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Cellular and Molecular Signature of Oral Squamous Cell Carcinoma

Thesis submitted in partial fulfilment of the requirements of the Degree of Doctor of Philosophy

by

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Declaration

I, Dr Fatima Qadir, confirm that the research included within this thesis is my own work or that where it has been carried out in collaboration with, or supported by others, that this is duly acknowledged below and my contribution indicated. Previously published material is also acknowledged below.

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Abstract

Oral squamous cell carcinoma (OSCC) is the sixth most common cancer worldwide. It is a result of numerous aetiological factors such as genetic predisposition, smoking, excessive alcohol consumption and viruses such as the human papilloma virus. Due to late diagnosis it has a high mortality and morbidity rates which has remained unchanged over the last 5 decades.

Currently no screening is available for high risk patients for better monitoring. Diagnosing OSCC relies on histopathology of biopsy tissue, reviewed for dysplasia and advancing lesions. Although the technique has been used for decades for successful diagnosis it fails to identify the molecular signature of OSCC which appears much before the visual signs. It also falls short in predicting the malignant transformation of pre-malignant oral lesions. Identifying the molecular and genetic changes leading to OSCC lesion will aid in more specific (quantitative) and early diagnosis of the disease reducing the financial burden of treating late-stage OSCC patients on the healthcare system.

This study focuses on developing new adjuncts which can be used alongside histopathology for early diagnosis. There is a need to monitor high risk patients through non-invasive methods causing less patient discomfort. We therefore explored the potentials of exosomes which are extracellular vesicles secreted by normal and tumour cells. They can be isolated from body fluids such as blood and saliva.

In cancer biology exosomes offer both diagnostic and therapeutic advantage. Their involvement in cell-cell communication indicates their influence in tumour development, progression, metastasis and therapeutic efficacy. Exosomes released by cancerous cells carry numerous biomarkers, which are passed on to healthy cells via microenvironment, causing stromal and angiogenic activation along with immune escape.

In this study exosomes were successfully isolated from body fluids (blood, saliva and plasma) and cell line supernatant through ultracentrifugation and characterised by visual and particle size quantification techniques including Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM), Zetasizer and Nanosight Tracking Analysis (NTA). Exosomal specific membrane proteins were identified through Western blotting.

We report the presence of a potential protein biomarker located exclusively on the outer membrane of cancer exosomes. Since body fluids consist of a heterogeneous population of exosomes derived from multiple cell types, such surface biomarker can potentially be used to isolate OSCC exosomes.

Characterisation of exosomal mRNA cargo was done using Agilent Bioanalyzer (for RNA quantity and quality assurance) and reverse transcription-quantitative PCR (RT-qPCR; for gene specific quantitation). Functional significance of exosomes was studied by transfecting normal oral keratinocyte cells with self and cancer-derived exosomes. Through gene-expression microarray and subsequent RT-qPCR verification, we report a panel of differentially expressed genes involved in essential cellular functions being modulated by exosome transfection.

A previously developed molecular diagnostic system by our research group called quantitative malignancy index diagnostic system (qMIDS) based on FOXM1 oncogene and its downstream targets was validated on archival formalin fixed paraffin embedded OSCC patient biopsy samples. We report that qMIDS index successfully correlates with the disease stages including dysplasia, tumour and lymph node metastasis.

Furthermore, through meta-analysis of 8 OSCC microarray studies we identified a panel of six genes including PLAU, FN1, CDCA5, CRNN, CLEC3B and DUOX1 (q6) which are able to identify two clinically distinct sub-groups of OSCC patient population. Through RT-qPCR the expression of q6 biomarkers was established in 100 OSCC biopsy samples. This information can be of immense importance in developing personalized treatment strategies based on the molecular makeup of the presenting tumour.

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Abbreviations

ACTB	β Actin
Ago2	Argonaute2
Alix	Apoptotic linked gene 2 interacting protein X
ALK	Anaplastic lymphoma Kinase
APC	Antigen presenting cells
BM	Basement membrane
CD	Cellular Debris
CDH1	Cadherin1
cDNA	Complementary DNA
CEP55	Centrosomal protein 55
DAPK1	Death associated protein kinase1
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
dNTP	Deoxynuceotide
dT/rp	Cocktail of oligo(dT) primer and random primer
EABR	ESCRT and ALIX binding region
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transformation
EMT	Epithelial to mesenchymal transition
ESCRT	Endosomal sorting complex required for transport
EV	Extracellular Vesicles
EXO	Exosomes
FCS	Foetal calf serum
FOXM1	Forkhead Box M1
HIFs	Hypoxia inducible factors
HNSSC	Head and neck squamous cell carcinoma
HRS	Hepatocyte growth factor regulated tyrosine kinases substrate
ILV	Intra luminal vesicle
MGMT	O^6-methylguanine DNA methyltransferase
MHC	Major histocompatibility complex
miRNA	Micro RNA

MMPs	Matrix metalloproteinases
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
MV	Microvesicles
MVB	Multi vesicular body
NTA	Nano particle tracking analysis
OSCC	Oral squamous cell carcinoma
PFA	Paraformaldehyde
piRNA	Piwi-interacting RNA
pRb	Retinoblastoma protein
Pre miRNA	Precursor micro RNA
RICS	RNA induced silencing complex
RNA	Ribonucleic acid
RNase	Ribonuclease
RNasin	Ribonuclease inhibitor
RT	Reverse transcription
RT-qPCR	Quantitative real time polymerase chain reaction
SEM	Scanning electron microscopy
SFM	Serum free medium
siRNA	Short interfering RNA
SNAP	Soluble NSF Attachment Protein
SNARE's	SNAP (Soluble NSF Attachment Protein) Receptor
TEM	Transmission electron microscopy
TGF-β	Transforming growth factor
TIF	Tumour invasion front
TRBP	Trans activating response RNA binding protein
TSAP6	Tumour suppressive activated pathway 6
TSG101	Tumour susceptibility gene 101
VEGF	Vascular endothelial growth factor
VPS4	Vascular protein sorting associated protein 4
WNT	Wingless homeobox genes

Chapter 1

Introduction

1.1 Head and neck squamous cell carcinoma

Office for National Statistics has reported head and neck squamous cell carcinoma (HNSCC) as the eighth most common cancer in the UK (2014). It is the fourth most common cancer in males and the 12th most common cancer in females in the UK (Office for national statistics, 2017). Historically, the male to female ratio was 3:1 which has recently changed to 2:1, due to increase in smoking by women and their long life expectancy (Joseph A. Regezi et al., 2017).

Global incidence of HNSCC is 500,000 new cases annually with 350,000 associated deaths (Marur and Forastiere, 2016). HNSCC has a high morbidity rate and can result in difficulty with eating, swallowing, and speech. It may also interfere with breathing when obstructing the airway (Li et al., 2015). In addition, treatments with chemo and radiotherapy are associated with high morbidity leading to long-term decrease in quality of life (Bar-Ad et al., 2014).

According to cancer research UK in the year 2014, 11,449 new cases of HNSCC were reported. Since 1999 to now there is a 30% increase in the rate of HNSCC incidence. The mortality rate was reported as 2,386 deaths in the year 2014, accounting to 1% of affected population. Only 19 to 59% of patients showed a 5 to 10 year survival rate, while 90 to 93% of cases were considered as preventable (Cancer research UK, 2014).

1.2 Oral squamous cell carcinoma (OSCC)

HNSCC is an aggressive and heterogeneous disease which can occur in oral cavity, pharynx, larynx and paranasal sinuses. Among different types of HNSCC, in the oral cavity 90% of cases accounts for oral squamous cell carcinoma (Brocic et al., 2009). Oral squamous cell carcinoma (OSCC) has been estimated to be the sixth most common cancer worldwide (Irani, 2016). It originates from keratinocytes of the oral cavity's stratified squamous epithelial lining. Clinically they present as non-healing ulceration due to tissue destruction, induration due to excessive keratin formation within tumour stroma, fixation of underlying tissue due to submucosal invasion and raised, everted tumour margins (Steel, 2013). It is not surprising that oral epithelial cells are vulnerable to cancerous changes, as they act as the first defensive barrier against pollutants and carcinogens.

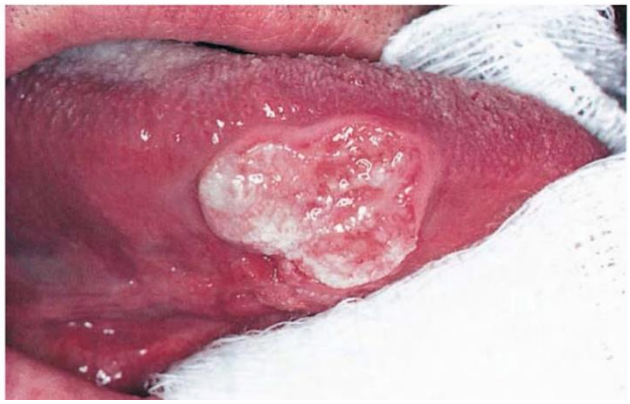


Fig 1.1: *Clinical presentation of Oral Squamous cell carcinoma lesion at the lateral border of the tongue (Brad W. Neville et al., 2003).*

Based on the evidence from multiple longitudinal studies it has been established that OSCC lesions can progress from potentially malignant lesions such as erythroplakia and less commonly leukoplakia (Warnakulasuriya et al., 2007). Developing OSCC arises from clonal expansion and aberrant growth of a single stem cell or from a few tumour initiating cells that acquire self-renewal capability. These cells escape normal growth control and gain growth advantage allowing clonal expansion which replaces normal epithelial tissue. As the lesion enlarges, additional genetic damage gives rise to sub clones resulting in an invasive cancer (Feller et al., 2013). Metastasis in OSCC is fast due to close proximity of several lymph nodes and late diagnosis, resulting in lower survival and poor prognosis.

Our study focuses on OSCC and aims to propose new aids in early diagnosis and screening leading to better patient survival. In order to achieve this aim, better understanding is required of the disease pathology and risk factors associated with it.

1.3 Aetiology of OSCC

Apart from genetic predisposition many environmental factors are associated with the development of OSCC including smoked and smokeless tobacco, alcohol, betel quid /areca nut chewing and microbes including Human Papilloma Virus (HPV), Epstein bar virus and ultraviolet light can all cause OSCC involving mucosa, lip and skin (Joseph A. Regezi et al., 2017).

Further, a compromised immune system due to diseases such as HIV and organ transplants including bone marrow and kidney also contribute to the occurrence of OSCC (Joseph A. Regezi et al., 2017).

1.31 Tobacco

Many factors are associated with developing OSCC of which the most important is tobacco smoking. The trend in the use of tobacco varies in different regions of the world. In the western countries tobacco is mostly used in the form of cigarette, cigar and pipe smoking. In some European countries tobacco is used in the form of snuff which is chewed.

In the east, especially in South Asian countries including India, Pakistan, Bangladesh and Nepal tobacco is consumed in the form of “Birhi” which is smoked without a filter and “Paan” which is a form of chewing tobacco (Scully et al., 2005). In addition to tobacco, Paan also contains betel quid which is a known carcinogen for OSCC (Chattopadhyay and Ray, 2016). It is usually placed in the buccal sulcus for longer time durations, resulting in a site specific lesion (Niaz et al., 2017).

The risk of developing OSCC due to smoking is dose dependent that is the number of packs smoked per year and the risk is reduced with smoking cessation (Morse et al., 2007). Carcinogenic substances in smoked tobacco modulate the mechanism of carcinogenesis; these cause DNA damage and P53 mutation leading to dysplasia and OSCC (Pfeifer et al., 2002).

Tobacco is known to have adverse effects on oral tissues, especially on wound healing after surgical and non-surgical procedures (Balaji, 2008). As a peripheral vasoconstrictor it causes delays in the rate of healing (Maddock and Coller, 1933). In addition combustion of tobacco produces carbon monoxide which reduces the capillary blood flow by 40% (Koszowski et al., 2015). Nicotine in tobacco increases platelet adhesiveness increasing the risk of microvascular occlusion and tissue ischemia (Al-Belasy, 2004). Smoking is also associated with catecholamine’s release, resulting in vasoconstriction and decreased tissue perfusion (McDaniel and Browning, 2014).

1.32 Alcohol

Historically alcohol was not believed to be a carcinogen itself but in combination with smoking was considered a risk factor of OSCC. Recent studies have shown that

alcohol drinking is an independent risk factor for the development of oral cancer, regardless of tobacco use (Maserejian et al., 2006). Similar to tobacco use, it has a dose dependent affect (Varoni et al., 2015). Alcohol has the ability to irritate the mucosa and to act as a solvent for carcinogens especially those in tobacco (McCullough and Farah, 2008). Alcohol also has the capacity to eliminate the lipid component of the protective barrier present in the oral cavity that surrounds the granules of the epithelial spinous layer (Squier et al., 1986). Further, contaminants and additives such as ethyl carbamate found in alcoholic drinks play a role in the development of OSCC (Lachenmeier et al., 2010). Alcoholic metabolite, acetaldehyde has been found to cause a carcinogenic risk by inducing DNA–protein crosslinks and sister chromatid changes (Zygogianni et al., 2011). It also directly interacts with DNA, producing DNA adducts, inhibits DNA repair enzymes and activates oncogenic transcription factors in oral keratinocytes (Brooks and Theruvathu, 2005).

1.33 Areca nut

Areca nut chewing is considered an independent risk factor in the development of potentially malignant oral sub-mucous fibrosis leading to oral and oesophageal SCC (Arakeri et al., 2017). The contact of its extract to oral mucosa caused erosion along with genotoxic and cytotoxic effects on oral keratinocytes and fibroblast. Areca-related oral carcinogenesis is considered mainly due to its active metabolite arecoline N-oxide (Lin et al., 2011).

The extract of areca nut causes stimulatory effects on cyclooxygenase-2 and prostaglandins, which act as inflammatory mediators during tumour initiation, development and metastasis (Jeng et al., 2000). While it inhibits the expression of tumour suppressor gene P53, impairs DNA repair and activate metalloproteinases 2, 8 and 9 accelerating tumour migration (Akhtar et al., 2012).

1.34 Microbes

The oral cavity hosts many bacterial, viral and fungal species which under physiological conditions coexist in harmony with each other and with the human immune host defence. Some of these species such as candida can be opportunistic causing fungal infections in pre-cancerous lesion (Sankari et al., 2015). It has been suggested to play a role in OSCC development by producing the carcinogen N-nitrosobenzylmethylamine causing cellular dysplasia (O'Grady and Reade, 1992).

Epstein barr virus has been linked to other HNSCC including Burkitt's lymphoma (Rowe et al., 2014) and nasopharyngeal carcinoma (Chu et al., 2008). The most widely known virus involved in OSCC is the human papilloma virus (HPV). Subtype 16 and 18 are found to be expressed in 6 to 10% of OSCC lesions while 60 to 70% of the tonsillar SCC shows positive expression of these subtypes (Golusinski, 2017). In HPV positive tumours the expression of cell cycle regulating protein P16 is found to over express and can prove as a valuable prognostic tool (Sritippho et al., 2015).

1.4 Pathogenesis

Majority of OSCC lesions are preceded by pre-cancerous lesions which are defined by the presence or absence of oral epithelial dysplasia categorised as mild, moderate, severe dysplasia and carcinoma in situ according to the presence and severity of cell atypia. This classification was designed to assist prediction of malignant transformation (Kujan et al., 2006) yet no objective method could be developed, as not all dysplastic lesions progress to OSCC. Through a binary classification of oral epithelial dysplasia, pre-cancerous lesions can be segregated into high and low risk of malignant transformation, improving sensitivity and specificity of detection (Nankivell et al., 2013)

Like most other malignancies, OSCC also arises from accumulation of genetic events leading to development of an invasive lesion. Most of these changes occur in genes which control basic cellular functions such as cell cycle, cell survival, cell motility and angiogenesis (Williams, 2000).

1.41 Oncogenes

Oncogenes are growth regulating and promoting genes that control cellular transduction pathways. A mutation in these genes leads to overproduction or an increase in the function of stimulatory proteins (Campo-Trapero et al., 2008). Numerous oncogenes have been implicated in oral carcinogenesis including epidermal growth factor receptor (EGFR) which is found to be upregulated in 40-80% of OSCC cases and has been proposed to have prognostic value implying higher likelihood of recurrence and worse survival (Grandis and Tweardy, 1993). Other oncogenes implicated in OSCC include c-myc which regulates cell proliferation, cyclin D1 which controls the cell cycle and fibroblast growth factor 3 regulating the proliferation of the stroma (Williams, 2000).

In our lab extensive amount of research has been done on FOXM1 transcription factor, regulating timely mitotic progression. Previous studies have shown that upregulation of FOXM1 (isoform B) is an early event in OSCC development where it causes genomic instability in oral keratinocytes in the form of loss of heterozygosity and copy number variations (Gemenetzidis et al., 2009). Furthermore, FOXM1 upregulation promoted expansion of progenitor keratinocyte stem cells to induce epithelial hyperplasia thereby increasing susceptibility to oncogenic transformation (Teh et al., 2010). Overexpression of FOXM1 triggers downstream targets such as centrosomal protein CEP55, essential for cytokinesis and Helicase (HELLS) involved in cellular proliferation and epigenetic regulations (Waseem et al., 2010).

1.42 Chromosomal instability

Cytogenetic analysis has shown a series of consistent chromosomal alterations in OSCC, including loss of heterozygosity at 9p21, the site of tumour suppressor genes P16 and P14ARF, an early event in 70% of OSCC lesions (Kim and Califano, 2004). P16 inhibits CDK2 and CDK6, preventing initiation of cell cycle causing arrest at G1 (el-Naggar et al., 1995). Mutations at 17p13, the site of P53 tumour suppressor gene has been noted in 79% of OSCC lesions and is considered a late event in progression of OSCC (Balz et al., 2003).

Further, amplification of 11q13 region which encodes proto-oncogene cyclin D1, functions to activate retinoblastoma protein (pRb) by phosphorylation and facilitates progression of the cell cycle from the G1 to S phase (Kim and Califano, 2004). Hence

D1 cyclin activation and P16 inactivation results in the same effect of increased cellular proliferation.

Stem cells are perfect targets for OSCC initiation due to their clonogenic potential and plasticity. A study using human organotypic 3D culture system has shown that FOXM1 oncogene (isoform B) regulates human adult epithelial stem cell renewal and differentiation (Gemenetzidis et al., 2010) and also maintains pluripotency genes such as Oct 4, Nanog and Sox2 (Xie et al., 2010). Hence, it was suggested that FOXM1 oncogene may be responsible for the replicative properties of stem cells and initiates an oncogenic process.

This indicates that tumour may begin from pre-cancer stem cells which have attained high levels of FOXM1 and give rise to progeny cancer stem cells with elevated FOXM1 expression **(Fig 1.2)**. Hence aberrant FOXM1 expression causes stem cell compartment expansion resulting in the initiation of hyperplasia.

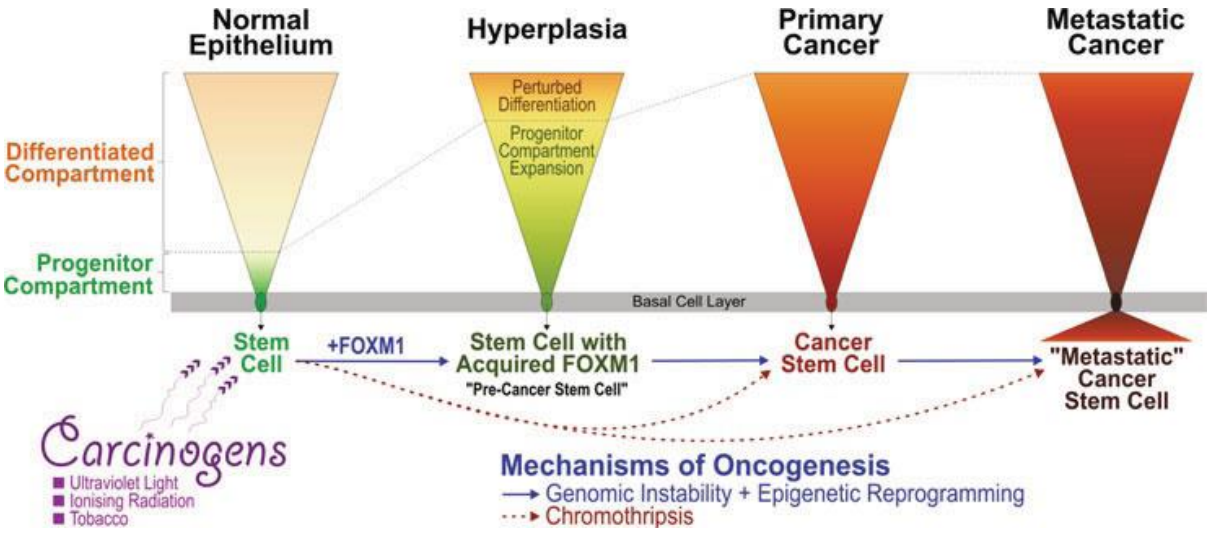


Fig 1.2 : *A model system illustrating the role of FOXM1 in human epithelial cancer initiation and progression (Teh, 2012a).*

It would be interesting to design therapeutics against cells which have abnormally high expression levels of FOXM1 and sparing cells with normal levels to avoid cell toxicity (Teh, 2012b). Due to the roles of FOXM1 in tumour initiation (Teh et al., 2010)

and progression (Gemenetzidis et al., 2009), its expression could be exploited as a biomarker for early cancer diagnosis (Teh et al., 2013).

1.43 Epigenetic alteration

These alterations affect or inactivate gene function by methylation of the promoter region, without changing the structure or sequence of the gene. Methylation is an epigenetic modification by which gene activity is controlled by addition of methyl group (CH_3) to certain DNA cytosine (Shaw, 2006). Both hypermethylation which inactivates suppressor gene and hypomethylation by inappropriate oncogene activation aids in carcinogenesis (Auerkari, 2006).

In OSCC methylation of $P16^{INK4a}$, CDH1(cadherin-1), MGMT (O^6-methylguanine-DNA methyltransferase) and DAPK1 (Death-Associated Protein Kinase 1) have been reported (Ha and Califano, 2006). Hypermethylation of cell cycle regulating gene promoter P16 was found frequently in OSCC patients, although no correlation was found with clinic pathological characteristics or with prognosis (Viswanathan et al., 2003). E-cadherin hypermethylation is associated with a more severe histological grade and poor patient survival rate (Shaw, 2006). MGMT helps in the removal of DNA adducts on the guanine base, thereby helping to maintain normal cell physiology and genomic stability (Gerson, 2004). DNA promoter hypermethylation of *MGMT* is associated with recursive mutagenesis and is a promising biomarker for OSCC prediction (Jayaprakash et al., 2017a). *DAPK1,* a mediator of ɣ – interferon induced programmed cell death, is a vital gene in signal transduction and apoptosis (Lin et al., 2010). Hypermethylation of DAPK1 has not only been associated with OSCC but has also been proposed as a potential diagnostic marker (Jayaprakash et al., 2017b).

We have previously shown that FOXM1 oncogenes regulate HELLS, a SNF/helicase involved in DNA methylation, implicating FOXM1 in epigenetic regulation (Waseem et al., 2010). Upregulation of FOXM1 supresses the expression of tumour suppressor gene $P16^{INK4A}$ through promoter hypermethylation. Through non-bias genome wide promoter methylation microarray, it was shown that inducing upregulation of FOXM1in normal oral keratinocytes causes global hypomethylation similar to that found in HNSCC (SCC15) cell line (Teh et al., 2012).

1.44 Tumour-stroma Interactions

The ability of tumour cells to disintegrate the extracellular matrix (ECM) and infiltrate surrounding tissue aids in metastasis. Matrix metalloproteinases (MMPs) play a major role in ECM degradation, leading to tumour invasion and metastasis (Sorsa et al., 2004). In OSCC increased expressions of MMP2, MMP9 and MMP3 have all been found to be related to tumour invasion, a more aggressive growth and a worse prognosis (Zhang et al., 2013). Expression of these genes could be used as tumour progression markers (Andisheh-Tadbir et al., 2016).

Over expression of VEGF (vascular endothelial growth factor) expression in OSCC was increased in insufficiently differentiated invasive carcinomas and was overexpressed in invasive oral squamous cell carcinoma but not in intraepithelial carcinoma tissues. These findings suggest that VEGF likely plays a role in angiogenesis of oral squamous cell carcinoma (Kim et al., 2015).

1.45 Tumour suppressor genes

Oncogenes alone are unable to cause cancer; the crucial event of transforming pre-malignancy into malignancy requires inactivation of negative cell regulators called tumour suppressor genes. These genes are frequently inactivated by point mutations, deletions and rearrangements in both copies of the gene (Bascones-Ilundain et al., 2008).

The most widely studied tumour suppressor genes include retinoblastoma, P53 and INK4-ARF encoding retinoblastoma protein (pRB), P53 and P16 proteins respectively (Gonzalez-Moles et al., 2007, Ha and Califano, 2006, Pande et al., 1998). These proteins regulate the cell cycle and are involved in the inhibition of defective cell proliferation. Inhibition of tumour suppressor genes and overexpression of oncogenes such as cyclin D1 increases the activity of cyclin-dependent kinases (CDK4 and CKD6) resulting in uncontrolled cellular proliferation (Gonzalez-Moles et al., 2007).

