to confluence and then wound were scratched within the confluent cell layer using the fine end of a 200 µl (time 0) and then treated with exosomes. The images of migrating cells collected from the wounded region for different time-points. The rate of wound closure increased in hypoxic exosome treated cells at 12h and 24h but decreased for later time points (Figure 13).

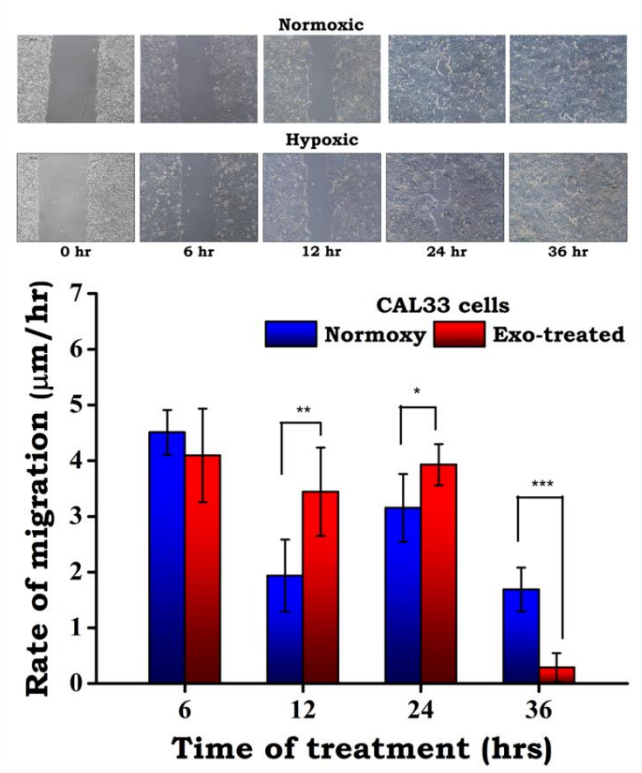


Figure 13: Rate of migration Cal33 upon treatment with ExoNormoxic and ExoHypoxic exosomes. Images are analyzed using imageJ software (Scale – 100μm) ***=p<0.001, **=0.001<p<0.01, *=0.01<p<0.05

To study the role of exosomes conferring drug resistance in HNC cells, we have exposed the Cal33 cells with hypoxic exosomes for interval 12 h, followed by IC_{50} dose (7.79 μM) of Cisplatin. The data exhibited significant restoration of cell viability in the cells pre- exposed to exosomes followed by IC_{50} dose of cisplatin (Figure 14). This indicates that exosomes can influence the efficacy of chemotherapeutic agents either directly or indirectly interfering with the action of drug.

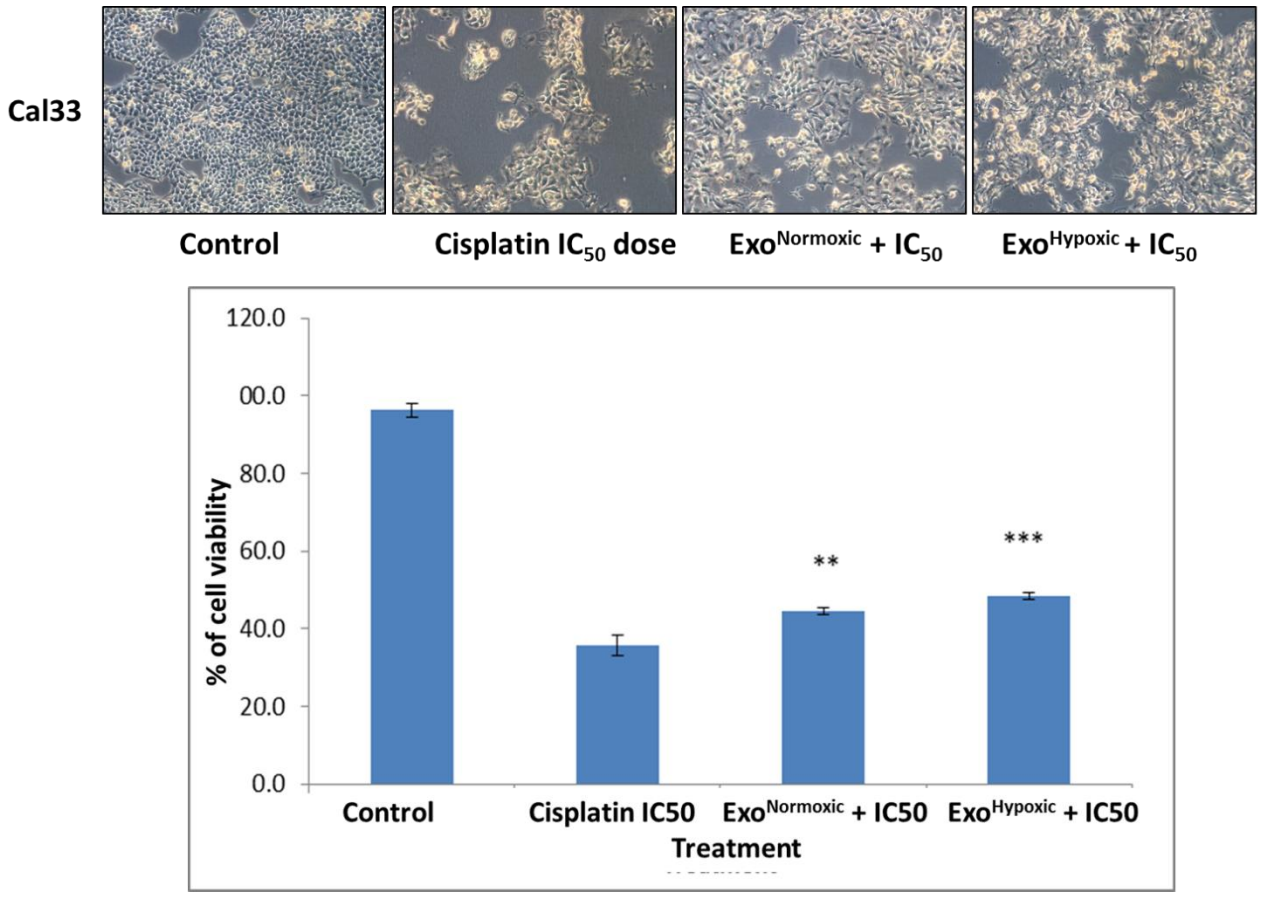


Figure 14 Cell viability of Cal33 cells pre-treated with exosomes for 12h followed by treatment with IC_{50} dose of Cisplatin. **p value < 0.0003; ***p value< 0.0001

8. Exosomal Proteome

We have also analyzed the proteome of exosomes isolated from Cal33 and FaDu cells exposed to hypoxia (3-5%) using LC-MS. From the chromatogram of the exosome proteome profile we have identified 773 and 518 proteins specific for normoxic and hypoxic conditions in Cal33 and 643 and 708 proteins in FaDu specific for normoxic and hypoxic conditions respectively. (Figure 15, 16 &17).

Further, the proteome analysis of Cal33 cells normoxic vs hypoxic conditions, we observed 21 proteins downregulated and 17 proteins upregulated (Fold change <0.5 is considered as downregulated and fold change >2.0 is considered as upregulated). The top five upregulated proteins are: Proteasome adapter and scaffold protein ECM29 (Fold Change 48.424), Myogenic factor 5 (Fold Change 48.424), A disintegrin and metalloproteinase with thrombospondin motifs 20(Fold Change 48.424) , Disintegrin and metalloproteinase domain-containing protein 23 (Fold Change 48.424), AT-hook DNA-binding motif-containing protein 1 (Fold Change 48.424) and downregulated proteins are: Protein jagged-1 (Fold Change 0.139), Tenascin (Fold Change 0.143), Pecanex-like protein 3(Fold Change 0.162), Trimethylguanosine synthase (Fold Change 0.177), Voltage-dependent L-type calcium channel subunit alpha-1D (Fold Change 0.179). The pathway analysis of upregulated and downregulated proteins in Cal 33 HNC cells exhibited activation of several pathways in response to hypoxia. The top pathways identified based on p-value that might play critical role in mitigating hypoxia are: RNA transport (p-value 3.43E-08), Amplification and Expansion of Oncogenic Pathways as Metastatic Traits (p-value 0.000491073), ECM-receptor interaction

(p-value 0.000635776), Focal Adhesion (p-value 0.000822734), O-glycosylation of TSR domain-containing proteins (p-value 0.002944489), Focal Adhesion-PI3K-Akt-mTOR-signaling pathway (p-value 0.003250681), Human papillomavirus infection (p-value 0.004002455), ECM proteoglycans (p-value 0.005988132), Beta1 integrin cell surface interactions -(p-value 0.008235007), Integrin cell surface interactions (p-value 0.008235007).