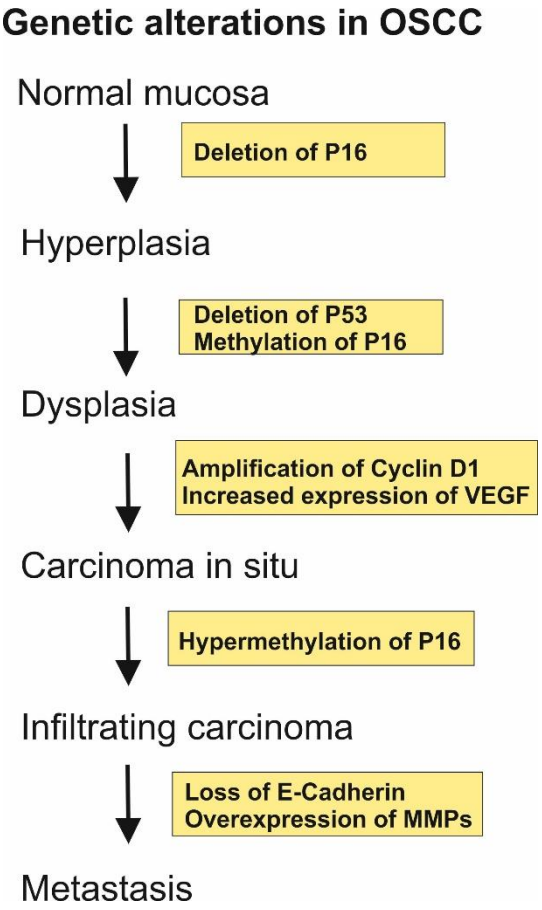


Fig 1.3: *Summary of some of the genetic changes involved in developing OSCC and its progression to a metastatic lesion.* *Modified from (Campo-Trapero et al., 2008)*

1.5 OSCC Screening

Oral cancer screening refers to detecting early signs of disease in an asymptomatic individual. OSCC screening is of immense importance for individuals predisposed to OSCC due to co-factors such as heavy smoking, alcohol consumption and genetic pre-disposition. Screening aims to segregate low risk individuals from high risk, this in turn will lead to early diagnosis resulting in favourable prognosis. A cohort study on colorectal cancer has shown that high risk patients benefitted by early diagnosis and better prognosis from screening and surveillance, while the lower risk individuals may only require just one follow-up (Atkin et al., 2017). In breast cancer it has been recommended that high risk patients expressing biomarkers BRCA1, BRCA2, ATM, CHEK2 and PALB2 should be offered breast MRI (magnetic resonance imaging) annually to facilitate early detection (Macklin et al., 2017).

In addition, reliable screening can reduce the burden of excessive cost from national health services used in the treatment of advanced lesions due to late diagnosis. These funds can be better utilized to develop personalised treatments for advanced lesion patients based on the genetic makeup of the tumour.

Currently, high risk individuals predisposed to developing OSCC are not screened. If the patient is asymptomatic no advice or screening is sought from the dentist. If the individual develops a lesion which is painless, again delays are made in making dentist appointments. According to the NICE guidelines any suspected lesions that persist for more than two weeks is referred for further investigation causing further delays.

An ideal screening adjunct should be non-invasive causing least discomfort to the patient. Here we concisely discuss a number of screening techniques and their limitation, highlighted in the literature which might be useful in OSCC patients which may or may not be symptomatic.

1.51 Toluidine blue

Toluidine blue also known as tolunium chloride is an acidophilic metachromatic dye that selectively stains acidic tissue components including nucleic acids and abnormal tissue (Sridharan and Shankar, 2012). Currently it is used by surgeons as an aid in demarcating the extent of the lesions prior to excision (Lingen et al., 2008).

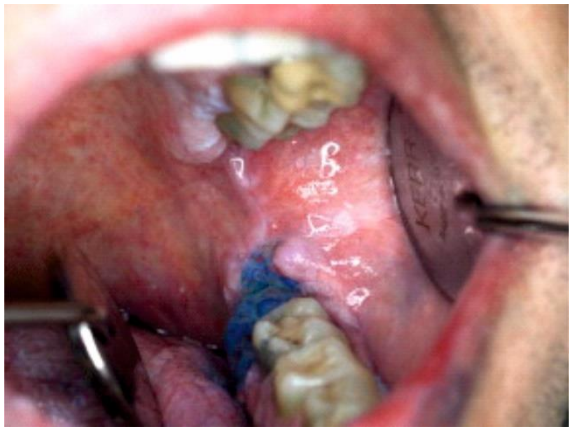


Fig 1.4: *Toluidine blue staining in oral squamous cell carcinoma. Adapted from (Seoane Leston and Diz Dios, 2010)*

The lack of use of toluidine blue as an adjunct to clinical examination for the identification of premalignant lesions is due to its higher sensitivity to detect carcinomas, than dysplasia (Seoane Leston and Diz Dios, 2010). In addition, the effectiveness of toluidine blue has only been evaluated in secondary care units on high risk population presenting a lesion visible to the naked eye (Gray et al., 2000). Hence, its efficiency to identify pre-malignancy in high risk population without presenting a lesion has not been tested.

In a study by Barrellier and colleagues on 235 patients with past history of oral cancer showed as many as 58% of patients had a false positive toluidine blue stain (Barrellier et al., 1993). Due to these limitations the use of toluidine blue as a screening adjunct is inadequate.

1.52 Light based detection systems

Tissue reflectiveness has been used for many years in diagnosing cervical and oral malignant and premalignant lesion (Lingen et al., 2008). ViziLite Plus (Epstein et al., 2008) and MicroLux (McIntosh et al., 2009) are two of the products currently available in the market, intended to enhance the identification of mucosal abnormalities.

Prior to screening, patient rinse the mouth with 1% acetic acid which helps to remove surface debris and increase the visibility of epithelial cell nuclei due to cellular dehydration. This is followed by direct visual examination under blue-white light. Normal epithelium appears lightly bluish while abnormal epithelium appears distinctly white (acetowhite) (Lingen et al., 2008).

Major limitation of this system include low specificity, expensive, the efficiency has not been tested on asymptomatic individuals in primary care setup (Seoane Leston and Diz Dios, 2010).

In addition the effectiveness of ViziLite was tested in a clinical survey of 150 patients to examine benign, premalignant and malignant lesions. It was found that 32 cases of benign leukoedema came positive (actetowhite), representing false positive. Similarly 2 of 14 cases of frictional keratosis also came positive. Further, brush biopsy of these lesions showed normal cytology (Huber et al., 2004).

1.53 Exfoliative cytology

Exfoliative cytology or brush biopsy is the study and interpretation of the characteristics of cells that flake off, naturally or artificially from the oral mucosa (Perez-Sayansm et al., 2010). It has been well established that DNA aneuploidy could predict malignancy prior to histopathology (Remmerbach et al., 2003). Exfoliative cytology is a non-invasive, easy, fast and low-cost examination for initial screening and early diagnosis of OSCC, with high sensitivity and specificity (Hashemipour et al., 2013).

Brush biopsy is beneficial for patients presenting multiple lesions in the oral cavity who are unlikely to consent for multiple scalpel biopsies. It is also useful for non-compliant patients who are unlike to come back for a follow-up (Babshet et al., 2011). Similar to other screening methods brush biopsy has been reported to overestimate dysplastic lesions and produce high number of false positive results (Bhoopathi et al., 2009, Liu et al., 2017). Ongoing research is being done to improve this method of early detection.

1.54 Saliva

Saliva is another screening tool which has gained much attention because it is non-invasive and can be collected easily and is cost effective. A recent study has shown that good quality mRNA can be extracted from saliva. This advancement will aid in identifying molecular biomarkers that appear at the initial stages of the disease. Although this study has been done on a small group of samples, further validation will be required for saliva to be used as a stable diagnostic tool (Li et al., 2004).

1.55 Blood serum

Blood serum is still being studied to be used as a successful screening tool. A metabolic profile can be obtained for early stage disease detection. A distinct signature of altered energy metabolism is found in late stage OSCC patients, including lipolysis (an accumulation of ketone bodies), a distorted Krebs cycle and amino acid catabolism. Some markers such as choline, betaine and carnitine allow differentiation between early and late stage disease (Tiziani et al., 2009).

1.6 OSCC Diagnostics

Most of the oral cancers arise as asymptomatic lesions and only when the patients or clinicians notice an abnormal growth the formal diagnostic procedures begin, resulting in late diagnosis and poor prognosis.

One of the biggest challenges in diagnosing oral cancer is the lack of patient awareness. Patients are not well informed that any oral lesion that does not heal within two weeks need to be examined by a dentist, who are trained to recognize early changes of a pre-cancerous lesion. Due to the presence of multiple lymph nodes in close vicinity to the oral cavity, delay in diagnosis and treatment often results in tumour metastasis and poor patient outcome.

Hence general public screening is of immense importance in segregating high risk population from low risk. Based not only on clinical examination but also on risk factors such as tobacco and alcohol consumption, betel nut chewing and exposure to microbes (Scully, 2008).

Currently, histopathology is the gold standard technique used for OSCC diagnosis. Histologically, the lesion passes through various phases (pre-neoplastic damage) until the ultimate establishment of cancer. This carcinogenesis may be associated with precancerous lesions such as leukoplakia and erythroplakia. However, it is necessary to consider that not all reactional lesions or potentially malignant lesions result in the subsequent development of malignant neoplasms (Ur Rahaman and Ahmed Mujib, 2014).

Most OSCC are moderately or well differentiated lesions. According to their histological appearance, lesions are classified according to epithelial changes in

which keratin pearls and individual cell keratinization are usually evident (Joseph A. Regezi et al., 2017). In addition, pre neoplastic lesions show dysplastic changes which can be mild, moderate or severe (Wang et al., 2009) prior to the establishment of an invasive carcinoma. These dysplastic changes, subsequently degrades the sub epithelial basement membrane (BM). Degradation of the BM results in local destruction and distant invasion via metastasis. Local invasion to the underlying tissue occurs via the islets and cords of epithelial cells (Fuentes et al., 2012)

Currently, two systems are used to histologically classify tumour lesions (Rivera and Venegas, 2014); the International Histological Classification of Tumours (WHO) based on the degree of tumour differentiation (well, moderately and undifferentiated) which is essential to evaluate the tumours growth rate and ability to metastasize (J.J.Pindborg et al., 1997) and the Tumour Invasion Front (TIF) which constitutes the area of the lesion with the greatest depth of invasion and progression into the surrounding tissues (Sawair et al., 2003). Since OSCC is a heterogeneous disease, cells of the TIF have different molecular characteristics when compared with the superficial cells of the tumour (Bankfalvi and Piffko, 2000).

In practice, diagnosis of OSCC is a combination of clinical and pathological information. The TNM staging system (T, tumour size; N, regional lymph node compromise; and M, metastasis) (Oliveira et al., 2008) and the degree of tumour differentiation (J.J.Pindborg et al., 1997) combined, are the predominant factors that determine the treatment strategy.

The limitation of current diagnostic system is partly responsible for poor prognosis and an unchanged 5 year survival rate of OSCC. The histopathological changes in a biopsy sample become evident when the disease is at an advanced stage. In addition the chances of misdiagnosis are considerable depending on the site from where the biopsy is taken.

Screening aids such as buccal scrapes, saliva and blood are non-invasive, easy to collect and cause lower patient discomfort. But none of these methods have been proven to give a definitive diagnosis (Scully et al., 2008). For that purpose the clinicians still rely on histopathology of the biopsied sample, however there are a number of limitations such as exceedingly time consuming, requires pathologists interpretation and early stages such as dysplasia can be undetected due to absence of histopathological changes.

Since cancer is a disease caused by genetic mutations a more sensitive and efficient diagnostic system is required. A system based on genetic biomarkers would reflect on early molecular changes not visible to the naked eye and will aid in better diagnosis and prognosis (Teh et al., 2013).

1.61 Use of Molecular Markers in Oral Cancer Detection

Although, much progress has been made in understanding the molecular changes leading to an aggressive OSCC lesion, none of these changes are given much account while diagnosing the disease due to which the molecular make-up of the tumour is much ignored. Technological advancements in genomics and proteomics have led to identification and revelation of different genomic and epigenomic alterations which form cascading pathways in the formation and progression of the tumour (De Cecco et al., 2015).

Over the last decade many molecular changes have been reported in the developing OSCC. Some of the major events include aberrant expression of P53, transforming growth factor- β (TGF-β), epidermal growth factor (EGFR) and cyclins. In addition dysregulation of certain pathways such as anaplastic lymphoma kinase (ALK), wingless homeobox genes (WNT) and mammalian target of rapamycin (mTOR) have also been reported (Su et al., 2017).

Diagnosis based on molecular signature offers advantage over conventional histopathology as it incorporates an almost complete landscape of gene expression within a tumour. Analysis of molecular biomarkers such as oestrogen and progesterone receptors and the expression of HER2 in breast tumours has led to great advancements in diagnostics leading to better prognosis through targeted therapy (De Abreu et al., 2013).

In the past our research group has translated the molecular make-up of the OSCC tumour biopsy samples into a quantitative malignancy index (qMIDs) values based of FOXM1 oncogene and its downstream targets (Teh et al., 2013). Through our current research we propose that the use to molecular biomarkers as an adjunct to histopathology will aid in detecting early molecular changes not visible to the naked eye assisting in early diagnosis. Further, this will lead to better outcomes by reducing the high mortality and morbidity load carried by OSCC patients.

1.62 Liquid biopsy

In majority of suspected OSCC patients a single site tumour biopsy is taken for histological diagnosis. The down side of such a biopsy is that tumour heterogeneity can be missed because only a small tissue sample is taken which might not be representative of the whole tumour. Evaluating and understanding the importance of heterogeneity within these tumours, may allow for better diagnostic methods and treatment protocols (Schmidt et al., 2016). Mutli-site and repeated tumour biopsies in order to assess tumour heterogeneity is not practical due to increased patient discomfort. Further, it has associated risks of complications, cost, spatial heterogeneity within the tumour and sampling bias (Visacri et al., 2015).

Comparison between single and multiple site biopsies of the same tumour tissue showed vast diversity in the tumour make-up (Zhang et al., 2016). This could explain why some cancers are resistant to treatment, or have much higher metastatic potential.

Targetable mutations may only be found in certain areas of the tumour, not assessable to the single biopsy site. Liquid biopsy based on circulating tumour extracellular vesicles such as exosomes consisting of DNA, RNA and proteins can provide an opportunity to address the issue of tumour heterogeneity. In comparison to a conventional biopsy, this would offer the advantage of identifying patients at risk of developing metastatic lesions based on molecular biomarkers. Currently, in cancers of other origins including pancreatic (Bettegowda et al., 2014), melanoma (Tsao et al., 2015), lung (Lebofsky et al., 2015), colorectal (Diehl et al., 2005), breast (Dawson et al., 2013) and prostate (Bettegowda et al., 2014) presence of circulating tumour DNA in liquid biopsies is being used as a biomarker to study tumour heterogeneity. In a cohort study of 93 OSCC patients circulating tumour DNA was detected in patient derived blood and saliva where it identified somatic mutations including TP53, PIK3CA, CDKN2A, HRAS and HPV (Wang et al., 2015). Further, assessing tumour heterogeneity will aid in tailoring personalized treatment options.

Since liquid biopsy based on blood or saliva would be a non-invasive method causing less patient discomfort, it can be used to track patient treatment outcomes and to monitor for residual disease.

1.7 OSCC prognosis

Current treatment of OSCC patients in early stages (I and II), aims for a single modality–either surgery or radiotherapy. In more advanced stages of the disease (III and IV) a combination of treatment modalities such as surgery and radiation or radiation and chemotherapy or all three are recommended (Levy et al., 2016). While for incurably advanced disease palliative chemotherapy or best support palliative care is offered (Economopoulou et al., 2015). Advanced stage cancer requires more aggressive treatment, resulting in increased morbidity and reducing the quality of life. Upscaling treatment provides no guarantee of cure (Grisanti et al., 2014). In these patients earlier diagnosis is desirable in order to reduce morbidity and likelihood of cure.

Based on the molecular changes in OSCC, studies have shown existence of sub-types among tumours. The diversity in tumours can be driven by ethnicity, exposure to different carcinogens and site of tumour development. It has been shown that members of the ras oncogene family are overexpressed in OSCC. Although loss of control of N-ras is an early step in oral carcinogenesis, with increased expression in dysplastic lesions, ras mutations are infrequent in western patients and detected in fewer than 5% of oral cancers (Williams, 2000). In contrast, 55% of lip cancers have H-ras mutation (Milasin et al., 1994), which is also present in 35% of OSCC in Asian populations associated with betel nut chewing (Kuo et al., 1994).

In OSCC till now four different groups of tumour sub-types have been identified based on their molecular signature. Chung and colleagues classified these groups as basal like (EGFR pathway signature), mesenchymal enriched type, normal epithelial subtype (high expression of cytokeratin 14) and antioxidant type expressing high levels of antioxidant enzymes (Chung et al., 2004). Unfortunately, in practice all the types are treated the same way with a combination of surgery, radiotherapy and chemotherapy. This can be one of the reasons as to why some tumours respond better to therapy while others do not. The standard treatment of all OSCC tumours is one of the major factors leading to drug and radio resistance leading to poor prognosis.

Platinum-containing chemotherapy drug such as cisplatin is the first generation anti-cancer compound used in the treatment of OSCC (Larkins et al., 2017). Treatment often fails due to rapid development of inherent and acquired chemoresistance (Wang and Lippard, 2005). A study by Long and colleagues demonstrated that increased

expression of β-catenin, an important adhesion molecule and key regulator of Wnt signalling pathway is associated with chemoresistance (Li et al., 2016b)

Radiotherapy is an inevitable component of OSCC management. The success or failure of radiotherapy is dependent on radiosensitivity of tumour cells and the limits imposed on treatment by the radiosensitivity of the surrounding normal tissue (Ishigami et al., 2007). In OSCC overexpression of COX-2 (Terakado et al.), glycerol (Imai et al., 2005b) and P53 DNA contact mutation (Yamazaki et al., 2003) has been associated to radioresistance, although the molecular mechanism is still largely unknown.

Studies have already highlighted the possible prognostic role of known biomarkers. It has been shown that tumours with nuclear P53 accumulation and loss of P16 is indicative of an aggressive disease with possible resistance to drug and radiotherapy (Shah et al., 2009).

Aims and objectives

We aim to explore different methods that can be used as adjunct to histopathology for early diagnosis and better prognosis of OSCC.

1. The aim of this thesis is to test the hypothesis that oral cancer cells secrete exosomes which can be isolated from cell culture supernatant and body fluids including saliva, blood and plasma. If this hypothesis is true a liquid biopsy based on exosomes can possibly overcome the limitation of tumour heterogeneity associated with conventional biopsy of OSCC tumours and will allow multiple biopsies to check treatment response and prognosis.

2. The second aim of this thesis is to test the hypothesis that oral cancer exosomes are taken up by normal cells and induce aberrant expression of genes vital for normal cellular function. If proven this inter cellular communication will highlight new pathways involved in the development and progression of OSCC.

3. Correlation between OSCC disease stage and a previously developed quantitative malignancy index diagnostic system (qMIDS) based on FOXM1 oncogene and its downstream targets was investigated. qMIDS formerly developed on fresh frozen OSCC biopsy tissue was validated on archival FFPE biopsy samples from OSCC patients.

4. New biomarkers were explored with the aim to differentiate between molecularly distinct sub-groups within OSCC patients. This will aid in defining molecular heterogeneity among tumour sub-types and developing personalized treatment suitable for individual patients.

Chapter 2

Methods and materials

2.1 Cell lines

Primary normal human oral keratinocyte cell lines OK113, NOK368 and NK4 were extracted from normal oral mucosa tissue donated by disease free individuals undergoing wisdom tooth extraction (kindly provided by Prof Daniela Costea, University of Bergen, Norway). Normal skin keratinocyte cell line N/TERT was derived from cells cultured from specimens of normal human epidermis (strain N). They have been immortalized by retroviral transduction of h-TERT, a telomerase catalytic subunit (Dickson et al., 2000). Normal primary cell lines were cultured in serum free medium (SFM) containing 15ng/ml of human recombinant epidermal growth factor (cat no. 10450-013), 62.5μg/ml bovine pituitary extract (cat no. 13028-014) and 1% penicillin/streptomycin (cat no. 15070-063 from Life technologies UK).

Normal fibroblast cell line NHOF-1 is derived from normal oral mucosa. They were separated from keratinocytes by treatment with 0.02% EDTA for 30s followed by addition of equal volume of PBS and vigorous mixing to promote detachment (Lim et al., 2011). Primary HNSCC derived cell lines Ca1, CaLH2, SqCC/Y1 are well characterized and have been used in multiple studies in our lab to represent disease state (Gemenetzidis et al., 2009, Teh et al., 2010). SVpgC2a is a Simian virus 40 T-antigen-immortalised human buccal keratinocyte cell line. SVpgC2a cells retain a non-tumourogenic phenotype and can be used as a model of premalignant oral epithelium (Staab et al., 2004). They express low levels of FOXM1 oncogene (Gemenetzidis et al., 2009, Teh et al., 2010). SVFN10 cell line has been transformed from SVpgC2a by nicotine exposure and FOXM1 retroviral transduction (as a fusion EGFP-FOXM1B gene) hence overexpressing high levels of FOXM1 oncogene (Gemenetzidis et al., 2009). Normal fibroblast, primary HNSCC and transformed cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% foetal bovine serum (FBS) (cat no. 02-00-850, from First Link Ltd UK) and 1% penicillin/streptomycin. All cells were cultured in a humidified incubator with 5% CO_2 and 95% atmospheric air at 37°C.

2.2 Isolation of Exosomes by Ultracentrifugation

It is critical to isolate exosomes without any contamination from non-vesicular components, including serum abundant proteins, apoptotic bodies, micro vesicles

and protein aggregates. Therefore after isolation, validation and characterisation of the purified vesicles is essential.

Exosomes can be isolated by various methods such as immune isolation and repetitive density gradient ultracentrifugation (Thery et al., 2006). Immune isolation requires the use of monoclonal antibodies bound to magnetic beads against exosome surface membrane proteins (Clayton et al., 2001). Isolation by this method has the advantage of being independent of ultracentrifugation but since not all surface markers are known this method limits the separation of the whole exosome population, only isolating a specific group.

Repetitive density gradient ultracentrifugation requires all the steps of ultracentrifugation along with an additional purification step in which a 30% sucrose gradient is used to separate the exosomes from other contaminant based on their floating density of 1.15 to 1.19 g/m (Chiou and Ansel, 2016). Apart from the excessive time required, repeated ultracentrifugation can result in the loss of a significant proportion of the total exosome yield while the effects of 30% sucrose on exosome morphology and functionality are unknown.

Differential ultracentrifugation is based on the principle of centrifugation of enriched cell culture supernatant or a body fluid sample containing exosomes at different speeds to isolate different fractions of extracellular vesicles. It is the most widely used method (Gould and Raposo, 2013) as it offers the advantage of isolating the entire exosome population irrespective of membrane surface markers from the enriched conditioned medium collected from cells and body fluids. This method was chosen based on the benefits mentioned above.

Cells were seeded in T175 flask of surface area 175 cm^2 in DMEM with 10% FCS and 1% penicillin/streptomycin. Once the flasks became 90% confluent with approximately 18 x 10^6 numbers of cells the medium was removed and the cells were washed with 1×PBS in order to remove any cell debris or remains of serum containing medium. Cells were then cultured in SFM for 3 days to allow exosome secretion into the supernatant. The conditioned SFM supernatant was collected and centrifuged in 50 mL tubes at the speed of 500×g for 10 min to remove cellular debris and apoptotic bodies. The supernatant was then centrifuged in Sorvall discovery 100SE ultra-centrifuge with a fixed angle rotor T-865 at the speed of 16500×g for 20 min to collect microvesicles. Special polycarbonate high speed centrifuge tubes from Thermo Scientific (cat. no. 314348) with screw cap lids (cat no. 314347) were used for high speed ultracentrifugation. The supernatant was filtered through a 0.22 µm filter to

remove protein and debris prior to centrifugation at 118000×g to obtain the final pellet of exosomes **(Fig 2.1)**. For every new cycle of exosome isolation, ultracentrifuge tubes and caps were cleaned with 70% ethanol and rinsed in distilled water.

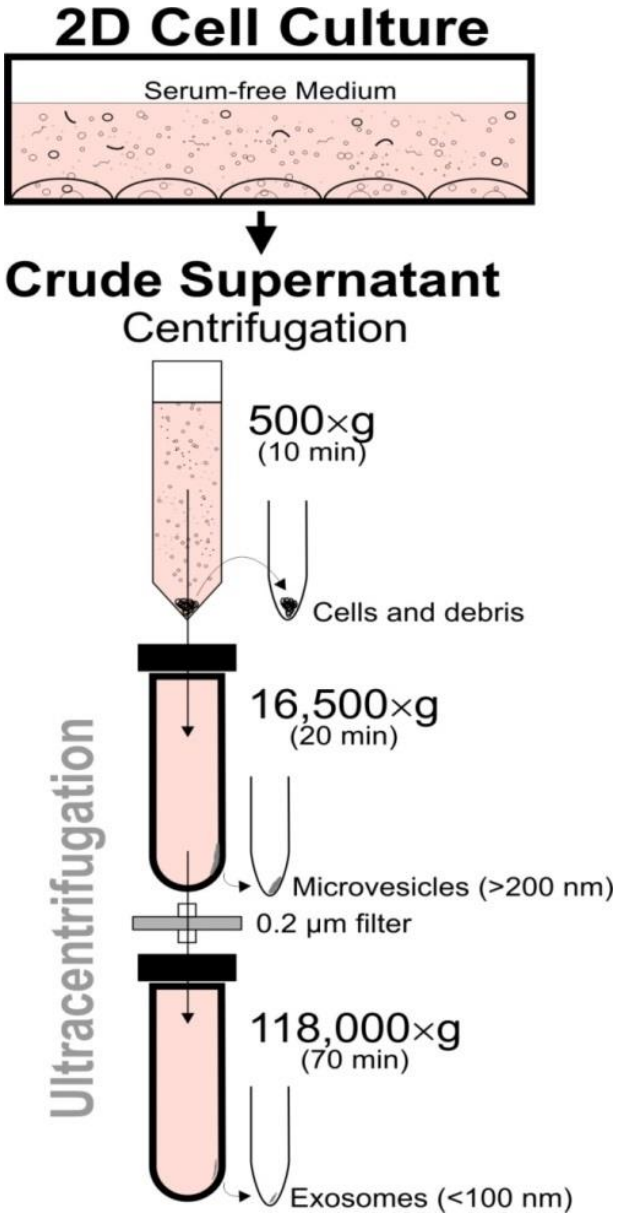


Fig 2.1: Diagrammatic representation of differential ultracentrifugation protocol for exosome isolation from cell culture supernatant. *Cells were incubated in serum free medium for 72 hrs. Different fractions of extracellular vesicles including apoptotic bodies, micro vesicles and exosomes were isolation at the speed of 500xg, 16,500xg and 118000xg respectively. Contamination by large protein aggregates was avoided by filtration through a 0.22 µm filter before isolating the final product of exosomes.*

2.3 Exo-spin™ (EXO) systems

Although ultracentrifugation is the most frequently used method for isolating exosomes, it has the disadvantage of being labour intensive and time consuming. Secondly, the small invisible pellet of exosomes obtained through ultracentrifugation can be missed while collecting the final pellet. In order to overcome these limitations, we tested a commercially available exosome isolation kit called EXO-spin™, exosome purification kit from Cell Guidance Systems (cat no. EXO1-25). The kit is based on incubation of conditioned supernatant containing exosomes with buffer A comprising ploy-ethylene glycol (Lane et al., 2015) followed by column purification.

Conditioned medium was collected from SVpgC2a and SVFN10 cell lines and centrifuged at 300×g for 10 min, followed by 20,000×g for 30 min to remove cells and debris. Supernatant was transferred to a new centrifuge tube and incubated with half a volume of buffer A and mixed well. The samples were incubated overnight at 4°C, followed by centrifugation at 20,000×g for one hour. The pellet obtained was suspended in 100 µl of 1xPBS. The spin column containing PBS was prepared by removing outlet plug and centrifuged at 50×g for 30s. This allowed the PBS to enter the column bed. Another 200 µl of PBS was added and spun down at 50×g for 30s to equilibrate the column. Exosome pellet suspended in 100 µl of 1xPBS was added to the column and centrifuge at 50×g for 30s, elute was discarded. 200 µl of 1xPBS was added and the column was centrifuged at 50×g for 1min to elute 200 µl of purified exosomes. The use of this kit was further discontinued due to lower particle/ml concentration of exosomes. In addition the exosomes were larger in size compared to isolation through ultracentrifugation. This might be the result of merging of outer lipid bi-layer of exosomes **(Appendix Fig: 8.1, 8.2, Page: 189, 190)**.

2.4 Isolation of exosomes from blood by ultracentrifugation

Isolation of exosomes from body fluids requires dilution of samples to a workable viscosity along with extended duration of centrifuge with additional filtration and washing (Thery et al., 2006).

Before the start of this study whole blood was collected from cancer patients and frozen down for future experiments. For exosome isolation the samples were defrosted and diluted to a workable viscosity. To facilitate ease of handling, 1.5 ml of

whole blood was diluted in 18.5 ml of 1xPBS. The sample was centrifuged at 2000×g for 10 min to get rid of cellular debris. It was further transferred into clean ultracentrifuge tubes and centrifuged at 20,000×g for 40 min to isolate micro vesicles. The diluted whole blood was filtered through 0.22 µm filter and centrifuged again at 118,000×g for 70 min. The exosome pellet was suspended in 20 ml of 1×PBS and was filtered again through 0.22 µm filter and centrifuged again at 118,000×g for 70 min for a final wash **(Fig 2.2)**. The final pellet of exosomes was verified by NanoSight tracking analysis (NTA, section 2.10 below).

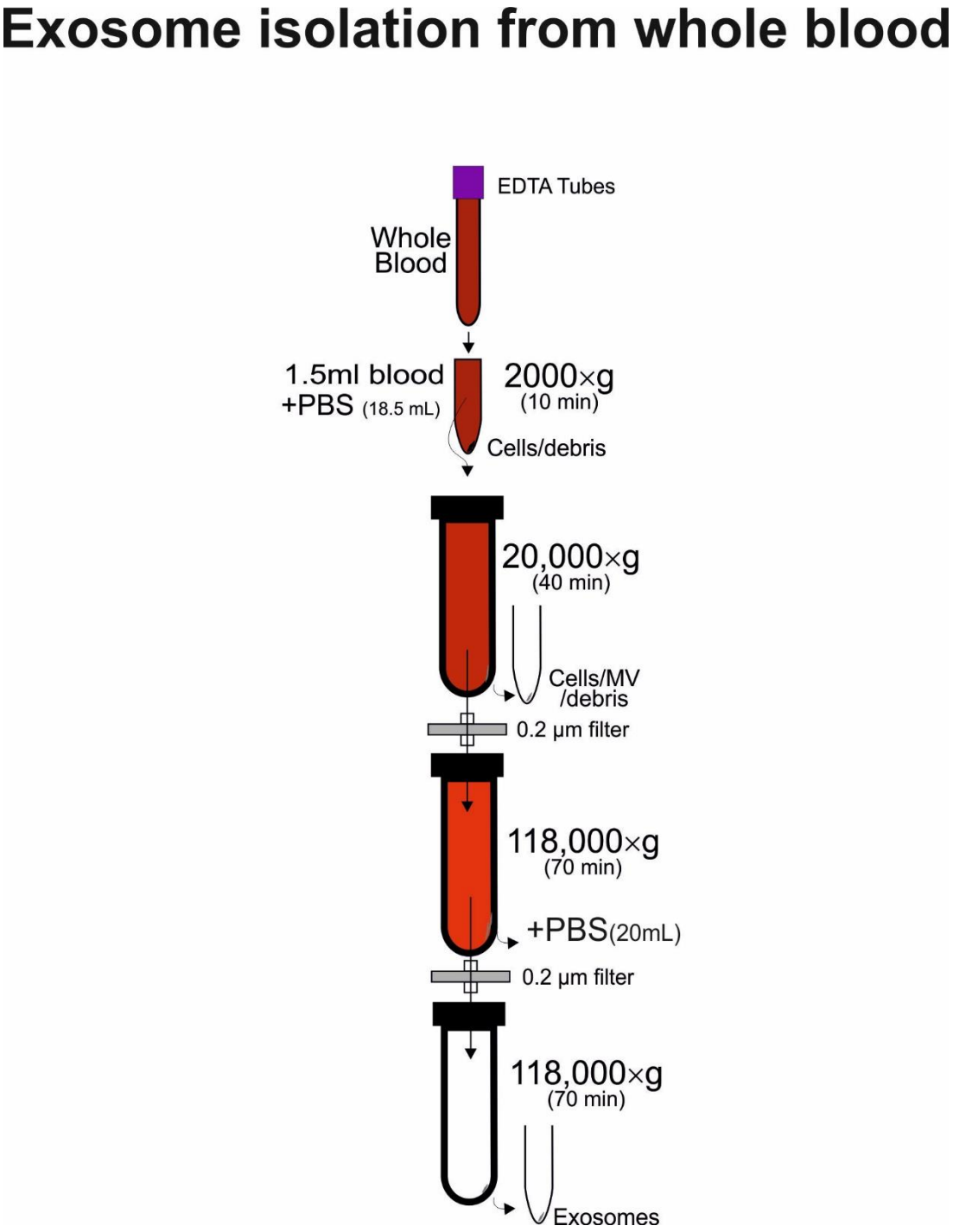


Fig 2.2: Graphic illustration of exosome isolation from whole blood through differential ultracentrifugation. *Frozen whole blood was defrosted and diluted in 1xPBS to a workable viscosity and centrifuged at 2000xg for 10 min to exclude cellular debris. Supernatant was further centrifuged at 20,000xg for 40 min to separate micro vesicles. In contrast to the ultracentrifugation protocol for cell culture supernatant, whole blood exosome isolation needed addition filtration through a 0.22 µm filter and washing for 70 min.*

2.5 Isolation of exosomes from Saliva by ultracentrifugation

Similar to whole blood, saliva sample was also diluted to a workable viscosity by N-Acetylcysteine (NaC), a drug derived from amino acid L-cysteine. In the past it has been used for effectively diluting patient saliva samples (Ericson et al., 1988) and in the treatment of Sjogren's syndrome (Walters et al., 1986).

Healthy volunteers were used to collect 4 ml of saliva in which 8 ml of NaC (5mM) was added to decrease viscosity. The suspension was centrifuged at 2000×g at 4˚C for 10 min to get a pellet of cellular debris. Carefully without disturbing the pellet, the supernatant was taken out and diluted with 8 ml of 1xPBS. The sample was transferred to clean ultracentrifuge tubes and centrifuged at 20,000×g for 40min. It was further filtered through 0.22 µm filter and centrifuged at 118,000×g for 70 min. The pellets were suspended in 1 ml of 1xPBS and pooled together into one tube. To attain the final volume of 20ml the tube was topped up with 18 ml of 1xPBS. The sample was filtered once gain through 0.22 µm filter and centrifuged at 118,000×g **(Fig 2.3)**. The final pellet was suspended in 500 µl of distilled H_2O and used for NTA.

Attempt was made to isolated exosomes from 500 µl of cancer patient saliva but the starting volume was very low for the exosomes to be detected by NTA.

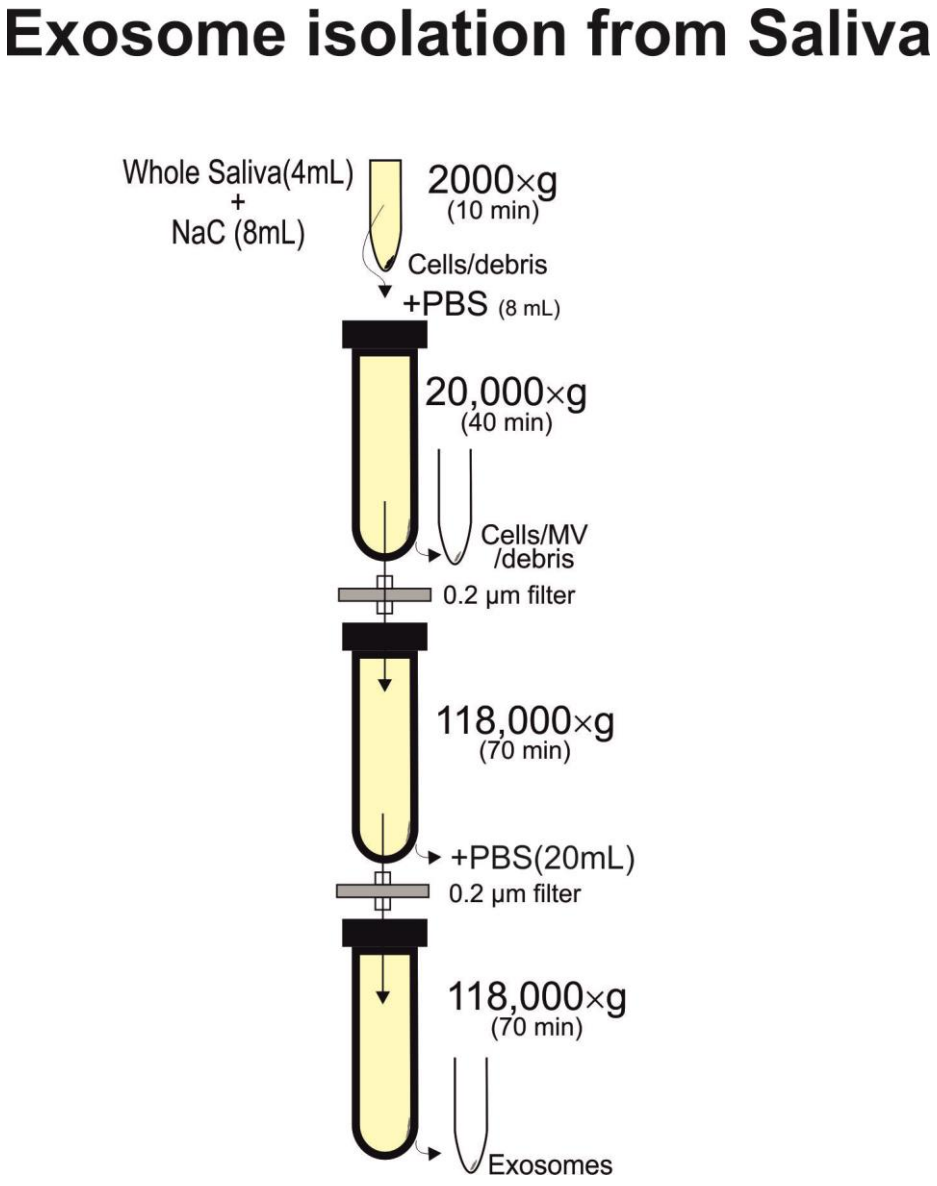


Fig 2.3: Graphic illustration of exosome isolation from normal saliva sample through differential ultracentrifugation. *Fresh saliva was diluted to a workable viscosity by addition of N-acetylcysteine (NaC) dissolved in double distilled water. The sample was centrifuged at 2000xg for 10 min to exclude cellular debris. Microvesicles were separated at 20,000xg for 40min. Followed by size exclusion filtration with a 0.22 µm filter and centrifuged at 118,000xg for 70 min. The final pellet of exosomes was washed in 1xPBS at 118,000xg for 70 min.*

2.6 Isolation of exosomes from plasma

Healthy volunteer's plasma was used for optimisation of exosome isolation protocol. Unfortunately for this study fresh blood from cancer patients was not available for separation of plasma.

Healthy volunteer's whole blood was collected in EDTA treated blood collection tubes. Whole blood (20 ml) was diluted in 10 ml of RPMI medium to obtain a workable viscosity. Plasma was separated from whole blood by adding 15 ml of Ficoll^{TM} in a 50 ml tube, to which 15 ml of diluted blood was added. The tubes were centrifuged at 650×g at 4°C for 20 min resulting in separation of plasma from red blood cells and the buffy coat containing white blood cells. Plasma was carefully collected from the top layer.

Plasma (2ml) was diluted in 18 ml of PBS in a 50 ml tube and centrifuged for 30 min at 2000×g at 4°C to exclude cellular debris. The supernatant was carefully transferred into ultracentrifuge tubes without disturbing the pellet and centrifuged at 20,000×g for 40 min to isolate microvesicles. The supernatant was filtered through 0.22 µm filter and centrifuged at 118,000×g for 70 min to obtain a pellet of exosomes. The exosome pellet was suspended in 20 ml of 1×PBS and was filtered again through 0.22 µm filter and centrifuged at 118,000×g for 70 min for a final wash **(Fig 2.4)**. The final pellet obtained was used in downstream experiments including NTA and immune TEM.

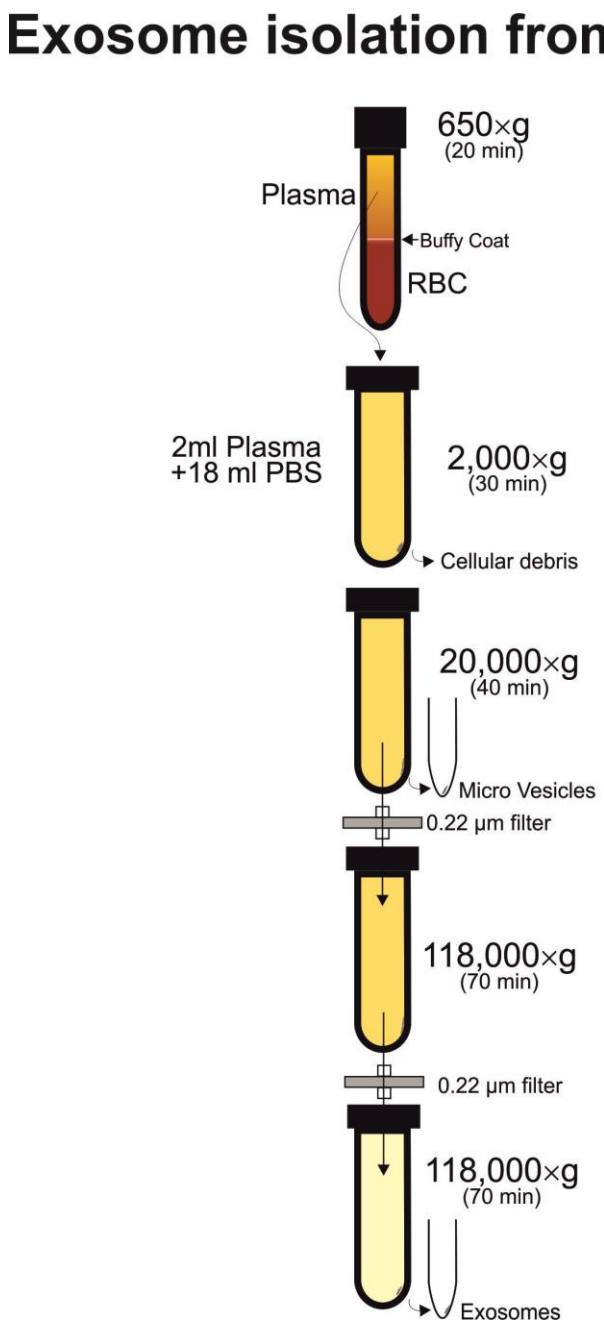


Fig 2.4: Diagrammatic representation of exosome isolation from plasma. *Whole blood was carefully poured on top of a Ficoll layer in a centrifuge tube and centrifuged at a slow speed of 650xg for 20 min; separating plasma from red blood cells and peripheral mononuclear cells. It was further diluted and spun at 2000xg and 20,000xg to segregate cellular debris and micro vesicles respectively. Size exclusion of bigger particles was achieved by filtration through 0.22 µm filter. Diluted plasma was centrifuged at 118,000xg for exosome isolation. Exosome pellet was further washed in 1xPBS, filtered through 0.22 µm filter and centrifuged at 118,000xg for 70 min to ensure a purified pellet of exosome.*

2.7 Scanning Electron Microscopy (SEM)

Given the nano-size of exosomes, they are not visible under a conventional light or confocal microscope as these apparatuses are not able to resolve below 20 µm (Alberts B et al., 2002). Hence, visualizing exosomes after ultracentrifugation is important to confirm successful isolation and characterization of exosomes.

SEM produces images of a sample by scanning it with a focused beam of electrons. The electrons interact with atoms in the sample, producing signals that can be detected and contain information about the sample's surface topography and composition. Biological specimen normally requires being completely dry, as the specimen chamber is maintained at high vacuum.

To facilitate fixation of exosome pellet, along with 100 µl of spun supernatant used as a control was suspended in 90 µl of 1xPBS and 10 µl of 25% glutaraldehyde and incubated for one hour prior to a wash step by ultracentrifugation at 118000×g for 90 min with 20 ml of 1xPBS. The fixed pellets obtained were suspended in 100 µl of 1xPBS. In order to aid attachment, 13 mm coverslips were incubated for 30-45 min at room temperature with 100 µl of fibronectin (0.5 mg/ml) suspended in medium. Excess was removed with an aspirator. Without allowing the coverslips to dry, the samples were placed in an incubator overnight at 37°C. Further, the coverslips were dehydrated by washing in ascending series of ethanol dilutions starting with 30%, 50%, 70%, 80%, 90% and 95% for 5 min, followed by two 5 min washes in 100% ethanol. The sample were chemically dried in 100% HMDS (Hexamethyldisilazane) for 3 min. Excess was removed and the samples were placed at 37°C for 30 min.

Double-sided adhesive carbon conductive tape was used to secure the samples onto the SEM stubs. A carbon conducting cement was used to aid a conducting pathway between the stub and the coverslips. The cement was allowed to dry for 24 hours.

The samples were analysed with both gold and carbon coating. Scanning electron microscope FEI Inspect F was used at 0.5 to 30 kV to study the samples. Higher kV was required for lower magnification. The xT microscope control software was used to control the operation of the microscope while the image capturing software was used to obtain images. SEM was supervised by Dr Russell Bailey at the Nano Vision centre, QMUL.

2.8 Transmission Electron Microscopy (TEM)

Although, SEM is a standard technique used to visualize exosomes, the phenomena of agglomeration is commonly observed (Sokolova et al., 2011). This hinders in observing the individual morphology of the exosomes. In order to characterize exosomes through morphological features previously reported in the literature (Thery et al., 2006), TEM was performed to obtain better resolution of exosomes.

TEM operates on the same basic principles as the light microscope but uses electrons instead of light. Due to smaller wavelength of electrons in comparison to light, optimal resolution attainable for TEM images is many orders of magnitude better than that from a light microscope. Thus, TEM can reveal the finest details of internal structure. A high energy beam of electrons passes through a very thin layer of sample, and the interactions between the electrons and the atoms can be used to observe different features of the sample such as surface membrane morphology.

In order to visualize exosomes using TEM, final pellet of SVpgC2a and SVFN10 exosome along with 100 µl of filtered supernatant (used as a control) was suspended in 100 µl of 4% paraformaldehyde for 10 min. Resuspended pellet (5µl) was placed on carbon coated EM grids (Catalogue no. S160-4 carbon film, 400 Mesh Cu by Agar scientific) for 20 min in dry environment.

A drop of 100 µl of 1xPBS on parafilm sheet was used to wash the grid (membrane side down). The grids were transferred to a 50 µl drop of 2.5% gluteraldehyde for 10 min, followed by eight washes in 100 µl of distilled water (Thery et al., 2006). The samples were stained by 0.4% lead citrate for 1 min. Stained grids were washed twenty times with distilled water and air dried on filter paper (Venable and Coggeshall, 1965). TEM was supervised by Dr Russell Bailey from Nano Vision centre QMUL.

2.9 Particle size verification by Zetasizer

Imaging techniques such as SEM and TEM are costly, time consuming and require fixation of exosomes making them unusable for further experiments. Particle size measurement through Zetasizer does not require fixation and hence the samples can be preserved.

Zetasizer accurately estimates particle size by measuring the Brownian motion of particles in a sample using Dynamic Light Scattering. Conditioned medium (80 ml)

was collected from SVpgC2a and SVFN10 cell lines and centrifuged at required speed to collect fractions of cellular debris (CD), micro vesicles (MV), supernatant (from final spin) and exosomes.
Pellet of CD, MV and exosome were suspended in 1ml of distilled water and 1 ml of supernatant was collected as a control after the final centrifuge. The samples were transferred to disposable transparent cuvettes and read on the Zetasizer Nano-ZS Malvern instrument (Model: ZEN3600, Serial no. MAL500457, Software: Zetasizer) to measure the particle size in each fraction. All samples were read at 4°C and particle size was measured as intensity percent with respect to diameter in nanometre (nm).

2.10 Particle size verification by NanoSight

Although Zetasizer is informative of particle size, it does not provide a visual output and a particle/ml concentration. Hence, variation in size measurements due to formation of aggregates cannot be identified. To overcome this limitation of Zetasizer, exosomes were analysed using Nanosight Tracking Analysis (NTA) LM10 instrument by Malvern.

NTA is a unique method of visualizing and analysing particles in liquid suspension with or without fluorescent labels, it can measure the size of particles ranging from 10-2000 nm. The particles are observed under Brownian motion; the rate of movement is related only to the viscosity of the liquid, the temperature and size of the particle and is not influenced by particle density or refractive index.

The particles contained in the sample are visualized by virtue of the light they scatter when illuminated by laser light. The light scattered by the particles is captured using a scientific digital camera with 20x magnification. The motion of each particle is tracked from frame to frame by the specially developed software. Also, since video clips form the basis of the analysis, accurate characterisation of real time events such as aggregation and dissolution of particles was possible.

Conditioned supernatant from cell lines NK4, OK113, NOK368, Ca1, CaLH2, SqCC/Y1, SVpgC2a and SVFN10 were centrifuged to obtain the pellet of exosomes and filtered supernatant collected after final centrifugation at 118000×g for 70 min. Exosome pellets were suspended in 500 µl of RNA free water. Sterile 1 ml syringe was used to load 200 – 300 µl of the sample on NanoSight LM10 sample chamber. All measurements were performed at room temperature. For capturing and analysing

the data NTA 2.0 software was used. The samples were observed for 60s with manual shutter and gain adjustments. Particle size verification of exosomes by NTA LM10 instrument was carried out in School of Pharmacy, University College London.

2.11 Western blotting

For further characterisation of exosomes from cell lines NOK368, NK4, OK113, Ca1, SqCC/Y1, CaLH2, SVpgC2a and SVFN10 western blotting for exosome specific proteins was done. Exosomal pellet and parental cells were lysed in sample buffer (4% SDS, 20% glycerol and 0.125M Tris HCL at pH 6.8). Samples were heated at 95°C for 5 min to denature the protein. Total protein concentration was measured using a BSA standard and the *DC*™ Protein Assay (Bio-Rad, Hamel Hampstead, UK). The total protein was analysed using 10% 2-merceptoethanol and 0.004% bromophenol blue to the lysate and separated by SDS-PAGE (NuPage Novex 10% bis tris protein gels, 1.0mm, 10 wells). For both gel electrophoresis and the transfer, Novex mini cell system was used (Invitrogen, Paisley, UK). The membranes were blocked with 5% fat-free milk in TBS-T buffer for 1 hour to avoid non-specific binding and washed 3 times for 10 min each with TBS-T (20 mM Trizma base, 150mM NaCl, 1% Tween 20). Membranes were than probed with primary antibody (diluted in blocking buffer) overnight at 4°C and washed with TBS-T to prepare for incubation with secondary antibody (diluted in blocking buffer) for 1 hour at room temperature **(Table 2.1 and 2.2)** . Membranes were washed again with TBS-T. Proteins were detected with Amersham ECL prime western blotting detection reagent (GE healthcare life sciences, Little Chalfont, UK). Peroxidase activity was measured in the dark room with autoradiography film (Amersham Hyperfilm ELC, GE Healthcare, Little Chalfont Bucks, UK)

Table 2.1: Primary Antibodies used for western blotting

Antigen	Type	Host	Catalogue no.	Dilution
Alix(3A9)	Monoclonal	Mouse	mAb#2171, Cell Signaling	1:1000
CD9	Monoclonal	Rabbit	ab92726, Abcam	1:200
CD63 (H-193)	Polyclonal	Rabbit	sc-15363, Santa Cruz	1:1000
CEP55	Monoclonal	Rabbit	ab170414, Abcam	1:10000
Calnexin	Polyclonal	Rabbit	ab22595, Abcam	1:1000
Glypican 1	Polyclonal	Rabbit	ab55971, Abcam	1:500
FOXM1	Polyclonal	Rabbit	sc-502, Santa Cruz	1:500
GAPDH	Monoclonal	Mouse	ab8245, Abcam	1:10000
HSC70	Monoclonal	Mouse	sc-7298, Santa Cruz	1:10000

Table 2.2: Secondary Antibodies used for western blotting

Antibody	Type	Host	Catalogue no.	Dilution
Anti rabbit	Polyclonal	Goat	AP#132P, Millipore	1:1000
Anti mouse	Polyclonal	Goat	A0168, Sigma	1:10000

2.12 Gene Knockdown by siRNA

Result of gel electrophoresis showed that anti-CEP55 antibody produced two bands very close to the target molecular weight of 55 kDa **(Fig:3.8, Page no. 95)**. In order to identify the true band and check the specificity of the antibody siCEP55 transfection was done to knockdown the protein from SVFN10 cell line.