The number of different proteins involved various biological process are Biological regulation (18), metabolic processes (17), response to stimuli (15), cell communication (11), developmental process (13), cellular localization (11), cellular component reorganization (13), cell proliferation (4) and cell growth (4). The number of proteins associated with important cellular structures such as membrane (20), nucleus (10), macro-molecular complex (10), nucleus (10), vesicles (7), endomembrane system (6), extracellular matrix (5), membrane enclosed lumen (5), extracellular space (3), endosomes (3), Golgi apparatus (3), cytosol (3), cell projections (2), ER (2), cytoskeleton (2) and unclassified (5) (Figure 17 &18, Table 1, 2).

The proteome analysis of FaDu cells normoxic vs hypoxic conditions, exhibited 27 proteins downregulated and 20 proteins upregulated (Fold change <0.5 is considered as downregulated and fold change >2.0 is considered as upregulated). The top ten down regulated proteins were Pro-epidermal growth factor (Fold Change 0.082), Olfactory receptor 8U9 (Fold Change 0.106), MTSS1-like protein (Fold Change 0.115), ATP-binding cassette sub-family A member 5 (Fold Change 0.120), Sickle tail protein homolog (Fold Change 0.139), Junction-mediating and -regulatory protein (Fold Change 0.143), Protein TASOR (Fold Change 0.179), Cilia- and flagella-associated protein 44 (Fold Change 0.205), A disintegrin and metalloproteinase with thrombospondin motifs 7 (Fold Change 0.223), RNA-binding protein 25 (Fold Change 0.227) and the top ten upregulated proteins were Protein TALPID3 (Fold Change 3.525), Signal peptide, CUB and EGF-like domain-containing protein 3 (Fold Change 3.743), Cilia- and flagella-associated protein 221(Fold Change 3.896), Integrator complex subunit 1((Fold Change 3.935), HIV Tat-specific factor 1(Fold Change 5.584), UPF0577 protein KIAA1324 (Fold Change 6.488), ATP-binding cassette sub-family A member 8 (Fold Change 8.846), Transport and Golgi organization protein 1(Fold Change 10.697), Dedicator of cytokinesis protein 2(Fold Change 12.935), Serine/threonine-protein kinase 11-interacting protein(Fold Change 36.598).

The top pathways identified based on the p-value were RNA transport (p-value 0.000715387), prion pathway (p-value 0.000792099), MET activates PTK2 signaling (p-value0.000889735), Laminin interactions (p-value0.001459935), MET promotes cell motility(p-value 0.002012969), Resolution of D-loop Structures through Synthesis-Dependent Strand Annealing (SDSA) (p-value 0.002012969), Resolution of D-loop Structures through Holliday Junction Intermediates (p-value
0.003000143), Resolution of D-Loop Structures (p-value 0.003370429), Homologous recombination (p-value 0.00460219), Non-integrin membrane-ECM interactions(p-value 0.004824859), Homologous DNA Pairing and Strand Exchange(p-value 0.004824859), a6b1 and a6b4 Integrin signaling(p-value 0.005522166), ABC transporters(p-value 0.005522166), Fanconi anemia pathway(p-value 0.005764288), agrin in postsynaptic differentiation(p-value 0.007872221), Fanconi anemia pathway(p-value 0.006262921), ABC-family proteins mediated transport(p-value 0.009340463), Signaling by MET(p-value 0.009959429).

The number of different upregulated and downregulated proteins involved various biological **p**rocess are Biological regulation (28), response to stimuli (22), cellular localization (22), cellular component organization (21), metabolic process (19), multicellular organismal

process (18), development process (13), cell communication (7), cell proliferation (5), growth (2), reproduction (2), unclassified (2). The number of proteins associated with important cellular structures such as membrane (23), nucleus (13), membrane enclosed lumen (13), endomembrane system (12), macromolecular complex (11), cell projection (11), cytoskeleton (9), vesicles (8), Golgi body (4), vacuole (4), cytosol (4), chromosome (3), ER (3), ECM (3), envelop (3), extracellular space (2), endosome (2), mitochondria (1). (Figure 18, Table 3 & 4)

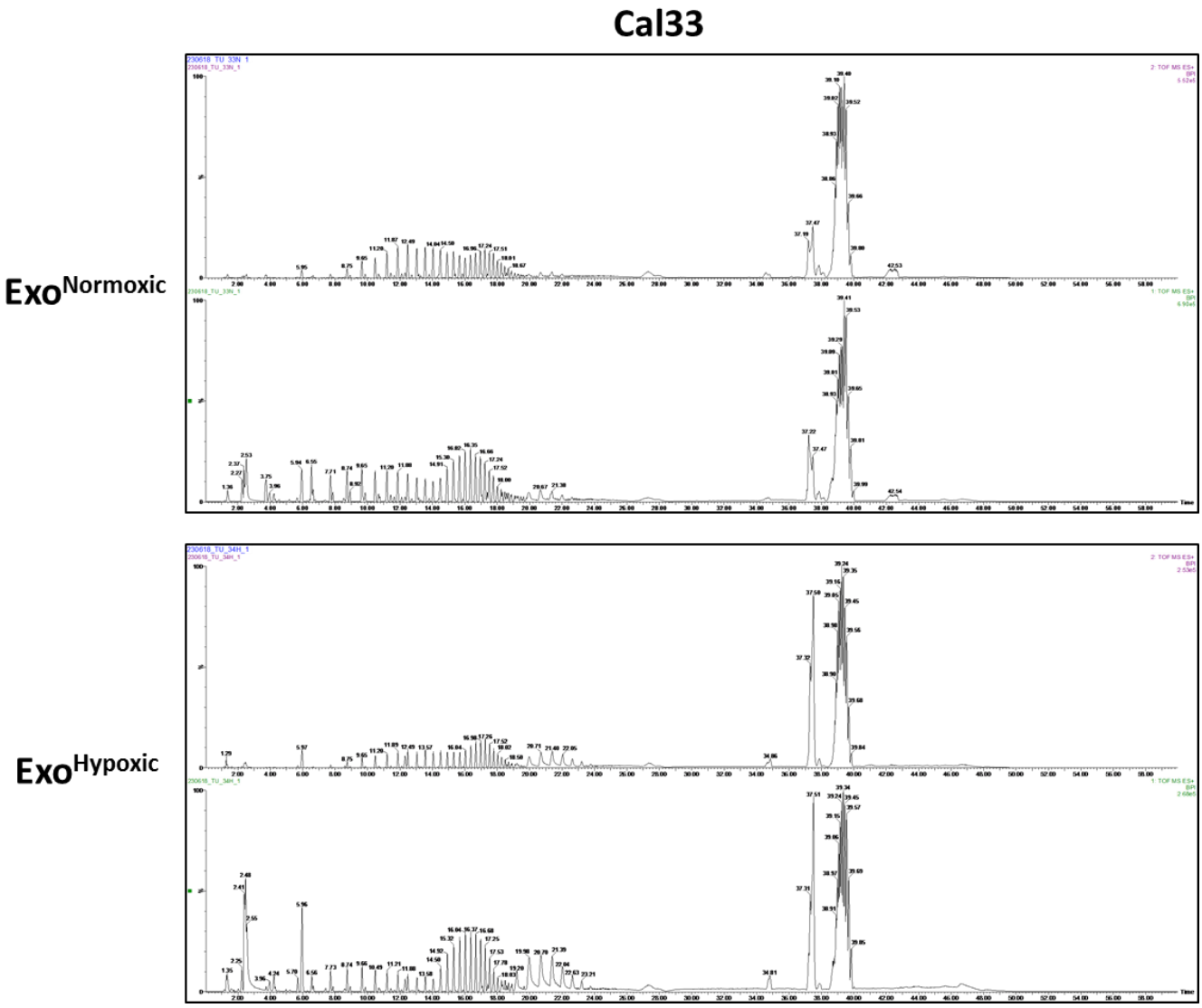


Figure 15 Chromatogram of Exosomal proteome profile of Cal33 exposed to normoxic and hypoxic conditions