SVFN10 cells were seeded in the density of 3×10^5 in three wells of diameter 34.8 mm. The cells were incubated overnight in DMEM medium containing foetal calf serum (FCS), excluding antibiotics at 37˚C in 5% CO_2.

For transfection, 5 µM siRNA (CEP55) solution was prepared in RNase-free solution. In a 1.5 ml Eppendorf (Tube 1) 10 µL of 5 µM siCEP55 solution was added along with 190 µL of serum free medium (DMEM without FCS). In another 1.5 ml Eppendorf (Tube 2) 194 µL of serum free medium (DMEM without FCS) was added to 6 µL of DharmaFECT. The contents of both the tubes were mixed by pipetted up and down and incubated for 5 min at room temperature. Further, the contents of tube 1 were added to tube 2 and mixed by pipetting and incubated for 20 min at room temperature.

Antibiotic free medium (1.6 ml) was added to the mix to make up the final volume of 2 ml **(Table: 2.3)**.

The culture medium from SVFN10 cells was removed and the mixture containing the content of tubes 1 and 2, and antibiotic free medium, was added to the cells. The cells were incubated at 37˚C with 5% CO_2 overnight for 48 hours. The knockdown of CEP55 mRNA and protein were analysed by RT-qPCR and western blotting, respectively.

A similar method was used for control sample which was SVFN10 cells transfected with siCtrl solution. Untreated SVFN10 cells were used as an additional control.

Table 2.3: Experimental design of siCEP55 transfection in SVFN10 cells.

Sample	Tube 1		Tube 2		
	5μM siRNA stock	Serum-free medium	DharmaFECT 1 Reagent	Serum free medium	Complete medium
Targeted siRNA	10μL	190μl	6μl	194μl	1600μl
siCTRL	10μL	190μl	6μl	194μl	1600μl
Untreated		200μl		200μl	1600μl

2.13 Immuno-gold labelling of exosomes

Exosomes were immuno-labelled with antibody against protein CEP55 to investigate its presence at the membrane and to compare the level of expression in normal and cancer exosomes.

Exosomes were isolated by ultracentrifugation **(Section 2.2, page no. 40)** from normal oral keratinocyte cell line OK113, tumour derived cell lines SqCC/Y1 and normal plasma. The exosome pellet was fixed in 100 µl of 4% Paraformaldehyde (PFA) from which 5 µl was placed on carbon coated electron microscopy grids. The grids were covered and left for 20 min to facilitate adsorption. Further, the grids were washed in 100 µL drops of 1×PBS and transferred in PBS/50mM ammonium chloride (NH_4Cl) for 3 min. The grids were transferred to blocking buffer (10% foetal calf serum) for 10 min followed by a transfer to 5 µl drops of CEP55 antibody in the dilution of 1:50 in blocking buffer for 30min. The grids were washed multiple times in washing buffer for 3 min. The grids were incubated with secondary (bridging) antibody diluted

in blocking buffer for 30 min and transferred to 100 µl drops of PBS/0.5% BSA and washed 3 times.

Further, the exosomes were incubated in 5 µl drops of protein A-gold conjugates diluted in blocking buffer for 20 min followed by multiple washes (7 times) in 1×PBS. The grids were transferred to 50 µL drops of 1% glutaraldehyde for 5 min to stabilize immunoreaction. The grids were washed multiple times (7 times) in 100 µl drops of double distilled water, each time for 2 min. The samples were contrasted using 4% uranyl oxalate at pH 7 for 5 min.

Immuno-gold labelling was done by Dr Giulia Mastroianni (TEM Facility Manager) at QMUL, following the protocol published in "Isolation and characterization of exosomes from cell culture supernatant and biological fluids." (Thery et al., 2006). The CEP55 primary antibody used was the same as used for western blotting **(Table 2.1)**.

2.14 Detection of fluorescent labelled exosomes by NTA

The NTA instrument NS500 has the ability to operate in fluorescence mode with a range of excitation wavelengths allowing visualization, sizing and concentration measurement of fluorescently labelled exosomes. This capability of NTA was used to quantify the presence of CEP55 on the membranes of exosomes derived from normal oral keratinocyte cell line OK113, tumour derived cell line CaLH2 and transformed malignant cell line SVFN10. ALIX an exosomal specific surface protein (Henne et al., 2011) was used as a positive control for exosomes.

The exosomes pallets were suspended in 110 µl of 1xPBS and divided in to two equal halves of 50 µl. Each half of the sample was incubated with 1 µl of ALIX and CEP55 antibody **(Table 2.1)** respectively. After an overnight incubation with primary antibody 0.5 µl of secondary antibody goat anti-mouse Alexa Fluor 488 (catalogue no. A-11029, Life technologies) and goat anti-rabbit (catalogue no. A-11070, Life technologies) were added to the samples.

At the time this study was conducted NTA instrument NS500 was not available in Queen Mary University hence our prepared samples were shipped to Malvern Company for analysis.

2.15 Exosomal RNA Cargo

To investigate the presence of RNA within exosomes, the final exosome pellet was subjected to treatment with RNase A, Proteinase K and triton X. The treatment was designed to eliminate contaminating proteins and RNA that spins down with the exosomes during ultracentrifugation.

Exosome pellet was collected from SVpgC2a and SVFN10 cell lines. Each pellet was dissolved in 260 µl of RNA free water and divided into five equal parts of 50 µl each. Exosomes treated with RNase free water and only RNase A (0.6 µg/ml) was used as controls. Rest of the samples were treated with proteinase K (1U/ml), triton X (2%) and a combination of two followed by RNase A treatment **(Fig 2.5)**.
Treatment with proteinase K required the samples to be incubated at 55°C for 10 min in order to activate proteinase K, followed by 5 min incubation at 95°C for deactivation. Further, RNase A was added to all the samples except the RNase free water treated controls and incubated for 30 min at 37°C **(Fig 2.5)**. Total RNA was extracted using RNeasy micro kit (74004) from Qiagen, followed by verification through Agilent BioAnalyzer and RT-qPCR.

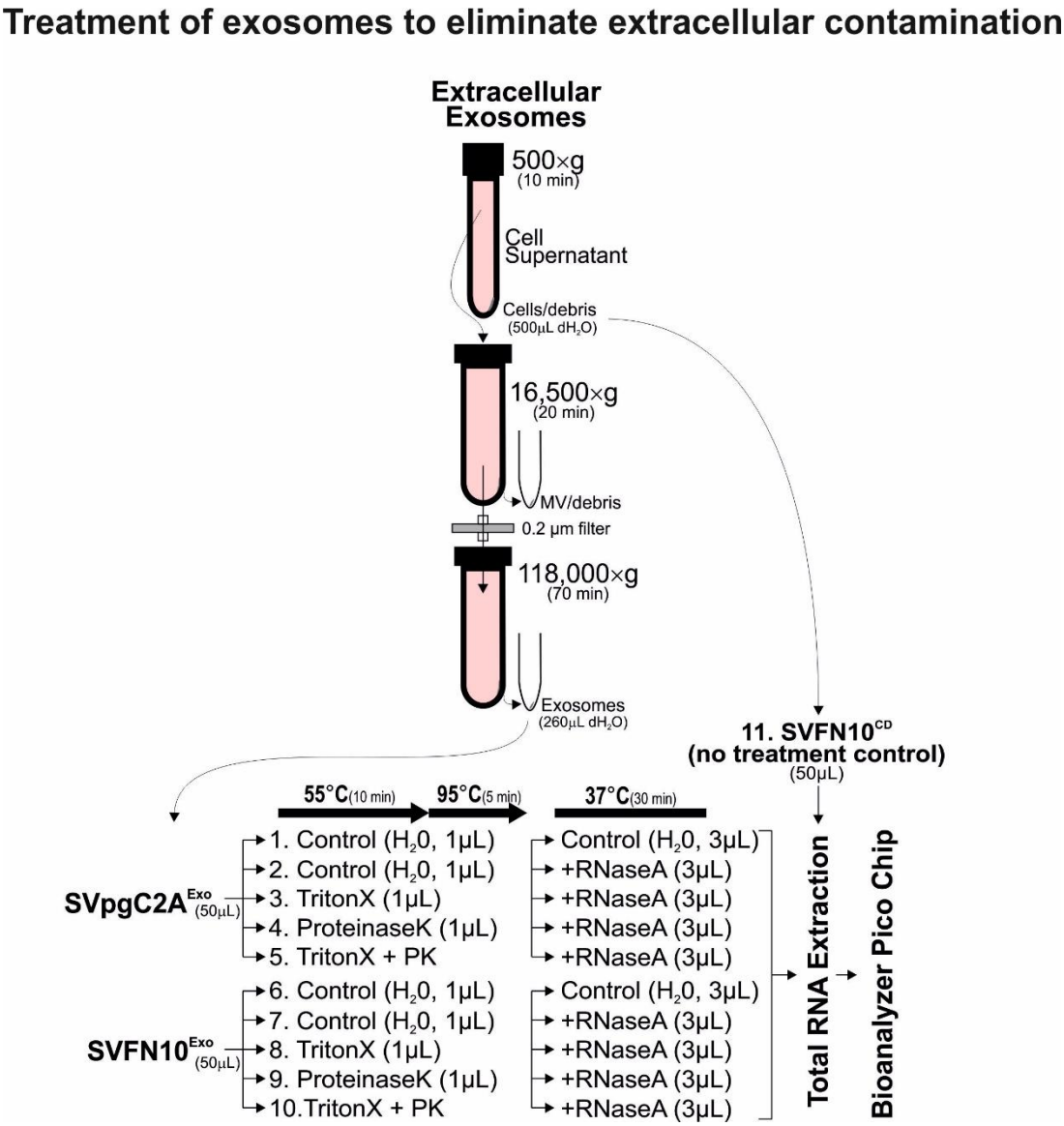


Fig 2.5: *Diagrammatic representation of exosomes treated with proteinase K (1U/ml), triton X (2%) and RNase A (0.6 µg/ml) to eliminate extracellular RNA contamination that can settle with exosomes during isolation through ultracentrifugation.* *Cellular debris from SVFN10 cells, SVpgC2a and SVFN10 exosomes treated with RNase free water and only RNase A were used as controls.*

2.16 Total RNA extraction from exosomes and cell lines

RNeasy Micro Kit (74004, Qiagen) was used to extract total RNA from treated exosomes and cell lines. The kit is based on selective binding properties of silica based membrane to RNA in combination with high speed micro spin technology. Lysis buffer (RLT) containing guanidine thiocyanate and ethanol, added to the samples create conditions that promote selective binding of RNA to RNeasy Mini Elute spin columns

Due to the small size of exosomes, they are known to contain short fragments of RNA measuring less than 120 nucleotides. For isolation of short fragments 1 volume of 100% ethanol was added to lysed exosomes in RLT lysis buffer as suggested in literature (Valadi et al., 2007). The samples were centrifuged in RNeasy MinElute spin columns for 15s at >8000xg and the flow-through were discarded.

Further according to manufacturer's protocol 1 volume of 70% ethanol was added to the spin columns and spun for 15s at >8000xg and the flow-through were discarded. Buffer RW1 (350μl) was added to the spin column and centrifuged for 15s at >8000xg again the flow through was discarded.

Any possible DNA contamination was removed by treatment with DNase1 and the samples were washed again in RW1 buffer. Buffer RPE (500 μl) containing ethanol was added to the spin columns and centrifuged for 15s at >8000xg. Further 500μl of 80% ethanol was added to the spin columns and centrifuged for 2 min at >8000xg again the flow-through was discarded. The membranes of the spin columns were made dry by opening the lids of the spin columns and centrifugation at full speed for 5 min. High quality of total RNA was eluted in 15 μl of RNase free water and centrifuged at full speed for 1 min to collect 13 μl of total RNA.

Reverse transcription reaction was run to convert total RNA to cDNA. To every 13 μl of total RNA 7.5 μl of cDNA synthesis mix was added containing: 5×Buffer (4.0 μl), dNTP (2.0 μl), dT/rP (0.8 μl), RNasin (0.4 μl), RT enzyme (0.4 μl). Samples were placed in a thermocycler at 42°C for 30min followed by 85°C for 5 min, cDNA was stored at 4°C.

2.17 Agilent BioAnalyzer

Agilent RNA 6000 pico kit (catalogue no. 5067-1513) containing chips and reagents were used to analyse total RNA quality of treated exosomes. Each RNA chip contains an interconnected set of micro-channels that are used for separation of nucleic acid fragments based on their size as they are driven through electrophoresis. They are designed to be used with Agilent 2100 BioAnalyzer instrument. One chip can analyse 11 samples and require 1 µl of sample volume. A gel-dye mixture was prepared by adding 1 µl of dye in 65 µl of filtered gel. It was vortexed and spun at 13000×g for 10 min at room temperature to ensure a homogenous solution.

Total RNA (1 µl) extracted from treated SVpgC2a and SVFN10 exosomes **(Fig 2.5)** along with the gel-dye mix was added to the allocated well and the chip was pressure primed by using a priming station connected to a 1ml syringe. The plunger was pressed for 30 sec and then slowly pulled back to the starting position. RNA conditioning solution, marker and ladder was added in respective wells. After adding 1 µl of the sample, the chip was placed horizontally in an IKA vortex and vortexed for 1min at 2400 rpm. The chip was run in Agilent 2100 bioAnalyzer instrument within 5 min.

2.18 Functional exosome transfer assay

In order to investigate any functional changes caused by exosomes on recipient cells. The cells were transfected with normal, pre-cancerous and cancerous exosomes, for different time durations in order to determine a suitable time of exposure that would cause an effect.

Recipient cells in the density of $1x10^7$ were seeded in culture wells of 22 mm in diameter (6 well plate), with 500 µl of DMEM medium with 10% FCS and 1% Pen/strep. Once the cells were attached to the culture plate 200 µl of the medium was carefully removed and exosome pellet dissolved in 200 µl of SFM was added to the cells. Cells were also treated with 200 µl of RNase free water and depleted medium as controls. The samples were incubated for 24, 48 and 72 hours at 37°C and 10% CO_2.

The concentration of exosomes was determined through NTA and normalized so each sample had approximately the same number of exosomes. Total RNA was extracted using RNeasy Micro Kit (74004, Qiagen) as per manufacturer's protocol. Quantity and quality of RNA was analysed using nano drop.

For functional assay's carried out on normal oral keratinocytes, the recipient cells were grown in SFM without 10% FCS. Through RT-qPCR expression levels of multiple senescence and differentiation markers were tested.

2.19 Quantitative Polymerase Chain Reaction (RT-qPCR)

RT-qPCR is based on amplification of DNA, it can precisely distinguish and quantify small amount of specific nucleic acid sequence present in a sample. It is a robust and widely used technique with major application in assessment of changes in gene expression as a result of deregulation of normal biological processes.

In the exosome study RT-qPCR was performed using both Taqman probe and SYBR green dye. Taqman probe uses a fluorogenic probe specific to target gene to detect target as it accumulates during PCR. It offers higher sensitivity and specificity but is a costly method. SYBR green dye binds to double stranded DNA and is detected during extension phase of RT-qPCR. It is the most widely used method, although it does not offer the same level of sensitivity as Taqman probe it is cost effective, allowing scanning through numerous genes.

Since exosomes contain less RNA compared to cells, more specific Taqman gene expression assay was used for detection of exosomal RNA. Taqman gene expression assays for FOXM1 (Hs1073586_m1), CEP55 (Hs01070181_m1) and β-Actin (Hs01060665_g1) were performed using LightCycler® 480 Probes Master (#04707494001, Roche Diagnostics) with a pre-incubation of 50ºC, 2 min and 95ºC, 10 min hot-start followed by 50 cycles of 95ºC, 10s and 60ºC, 60s.

RT-qPCR was performed using forward and reverse primers **(Table 2.4, Page no. 62)** and SYBR green dye on exosome transfected cells in order to evaluate any functional changes caused by exosomes. Amplification protocol included denaturation at 95°C for 10 min followed by 45 cycles of 95("Stanford Microarray Database. [http://smd.stanford.edu/],")° for 10 s, 60° annealing for 6 s and 72°C elongation (incubation time depends on the length of the product ; 1s/25 base pairs). Fluorescence readings were acquired at 75°C following the elongation step. At the end of 45 cycles, samples were subjected to a melting analysis to confirm amplification specificity of the PCR product.

Table 2.4: List of all the genes along with their forward and reverse primer sequence

Target genes	Forward primers sequence (5'-3')	Reverse Primers sequence(5'-3')
ADAM9	tcccccaaattgtgagactaa	tccgtccctcaatgcagtat
ANXA8	gaggacagcatcaagagtgaga	gaggttttgggtgcatttca
AURKA	cgccctgtaggatactgctt	caaatatccccgcactctg
BBOX1	ccagagcaaagctccaaaga	tccaaagtgggtagctggag
BMI1	tgtaaaacgtgtattgttcgttacc	caatatcttggagagttttatctgacc
CBX7	cgagtatctggtgaagtggaaa	gggggtccaagatgtgct
CCNB1	catggtgcactttcctcctt	aggtaatgttgtagagttggtgtcc
CDCA5	agacatgactctccctggaatc	cactcatccagctccgtttt
CDCP1	cactgctaggggttctgctg	cgtggcagagcaatctca
CENPA	gcacccagtgtttctgtcagt	ccagacagcatcgcagaat
CEP55	tgaagagaaagacgtattgaaacaa	gcagtttggagccacagtct
CLEC3B	agcagcatggagctctgg	ctcctcaaacatctttgtgttca
CORN	tcactgttgcagcatgagttc	tggcaaggctgtttcacc
CREG1	agctctccgtgagcaacc	tgtgccaaagtcatggtcag
CRNN	gagcaagagtttgccgatgt	tccacagtccctgtgtggt
CTSC	ccctgggagatatgattaggaga	cagtcagtggtgcaggtttg
DKK3	agagcctgatggagccttg	ggcttgcacacatacaccag
DNMT1	gccaaagcccgagagagt	cagtgcatgttggggattc
DNMT3B	agagggacatctcacggttc	ggttgccccagaagtatcg
DUOX1	ggaggtttggcaagaaggt	gcgcttgaactgttgcac
EEF2K	caccacacagccagaagcta	tgccatgttatcgaggtcac
EEFMP1	aacccttcccaccgtatcc	tgcagtgcactcgtctatgtc
ELP3	ggattgttttgtggctgtca	agcagggctgagatctcctt
FEZ1	caggacccgcacagataaac	gggcctccattcttgctc
FLG	caagtccaggagagagacacg	tgcagatgaagcttgtccac
FN1	aacgtgggagaagccctac	ttgtgtcctgatcgttgcat
FOXM1B	ccaggtgtttaagcagcaga	tcctcagctagcagcaccttg
HELLS	ggatggctgaattcaaaagatt	caaagtccctttccgtttgt
HOXA7	ctggatgcggtcttcagg	ggtagcggttgaagtggaac
IGFBP3	aacgctagtgccgtcagc	cggtcttcctccgactcac
ITGB1	cgatgccatcatgcaagt	agtgaaacccggcatctg
IVL	tgcctgagcaagaatgtgag	ttcctcatgctgttcccagt
K1	cggaactgaagaacatgcag	catataagcaccatccacatcc

Target genes	Forward primers sequence (5'-3')	Reverse Primers sequence(5'-3')
K10	aaaccatcgatgaccttaaaaatc	gcgcagagctacctcattct
K13	agtcccagctgagcatgaa	ctgctgatgagtccctggat
KLF6	aaagctcccacttgaaagca	ccttcccatgagcatctgtaa
LAMP2	gctcgttctggtctgccta	tgtcaaattaagttccaatgcataa
LORI	gtccagtgccagagctacg	ccagagtagccgcagacag
LRP11	cagtgtcctgatgggtctga	gttaccatcttgcggtccag
MAPK8	gggcagccctctccttta	cattgacagacgacgatgatg
MMP13	tgagctggactcattgtcgg	aggtagcgctctgcaaactg
MMP9	gaaccaatctcaccgacagg	gccacccgagtgtaaccata
NAV2	cggctcagaccagtctcc	cttcccaccaaagagtctgc
NEK2	cattggcacaggctcctac	gagccatagtcaagttctttcca
OAS1	cgcctagtcaagcactggta	ccaagcatagaccgtcagg
OTUD1	gacgagaagctggccctatac	ctgccgcagatacttgtcct
P16	tgcccaacgcaccgaatag	caccagcgtgtccaggaag
P21	tcactgtcttgtacccttgtgc	ggcgtttggagtggtagaaa
P53	aggccttggaactcaaggat	ccctttttggacttcaggtg
PGAM1	gcatggaacctggagaacc	tgtcaaactcatagccagcatc
PLAU	tcactggctttggaaaagaga	tggtgacttcagagccgtag
POLR2A	gcaaattcaccaagagagac	cacgtcgacaggaacatcag
PUS7	cgaaagatcattattcgtcctca	tgggatcatcatatgcaacg
RAB6	agcacacaggacagaagcag	ggttgaagatgacatgggagat
RRP12	gctgaaccttgtcctgaagc	tgaaggctttggaggtatcag
S100A16	caagatcagcaagagcagctt	gagcttatccgcagccttc
SEMA3F	gctgttccagcgagatcct	gagcaacaagccctgctc
sFOX	actttaagcacattgccaagc	cgtgcagggaaaggttgt
SPARC	ttccctgtacactggcagttc	aatgctccatggggatga
SPRR2E	tggtacttgagcactgatctgc	tgcactgctgctgttgataa
SRPX	cagcccgaaacctcctttac	gtcgaagatcaaggccacac
TCTN1	cactatgcaatctggctgtaaact	gccacgtaatctgggaagc
TDP1	tttcctcccttcagcatttg	acaggaaaggtggccattg
TGM1	ggactcagtactgcggttgc	tggagatggcgtggtagg
TNKS1BP1	aacggggacctggctaag	ggctggacttgagggaatc
TSCC22D3	tccgttaagctggacaacagt	atggcctgttcgatcttgtt
TUBB6	tgcagctggagagaatcaac	gtgcctggctctaagtccac
VIM	aggtggaccagctaaccaac	tttcggcttcctctctctga
YAP1	cccagatgaacgtcacagc	gattctctggttcatggctga
ZMAT3	ccaggaaagaagggaatgagt	gcggggattgaagtaaggac

2.20 β- Galactosidase staining

Normal cells can permanently lose the ability to proliferate when challenged by potential stress, a process termed cellular senescence. Senescence-associated beta-galactosidase (SA-βgal) activity permits the identification of senescent cells in culture. The LacZ gene from E.coli is one of the most commonly used reporter genes for testing the efficiency of expression vector mediated gene transfer and for studying the regulation of promoters of genes. The LacZ gene encodes the enzyme β-galactosidase, which is very stable, resistant to proteolytic degradation, can utilize a variety of substrates and can be easily assayed in situ. The β_Galactosidase staining kit from BioVision (catalogue no. K802-250) utilizes X-gal as the substrate.

Cells were first treated with chemotherapeutic drugs causing senescence including Dimethyl sulfoxide (DMSO), Azacitidine (5AZA), Trichostatin A (TSA) and Etoposide (ETO) to check if senescence can be induced **(Fig 2.6)**.

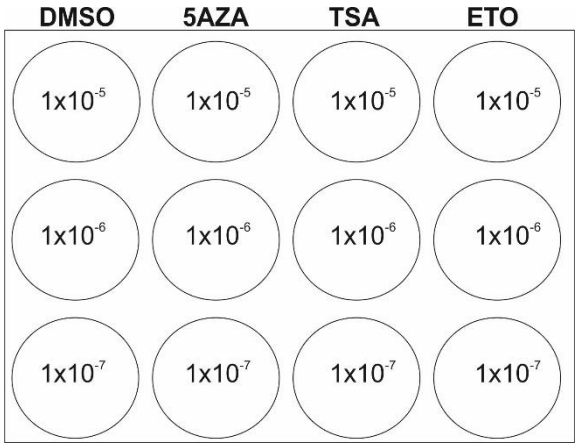


Fig 2.6: Drug treatment of SVpgC2a cells to induce senescence. *Each well contains approximately 0.1x10^6 of SVpgC2a cells in 1 ml of DMEM medium. Drugs were added to provide final concentrations of 1x10^{-5}, 1x10^{-6} and 1x10^{-7} M as indicated.*

After 24 hrs of drug treatment, cells were prepared for X-gal staining. The medium was removed and the cells were washed in 1xPBS followed by addition of 0.5ml of fixative solution for 10-15 min at room temperature. Fixed cells were washed twice in 1xPBS before adding 0.5 ml of staining solution mix in each well containing 470 µl of staining solution, 5 µl of staining supplement and 25 µl of 20 mg/ml X-gal in DMF

(Dimethylformamide). The plate was covered and incubated overnight at 37°C. The cells were observed under a microscope for development of blue colour.

The treatment with Etoposide in the concentration of $1x10^{-7}$ M for 24 hours was able to senesce cells. Once senescence was established in SVpgC2a cells, they were treated with self and SVFN10 exosomes for 48 hrs. The exosome transfected cells were stained for X-gal in the similar way as mentioned above.

2.21 Microarray

OK113 cells in the density of 1×10^5 / well were seeded in 6-well plates. The cells were grown in SFM (as mentioned earlier). Once the cells were 70% confluent they were transfected with exosomes from cell lines OK113, NK4, NOK368, CA1, CALH2, SQCC/Y1, SVpgC2a and SVFN10. The particle/ml concentration of exosomes, measured on Nanosight was normalized so that each well contained $2x10^{10}$ exosomes **(Table 2.5)**. Untreated OK113 cells were used as a control. After 48 hrs of incubation the cells were washed with 1×PBS and lysed in lysis buffer (RLT buffer) for total RNA extraction using RNeasy Micro Kit from Qiagen (catalogue no. 74004). Quality and quantity of total RNA was analysed on Nano drop 1000 spectrophotometer and Agilent Bioanalyzer.

Table 2.5: Normalized concentration of exosomes measured by NTA.

Normalization of exosome concentration

Exosomes	NTA	Dilution used for NTA	Particle/ml	Dilution	Exo volume (µl)	Particles/well
OK113	1.19E+09	50	5.95E+10	1.68	336	2.00E+10
NK4	1.07E+09	50	5.35E+10	1.87	374	2.00E+10
NOK368	4.08E+09	50	2.04E+11	0.49	98	2.00E+10
CA1	1.15E+10	16.66666667	1.92E+11	0.52	104	2.00E+10
CALH2	5.55E+09	50	2.78E+11	0.36	72	2.00E+10
SQCC/Y1	3.74E+09	50	1.87E+11	0.53	107	2.00E+10
SVpgC2A	7.06E+09	50	3.53E+11	0.28	57	2.00E+10
SVFN10	6.40E+09	50	3.20E+11	0.31	63	2.00E+10

An Illumina genome wide gene expression array for human samples HT12v4 exploring 47,231 genes was performed by Eva Wozniak, Senior Research Technician at Barts and The London Genome Centre, Queen Mary University of London. Illumina arrays target the 3' end of genes with 50 bp probes bound to microbeads. It is a robust

method for surveying global gene expression of protein coding genes. The data from microarray was analysed on Genome studio version 3. The results of microarray were verified by RT-qPCR.

2.22 Clinical specimens

The use of fresh biopsy clinical specimens collected from UK and FFPE samples from Norway was approved by relevant Research Ethics committees at each institute UK NREC: 06/MRE03/69 (head and neck) and Norway REK Vest: 2010/481-7 (head and neck). The samples were collected according to local ethical committee approved protocols and informed patient consent was obtained from all participants. The histopathology report of each sample was collected from collaborating clinicians.

The fresh biopsy specimens were stored in RNA later solution (#AM7002, Ambion, Applied Biosystems, Warrington,UK) at -20^{o}C. For cDNA synthesis Transcriptor cDNA synthesis kit by Roche was used.

2.23 Total RNA extraction and analysis of FFPE samples

RNA extraction from FFPE samples is challenging because fixation and embedding conditions affect nucleic acid, making them fragmented and chemically modified by formaldehyde. Resulting in lower molecular weight than those obtained from fresh or frozen samples. The RNeasy FFPE kit (Qiagen catalogue no. 73504) was used to purify total RNA from FFPE samples, isolating RNA molecules longer than 70 nucleotides.

Fifteen samples were tested 3 normal, 4 dysplastic, 4 OSCC and 4 from tumours that have metastasized to lymph nodes. Deparaffinization was done by treating samples with xylene. Sample incubation in lysis buffer containing proteinase K releases RNA from 5 micron FFPE sections. 15 min incubation at 80°C partially reverses formalin crosslinking from nucleic acids giving a good quality and a high yield of RNA. Treatment with DNAse eliminates all genomic DNA including small segments that persist in FFPE samples due to prolonged formalin fixation and storage.

Buffer RBC containing ethanol was added to provide optimal binding conditions for RNA. In RNeasy Mini Elute columns, RNA binds to the membrane while the contaminants were washed away. RNA was eluted in 15 µl of RNase-free water giving

13 µl of total RNA, where 2 µl accounts for dead volume of RNeasy Mini Elute column. Reverse Transcription reaction was run to convert RNA into cDNA. To every 13 µl of RNA 7.5 µl of cDNA synthesis master mix was added containing 5×Buffer (4.0µl) , dNTP (2.0µl) , dT/rP (0.8µl) , RNasin (0.4µl) , RT enzyme (0.4µl).

Setting up qMIDS assay in a 384 well plate

To set up qMIDS assay in a 384 well plate 2 µl of forward and reverse primers (2.5µM) of 16 genes were loaded at the bottom of each well using a 16 channel pipette. The primers were arranged in the following order **(Fig 2.7)**

qMIDS™ Biomarker Standard Curve Metadata and Primer Sequences

	Gene	Loci	Efficiency[a]	Slope[b]	Error[c]	Forward Primer	Reverse Primer	Tm[d]	Bp[e]
A	HOXA7	7p15-p14	1.919	-3.533	0.003	ctggatgcggtcttcagg	ggtagcggttgaagtggaac	87	101
B	MAPK8	10q11.22	1.659	-4.547	0.001	gggcagccctctccttta	cattgacagacgacgatgatg	83	78
C	CCNB1	5q12	1.941	-3.471	0.004	catggtgcactttcctcctt	aggtaatgttgtagagttggtgtcc	81	102
D	AURKA	20q13.2-3	1.953	-3.439	0.005	cgccctgtaggatactgctt	caaatatccccgcactctg	83	80
E	CENPA	2p24-p21	1.936	-3.486	0.003	gcacccagtgtttctgtcagt	ccagacagcatcgcagaat	85	104
F	NEK2	1q32.2	1.865	-3.694	0.005	cattggcacaggctcctac	gagccatagtcaagttctttcca	85	90
G	CEP55	10q23.33	1.705	-4.316	0.011	tgaagagaaagacgtattgaaacaa	gcagtttggagccacagtct	81	112
H	BMI1	10p11.23	1.731	-4.195	0.018	tgtaaaacgtgtattgttcgttacc	caatatcttggagagttttatctgacc	81	121
I	HELLS	10q24.2	1.900	-3.586	0.006	ggatggctgaattcaaaagatt	caaagtccctttccgtttgt	77	113
J	DNMT1	19p13.2	1.979	-3.374	0.009	gccaaagcccgagagagt	cagtgcatgttggggattc	85	114
K	DNMT3B	20q11.2	1.902	-3.582	0.003	agagggacatctcacggttc	ggttgccccagaagtatcg	83	96
L	FOXM1B	12p13	1.888	-3.623	0.006	ccaggtgtttaagcagcaga	tcctcagctagcagcaccttg	89	279
M	IVL	1q21	1.888	-3.624	0.005	tgcctgagcaagaatgtgag	ttcctcatgctgttcccagt	85	83
N	ITGB1	10p11.2	1.807	-3.890	0.004	cgatgccatcatgcaagt	agtgaaacccggcatctg	83	95
O	YAP1	11q13	1.948	-3.454	0.002	cccagatgaacgtcacagc	gattctctggttcatggctga	84	83
P	POLR2A	17p13.1	1.970	-3.396	0.003	gcaaattcaccaagagagac	cacgtcgacaggaacatcag	82	73

Absolute qPCR gene standard curve efficiency[a], slope[b] and statistical P-value error[c] were determined using the LightCycler 480 Absolute Quantitation software internal algorithm (second derivative maximum). Tm[d] indicates the melting temperature of primer pairs and Bp[e] indicates the basepair length of each PCR product. Red, green and black fonts indicate upregulated, downregulated and reference genes, respectively.

Fig 2.7: *Order in which primers were loaded into the 384 RT-qPCR plates along with their forward and reverse sequence. The diagram was provided by Dr Muy-Teck Teh.*

Each qMIDS assay plate measures 10 unknown samples in duplicates with calibrator and standard. To each cDNA sample 90 µl of 2 × SYBR Green I Master mix was added and 3 µl loaded in to each well, each sample was run in duplicates. RT-qPCR 384 plate was sealed with Roche's LC480 RT-qPCR sealing foil. The edges were pressed with a plastic spreader and the tray was centrifuged to allow the contents to flow to the bottom of the well.

RT-qPCR was run on LightCycler LC480 instrument; the touch-down cycles were increased from 8 to 13 to allow amplification of samples with high crossing point (Cp) values. The samples were labelled and the data was analysed.

2.24 RNA extraction for fresh frozen biopsies

Different methods of RNA extraction were tested to affirm a method that would yield high quality RNA from heterogeneous fresh frozen biopsy tissue and would aid in making the protocol for qMIDS assay concise. Roche mRNA capture kit (Reference number 11787 896 001) and Qiagen RNeasy micro kit (catalogue number 74004) were compared to the original method used for qMIDs, Dynabeads mRNA direct kit.

Dynabeads mRNA Direct Kit (Invitrogen catalogue number 610.12) was used for mRNA extraction from fresh frozen tissue biopsy samples from OSCC patients. Samples were selected in pairs of margin and core tissue from the same patient and stored in RNA later at -80^{o}C.

Tissue digestion

Frozen samples were defrosted at room temperature and sample ID's along with the date of biopsy were noted down. Tissue was digested in digestion buffer containing lysis buffer and proteinase K (1U/ml). A sharp scalpel was used to cut 1-2 mm^3 sections from biopsy sample. The small piece of tissue was minced into further small pieces to aid quick digestion and incubated at 60°C for 20 min.

mRNA extraction

To the digested tissue 40 µl of Dynabead was added and triturated to reduce the viscosity by shearing genomic DNA. Samples were rotated for 10 min on a mixer and RT master mix was prepared using the same quantity as mentioned earlier for cDNA synthesis. 7.5 µl of the master mix was allocated in each PCR tube.
The rotated samples were placed on a magnetic rack. The tubes were inverted a couple of times to collect all the beads from the cap. Removing the magnetic rack the samples were triturated once more to mix the contents.

The beads were concentrated using the magnetic rack to collect the supernatant. Removing the magnetic rack 400 µl of buffer A was added and the samples were triturated. Placing the samples back on the magnetic rack buffer A was removed.

Same process was repeated with buffer B. Beads were suspended in 13µl of tris HCL and placed on the heat block for 2 min at 82°C to elute the mRNA from the beads. The samples were immediately placed on the magnetic rack and 13 µl of mRNA was collected and added to 7.5 µl of the total master mix. The tubes were capped and placed into the thermocycler for cDNA synthesis.

2.25 Statistical analysis

For all the data in this thesis statistical significance was calculated by One - way ANOVA and t-test on Graph Pad Prism software and Microsoft excel. A Mann Whitney test was also carried out on SPSS software.

Chapter 3

Characterisation and biomarker potential of oral cancer exosomes.

3.1 Introduction

Oral squamous cell carcinoma (OSCC) is the most common malignant neoplasm of the oral cavity. Currently, the gold standard of diagnosing OSCC depends on tissue biopsy which is an invasive method performed by a highly skilled oral surgeon and the biopsy is subjectively verified by a trained pathologist. There is a need to develop an objective and less or non-invasive system of detecting the disease at an early stage. This aim led us to investigate the potential of salivary and blood extracellular vesicles (EVs) which may contain surrogate cancer biomarkers.

In the past, EVs were considered as cellular junk but with advancing technology it was established that EVs have an important role of maintaining homeostasis and regulation of physiological functions (Yanez-Mo et al., 2015).

Today we know that EV's are present in nearly all body fluids such as blood, saliva (Michael et al., 2010), urine (Gonzales et al., 2010), breast milk (Zhou et al., 2012), ascites (Peng et al., 2011), amniotic fluid (Asea et al., 2008), broncoalveolar fluid (Rodriguez et al., 2014), cerebrospinal fluid (Teplyuk et al., 2012), seminal fluid (Vojtech et al., 2014) and synovial fluid (Skriner et al., 2006). They are multi component delivery vehicle for RNA, DNA and proteins. Hence, they have the potential to be used as non-invasive biomarkers for early OSCC detection.

Most cells release varied types of extracellular vesicles into their environment. They are important for intercellular communication by providing a mode of transfer for cytosolic proteins, lipids, regulatory micro RNA, mRNA and genomic DNA (Balaj et al., 2011, Kahlert et al., 2014). This transfer of information can change the fate of target cell by regulating gene expression, causing differentiation or dedifferentiation (Lakkaraju and Rodriguez-Boulan, 2008).

3.2 Different types of EV's

Extracellular vesicles are differentiated from each other on the basis of size and mechanism of formation.

Apoptotic bodies:

Apoptotic bodies released during apoptosis (Hristov et al., 2004) are the largest measuring 50-5000 nm (Akers et al., 2013). They are formed by nuclear condensation

and cytoplasmic shrinkage of the apoptotic cell, followed by blebbing of cytoplasm and disintegration of the cell into membrane bound bodies (Catchpoole and Stewart, 1995).

Microvesicles:

Microvesicles or ectosomes are membranous vesicles with a diameter of 100-1000 nm, they are produced by the budding of plasma membrane **(Fig 3.1)** (Gyorgy et al., 2011, Crescitelli et al., 2013).

Exosomes:

Exosomes are nano-particles, discovered almost 30 years ago (Trams et al., 1981). They are plasma membrane bound; endosomal in origin, measuring 40 to 100 nm in size (Kalra et al., 2012, Raposo and Stoorvogel, 2013). They float at the density of 1.15-1.19 g/ml hence can be separated in the laboratory via centrifugation and ultra-filtration (Robbins and Morelli, 2014).

In recent years, exosomes have become the focus of multiple studies due to their potential to be used as non-invasive biomarkers, their ability to be bioengineered for vaccines and clinical applications such as intracellular drug delivery (Andreu and Yanez-Mo, 2014). Their composition depends on the parental cell type and its site of origin. Exosomes reflect the physiological or pathological state of parental cells. For example epithelial exosomes released from apical and basolateral surfaces are different from each other in composition and content (Sreekumar et al., 2010). Similarly, exosomes released from T cells and B cells carry cell type specific markers indicating their origin (McLellan, 2009, Wahlgren et al., 2012).

Protein composition of exosomes is informative of any existing pathology as they can carry tumour antigens and inflammatory mediators. They also carry customary proteins including HSC70, TSG101 and tetraspanins (Choi et al., 2014), in addition they carry specific proteins which are involved in vesicle formation and trafficking such as ALIX (Apoptosis linked gene 2-interacting protein X) (Baietti et al., 2012).

Exosomes are enriched in tetraspanins, a family of proteins that organizes membrane microdomains called tertraspanin enriched microdomains, by forming clusters and interacting with transmembrane and cytosolic signalling proteins (Hemler, 2005). Among tetraspanin CD9, CD63, CD81, CD82 and CD151 have a broad tissue

distribution. They are involved in biological processes including cell adhesion, motility, membrane fusion, signalling and protein trafficking (Hemler, 2001).

Similarly, the lipid composition of exosomes is representative of their cellular origin. They are enriched in lipids such as sphingomyelin, phosphatidylserine, gangliosides and cholesterol as compared to plasma membrane and other intracellular membranes (Subra et al., 2007).

In recent years multiple studies have focused on the RNA content of exosomes, the nucleotide sequence, and its ability to be transferred into neighbouring cells and function within these cells. Human (HMC-1) and mouse (MC/9) mast cell lines release exosomes that contain both mRNA and small RNA including miRNA. The mRNA content is found to be transferable to neighbouring cells and is functional in its new location (Valadi et al., 2007).

3.3 Exosome formation

Exosomes are formed by the invagination of plasma membrane that pinches off and becomes intraluminal vesicle (ILV) , which later transforms into multi-vesicular body (MVB) consisting of numerous free vesicles (Thery et al., 2002) **(Fig 3.1).**

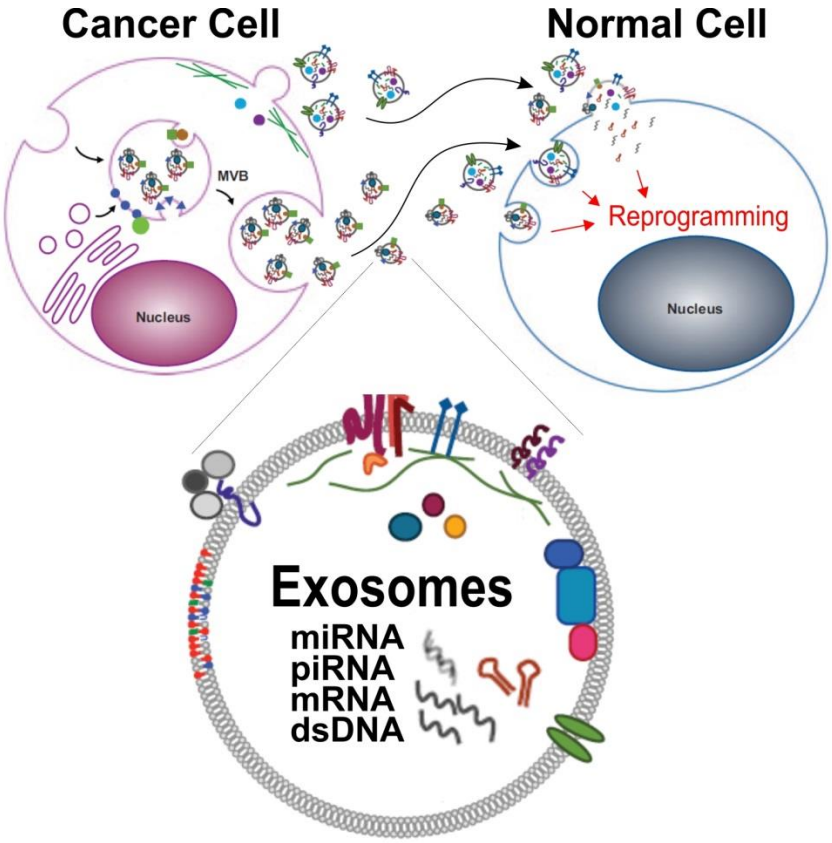


Fig 3.1: Different mechanism of formation and release of microvesicles (ectosomes) and exosomes. *The cancer derived microvesicles and exosomes are taken up by normal cells causing genetic reprogramming. Adapted from (Choi et al., 2014).*

Biogenesis of ILV involves endosomal sorting complex required for transport (ESCRT). ESCRT consist of approximately 20 proteins that assemble into four complexes ESCRT-0, I, II and III with associated proteins VPS4 (Vacuolar protein sorting- associated protein 4), VTA1 (vesicle trafficking 1) and ALIX forming ESCRT accessory complex (Henne et al., 2011). ESCRT-0 complex recognizes and segregates ubiquitylated proteins in endosomal membrane. ESCRT I and II deform

the membrane into buds with sequestered cargo. ESCRT III is responsible for cleavage into free vesicles (Colombo et al., 2013). The mechanism by which ESCRT III complex detaches ILV into MVB is similar to final cut between two dividing daughter cells (Raiborg and Stenmark, 2011). Recent studies have shown formation of a helix with a centrosomal protein (CEP55), which trans locates to the mid-body during the late phase of cell division and functions as a scaffold for components of the abscission machinery **(Fig 3.2)**. CEP55 interacts with ESCRT and Alix binding region (EABR) (Lee et al., 2008). Previous study from our lab have shown that CEP55 is a downstream target of FOXM1, an oncogene that regulates cell cycle, DNA repair and maintenance of genomic stability (Waseem et al., 2010, Gemenetzidis et al., 2009).

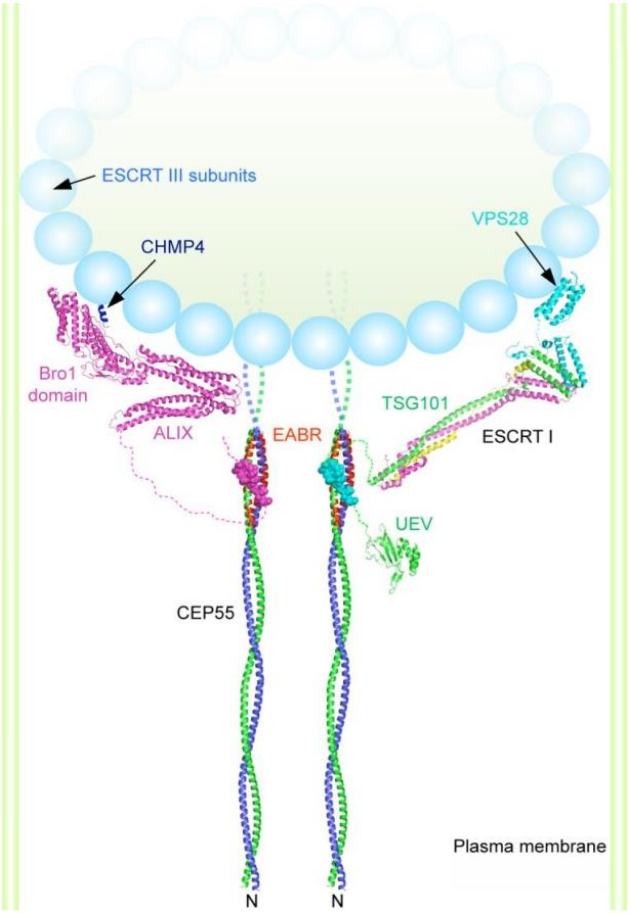


Fig 3.2: A model for the organization of CEP55-ESCRT-ALIX complex *adapted from (Lee et al., 2008).*

In oligodendrocytes the mechanism of exosome formation is independent of ESCRT complex, requiring inclusion of sphingolipid containing lipid raft in the MVB membrane. Sphingolipids releases ceramide through breakdown by sphingomyelinases. Ceramide has a role in IVL budding. MVBs formed independent of ESCRT complex express high levels of tetraspanins enriched microdomain (Trajkovic et al., 2008).

3.4 Exosome release pathway

MVB either follows a secretory or a degradative pathway. In the secretory pathway MVB is transported to the cell periphery with coordinated action of cytoskeleton, the molecular motors and vesicle fusion machinery, where it binds to the plasma membrane and releases the vesicles now called extracellular vesicles. RAB family of small GTPases controls MVB trafficking to cell periphery. RAB27A anchors and fuses MVB to plasma membrane, whereas RAB27B participates in mobilization of MVBs to the actin rich cortex under the plasma membrane (Ostrowski et al., 2010). RAB11 promotes the fusion of MVB to plasma membrane in response to increased cytosolic calcium (Savina et al., 2005). MVBs fuse to the plasma membrane with the help of SNAP receptors (SNAREs). These proteins regulate the fusion and target specificity in intracellular trafficking (Fader et al., 2009). The population of MVBs that are low in cholesterol and high in lysobisphosphatidic acid are targeted to lysosomal pathway (Buschow et al., 2009).

Accessory proteins including TSG101 (Tumour susceptibility gene 101), HRS (Hepatocyte growth factor regulated tyrosine kinase substrate) and ALIX are necessary for the secretion of exosomes. Evidence suggests that syndecan heparan sulphate proteoglycans and their cytoplasmic adaptors syntenin control the biogenesis of exosomes. Syntenin interacts directly with ALIX and support the intraluminal budding of endosomal membrane (Baietti et al., 2012).

3.5 Physiological function of exosomes in regulating immune response

Extracellular vesicles released from immune and non-immune cells have important role in regulation of immunity. They can stimulate or suppress immune response and hence are important factors in causing inflammatory, auto immune and infectious diseases. (Robbins and Morelli, 2014).

Exosomes are capable of direct and indirect antigen presentation. Exosomes released by B cells carry MHC class II, co- stimulatory and adhesion molecule and have been found to directly activate $CD4^+$T cells (Raposo et al., 1996). Exosomes released by antigen presenting cells (APC) carry both MHC I and II can activate both $CD8^+$ and $CD4^+$ cells. However, in vitro T cell activity caused by exosomes is 10-20 fold less efficient than that of parent APC (Schneider and Simons, 2013), this might be due to their small size and vesicle dispersion caused by Brownian motion. Studies have shown that when exosomes were immobilized at high concentration on latex beads or when the number of peptide MHC complex was increased per vesicle it increased their T cell stimulatory capacity (Hsu et al., 2003).

Indirect antigen presentation by exosomes involves transfer of antigenic peptides to APC (Mallegol et al., 2007). Exosomes bind to dendritic cells via integrins and intercellular adhesion molecule (ICAM1/CD54), depending on cellular linage and activation stage of parent cell. Ligands and adhesion molecules such as tetraspanins and externalized phosphatidylserine are constitutively present on exosomes (Morelli et al., 2004). Exosomes internalized by DCs, degrade antigen-MHC complex carried by the exosome and is used as a source of peptide to indirectly interact with T cell (Montecalvo et al., 2008).

Another mechanism of antigen presentation by exosomes called cross dressing involves transfer of internalized exosomal antigenic peptides to MHC molecule of host APC. The host MHC molecules that are loaded with exosomes derived peptide are then transported to the APC surface for presentation to T cells. Study by Smyth and colleagues suggests that following allograft transplantation, cross dressing of recipient APC with donor MHC molecule could be mediated through exosomal transfer (Smyth et al., 2007).

3.6 Role of exosomes in developing oral cancer

Tumour progression is a result of active partnership between cancer cells and their microenvironment. This requires efficient exchange of information by cell to cell contact, secretion of signalling molecules and release of cargo vesicles such as exosomes into the extracellular environment (Kucharzewska and Belting, 2013). Exosomes are unique as they are capable of affecting locally and to distant sites via body fluids.