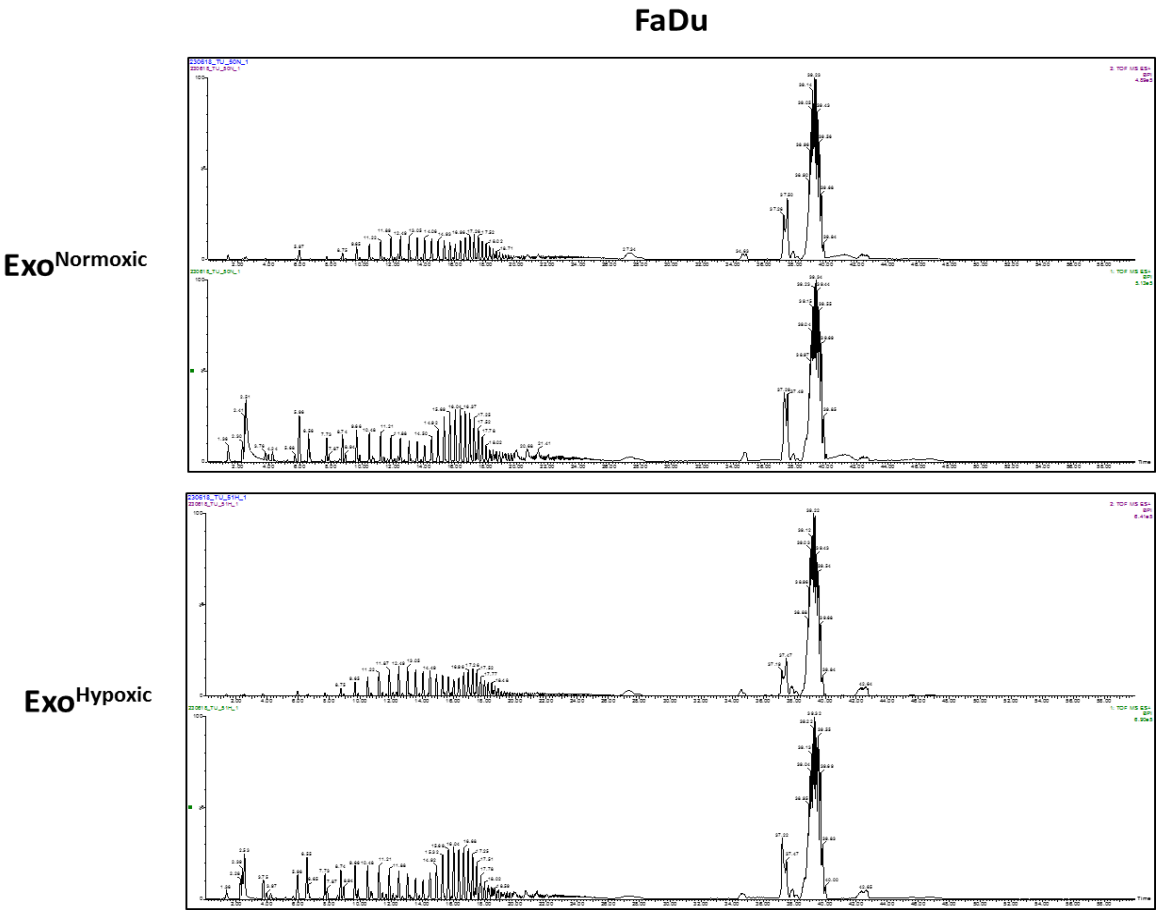


Figure 16 Chromatogram of Exosomal proteome profile of FaDu exposed to normoxic and hypoxic conditions

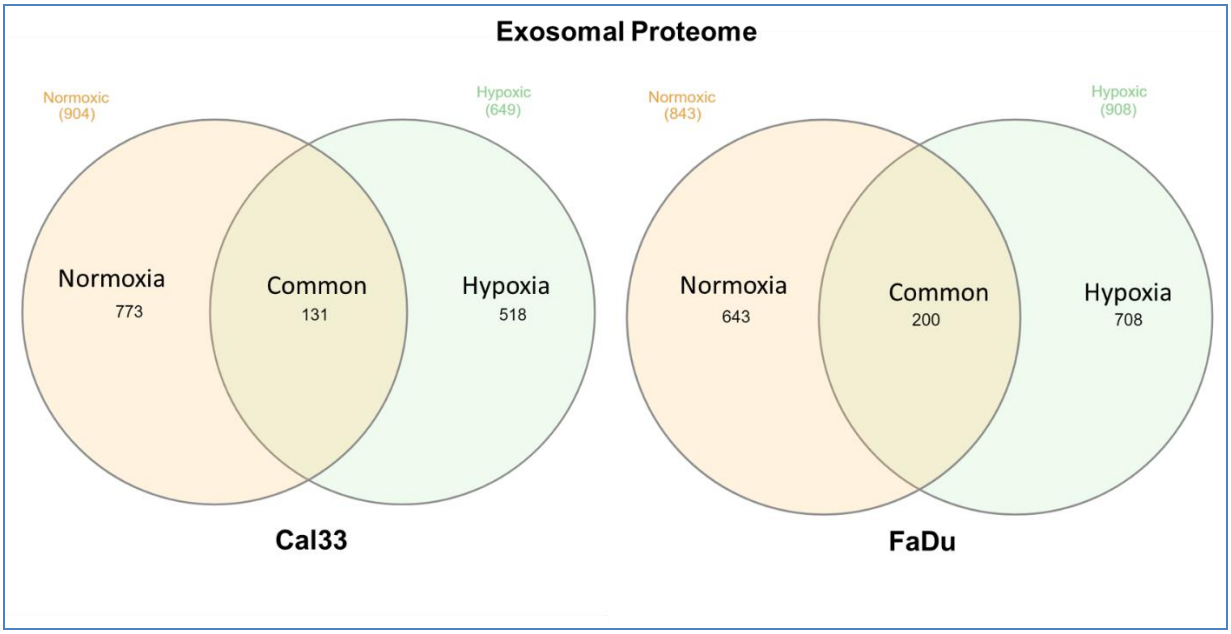


Figure 17 Venn diagram showing Exosomal proteome of Cal33 and FaDu cells exposed to normoxic and hypoxic conditions

Table 1: Exosomal proteins upregulated/down regulated in Cal 33 head and neck cancer cell line exposed to 3-5% hypoxic condition. Fold change <0.5 is considered as downregulated and fold change >2.0 is considered as upregulated

S.No.	Accession No	Description	Score	Fold Change	Remarks
1	P78504	Protein jagged-1 OS=Homo sapiens OX=9606 GN=JAG1 PE=1 SV=3	97.61	0.139	Down regulated
2	P24821	Tenascin OS=Homo sapiens OX=9606 GN=TNC PE=1 SV=3	40.03	0.143	Down regulated
3	Q9H6A9	Pecanex-like protein 3 OS=Homo sapiens OX=9606 GN=PCNX3 PE=1 SV=2	87.35	0.162	Down regulated
4	Q96RS0	Trimethylguanosine synthase OS=Homo sapiens OX=9606 GN=TGS1 PE=1 SV=3	28.23	0.177	Down regulated
5	Q01668	Voltage-dependent L-type calcium channel subunit alpha-1D OS=Homo sapiens OX=9606 GN=CACNA1D PE=1 SV=2	87.87	0.179	Down regulated
6	Q9BZV3	Interphotoreceptor matrix proteoglycan 2 OS=Homo sapiens OX=9606 GN=IMPG2 PE=1 SV=3	119.04	0.267	Down regulated
7	P20815	Cytochrome P450 3A5 OS=Homo sapiens OX=9606 GN=CYP3A5 PE=1 SV=1	137.15	0.267	Down regulated
8	P35442	Thrombospondin-2 OS=Homo sapiens OX=9606 GN=THBS2 PE=1 SV=2	112.98	0.280	Down regulated
9	Q96R84	Putative olfactory receptor 1F2 OS=Homo sapiens OX=9606 GN=OR1F2P PE=5 SV=2	231.01	0.310	Down regulated
10	O14715	RANBP2-like and GRIP domain-containing protein 8 OS=Homo sapiens OX=9606 GN=RGPD8 PE=1 SV=2	26.27	0.316	Down regulated
11	A6NKT7	RanBP2-like and GRIP domain-containing protein 3 OS=Homo sapiens OX=9606 GN=RGPD3 PE=3 SV=2	26.73	0.316	Down regulated
12	Q6IE37	Ovostatin homolog 1 OS=Homo sapiens OX=9606 GN=OVOS1 PE=2 SV=2	76.65	0.316	Down regulated
13	Q99666	RANBP2-like and GRIP domain-containing protein 5/6 OS=Homo sapiens OX=9606 GN=RGPD5 PE=1 SV=3	26.27	0.319	Down regulated
14	Q7Z3J3	RanBP2-like and GRIP domain-containing protein 4 OS=Homo sapiens OX=9606 GN=RGPD4 PE=2 SV=3	26.73	0.319	Down regulated
15	P0DJD0	RANBP2-like and GRIP domain-containing protein 1 OS=Homo sapiens OX=9606 GN=RGPD1 PE=2 SV=1	29.13	0.336	Down regulated
16	P51805	Plexin-A3 OS=Homo sapiens OX=9606 GN=PLXNA3 PE=1 SV=2	45.16	0.339	Down regulated
17	P0DJD1	RANBP2-like and GRIP domain-containing protein 2 OS=Homo sapiens OX=9606 GN=RGPD2 PE=2 SV=1	78.32	0.343	Down regulated
18	Q9UQP3	Tenascin-N OS=Homo sapiens OX=9606 GN=TNN PE=1 SV=2	56.16	0.371	Down regulated
19	Q149N8	E3 ubiquitin-protein ligase SHPRH OS=Homo sapiens OX=9606 GN=SHPRH PE=1 SV=2	22.44	0.444	Down regulated
20	O75970	Multiple PDZ domain protein OS=Homo sapiens OX=9606 GN=MPDZ PE=1 SV=2	73.2	0.467	Down regulated
21	Q5IJ48	Protein crumbs homolog 2 OS=Homo sapiens OX=9606 GN=CRB2 PE=1 SV=2	78.59	0.477	Down regulated
22	Q96KG7	Multiple epidermal growth factor-like domains protein 10 OS=Homo sapiens OX=9606 GN=MEGF10 PE=1 SV=1	93.09	2.095	Up regulated
23	Q9ULH0	Kinase D-interacting substrate of 220 kDa OS=Homo sapiens OX=9606 GN=KIDINS220 PE=1 SV=3	72.66	2.247	Up regulated
24	O14830	Serine/threonine-protein phosphatase with EF-hands 2 OS=Homo sapiens OX=9606 GN=PPEF2 PE=1 SV=2	25.31	2.363	Up regulated
25	Q8IWU2	Serine/threonine-protein kinase LMTK2 OS=Homo sapiens OX=9606 GN=LMTK2 PE=1 SV=2	116.12	2.363	Up regulated
26	Q8IV36	Protein HID1 OS=Homo sapiens OX=9606 GN=HID1 PE=1 SV=1	50.28	2.484	Up regulated
27	Q8WWL7	G2/mitotic-specific cyclin-B3 OS=Homo sapiens OX=9606 GN=CCNB3 PE=1 SV=2	125.07	2.829	Up regulated
28	P98175	RNA-binding protein 10 OS=Homo sapiens OX=9606 GN=RBM10 PE=1 SV=3	93.85	2.886	Up regulated
29	Q16760	Diacylglycerol kinase delta OS=Homo sapiens OX=9606 GN=DGKD PE=1 SV=4	88.31	3.126	Up regulated
30	A6NKB5	Pecanex-like protein 2 OS=Homo sapiens OX=9606 GN=PCNX2 PE=2	22.09	3.455	Up regulated

		SV=3			
31	Q8TDI0	Chromodomain-helicase-DNA-binding protein 5 OS=Homo sapiens OX=9606 GN=CHD5 PE=1 SV=1	43.69	3.669	Up regulated
32	Q9NZB2	Constitutive coactivator of PPAR-gamma-like protein 1 OS=Homo sapiens OX=9606 GN=FAM120A PE=1 SV=2	19.68	4.526	Up regulated
33	Q13349	Integrin alpha-D OS=Homo sapiens OX=9606 GN=ITGAD PE=1 SV=2	144.01	4.618	Up regulated
34	Q5TGY3	AT-hook DNA-binding motif-containing protein 1 OS=Homo sapiens OX=9606 GN=AHDC1 PE=1 SV=1	40.06	5.528	Up regulated
35	O75077	Disintegrin and metalloproteinase domain-containing protein 23 OS=Homo sapiens OX=9606 GN=ADAM23 PE=1 SV=1	87.07	10.485	Up regulated
36	P59510	A disintegrin and metalloproteinase with thrombospondin motifs 20 OS=Homo sapiens OX=9606 GN=ADAMTS20 PE=2 SV=2	64.79	15.642	Up regulated
37	P13349	Myogenic factor 5 OS=Homo sapiens OX=9606 GN=MYF5 PE=2 SV=2	50.04	20.085	Up regulated
38	Q5VYK3	Proteasome adapter and scaffold protein ECM29 OS=Homo sapiens OX=9606 GN=ECPAS PE=1 SV=2	28.9	48.424	Up regulated

Table 2: Pathway Analysis Exosomal proteins upregulated/down regulated in Cal 33 head and neck cancer cell line exposed to 3-5% hypoxic condition.

p-value	q-value	pathway	source	external_id
3.43E-08	9.61E-07	RNA transport - Homo sapiens (human)	KEGG	path:hsa03013
0.000491073	0.00575914	Amplification and Expansion of Oncogenic Pathways as Metastatic Traits	Wikipathways	WP3678
0.000635776	0.00575914	ECM-receptor interaction - Homo sapiens (human)	KEGG	path:hsa04512
0.000822734	0.00575914	Focal Adhesion	Wikipathways	WP306
0.002944489	0.015169846	O-glycosylation of TSR domain-containing proteins	Reactome	R-HSA-5173214
0.003250681	0.015169846	Focal Adhesion-PI3K-Akt-mTOR-signaling pathway	Wikipathways	WP3932
0.004002455	0.016009821	Human papillomavirus infection - Homo sapiens (human)	KEGG	path:hsa05165
0.005988132	0.020958463	ECM proteoglycans	Reactome	R-HSA-3000178
0.007906228	0.020961836	Focal adhesion - Homo sapiens (human)	KEGG	path:hsa04510
0.008235007	0.020961836	Beta1 integrin cell surface interactions	PID	integrin1_pathway
0.008235007	0.020961836	Integrin cell surface interactions	Reactome	R-HSA-216083