3.61 Cancer niche

Tumour cells tend to secrete more exosomes in comparison to normal proliferating cells (Taylor and Gercel-Taylor, 2008). This can be the result of stimulation in response to stressful conditions due to excess growth and cell damage (de Jong et al., 2012). It has been shown that activation of pro-apoptotic protein p53 results in overexpression of tumour suppressive-activated pathway 6 (TSAP6) which increases exosome production (Yu et al., 2006). The enzyme heparan sulphate along with syndecan-1 is upregulated in many cancer cell lines, also aid in increased exosomal release (Thompson et al., 2013). Free exosomes in the extracellular environment regulate the release of new exosome hence creating a negative feedback control loop (Riches et al., 2014).

Cells of stroma release exosomes to communicate with each other and with tumour cells. It is thought that tumour cells release exosomes making the environment more tumour promoting. Most commonly affected stromal cells are fibroblasts, vascular endothelial cells and immune cells. Cancer exosomes trigger fibroblastic transformation into myofibroblasts through TGFβ/Smad pathway to increase cancer aggression (Webber et al., 2010, Webber et al., 2015).

Tumour derived exosomes aid in evading the immune system by influencing myeloid progenitor cells to differentiate into myeloid derived suppressor cells, that promote tumour progression (Xiang et al., 2009). In addition, tumour exosomes decrease T cell proliferation and effector functions, cancelling the natural cytotoxic responses mediated by natural killer cells (Iero et al., 2008).

3.62 Angiogenesis

Exosomes can induce endothelial cell activation and transfer metastatic capacity (Tickner et al., 2014). They have the capability to increase angiogenesis. As hypoxia promotes in the tumour, cancer exosomes carrying hypoxia inducing proteins, are taken up by normal endothelial cells. This uptake stimulates new tubule formation, creating a network of blood vessels (Skog et al., 2008).

3.63 Metastasis

Tumour metastasis requires cells to undergo epithelial to mesenchymal transition (EMT) allowing migration to gain access to vascular or lymphatics channels (Fidler,

2002). As cancer progresses exosomes gain metastatic capability by becoming enriched with EMT protein such as vimentin and annexin A2 (Jeppesen et al., 2014b). Furthermore, in a study of breast and prostate cancer it was found that hypoxic tumour microenvironment induces expression of factors (HIFs) that aid in metastasis and are linked to poor prognosis in patients (Kimbro and Simons, 2006). HIFs along with tumour exosomes have been found to be involved in formation of a premetastatic niche, with ideal tumour growth conditions (Thuma and Zoller, 2014).

3.64 Chemoresistance

Exosomes also play a contributing role in chemoresistance. In a breast cancer study it was found that tumour cells transmit resistant properties via exosomes to sensitive parental cells, making cancer treatment ineffective. This mechanism involves intracellular transfer of selective miRNA that alter cell cycle distribution and affect apoptosis pathways to decrease drug susceptibility (Chen et al., 2014). Tumour cells evade the cytotoxic effects to chemotherapy drugs by packaging it into exosomes as soon as it is taken up by cell, thereby preventing the drugs from exerting its cytotoxic effects (Federici et al., 2014).

3.7 Exosomes as Oral Cancer Biomarkers

Evidence suggests that exosomes can be ideal candidates for early oral cancer diagnosis and prognosis, since a tumour tissue biopsy is not required. They can also aid in predicting and monitoring therapeutic response. They offer a non-invasive testing opportunity causing less patient discomfort. Exosomes can be isolated from body fluids such as blood and saliva. Multiple studies have found elevated levels of exosomes in cancers of different origins. They offer an enriched source of biomarkers as they are packed with biologically active molecules reflecting the pathological state of host cells (Brinton et al., 2015).

Tumour specific markers such as proteins, mRNA and microRNAs (miRNA) have been found in circulating exosomes. In colorectal cancer patients, ascites fluid derived exosomes show elevated level of claudin-3 a protein diagnostic biomarker (Choi et al., 2011). Similarly prostate cancer mRNA biomarker PCA-3 have been isolated from urine exosomes (Nilsson et al., 2009).

In addition circulating miRNA has gained considerable attention as promising biomarkers. miRNAs are small noncoding RNAs of 18-24 nucleotide in length that

regulate gene expression post transcriptionally (Bartel, 2009). Encapsulation of miRNA by exosomes is the primary mechanism by which it stays stable in extracellular environment (Takeshita et al., 2013). In a study on ovarian cancer, expression levels of eight exosomes derived miRNA were used to successfully distinguish between benign and malignant tumours (Taylor and Gercel-Taylor, 2008).

3.8 Oncogenes packaged in Exosomes

Tumour cells secrete exosomes in abundance, containing proteins, RNA and DNA which reflect the genetic status of the tumour (Raposo and Stoorvogel, 2013, Thakur et al., 2014). Recent studies have shown that cancer exosomes carry amplified oncogenes such as c-Myc and high levels of retrotransposon RNA transcript that can be transferred to normal cells affecting genomic stability (Balaj et al., 2011).

Exosomes released from breast cancer cells contain miRNA associated with RNA induced silencing complex (RISC) and are capable of independently processing precursor microRNA (pre-miRNA) into mature miRNA, more efficient in altering gene expression post transcriptionally. Cancer exosomes rapidly silence mRNA in recipient cells with the help of pre-miRNAs along with catalytic engine of RISC including Dicer (multidomain enzyme that generates small RNAs for gene silencing), Argonaute 2 (Ago2) and the transactivating response RNA-binding protein (TRBP). Hence cancer exosomes initiate tumourogenesis in a dicer-dependent manner (Melo et al., 2014).

This study aimed to isolate and characterised exosomes derived from OK113, NK4 and NOK368 (normal oral keratinocyte), Ca1, CaLH2 and SqCC/Y1 (OSCC tumour derived), SVpgC2a (oral epithelial pre-malignant) and SVFN10 (transformed malignant) cell lines (Teh et al., 2013).

Due to the very small size to exosomes which is comparable to a virus (Gyorgy et al., 2011), characterisation and visual verification of exosomes are of prime importance. Successful isolation of exosomes from cell lines supernatant and body fluids (saliva, whole blood and plasma) was verified using different techniques of visualisation including Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). Particle size was verified using Zetasizer and Nanosight Tracking Analysis (NTA). Exosomal specific membrane proteins were identified through Western blotting.

This study also aimed to explore the biomarker potential of oral cancer exosomes. Through Western blotting and immune TEM, we report the presence of a potential protein biomarker located exclusively on the outer membrane of cancer exosomes. Since body fluids consist of a heterogeneous population of exosomes derived from multiple cell types, such surface biomarker can be used to isolate OSCC exosomes from body fluids.

Results

3.9 Ultracentrifugation as the superior method of exosome isolation

Exosomes can be isolated by multiple methods including ultracentrifugation, density gradient separation and immune affinity capture (Thery et al., 2006). Different methods of exosome preparations can contain varying proportions of contamination from other membranous vesicles such as microvesicles and apoptotic bodies (Tauro et al., 2012), having consequences on downstream experiments.

All the isolation methods have some limitations such as immuno isolation will only segregate a population of exosomes depending on the surface protein chosen for capture (Thery et al., 2006), while a 30% sucrose gradient requires repeated ultracentrifugation steps resulting in significant loss of the total exosome yield (Lamparski et al., 2002). Ultracentrifugation is the most commonly used method of isolation (Gould and Raposo, 2013) as it offers the advantage of isolating the whole exosome population. The drawbacks of ultracentrifugation is that it is a time consuming process and large protein complexes can sediment with exosomes (Jeppesen et al., 2014a), although this can be overcome by additional filtration steps.

A well- established Thery exosome isolation protocol was followed (Thery et al., 2006), which required cells to be seeded in two T-175 flasks. Once the flasks were 90% confluent containing approximately $18x10^6$ cells the medium was changed to SFM with 40ml in each flask. The cells were further incubated for 72 hours. Enriched supernatant was centrifuged at 500xg for 10min to get rid of cell debris. The second centrifuge at 20,000xg for 20 min isolated micro vesicles, followed by filtration with 0.22µm filter. Finally the enriched supernatant was centrifuged at high speed of 118,000xg for 70 min to isolate the final pallet of exosomes **(Fig 2.1 in Methods, page no. 42).**

The process of exosome isolation is very time consuming, each batch takes up to almost 10 days. It can become even longer for primary cell lines which require much longer to grow. Collecting the final pallet of exosome is very tedious as it is invisible and can be very easily missed hence it is very prone to human error.

With growing interest in the potential use of exosome as disease diagnostic markers and drug delivery vehicles, commercially available new isolation methods are being introduced such as Invitrogen Total Exosome Isolation Kit (Life Technologies, USA) and ExoSpin™ Exosome Purification Kit (Cell Guidance Systems, USA). These kits claim to give higher yield with comparable size of vesicles using low speed centrifugation (10,000-20,000xg), minimizing the dependency on ultracentrifuge for exosome isolation.

In this study isolation method ultracentrifugation was compared with a commercially available Exo-spin™ by cell guidance systems. Exo-spin™ precipitates vesicles by using ploy-ethylene glycol (Lane et al., 2015). Particle size and concentration of isolated exosomes was verified using Nanosight Tracking Analysis system (NTA LM10).

In a pilot experiment, it was found that particle size measurement of SVpgC2a derived exosomes isolated through ultracentrifugation and Exo-spin™ system exhibit a mode values of 40 nm and 157 nm, respectively. The particle/ml concentration was found to be higher 21.65×10^8/ml through ultracentrifugation and 8.58×10^8/ml by Exo-spin™ **(Appendix Fig 8.1, 8.2, Page no. 189,190)**.

Similar results were seen for a malignant cell line (SVFN10) derived exosomes isolated by ultracentrifugation and Exo-spin™. A mode value of 62 nm and 145 nm was obtained, respectively. Ultracentrifugation yielded a higher particle/ml concentration of 25.16×10^8 as compared to Exo-spin™ which gave 12.54×10^8 particle /ml concentration **(Appendix Fig 8.1, 8.2, Page no. 189,190)**.

In conclusion, exosome particle size obtained through ultra-centrifugation were more similar to the observed size in most of the exosome literature, with majority measuring 30 to 100 nm (Raposo and Stoorvogel, 2013). While exosomes isolated by Exo-spin™ measured 150 to 200 nm. The larger size of exosomes might be because of the glycol component in the kit, fusing together the lipid bilayer membrane of exosomes. The particle/ml concentration of exosomes was also found to be higher through ultracentrifugation **(Appendix Fig 8.1, 8.2, Page no. 189,190)**. Based on these findings it was established that ultracentrifugation was a superior method of isolating exosomes.

3.10 Visual characterisation of Exosomes

Exosomes measure between 40-100 nm in size hence they are not visible under inverted light microscope (Ishikawa et al., 2014). Techniques allowing higher magnification such as electron microscopy were used to visualize exosomes from normal and tumour derived cell lines, whole cancer patient blood, normal plasma and normal saliva.

3.10(1) Electron Microscopy (EM)

Through Scanning Electron Microscopy (SEM) we were able to visualize exosomes from SVpgC2A (premalignant) and SVFN10 (transformed malignant) oral keratinocyte cell lines. The vesicles were found in clusters, in a size range of 30 nm to 157 nm. The technique worked best at lower magnification of 5 µm, which was sufficient to visualise clusters of exosomes, however individual morphology was less evident. It was difficult to visualise nano-size exosomes at higher magnification of 500 nm, which is a limiting factor of this technique **(Fig 3.3)**.

Therefore, subsequently Transmission Electron Microscopy (TEM) was done to better visualise exosomes. TEM is an established technique used for characterisation of exosomal structures due to its higher resolution. A typically flattened spherical, membrane bound particles were identified, confirming the presence of exosomes (Thery et al., 2006). Most of the particles measured between 40-100 nm in size (**Fig 3.4)**.

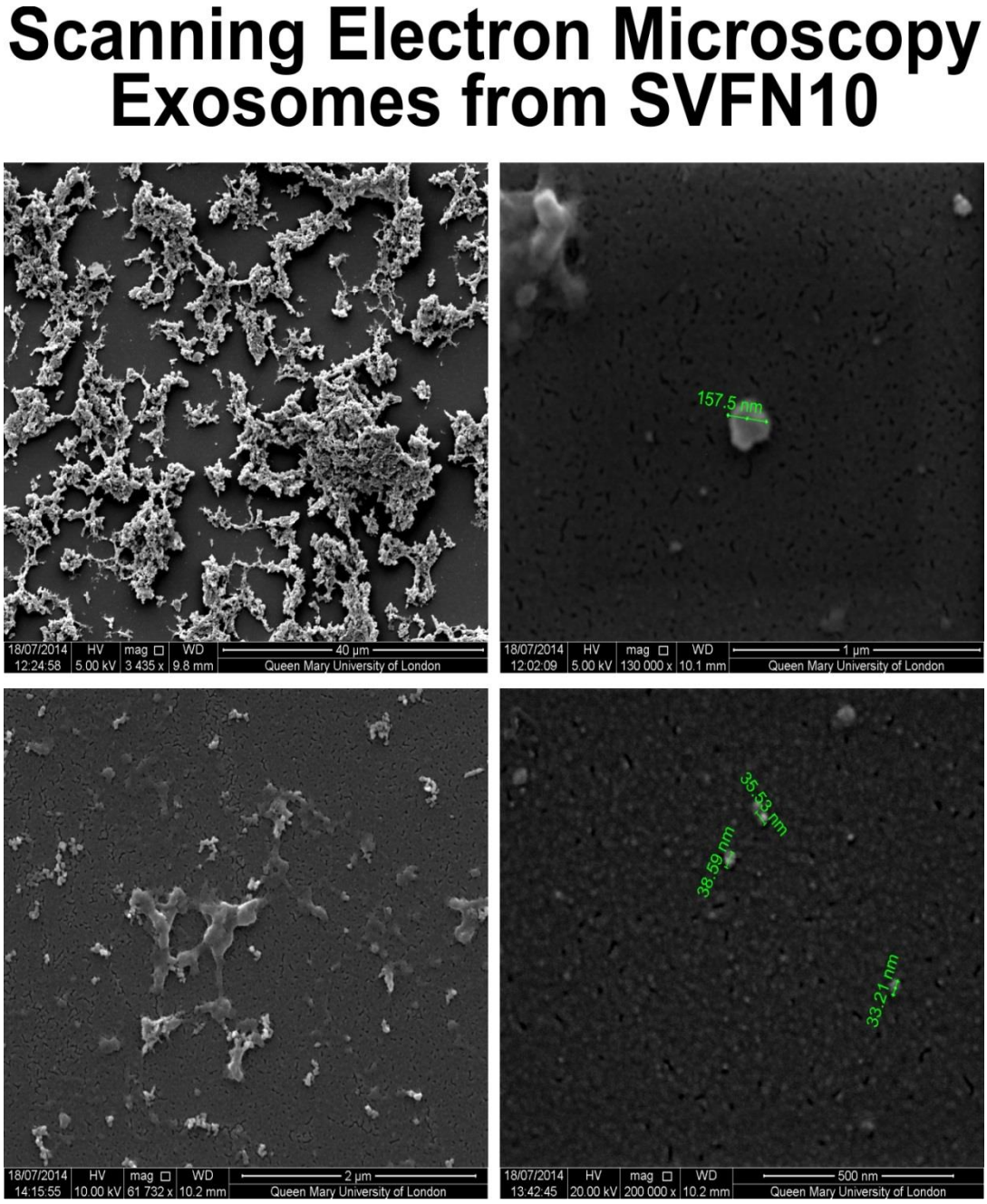


Fig 3.3: Scanning electron microscopy for SVFN10 exosomes. *Images above showed that exosomes appeared mostly in clusters from magnification of 40 to 2µm as indicated by respective scale bars within each image. Individual exosomes were visualized at the magnification of 1 µm and 500 nm but the flattened spherical morphology was not evident due to limited resolution.*

Transmission electron microscopy (TEM)

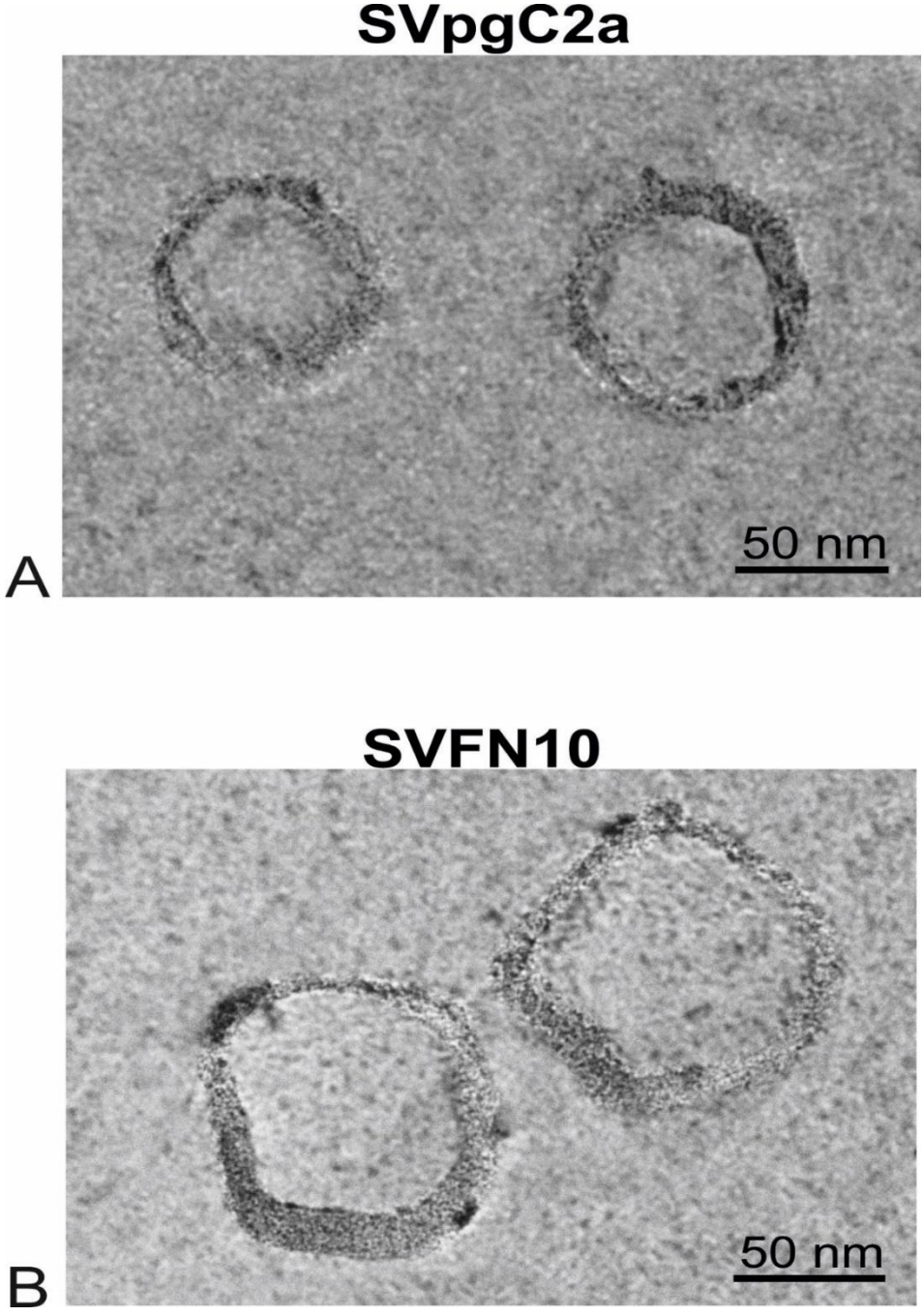


Fig 3.4: Transmission electron microscopy *Showing membrane bound exosomes from SVpgC2a (A) and SVFN10 (B) cell lines at the magnification of 50 nm, measuring between 80-100nm.*

3.11 Particle size measurement

EV's are classified into different subgroups based on their size and mechanism of origin. The most widely known are apoptotic bodies, microvesicles and exosomes. These subgroups are functionally and morphologically very different from each other and contamination of one subgroup with another will have an effect on the outcomes. Hence it is essential to verify the size of vesicles for any downstream experiment.

3.11(1) Zetasizer

The Zetasizer system determines particle size by measuring the Brownian motion of particles suspended in liquid, using dynamic light scattering (Wang et al., 2017). Zetasizer instrument ZS used in this study can measure particles in the range of 0.3 nm to 10 µm.

Differential ultracentrifugation yields various populations of EV at different speeds. This included cellular debris containing apoptotic bodies, microvesicles and exosomes. Particle size of these three populations was measured using Zetasizer to verify the size of exosomes and to authenticate the ultracentrifugation protocol **(Methods section 2.2, Fig 2.1, Page no. 42)**

It was found that majority of exosomes from SVpgC2a and SVFN10 cell lines were in the size range of 50 to 130 nm. Microvesicles measured from 200 nm to 600 nm and the cellular debris showed a particle size of 1000 to 5000 nm **(Fig 3.5)**. The findings were similar in three biological replicates.

Zetasizer

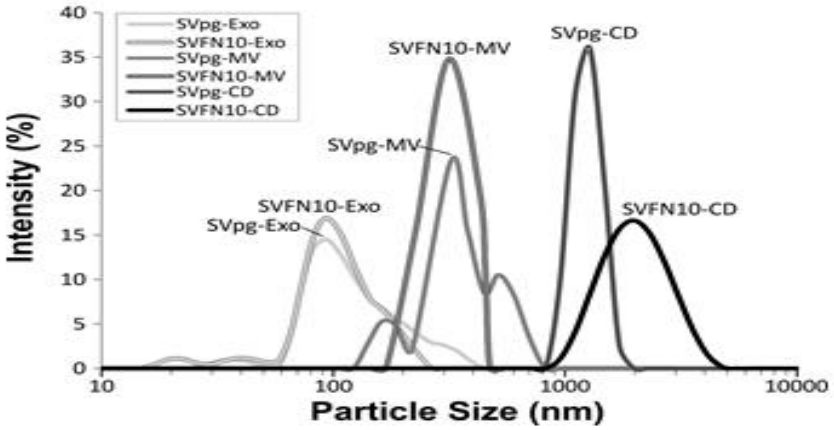


Fig 3.5: Particle size verification using Zetasizer *A range of particle size of cellular debris (SVpg-CD and SVFN10-CD) measuring 1000-5000 nm, microvesicles (SVpg-MV and SVFN10-MV) from 200-600 nm and exosome (SVpg-Exo and SVFN10-Exo) 50-130 nm isolated from SVpgC2a and SVFN10 cell lines at different stages of ultracentrifugation. This is a representative data of 3 independent experiments giving similar results.*

3.11(2) Nanosight Tracking Analysis (NTA)

Nanoparticle Tracking Analysis (NTA) utilizes the properties of both light scattering and Brownian motion in order to obtain the particle size distribution and per ml concentration of samples in liquid suspension. It is the most widely used technique for verification of exosomes (Soo et al., 2012, Fang et al., 2013, Gabriel and Giordano, 2010) as it offers the advantage of being inexpensive and less time consuming than TEM. It also allows visual verification of individual exosomes while formation and dissociation of aggregates can be noted in real time. Since no fixation of exosomes is required, they can be reused for further downstream experiments.

3.11(2a) Exosomes from Cultured Cells

Exosomes were isolated from normal oral keratinocyte cell lines (OK113, NK4 and NOK368), tumour derived cell lines (SqCC/Y1, Ca1 and CaLH2), transformed premalignant cell line (SVpgC2a) and transformed malignant cell line (SVFN10). NTA LM10 instrument (UCL, School of pharmacology) was used for size verification and particle/ml concentration of exosomes derived from all the above cell lines **(Fig 3.6)**.

Although it has been reported in the literature that cancer cells secrete higher concentration of larger size exosomes, sometimes referred as oncosomes (Minciacchi et al., 2015). In our study, exosomes from normal and cancer oral keratinocytes were approximately the same size **(Table 3.1)**.

The concentration (particle/ml) of exosomes was higher in cancer cell lines compared to normal **(Table 3.1)** suggesting that cancer cells yield more exosomes.