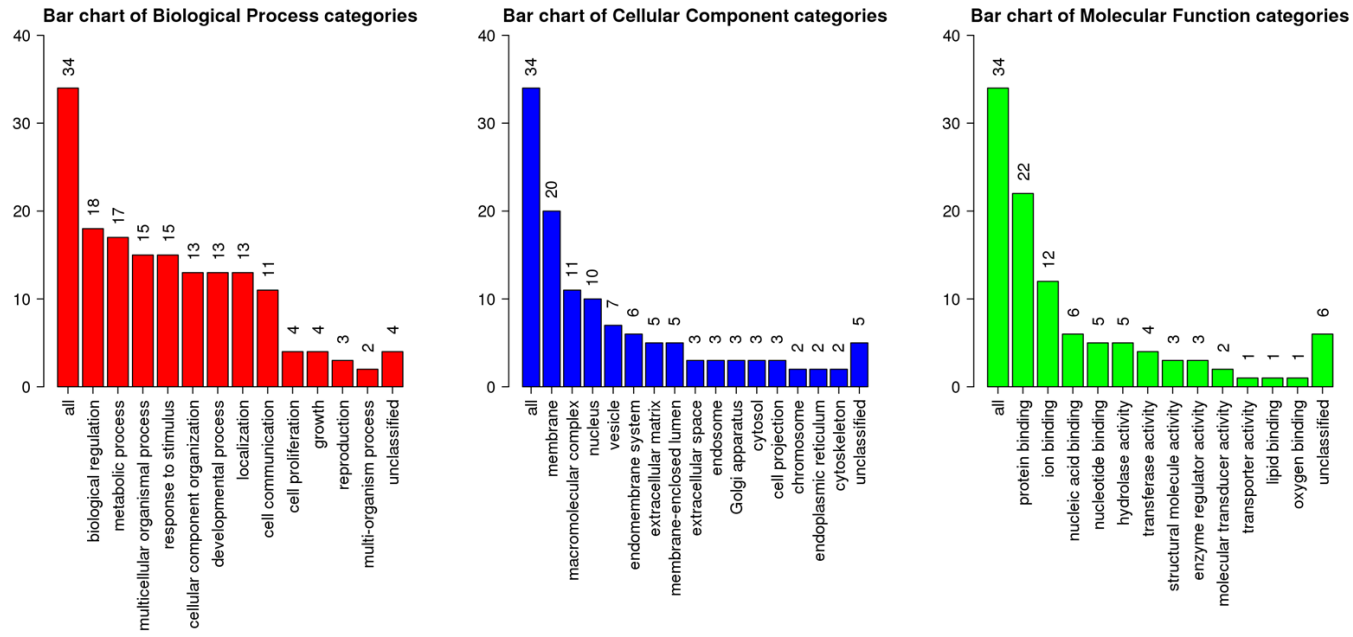


Figure 18: GoSlim summary exosomal proteins showing canonical pathways based on biological processes, cellular components and molecular functions for Cal 33 cells exposed to 3-5% hypoxia

Table 3: Exosomal proteins upregulated/down regulated in FaDu head and neck cancer cell line exposed to 3-5% hypoxic condition. Fold change <0.5 is considered as downregulated and fold change >2.0 is considered as upregulated

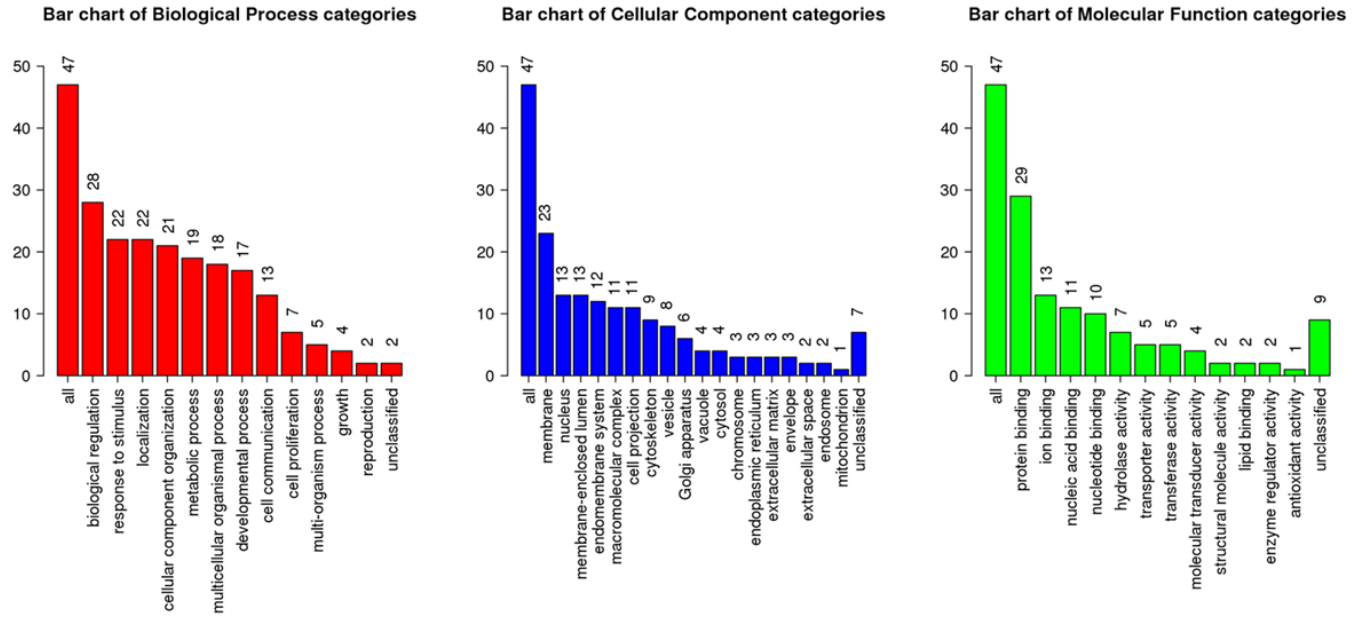
S.No.	Accession No	Description	Score	Fold Change	Remarks
1	P01133	Pro-epidermal growth factor OS=Homo sapiens OX=9606 GN=EGF PE=1 SV=2	73.3	0.082	Down regulated
2	P0C7N5	Olfactory receptor 8U9 OS=Homo sapiens OX=9606 GN=OR8U9 PE=3 SV=1	148.97	0.106	Down regulated
3	Q765P7	MTSS1-like protein OS=Homo sapiens OX=9606 GN=MTSS1L PE=1 SV=1	148.33	0.115	Down regulated
4	Q8WWZ7	ATP-binding cassette sub-family A member 5 OS=Homo sapiens OX=9606 GN=ABCA5 PE=2 SV=2	71.81	0.120	Down regulated
5	Q5T5P2	Sickle tail protein homolog OS=Homo sapiens OX=9606 GN=KIAA1217 PE=1 SV=2	46.77	0.139	Down regulated
6	Q8N9B5	Junction-mediating and -regulatory protein OS=Homo sapiens OX=9606 GN=JMY PE=1 SV=2	32.87	0.143	Down regulated
7	Q9UK61	Protein TASOR OS=Homo sapiens OX=9606 GN=FAM208A PE=1 SV=3	91.6	0.179	Down regulated
8	Q96MT7	Cilia- and flagella-associated protein 44 OS=Homo sapiens OX=9606 GN=CFAP44 PE=1 SV=1	27.92	0.205	Down regulated
9	Q9UKP4	A disintegrin and metalloproteinase with thrombospondin motifs 7 OS=Homo sapiens OX=9606 GN=ADAMTS7 PE=1 SV=2	145.57	0.223	Down regulated
10	P49756	RNA-binding protein 25 OS=Homo sapiens OX=9606 GN=RBM25 PE=1 SV=3	89.32	0.227	Down regulated
11	Q96KG7	Multiple epidermal growth factor-like domains protein 10 OS=Homo sapiens OX=9606 GN=MEGF10 PE=1 SV=1	87.77	0.232	Down regulated
12	Q9BX84	Transient receptor potential cation channel subfamily M member 6 OS=Homo sapiens OX=9606 GN=TRPM6 PE=1 SV=2	24.18	0.272	Down regulated
13	Q86X53	Glutamate-rich protein 1 OS=Homo sapiens OX=9606 GN=ERICH1 PE=1 SV=1	323.73	0.275	Down regulated
14	P0DJD0	RANBP2-like and GRIP domain-containing protein 1 OS=Homo sapiens OX=9606 GN=RGPD1 PE=2 SV=1	88.15	0.283	Down regulated
15	A6NKT7	RanBP2-like and GRIP domain-containing protein 3 OS=Homo sapiens OX=9606 GN=RGPD3 PE=3 SV=2	107.79	0.289	Down regulated
16	Q9Y6N6	Laminin subunit gamma-3 OS=Homo sapiens OX=9606 GN=LAMC3 PE=1 SV=3	97.75	0.304	Down regulated
17	Q7Z3J3	RanBP2-like and GRIP domain-containing protein 4 OS=Homo sapiens OX=9606 GN=RGPD4 PE=2 SV=3	107.79	0.329	Down regulated
18	P0DJD1	RANBP2-like and GRIP domain-containing protein 2 OS=Homo sapiens OX=9606 GN=RGPD2 PE=2 SV=1	94.67	0.343	Down regulated
19	Q9H987	Synaptopodin 2-like protein OS=Homo sapiens OX=9606 GN=SYNPO2L PE=2 SV=3	28.21	0.346	Down regulated
20	Q4ADV7	RAB6A-GEF complex partner protein 1 OS=Homo sapiens OX=9606 GN=RIC1 PE=1 SV=2	70.93	0.357	Down regulated
21	Q9BX63	Fanconi anemia group J protein OS=Homo sapiens OX=9606 GN=BRIP1 PE=1 SV=1	50.47	0.379	Down regulated
22	O60337	E3 ubiquitin-protein ligase MARCH6 OS=Homo sapiens OX=9606 GN=MARCH6 PE=1 SV=2	116.95	0.379	Down regulated
23	P07942	Laminin subunit beta-1 OS=Homo sapiens OX=9606 GN=LAMB1 PE=1 SV=2	48.5	0.386	Down regulated
24	P27708	CAD protein OS=Homo sapiens OX=9606 GN=CAD PE=1 SV=3	83.57	0.394	Down regulated

25	Q6ZV73	FYVE, RhoGEF and PH domain-containing protein 6 OS=Homo sapiens OX=9606 GN=FGD6 PE=1 SV=2	45.11	0.406	Down regulated
26	Q9Y5H6	Protocadherin alpha-8 OS=Homo sapiens OX=9606 GN=PCDHA8 PE=2 SV=1	236.88	0.491	Down regulated
27	Q6PHW0	Iodotyrosine deiodinase 1 OS=Homo sapiens OX=9606 GN=IYD PE=1 SV=2	101.97	0.491	Down regulated
28	Q6UXC1	Apical endosomal glycoprotein OS=Homo sapiens OX=9606 GN=MAMDC4 PE=2 SV=2	118.93	2.033	Up regulated
29	Q14028	Cyclic nucleotide-gated cation channel beta-1 OS=Homo sapiens OX=9606 GN=CNGB1 PE=1 SV=2	25.67	2.293	Up regulated
30	Q9UPW8	Protein unc-13 homolog A OS=Homo sapiens OX=9606 GN=UNC13A PE=2 SV=4	115.63	2.386	Up regulated
31	O94916	Nuclear factor of activated T-cells 5 OS=Homo sapiens OX=9606 GN=NFAT5 PE=1 SV=1	52.08	2.509	Up regulated
32	Q7Z6E9	E3 ubiquitin-protein ligase RBBP6 OS=Homo sapiens OX=9606 GN=RBBP6 PE=1 SV=1	68.76	2.585	Up regulated
33	Q8IY21	Probable ATP-dependent RNA helicase DDX60 OS=Homo sapiens OX=9606 GN=DDX60 PE=1 SV=3	57.67	2.611	Up regulated
34	Q92823	Neuronal cell adhesion molecule OS=Homo sapiens OX=9606 GN=NRCAM PE=1 SV=3	118.5	2.718	Up regulated
35	Q8TDI0	Chromodomain-helicase-DNA-binding protein 5 OS=Homo sapiens OX=9606 GN=CHD5 PE=1 SV=1	29.43	2.974	Up regulated
36	Q8TER0	Sushi, nidogen and EGF-like domain-containing protein 1 OS=Homo sapiens OX=9606 GN=SNED1 PE=2 SV=2	56.85	3.387	Up regulated
37	P52655	Transcription initiation factor IIA subunit 1 OS=Homo sapiens OX=9606 GN=GTF2A1 PE=1 SV=1	362.02	3.421	Up regulated
38	Q86YC2	Partner and localizer of BRCA2 OS=Homo sapiens OX=9606 GN=PALB2 PE=1 SV=1	104.78	3.490	Up regulated
39	Q9BVV6	Protein TALPID3 OS=Homo sapiens OX=9606 GN=KIAA0586 PE=1 SV=4	73.59	3.525	Up regulated
40	Q8IX30	Signal peptide, CUB and EGF-like domain-containing protein 3 OS=Homo sapiens OX=9606 GN=SCUBE3 PE=1 SV=1	84.3	3.743	Up regulated
41	Q4G0U5	Cilia- and flagella-associated protein 221 OS=Homo sapiens OX=9606 GN=CFAP221 PE=1 SV=2	35.95	3.896	Up regulated
42	Q8N201	Integrator complex subunit 1 OS=Homo sapiens OX=9606 GN=INTS1 PE=1 SV=2	36.52	3.935	Up regulated
43	O43719	HIV Tat-specific factor 1 OS=Homo sapiens OX=9606 GN=HTATSF1 PE=1 SV=1	171.34	5.584	Up regulated
44	Q6UXG2	UPF0577 protein KIAA1324 OS=Homo sapiens OX=9606 GN=KIAA1324 PE=2 SV=2	21.2	6.488	Up regulated
45	O94911	ATP-binding cassette sub-family A member 8 OS=Homo sapiens OX=9606 GN=ABCA8 PE=1 SV=3	46.7	8.846	Up regulated
46	Q5JRA6	Transport and Golgi organization protein 1 homolog OS=Homo sapiens OX=9606 GN=MIA3 PE=1 SV=1	65.72	10.697	Up regulated
47	Q92608	Dedicator of cytokinesis protein 2 OS=Homo sapiens OX=9606 GN=DOCK2 PE=1 SV=2	40.51	12.935	Up regulated
48	Q8N1F8	Serine/threonine-protein kinase 11-interacting protein OS=Homo sapiens OX=9606 GN=STK11IP PE=1 SV=4	99.82	36.598	Up regulated

Table 4: Pathway Analysis Exosomal proteins upregulated/down regulated in FaDu head and neck cancer cell line exposed to 3-5% hypoxic condition

p-value	q-value	pathway	source	external_id
0.000715387	0.019870738	RNA transport - Homo sapiens (human)	KEGG	path:hsa03013
0.000792099	0.019870738	prion pathway	BioCarta	prionpathway
0.000889735	0.019870738	MET activates PTK2 signaling	Reactome	R-HSA-8874081
0.001459935	0.022478153	Laminin interactions	Reactome	R-HSA-3000157
0.002012969	0.022478153	MET promotes cell motility	Reactome	R-HSA-8875878
0.002012969	0.022478153	Resolution of D-loop Structures through Synthesis-Dependent Strand Annealing (SDSA)	Reactome	R-HSA-5693554
0.003000143	0.027586237	Resolution of D-loop Structures through Holliday Junction Intermediates	Reactome	R-HSA-5693568
0.003370429	0.027586237	Resolution of D-Loop Structures	Reactome	R-HSA-5693537
0.00460219	0.027586237	Homologous recombination - Homo sapiens (human)	KEGG	path:hsa03440
0.004824859	0.027586237	Non-integrin membrane-ECM interactions	Reactome	R-HSA-3000171
0.004824859	0.027586237	Homologous DNA Pairing and Strand Exchange	Reactome	R-HSA-5693579
0.005522166	0.027586237	a6b1 and a6b4 Integrin signaling	PID	a6b1_a6b4_integrin_pathway
0.005522166	0.027586237	ABC transporters - Homo sapiens (human)	KEGG	path:hsa02010
0.005764288	0.027586237	Fanconi anemia pathway	PID	fanconi_pathway
0.006262921	0.027974383	agrin in postsynaptic differentiation	BioCarta	agrpathway
0.007872221	0.032964927	Fanconi anemia pathway - Homo sapiens (human)	KEGG	path:hsa03460
0.009340463	0.036812413	ABC-family proteins mediated transport	Reactome	R-HSA-382556
0.009959429	0.037071208	Signaling by MET	Reactome	R-HSA-6806834

Figure 18: GoSlim summary exosomal proteins showing canonical pathways based on biological processes, cellular components and molecular functions for FaDu cells exposed to 3-5% hypoxia



12. **Detailed analysis of results**.

1. The present findings showed increased levels of lipid droplet (LD) accumulation in FaDu cells in response to hypoxia. This alteration in the lipid metabolism could be used as a therapeutic intervention to target cancer cells using inhibitors lipid metabolism pathways that could also provide new strategies to sensitize the cancer cells.