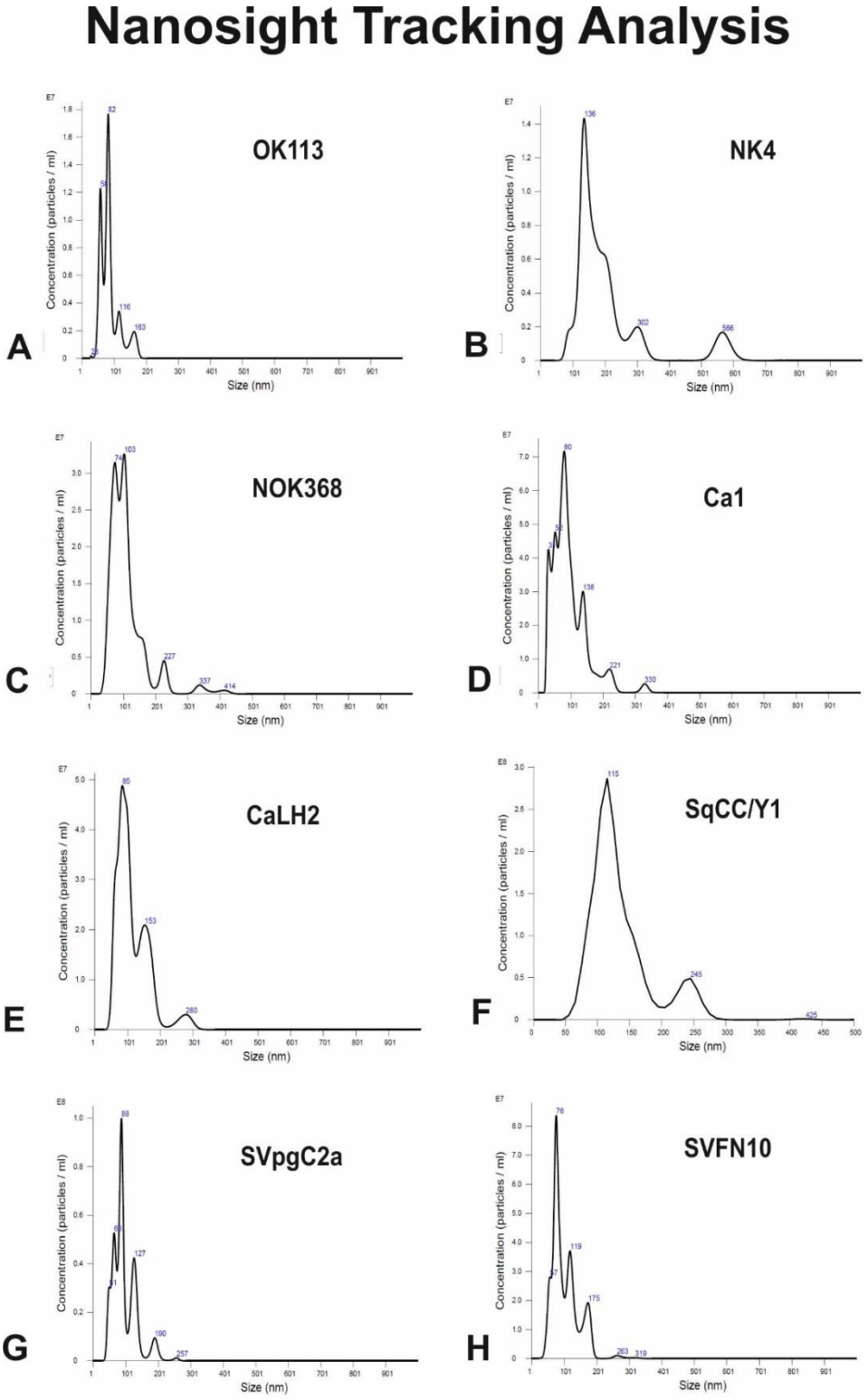


Fig 3.6 Exosome particle size verification using NTA LM10 instrument. *Exosomes isolated from normal oral keratinocytes* ***(A)*** *OK113* ***(B)*** *NK4* ***(C)*** *NOK368, tumour derived cell lines* ***(D)*** *Ca1* ***(E)*** *CaLH2* ***(F)*** *SqCC/Y1, premalignant cell lines* ***(G)*** *SVpgC2a and transformed malignant cell line* ***(H)*** *SVFN10.*

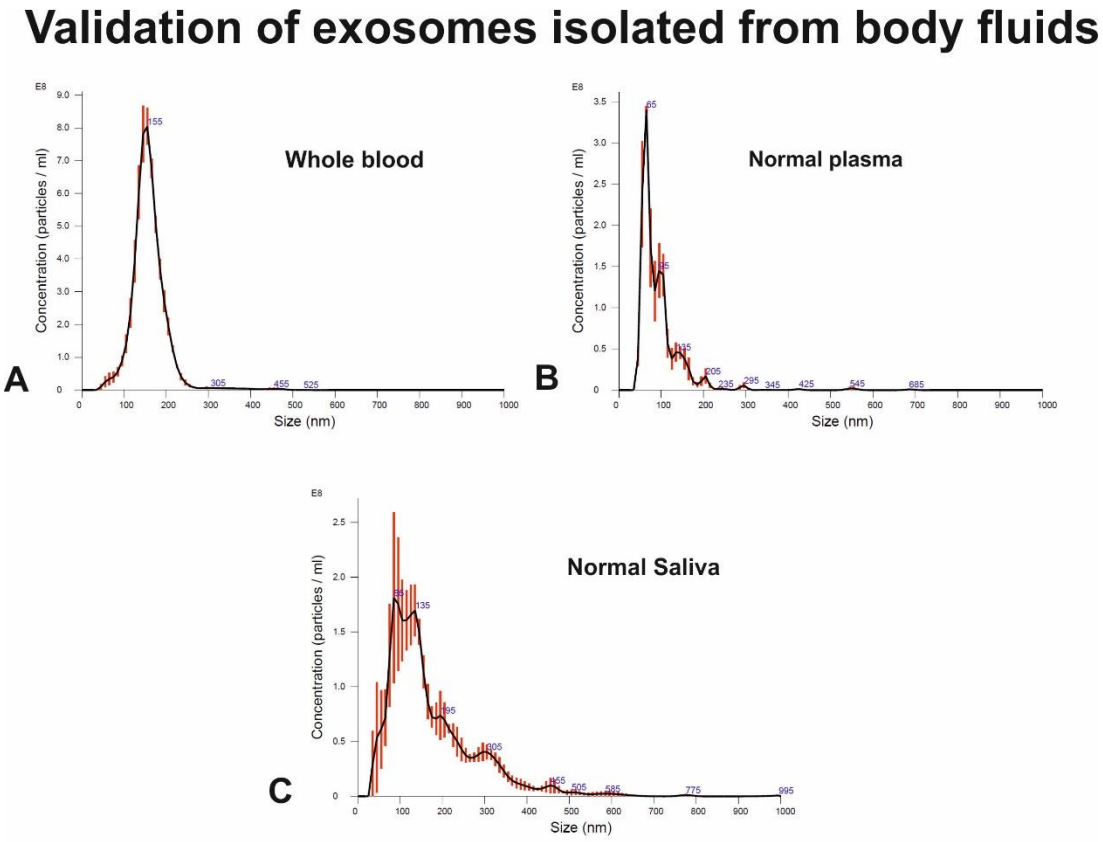
Table 3.1: Particle size and concentration (particle/mL) *Exosomes isolated from normal (OK113, NK4, NOK368) and cancer (Ca1, CaLH2, SqCC/Y1, SVpgC2a, SVFN10) cell line supernatant. Particle size (mode value) and concentration (particle/mL) were obtained using NTA. Each value represents the mean of mode values obtained from three independent experiments.*

Cell lines	Mode value	Particle/ml concentration
OK113	82.4nm	1.11×10^{9}
NK4	135.9nm	1.07×10^{9}
NOK368	103.4nm	4.08×10^{9}
Ca1	80.3nm	1.15×10^{10}
CaLH2	85.1nm	5.55×10^{9}
SqCC/Y1	113.9nm	1.91×10^{9}
SVpgC2a	88nm	7.06×10^{9}
SVFN10	76.3nm	6.40×10^{9}

3.11(2b) Exosomes from Body Fluids

Isolating exosomes from body fluids such as saliva, plasma and whole blood required the sample to be diluted to a workable viscosity. The ultracentrifugation protocol was modified by allowing longer duration and higher speed of centrifuge as recommended in literature (Thery et al., 2006). An extra filtration step was added as body fluids carry more cellular debris compared to cell culture supernatant and the purity of exosomes was verified through NTA **(Methods section 2.4,2.5,2.6 on page no. 45, 47 and 49).**

It was found that exosomes isolated from whole blood had a mean modal diameter of 151 nm with concentration of $5.61\text{x}10^{9}$ particles/ml **(Fig 3.7 A and D)**. Exosomes from normal saliva and plasma had mean modal diameter of 89 nm and 64 nm with concentrations of $1.53\text{x}10^{9}$ and $2.73\text{x}10^{9}$ particles/ml, respectively **(Fig 3.7 B, C and D)**. Visual verification on NTA showed no traces of cellular debris in any sample.



D

Body fluid	Mode value	Particles/ml concentration
Cancer patient whole blood	151.5nm	5.61×10^{9}
Normal plasma	63.8nm	1.53×10^{9}
Normal saliva	89.0nm	2.73×10^{9}

Fig 3.7: Verification of exosomes from body fluids using NTA ***(A)*** *Exosomes isolated from cancer patient's whole blood,* ***(B)*** *exosomes isolated from healthy volunteer's plasma* ***(C)*** *exosomes isolated from healthy volunteer's saliva.* ***(D)*** *Table showing size (mode values) and concentration (particle/ml) of respective exosomes. Each value represents the mean of three technical readings (n=3).*

3.12 Western blot

Protein component of exosomes was explored for exosome specific markers which validate not only successful isolation of exosomes but also reflects the purity of the sample. It was analysed by Western blotting (gel electrophoresis and immunoblotting). Exosomes were isolated as described above using ultracentrifugation from cell lines NOK368, OK113, NK4, SqCC/Y1, CALH2, Ca1, SVpgC2a and SVFN10. Cell lysate from parental cell lines was also collected to compare the differences in protein expression. Prior to western blotting protein concentration of exosomes and cell lysate were determined by Bradford BSA protein assay.

3. 12(1) Exosome-specific proteins

ALIX is an exosomal specific membrane protein and a component of ESCRTiii complex (Henne et al., 2011) and is involved in the biogenesis of exosomes (Baietti et al., 2012). It was expressed in exosomes derived from all the eight cell lines at the molecular weight of 102 kDa **(Fig 3.8)**, indicating successful isolation of exosomes. Heat shock protein HSC70 (70 kDa) and Glyceraldehyde 3-phosphate dehydrogenase GAPDH (37 kDa) were used as loading controls **(Fig 3.8)**.

In order to rule out extravesicular protein contamination, calnexin, an endoplasmic reticulum protein aiding in proper protein folding (Ellgaard and Helenius, 2003), was used to investigate the purity of exosomes. Ideally the expression of calnexin should be absent from exosomes since they lack the endoplasmic reticulum machinery. Calnexin was expressed in all cell lines at the molecular weight of 90 kDa, while the expression was low to absent in the exosome samples **(Fig 3.8)**, verifying that our exosomes are relatively clean and free from contaminations.

Tetraspanin proteins including CD63, CD9 and CD81 have been reported as exosomal specific membrane proteins (Andreu and Yanez-Mo, 2014). In this study expression of CD63 (63 kDa) and CD9 (25 kDa) was investigated. CD63 was expressed in cell lines but the expression was absent from the exosomes **(Fig 3.8)**. No expression of CD9 was found in cell lines or exosomes. Further exploration of the literature indicates that these markers were reported from studies carried out on immune exosomes derived from mast and dendritic cells (Escola et al., 1998, Thery et al., 1999). In conclusion, these surface protein markers are possibly not expressed in exosomes derived from oral keratinocyte cell lines.

3.12(2) Candidate cancer-exosome specific proteins

CEP55 (55 kDa) is a centrosomal protein involved in cytokinesis (van der Horst et al., 2009). It is a partner of Alix in ESCRTiii complex (Lee et al., 2008) . It is also a downstream target of FOXM1 oncogene (Waseem et al., 2010). The expression of CEP55 protein was exclusive to exosomes derived from malignant cell lines **(Fig 3.8)**. Within parental cell lines CEP55 expressed double bands very close to each other at the molecular weight of 55 kDa. In order to verify the specificity of our antibody and exclude non-specific binding, CEP55 was knocked down in SVFN10 cells by siRNA transfection. The successful knockdown of CEP55 mRNA in siCEP55 transfected SVFN10 cells was also validated by RT-qPCR **(Fig 3.9 A)**. Silencing of CEP55 protein by siCEP55 led to the disappearence of the top band **(Fig 3.9 B)** indicating that the top band was the correct CEP55 protein by Western blotting. Further attempts were made to explore the expression of exosomal CEP55 in oral cancer patient whole blood and normal plasma through western blotting. Unfortunately, the expression of loading controls HSC70 and GAPDH could not be established(result not shown). Due to the lack of clinical blood or plasma samples, this was not pursued further.

GPC1 is a cell-surface heparan sulphate proteoglycan, which acts as a co-receptor for heparin binding of mitogenic growth factors. It has a physiological role in cell growth and adhesion. GPC1 has been reported to be overexpressed in oesophageal squamous cell carcinoma (Hara et al., 2016). According to a recent publication, high levels of GPC-1 was found in pancreatic cancer cell lines and exosomes derived from them (Melo et al., 2015). In our study GPC-1 was expressed in all cell lines at the molecular weight of 62 kDa. In exosomes GPC-1 was only slightly detected in SVFN10 exosomes **(Fig 3.8)**. This can be due to high levels of FOXM1 causing increase in the cell cycle and aiding in cell growth.

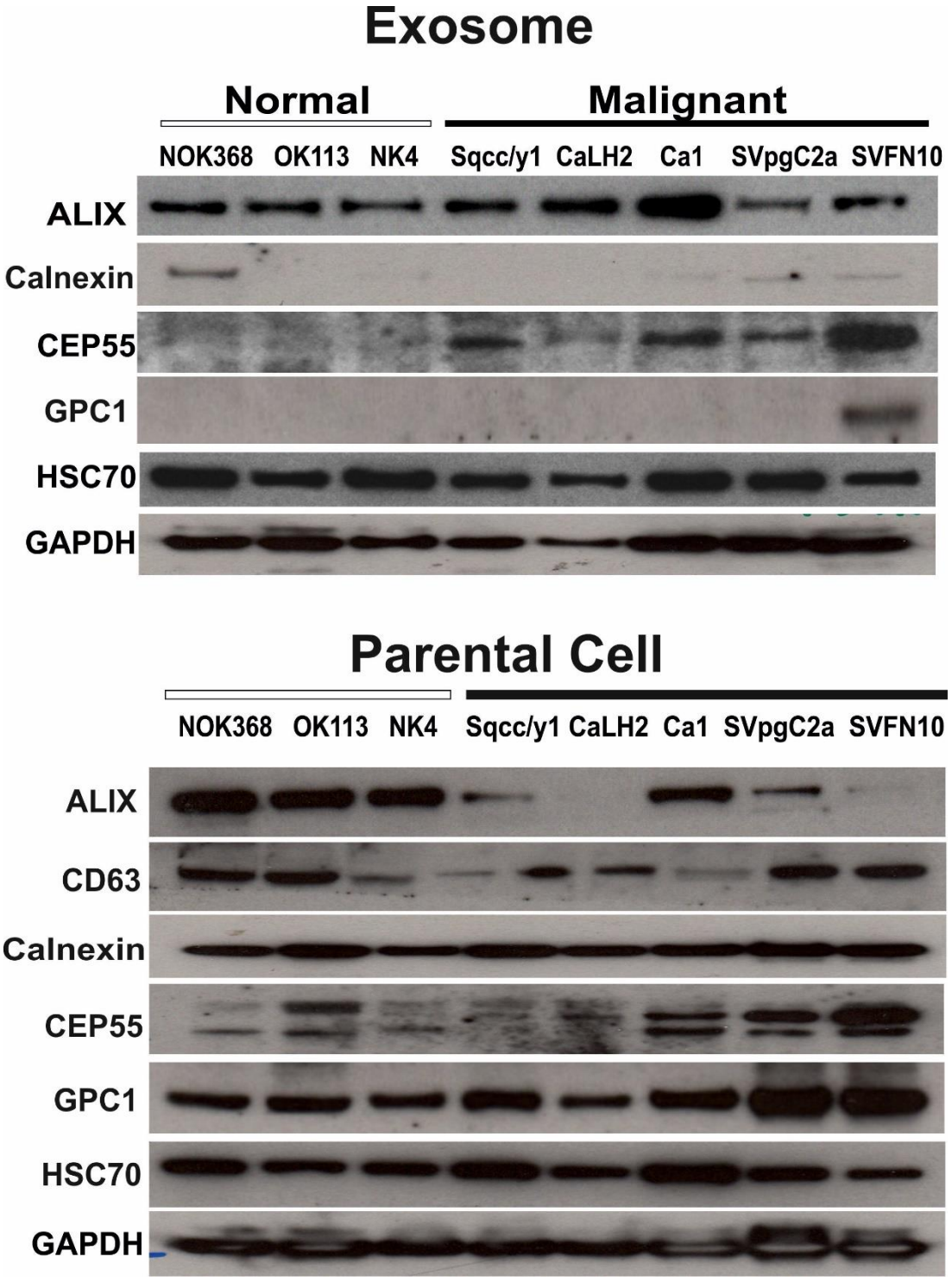


Fig 3.8: Western blotting for exosomal proteins and candidate cancer markers. *Protein expression of ALIX, CD63, Calnexin, CEP55, GPC-1, , HSC70 and GAPDH in exosomes (top) isolated from parental cell lines (bottom) including normal (NOK368, OK113, NK4) and cancer (SqCC/Y1, CaLH2, Ca1, SVpgC2a, SVFN10) oral keratinocytes.*

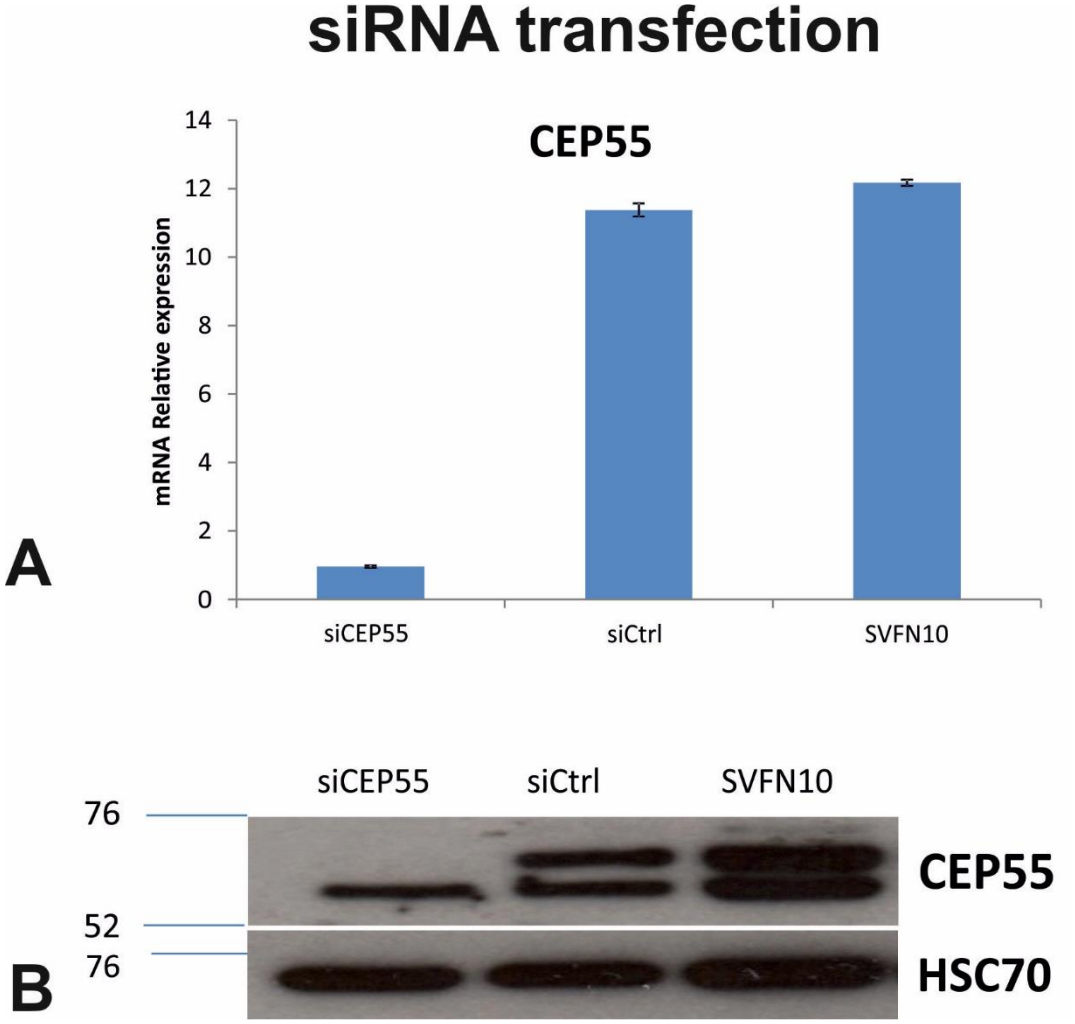


Fig 3.9: Specificity of CEP55 antibody verified by CEP55 knockdown *siRNA transfection of SVFN10 cells with siCEP55 and siCtrl. Untransfected SVFN10 cells were used as an additional control. (A) mRNA expression of CEP55 via RT-qPCR after successful siCEP55 transfection. (B) Western blotting showing loss of top band in siCEP55 transfected cells. HSC70 was used as a loading control.*

3.13 CEP55 as a potential biomarker of oral squamous Cell Carcinoma (OSCC)

Given that CEP55 protein was found to be enriched in exosomes from cancer cell lines, we further investigated if this protein was specific to OSCC exosomes or non-specifically co-purified with exosomes. Immune-gold TEM was performed on exosomes to directly visualise CEP55 protein in exosomes isolated from normal oral keratinocyte cell line OK113, tumour derived oral keratinocyte cell line SqCC/Y1 and normal plasma. Exosomes were incubated with CEP55 antibody and the gold labels were primed against CEP55 antibody. Immuno-TEM images showed that CEP55 gold labels appeared to be clustered around debris in normal plasma and OK113 exosomes while SqCC/Y1 exosomes showed CEP55 gold labels appeared on the outer membrane of exosomes **(Fig 3.10)**. Although these are not quantitative, it provided some qualitative confirmation that CEP55 could be a specific cancer exosomal membrane marker.

We concluded that although CEP55 membrane protein was found on SqCC/Y1 cancer exosomes this finding needs to be quantified using another technique such as single exosome sorting by nano FACS analysis. Since we did not have such a facility at our institute, attempt was made to quantify CEP55 expressing exosomes by immuno-NTA. The samples were prepared using rabbit anti-CEP55 and Alexa fluor 488 antibody and were sent to Malvern company for analysis. Report from Malvern was inconclusive and further optimisation of sample preparation was suggested (data not shown). Ideally the presence of CEP55 specific exosomes should be verified on OSCC patient plasma compared to healthy controls. Request for clinical samples has been forwarded to our clinical collaborator.

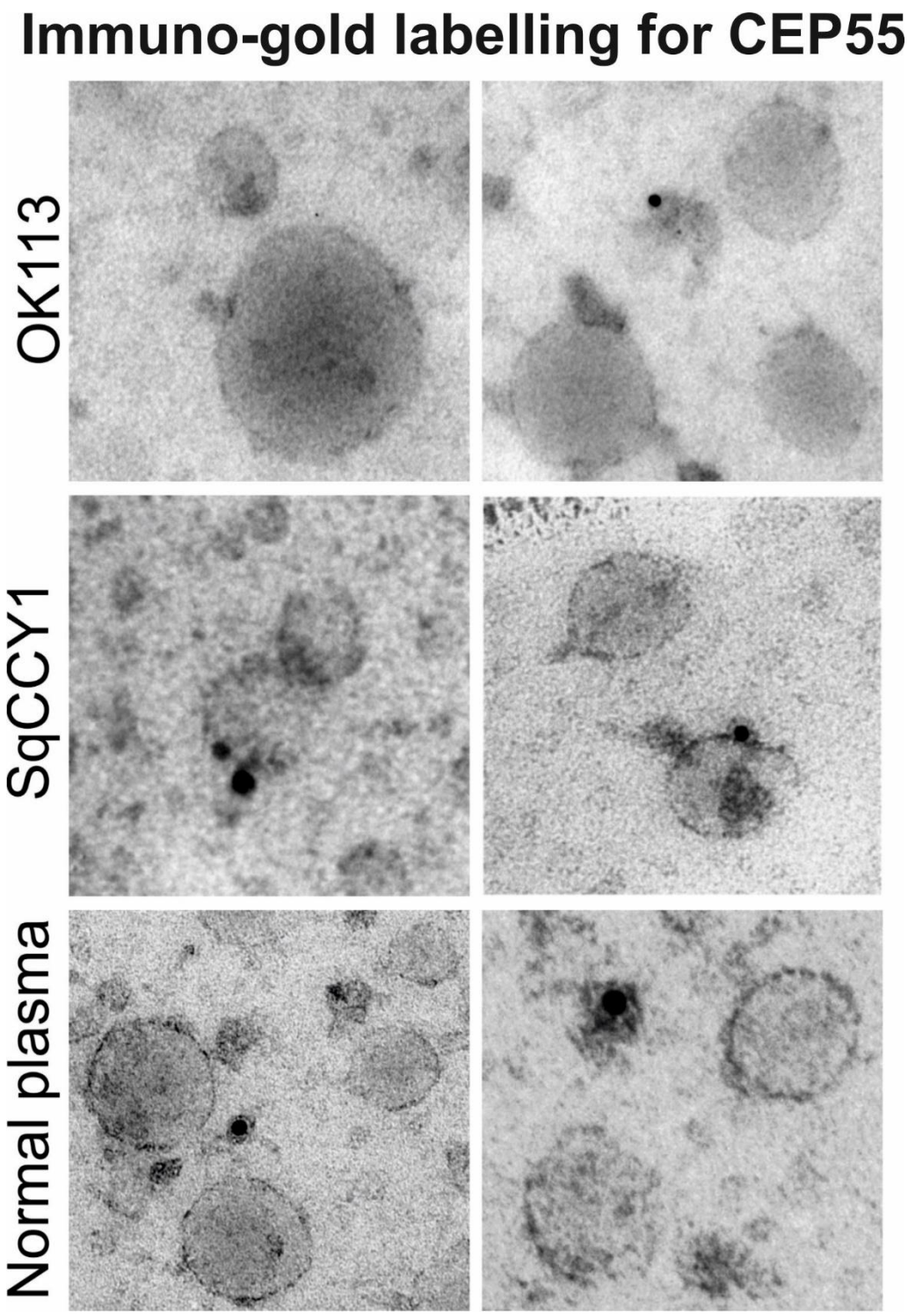


Fig 3.10: Immuno-gold labelling of exosomes on transmission electron microscopy. *Exosomes were isolated from OK113, SqCC/Y1 and normal plasma. The gold labels (black dots) indicated the localisation of CEP55 protein on the membrane of cancer exosomes from SqCC/Y1 cell line. No gold label or non-specific labelling was noted in normal exosomes (OK113 and normal plasma).*

3.14 Discussion

Oral squamous cell carcinoma (OSCC) is the most common oral malignancy, representing approximately 3% of all malignancies in the body and accounting for more than 300,000 newly diagnosed cancer cases each year worldwide (Siegel et al., 2013). The disease is related to high morbidity and mortality rate due to close proximity of cervical lymph nodes resulting in fast spread.