2. Several reports have shown that autophagy plays important role in reprogramming tumors cells and confers resistance to chemotherapeutic agents. Here we observed increase in the number of acid vesicles (AVOs) and the degree of acidity (DOA) in both Cal33 and FaDu cells in response to hypoxia. The induction of autophagy in response to hypoxia is suggested to be involved in chemo/radio resistance in tumor cells.

3. HNSCC cells co-cultured with hypoxic exosomes and then treated with IC50 dose (7.79 µmole) of cisplatin resulted in restoration of cell viability. This indicates that exosomes may influence the efficacy of chemotherapeutic agents either directly or indirectly interacting with the drug.

4. The proteome analysis of exhibited significantly upregulated and downregulated proteins in both Cal33 and FaDu cells. The functional studies of upregulated/downregulated proteins using knock down approach is needed to further establish their role in cell proliferation, migration and chemo-resistance in response to hypoxia.

13. A summary sheet of not more than two pages under following heads (Title, Introduction, Rationale, Objectives, Methodology, Results, Translational Potential)

Title: "Studies on Exosomal Proteome and Lipidome and their Clinical Implication in the Management of Drug Resistant and Recurrent Head and Neck Squamous Cell Carcinomas (HNSCC)"

Introduction:

Despite improved means of diagnosis and treatment, drug resistance and recurrence remains the biggest obstacle to long term survival in patients with HNSCC. Late stage diagnosis and high frequency of drug resistance are key contributors to the poor survival rates of HNSCC patients. This high- lightens the need for identification of novel drug resistance, diagnostic markers for better management of the disease. Recent studies suggest that phenotypic changes associated with cancer may be transferred from cell to cell via micro-vesicles/exosomes. Exosomes are nano-sized membrane bound vesicles of endocytic origin and has been implicated in proliferation, angiogenesis, immunesuppression; and more lately, in the preparation of pre-metastatic niches in secondary organs. Even though exosomes have been widely reported to mediate local and systemic cell communication through horizontal transfer of information such as proteins microRNAs, and mRNAs; limited information is available on the role of exosomal proteome and lipids in cancer. Also the role of exosomal proteins and lipidome in drug resistant and recurrent HNSCC are not well understood. In the proposed study we have examined proteome of exosomes derived from HNSCC cells exposed to hypoxia and their role in cancer associated phenotypic changes and drug response in HNSCC.

Methodology:

1 Human HNSCC cells FaDu will be procured from ATCC and Cal 33 was gifted by our collaborator at JNU, New Delhi. Drug resistant sublines will be developed by sequential exposure to the commonly used drug Cisplatin/Carboplatin. Detailed methodology for

development of drug resistant subline is described in Section 10 (a-c). But unfortunately we lost the cells due to sever contamination in the laboratory.

2. HNSCC cells will be cultured in the CO_2 incubator upto 30% confleuncy and followed by the exposure to hypoxia. Cells were then allowed to grow upto 90% confluency and thereafter exosomes were isolated from the conditioned media by precipitation method using commercially available ExoquickTM reagent (System Biosciences) according to the manufacturer's instructions. The exosomes isolated will be characterized for the size and structure by particle size analyzer and transmission electron microscopy.
3. Proteins will be extracted from the exosomes and their parent HNSCC cells using standard method. The proteins will be separated by 1D gel electrophoresis and identified by LC/MS/MS. Protein database searches of the spectra will be performed with Mascot v2.2 (Matrix Science). The functional network/pathway of the proteins will be carried out using STRING/KEGG databases and Cytoscape/Ingenuity software (Ramteke *et al*, 2013).
4. The role of hypoxic exosomes in the cell proliferation and drug response werestudied in the drug sensitive HNSCC in presence and absence of the drug. For this standard cell culture and treatment procedure will be used. The role of exosomes were also studied in cell migration using wound healing method.
5. Bioinformatics and statistical tools will be used for analyzing the proteome data as described above. All statistical analyses will be carried out with Sigma Stat software version 2.03. One-way or two-way ANOVA followed by Tukey-test will be used to determine the statistical significance. Each experiment will be performed in triplicate and repeated 3 times.

Results: Major finding are mentioned section 11 and 12.

Translational Potential:

The molecular characterization of hypoxic exosomal proteome in HNSCC have led to the identification of some new pathways that might paly important role in aggressive growth and drug response in HNSCC. Deciphering the role of identified molecules will provide in depth and novel understanding of the molecular mechanism underlying carcinogenesis and tumor progression, metastasis, drug.

14. Contributions made towards increasing the state of knowledge in the subject.

Hypoxia within tumor microenvironment is responsible for poor outcome of several malignancies including HNSCC. Hypoxia driven genes and proteins enhances EMT, remodels ECM and causes drug resistance in cancer. Here in the present study we have evaluated ROS and lipid accumulation in HNSCC cells exposed to hypoxia. Further we observed induction of autophagy in response to hypoxia. The present findings indicates close link between ROS, lipid accumulation and autophagy which might be responsible for modulation in drug response in HNSCC. Naïve HNSCC cells when exposed to hypoxic exosomes exhibited increased resistances towards chemotherapeutic agent suggesting that the exosomes might determine the drug response in the cancer cells. The proteome analysis of hypoxic exosomes led to identification several upregulated and downregulated proteins in HNSCC cells exposed to hypoxia. Further functional studies using knockout approach might establish the role of these proteins in cell proliferation, migration and drug response in HNSCC.

15. **Conclusions summarizing the achievements and indication of scope for future work**.

We conclude that hypoxia induced ROS level, lipid accumulation and autophagy might be closely linked and play important role in aggressive growth and chemo-resistance in tumors. We have also observed that exposure of hypoxic exosomes increased the restoration of cell viability in naïve HNSCC cells treated with IC50 dose of cisplatin. There are reports that showed increased secretion of exosomes under hypoxia. It is quite possible the increased level of exosome secretion might be related lipid accumulation and autophagy. The proteome analysis of hypoxic vs normoxic exosomes exhibited significantly upregulated and downregulated proteins in both Cal33 and FaDu cells. The functional studies of upregulated/downregulated proteins using knock down approach is needed to further establish their role in cell proliferation, migration and drug response of HNSCC to chemotherapeutic agents.

16. Science and Technology benefits accrued:

I. List of research publications with complete details: In process of communication

Authors, Title of paper, Name of Journal, Vol., page, year

II. Manpower trained in the project:

a.	Research Scientists or Research Fellows	02
b.	No. of Ph.Ds produced	NIL
c.	Other Technical Personnel trained	02

III. Patents taken, if any: NIL

IV. Products developed, if any. NIL

17. Abstract (300 words for possible publication in ICMR Bulletin).

Hypoxia is considered as one of the hallmark of tumor microenvironment and is responsible for poor outcome of several malignancies including HNSCC. Here in the present study we significant increase in ROS levels in HNSCC cells exposed to hypoxia. The increased levels of ROS might have led to ER stress disrupting various signaling pathways causing decreased rate of cell proliferation. There are evidences suggesting convergence of various signaling pathways to autophagy in response to hypoxia. Our study showed increased levels of AVOs (acid vesicles) and DOA (degree of acidity) in HNSCC exposed to hypoxia. This increase might be due to increased levels of ROS, wherein autophagy is known to remove ROS protecting cell from damage. The increased level of autophagy is suggested to be involved chemo/radio-resistance in various tumors which could possible therapeutic target for HNSCC. We also observed increased level of lipid accumulation in response to hypoxia in HNSCC. This possibly suggests link between lipid metabolism, autophagy and drug response. Recent reports suggests that exosomes released by the tumor cells can modulate the tumor microenvironment and drug response. Here we observed that the naïve HNSCC cells exposed hypoxic exosomes resulted in significant restoration in the cell viability in HSCC cell treated with IC50 dose of cisplatin suggesting that exosome may influence the efficacy of chemotherapeutic agents directly or indirectly by interacting with the drug. Hypoxic is also known to increase the secretion of exosomes to interfere with the drug action. It could be possible that increased secretion of exosomes might be related with elevated levels of lipid accumulation and autophagy. Further the proteome analysis data of hypoxic exosomes led to

identification of several proteins upregulated and downregulated in HNSCC. The function studies of these protein using knockout approach is needed to establish their role in modulation of tumor microenvironment and drug response in HNSCC.

18. **Procurement/usage of Equipment** NIL

a.

S. No	Name of Equipment	Make/ Model	Cost FE/₹	Date of Installation	Utilization rate %	Remarks regarding maintenance/breakdown

b. **Suggestions for disposal of equipment**. Not Applicable

Name and signature with date

1. _____ (Principal Investigator)

2. _____ (Co-Investigator)

3. _____ (Co-Investigator)

Annexure 4

FINAL REPORT

Project title: "Studies on exosomal proteome and lipidome and their clinical implication in the management of drug resistant and recurrent head and neck squamous cell carcinomas (HNSCC)"

Unique Project ID: R.F.C No. RCH/NER/4/2016-2017 dated 03/05/201

Submitted By

Principal Investigator
Professor Anand Ramteke
Dept. of Molecular Biology and Biotechnology
TEZPUR UNIVERSITY
PO Napaam, Tezpur, Assam 784028

Annexure-3a

Utilization Certificate of grant for the project for the period 17/05/2021 to 16/05/2022

1. Title of the Project **"Studies on exosomal proteome and lipidome and their clinical implication in the management of drug resistant and recurrent head and neck squamous cell carcinomas (HNSCC)"**
2. Name of the Institutions: **Tezpur University**
3. Principal Investigator **Dr. Anand M Ramteke**
4. ICMR letter No. and date sanctioning the project. **5/7/1501/2016-CH dated 17/05/2016;**

   R.F.C No. RCH/NER/4/2016-2017 dated 03/05/2016

5. Head of account as given in the original sanction letter

S. No.	Particulars	5th Year
1.	Staff	Rs 0.00
2.	Consumables	Rs 0.00
3.	Contingency	Rs 0.00
4.	Travel	Rs 0.00
5.	Overhead	Rs 0.00
	Grand Total:	Rs 0.00

6. Amount received during the year (Please give No. & Date of ICMR's sanction letter for the amount and period) **NIL**
7. Total amount that was available for expenditure (excluding commitments) during the year (Sl.No.6+7): **Rs 250037.00 (unspent amount of previous year)**
8. Interest earned (period is DOS i.e. 17/05/2016 to 16/05/2022) : **Rs 27802.00**
9. Actual expenditure (excluding commitments) incurred during the year. **NIL**
10. Balance amount available at the end of the year. **Rs 277839.00**
11. Amount already committed, if any: **NIL**
12. Amount to be carried forward to the next year (if applicable). Indicate the amount already committed with supporting documents. **NIL**

Ramteke

Format for Utilization Certificate
(Final)

Certified that out of **NIL** of grants-in-aid sanctioned during the year 2020-2021 in favour of **Dr. Anand M Ramteke** under ICMR **NIL** and **Rs 277839.00** on account of unspent balance of the year 2020-2021, a sum of **Rs NIL** has been utilized for the purpose of **project expenses** for which it was sanctioned and that the balance of **Rs 277839.00** remaining unutilized at the end of the year 2021-2022 is to be returned to funding agency ICMR, New Delhi.

Demand Draft No: 847021 dated 12/07/2022 in favour of Director General, ICMR

Signature of Principal Investigator with date
20/06/2022

Signature of Registrar/ of the Institute with date
Registrar
Tezpur University
28/7/22

Signature of Accounts Officer of the Institute with date
Finance Officer
Tezpur University

Annexure-3a

Check list for covering note to accompany Utilization Certificate of grant for the project for the period 17/05/2020 to 16/05/2021

1. Title of the Project **"Studies on exosomal proteome and lipidome and their clinical implication in the management of drug resistant and recurrent head and neck squamous cell carcinomas (HNSCC)"**
2. Name of the Institutions: **Tezpur University**
3. Principal Investigator **Dr. Anand M Ramteke**
4. ICMR letter No. and date sanctioning the project. **5/7/1501/2016-CH dated 17/05/2016;**

   R.F.C No. RCH/NER/4/2016-2017 dated 03/05/2016

5. Head of account as given in the original sanction letter

S. No.	Particulars	4th Year
1.	Staff	Rs 0.00
2.	Consumables	Rs 0.00
3.	Contingency	Rs 0.00
4.	Travel	Rs 0.00
5.	Overhead	Rs 0.00
	Grand Total:	Rs 0.00

6. Amount received during the year (Please give No. & Date of ICMR's sanction letter for the amount and period) **NIL**
7. Total amount that was available for expenditure (excluding commitments) during the year (Sl.No.6+7): **Rs 250037.00 (unspent amount of previous year)**
8. Actual expenditure (excluding commitments) incurred during the year. **NIL**
9. Balance amount available at the end of the year. **Rs 250037.00**
10. Amount already committed, if any: **NIL**
11. Amount to be carried forward to the next year (if applicable). Indicate the amount already committed with supporting documents. **Rs 250037.00**

Format for Utilization Certificate
(Annual)

Certified that out of **NIL** of grants-in-aid sanctioned during the year 2020-2021 in favour of **Dr. Anand Ramteke** under ICMR **NIL** and **Rs 250037.00** on account of unspent balance of the year 2019-2020, a sum of **Rs NIL** has been utilized for the purpose of **project expenses** for which it was sanctioned and that the balance of **Rs 250037.00** remaining unutilized at the end of the year is carried forward during the extension period of the project 2021-2022.

Signature of Principal Investigator with date

Signature of Registrar/ of the Institute with date
Registrar
Tezpur University

Signature of Accounts Officer of the Institute with date
Finance Officer
Tezpur University

Annexure-3

Final Statement of Accounts

(Period 17/05/2016 to 16/05/2022)

1. Sanction Letter — File No. **5/7/1501/2016-CH dated 17/05/2016;** **R.F.C No. RCH/NER/4/2016-2017 dated 03/05/2016**
2. Total Project Cost (1st, 2nd and 3rd Year) : **Rs. 1327452.00**
3. Sanction /Revised Project cost (if applicable) : NIL
4. Date of Commencement of Project : 17/05/2016
5. Proposed Date of Completion : 16/05/2022 (with extension)
6. Statement of Expenditure : From 17/05/2016 to 16/05/2022

S. No.	Sanctioned / Heads	Funds Allocated						Expenditure Incurred						Balance as on (Date 16/05/2021)
		1st Year	2nd Year	3rd Year	4th Year	5th Year	6th Year	1st Year	2nd Year	3rd Year	4th Year	5th Year	6th Year	
1.	Salaries	204480.00	174480.00	8743.00	0.00	0.00	0.00	153360.00	128368.00	72000.00	0.00	0.00	0.00	33975.00
2.	Equipments	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	0.00	Nil	Nil	Nil
3.	Consumables	350000.00	223458.00	200000.00	0.00	0.00	0.00	223458.00	435530.00	0.00	0.00	0.00	0.00	114470.00
4.	Travel	25000.00	0.00	25000.00	0.00	0.00	0.00	0.00	36542.00	0.00	58749.00	0.00	0.00	(-)45291.00
3.	Contingency	50000.00	13195.00	50000.00	0.00	0.00	0.00	13195.00	368.00	0.00	3156.00	0.00	0.00	96476.00
4.	Overhead Expenses	35224.00	31045.00	24260.00	0.00	0.00	0.00	29989.00	22675.00	0.00	0.00	0.00	0.00	37865.00
5.	Sample analysis	100000.00	62604.00	0.00	0.00	0.00	0.00	62604.00	87458.00	0.00	0.00	0.00	0.00	12542.00
6.	Interest earned	7052.00	1899.00	98.00	6251.00	6251.00	6251.00	0.00	0.00	0.00	0.00	0.00	0.00	27802.00
	Total	771756.00	506681.00	308101.00	6251.00	6251.00	6251.00	482606.00	710941.00	72000.00	61905.00	0.00	0.00	277839..00

Signature of Principal Investigator
with date 20/06/2022

Signature of Accounts Officer
With date 28/6/22
Finance Officer
Tezpur University

28/6/22
Signature of Head of the Institution
Registrar
Tezpur University

committed with supporting documents: NIL

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