Patients with OSCC are diagnosed only when the disease has reached an advanced stage, leading to a five year survival rate of only 20% (van der Waal, 2013). OSCC diagnosis is currently based on histopathology, which is an invasive method requiring biopsy samples. The limitation of this system is that it is unable to recognise early genetic changes aiding in the development and progression of the disease. Thus, the identification of biological biomarkers that assist in early diagnoses of OSCC is essential.

Advancement in technology has led to increased interest in EVs research. Today we know that EVs are released by all cell type. Out of all the different types of EVs (Apoptotic bodies, microvesicles, exosomes) exosomes have gained the most popularity because they initially form from intracellular compartments, having immense resemblance to their parental cells. They play an essential role in cell-to-cell communication by carrying their contents including proteins, metabolites, RNA (mRNA, miRNA, long non coding RNA), DNAs (mtDNA, ssDNA, dsDNA) and lipids (S et al., 2013, De Toro et al., 2015) which can be transferred to neighbouring and distant cells upon fusion with extracellular membrane.

Due to the presence of exosomes in body fluids they have great potential to serve as a liquid biopsy tool for various diseases. In pancreatic cancer Melo and colleagues have reported the presence of Glypican-1 as a cancer exosome biomarker (Melo et al., 2015). Similarly in ovarian cancer multiple potential exosome biomarkers have been reported (Tang and Wong, 2015).

Exosome based liquid biopsy offers many advantages in comparison to conventional diagnostic methods. A small sample size of tissue biopsy cannot provide the detailed information of genetic heterogeneity within a tumour. However, exosomes shed from heterogeneous tumour can be collected at once and provide the dynamic information from the tumour. Since exosome based biopsy is non-invasive it causes less patient discomfort.

In the field of OSCC, search for a non-invasive diagnostic aid is ongoing. This would offer an advantage of early disease screening segregating high risk individuals from low risk. The high risk individuals can be further kept under observation for detecting early changes to disease state.

Saliva is a very relevant body fluid associated with OSCC. Salivary diagnostics offer many clinical advantages including undemanding collection, as trained personnel are not need. It is non-invasive hence causing no discomfort to the patient. Saliva samples are easier to handle and store, unlike blood saliva does not clot.

To date only a few studies have shown the presence of OSCC bio-markers in saliva (Radhika et al., 2016, Gleber-Netto et al., 2016, Sahibzada et al., 2017). All these studies have proposed increased levels of cytokines (IL-8, IL-6 and TNF alpha) as markers of OSCC initiation. The limitation of cytokines as OSCC markers is that the levels are found to be significantly increased in inflammatory diseases.

With the aim to develop a non-invasive diagnostic test, which is able to report the genetic changes leading to disease development and progression, we looked into the subject of acquiring potential OSCC biomarkers from cancer exosomes in body fluids.

In this study exosomes were successfully isolated from normal and cancer oral keratinocyte cell lines (culture medium), saliva, plasma and blood through ultracentrifugation by optimizing Thery protocol (Thery et al., 2006).

Due to the very small size of exosomes, they are not visible under an inverted light microscope. Multiple visualisation and particle size measuring techniques were used for verification and validation of successful exosome isolation. These include SEM, TEM, zetasizer and NTA **(Fig 3.3, 3.4, 3.5, 3.6 and 3.7. Page no. 85,86,88, 90,92)**. Through NTA it was found that cancer cells released more exosomes compared to normal cells, presumably involved in facilitating cancer progression and spread **(Table 3.1, Page no.91)**. Similar finding has also been reported by Tickner and colleagues (Tickner et al., 2014).

Further, the presence of exosomal specific protein ALIX was verified in our collected samples through Western blotting indicating successful isolation **(Fig 3.8, Page no. 95)**. Expression of other exosomal specific proteins including CD63 and CD9 was not found in exosomes from oral keratinocytes.

We found our samples to be free of cellular contamination which was verified by absence of Calnexin (endoplasmic) protein **(Fig 3.8, Page no 95)**.

3.14(1) CEP55 as a potential biomarker of OSCC exosomes

In adult tissue, CEP55 is most highly expressed in germ cells (testes and ovaries) (Martinez-Garay et al., 2006). It is well known for its role in cytokinesis (Carlton and Martin-Serrano, 2007, Lee et al., 2008). More recently it has been found to regulate a major pro-survival pathway PI3K/AKT pathway (Jeffery et al., 2015) and hence is indispensable for embryonic development. CEP55 also regulates stemness and is found to promote tumorigenesis (Kuo et al., 2011).

Recently increased expression levels of CEP55 were found in human urinary bladder transitional cell carcinoma and prostate cancer (Singh et al., 2015, Kulkarni and Uversky, 2017). In gastric carcinoma along with increased expression levels, CEP55 was also found to regulate cell proliferation (Tao et al., 2014).It has also been found to play a role in development of laryngeal squamous cell carcinoma (Hui et al., 2015).

CEP55 is a direct transcriptional target of FOXM1 (Waseem et al., 2010, Gemenetzidis et al., 2009). FOXM1 transcriptional program is activated during G2/M to allow normal progression through mitosis in a timely fashion. FOXM1 is overexpressed in many cancers, where it is associated with chromosomal instability (Laoukili et al., 2005, Laoukili et al., 2007).

In OSCC, FOXM1 is found to be overexpressed, where it correlates with CEP55 overexpression in lymph node metastasis. In contrast, both genes are expressed at low levels in the basal layer of normal oral mucosa (Waseem et al., 2010). Furthermore, CEP55 has been reported as a driver of FOXM1 expression in OSCC cell lines where it up regulates FOXM1 levels in a dose dependent manner, resulting in increased transcription and activity of MMP-2 (Chen et al., 2009). These findings suggest that CEP55 exist in a positive feedback loop with FOXM1, where CEP55 promotes FOXM1 expression, which in turn increases transcription of CEP55 (Jeffery et al., 2016).

In our study expression of FOXM1 protein was not found in oral keratinocytes exosomes from normal and cancer cell lines, while the expression of CEP55 protein was exclusively found in cancer exosomes **(Fig 3.8, Page no. 95)**. Specificity of

CEP55 antibody was established through CEP55 knockdown in SVFN10 cell line **(Fig 3.9, Page no. 96)**. We further investigated this finding by immuno TEM and found CEP55 was expressed on the outer surface of cancer exosomes isolated from SqCC/Y1 tumour derived cell line and not on normal exosomes from OK113 cell line and healthy volunteer plasma **(Fig 3.10, Page no. 98)**. This finding suggests that CEP55 can be used as a potential OSCC biomarker to segregate cancer exosomes from normal in a diverse body fluid such as saliva and blood.

Immuno TEM is a subjective technique and a more quantitative method such as nano FACS needs to be done for a definitive conclusion. Furthermore, these findings need to be verified on OSCC patient plasma and saliva.

3.15 Limitations

Isolating exosome from cell lines and body fluids such as saliva and blood is a time consuming and laboursome process. It can take up to ten days to isolate exosomes from cell lines and almost double the time when collecting from primary cell lines, which grow at a much slower rate.

Isolating exosomes from body fluids requires diluting it to a workable viscosity. The duration and speed of centrifuge has to be increased and extra filtration is needed to get rid of debris. The final pellet of exosomes is invisible to the naked eye; hence it can be easily missed resulting in no to low yield. Novel nanoparticle isolation/detection technology may have to be developed to help resolve this problem.

OSCC is a heterogeneous disease involving majorly epithelial cells and surrounding supporting tissue. Developing a diagnostic or screening test for OSCC based on exosomes isolated from saliva has the limitation that saliva is a product of salivary glands, majorly made of secretory acini cells and not epithelial cells. Although some literature has suggested that circulating biomolecules that originate from the disease, entering bloodstream may eventually be transported in to the salivary glands resulting in changes in the composition of saliva (Schafer et al., 2014, Yoshizawa et al., 2013), this mechanism needs to be further investigated.

In addition, due to gaps in our knowledge currently we are unaware as to how long it would take for cancer exosomes to appear in the peripheral blood or saliva to be detected for early detection of the disease.

Chapter 4

Functional significance of Oral cancer exosomes

4.1 Introduction

The emerging role of exosomes as a liquid biopsy tool and a therapeutic delivery vesicle to cells has overshadowed its functional significance in health and disease state. Today we have limited knowledge regarding the effects on exosomes on it neighbouring cells. Since exosomes are known to participate in intercellular communication and are capable of transferring their content including mRNA (Janas et al., 2015), miRNA (Taylor and Gercel-Taylor, 2008), siRNA, piRNA (Baixauli et al., 2014), dsDNA (Thakur et al., 2014), proteins (Buschow et al., 2005) and lipids (Record et al., 2014) to neighbouring cells and to distant cells via the bloodstream (Taylor and Gercel-Taylor, 2013). It has been hypothesized that this transfer of information can possibly lead to cell fate determination. A detailed insight into the content of exosomes reported to date can be reviewed on ExoCarta database (http://www.exocarta.org/) (Keerthikumar et al., 2016) and vesiclepedia (Kalra et al., 2012).

Their involvement in cell-cell communication is indicative of their influence on tumour development, progression, metastasis and therapeutic efficacy (Kucharzewska and Belting, 2013). Exosomes released by cancerous cells carry numerous biomarkers, which are passed on to healthy cells via microenvironment, causing stromal and angiogenic activation along with immune escape (Tickner et al., 2014). They contribute to metastasis by aiding in epithelial to mesenchymal transition and formation of pre-metastatic niche (Jeppesen et al., 2014b). Exosomes also shield tumour cells from cytotoxic properties of chemotherapeutic drugs and transfer chemo-resistant effects to nearby cells (Federici et al., 2014).

Multiple studies have focused on the miRNA content of exosomes, as it is abundant and regulates the mRNA expression within recipient cells (Valadi et al., 2007). Study by Grange and colleagues have reported that exosomes from cancer stem cells contained miR29a, miR650 and miR15, all associated with tumour invasion and metastasis. In addition exosome bound miR19b, miR29c and miR151 are found to be up regulated in patients with renal carcinoma (Grange et al., 2011)

In a study on oesophageal squamous cell carcinoma (ESCC) serum exosomal miRNA-1246, associated with metastasis through tumour microenvironment has been found to be upregulated in ESCC cell lines. miRNA-1246 has been proposed as a diagnostic and prognostic biomarker in ESCC (Takeshita et al., 2013).

Studies have shown that the expression of miRNA is higher in exosomes compared to parental cells (Goldie et al., 2014) and many of the RNAs enriched in exosomes may not be abundant or even detectable in the parental cell or highly expressed within the cell and low or absent within exosomes (Taylor and Gercel-Taylor, 2013, Taylor and Gercel-Taylor, 2008), indicating sorting of specific RNAs into exosomes (Guduric-Fuchs et al., 2012). Although the mechanism of this selective sorting is unclear, some have proposed this selectivity relates to miRNA/RNA induced silencing complex (RISC) component (Taylor and Gercel-Taylor, 2013).

With so much work focused on miRNA, very little is known regarding the mRNA content of exosomes (Valadi et al., 2007, Tomasoni et al., 2013). Studies on transfer of reporter mRNA and their translation into proteins, suggests that mRNA delivered from exosomes to recipient cells are functional (El-Andaloussi et al., 2012, Tetta et al., 2013).

Exosomes derived from colorectal tumours (Silva et al., 2012) , lung (Rabinowits et al., 2009), and prostate (Bryant et al., 2012) cancer cells alter the phenotype of normal cells by transferring specific RNA subsets. In contrast, exosomes released from normal surrounding cells may modify cancer cell gene expression (Bryant et al., 2012).

To date only a hand full of studies have been conducted on OSCC exosomes. Winck and colleagues reported that salivary exosomes from OSCC patients were enriched with proteins responsible for molecular transport, cell growth and proliferation (Winck et al., 2015). While another study have suggested that Epstein Barr Virus infected cells package the virus in exosomes which manipulates the tumour microenvironment and contribute to tumour escape mechanisms by inducing apoptosis of immune cells (Principe et al., 2013).

Exosomes derived from HNSCC cell lines have been found to promote cell survival after ionizing radiation (Mutschelknaus et al., 2016) by triggering DNA repair as it has been shown that phosphorylation of critical DNA repair proteins is influenced by exosomes (Dutta et al., 2014).

In the field of oral cancer the miRNA content of exosomes have already been studied (Sakha et al., 2016). Through mi-RNA sequencing it was found that exosomes derived from Hypoxic oral squamous cell carcinoma deliver miRNA-21 to surrounding normal cells. Exosomal miRNA-21 markedly increased the expression of snail and vimentin gene giving way for Epithelial Mesenchymal Transition (EMT) to elicit a pro-

metastatic phenotype (Li et al., 2016a). Currently, not much is known about mRNA cargo of oral cancer exosomes and their implication on neighbouring healthy cells.

To our knowledge no study has been published on the functional significance of mRNA content of OSCC exosomes on recipient cells. Hence we took the opportunity to explore the effect of packaged mRNA within exosomes secreted by normal, pre-cancerous and cancerous oral keratinocytes cells on recipient cells.

In this study characterisation of exosomal mRNA cargo was done using RT-qPCR and Agilent Bioanalyzer. Functional significance of exosomes was studied by transfecting normal oral keratinocyte cells with self-derived and cancer-derived exosomes. Through gene-expression microarray and RT-qPCR, genes affected by exosome transfection were identified.

Results

4.2 RNA cargo protected within exosomes

Exosomes were isolated by ultracentrifugation **(Methods Fig 2.1, Page no. 42)**, there is a possibility that some free RNA or protein complexes containing RNA may be precipitated alongside exosomes. In order to validate that exosomal mRNA were protected within exosomes, we subjected exosomal pellet to various biochemical treatments, involving TritonX, proteinase K and RNase A **(Fig 4.1)**. Treatment with proteinase K and RNase A, revealed that RNA is protected within exosomes and is not associated with protein aggregates. Addition of TritonX which is a detergent resulted in rupturing of exosomal lipid bilayer, allowing complete degradation of RNA by RNaseA **(Fig 4.1).** This indicates that RNA is truly protected within exosomes. We verified this finding through Agilant BioAnalyzer **(Fig 4.2)** and RT-qPCR. Total RNA from treated exosomes was used to verify the size and quality of RNA by Agilent BioAnalyzer **(Fig 4.2).**

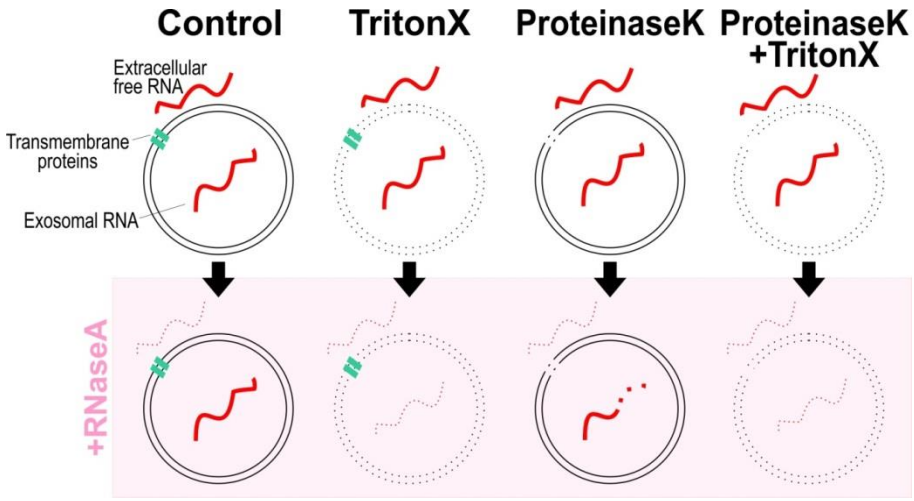


Fig 4.1: Experimental approaches for the validation of exosomal RNA

Diagrammatic representation of various biochemical treatment approaches using proteinase K (for protein digestion) and RNase A (for RNA digestion). Addition of TritonX breaks the protective lipid bi-layer exposing RNA to RNase A for degradation.

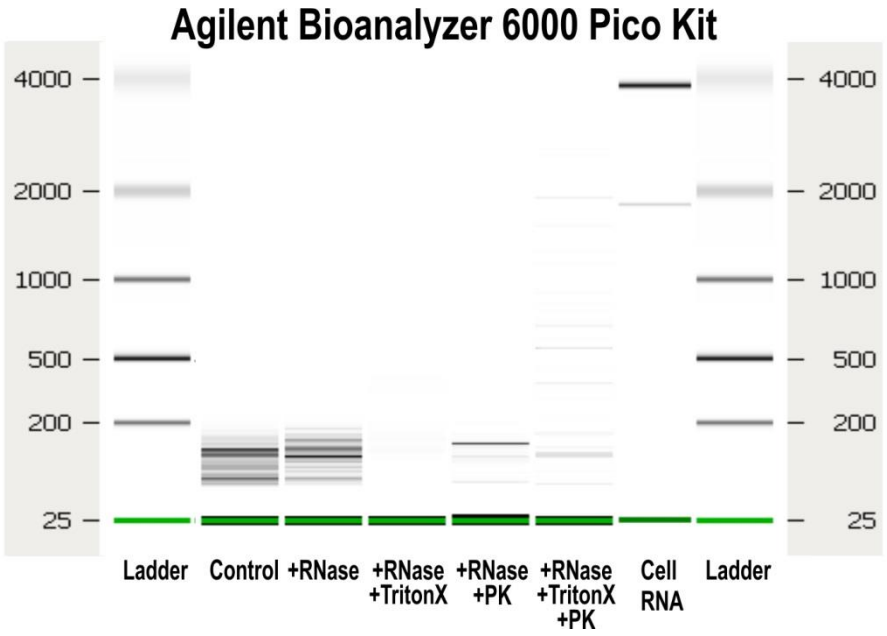


Fig 4.2: Exosomal RNA size and quality validation. *Total RNA isolated from exosomes, pre-treated with various combinations of RNase, TritonX and Proteinase K (PK) as indicated, analysed on BioAnalyzer 6000 Pico chip. Cell RNA, indicates untreated parental cell total RNA, was used as a control showing two ribosomal RNA bands at around 2000 and 4000 nucleotides.*

4.3 FOXM1 mRNA is found within exosomes derived from SVFN10

Having verified that our exosomes contain RNA cargos, we next investigated if cancer exosomes contain mRNA sequence of oncogenes. Total RNA was purified from exosomes prior to reverse transcription and cDNA was used to perform RT-qPCR for detection of FOXM1 mRNA in two cell lines. SVpgC2a expresses very low levels of FOXM1 mRNA whilst SVFN10 consitutively expresses high levels of FOXM1 (Gemenetzidis et al., 2009). We were able to detect high levels of FOXM1 mRNA in exosomes derived from SVFN10 compared to SVpgc2a cells **(Fig 4.3)**.

As SVFN10 cells expresses high levels of FOXM1 mRNA, to rule out the posibility that the FOXM1 mRNA were extravesicular, we subjected exosomes to various

treatments as described in Fig 4.1. Treatment with RNase alone did partially reduce FOXM1 mRNA levels (not significant), but pretreatment with RNase plus TritonX significantly abolished FOXM1 mRNA levels **(Fig 4.4)**. This indicates that large proportion of FOXM1 mRNA were protected within the exosomes. Similarly, pretreatment of exosomes with proteinase K and RNase A did not degrade FOXM1 mRNA indicating that it is not being protected as a protein-mRNA aggregages. Addition of TritonX, Proteinase K and RNase once again led to degration of all mRNA **(Fig 4.4)**, confirming that FOXM1 mRNA were protected within exosomes.

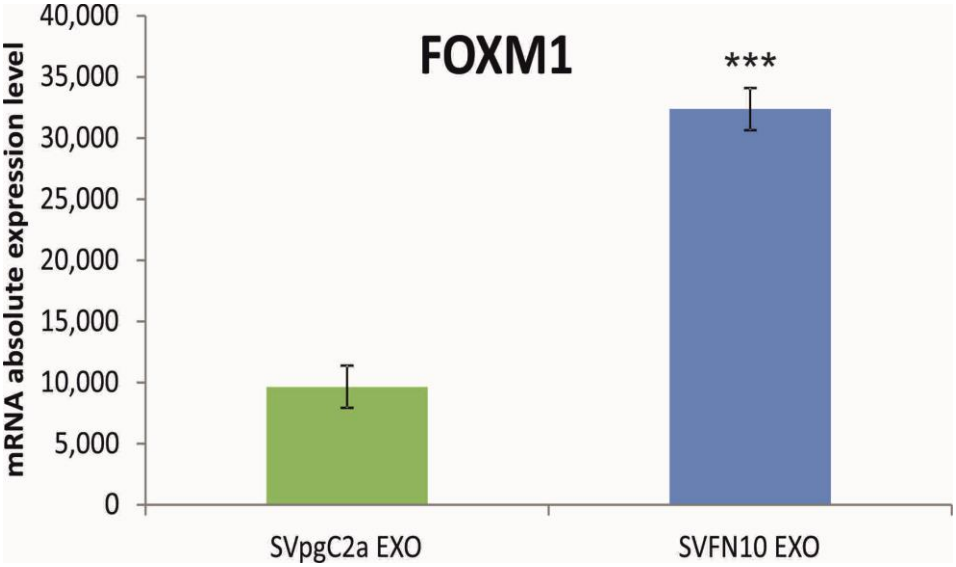


Fig 4.3: Presence of FOXM1 mRNA detected in SVFN10 exosomes through RT-qPCR. *mRNA expression levels of FOXM1 in exosomes derived from SVpgC2a (SVpgC2a EXO) and SVFN10 (SVFN10 EXO) cell lines. The error bars represent the standard error of the mean of three independent experiments (n=3). Significant P values are denoted by (***P<0.001).*

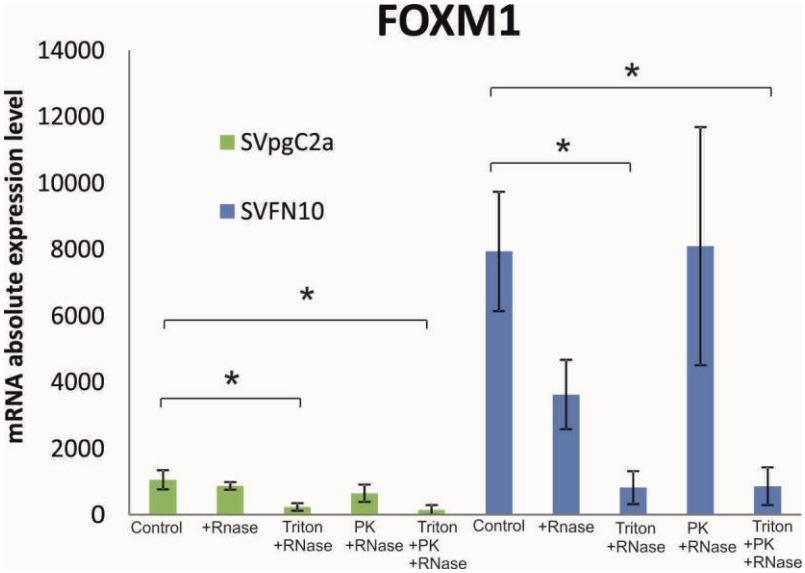


Fig 4.4: Validation of exosomal FOXM1 mRNA *Exosome pellets were pre-treated as shown in Figure 4.1 prior to RT-qPCR to investigate if FOXM1 mRNA were protected within exosomes. The error bars represent the standard error of the mean of three independent experiments (n=3). Significant P values are denoted by (*P<0.05).*

The same experiments were then repeated to measure reference gene, we have chosen β-Actin (ACTB) as it is expressed equally in exosomes from both cell lines **(Fig 4.5)**. Similarly, pre-treatment of exosomes with RNase A and proteinase K alone reduced slightly the levels of mRNA of ACTB while adding triton X significantly lowered the mRNA levels **(Fig 4.6)**. This indicates that the yield of exosomes in the two cell lines are equivalent and that the low levels of FOXM1 mRNA found in SVpgC2a exosomes (Fig 4.3) were not due to low exosome yield, but rather due to low level of FOXM1 gene expression in this cell line.

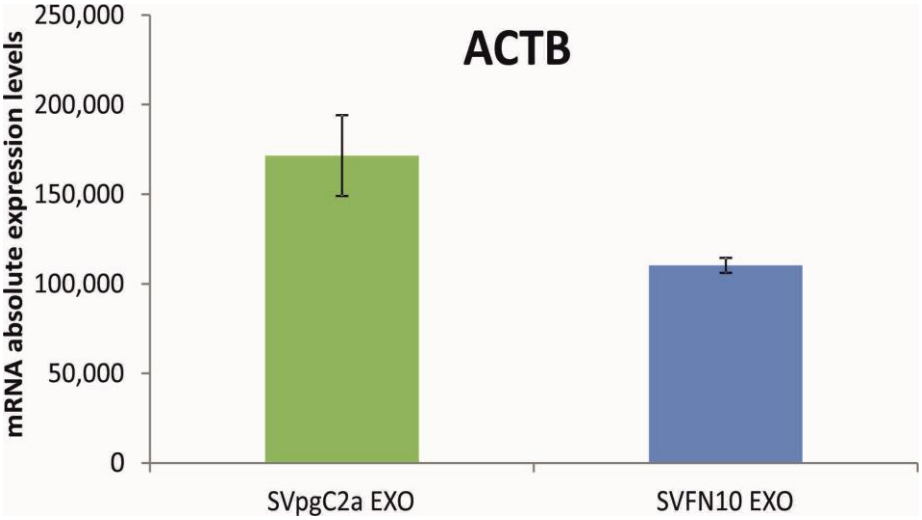


Fig 4.5: mRNA expression levels of β-Actin. *Nearly equal levels of ACTB mRNA was detected in SVpgC2a (green) and SVFN10 (blue) exosomes. The error bars represent the standard error of the mean of three independent experiments (n=3).*

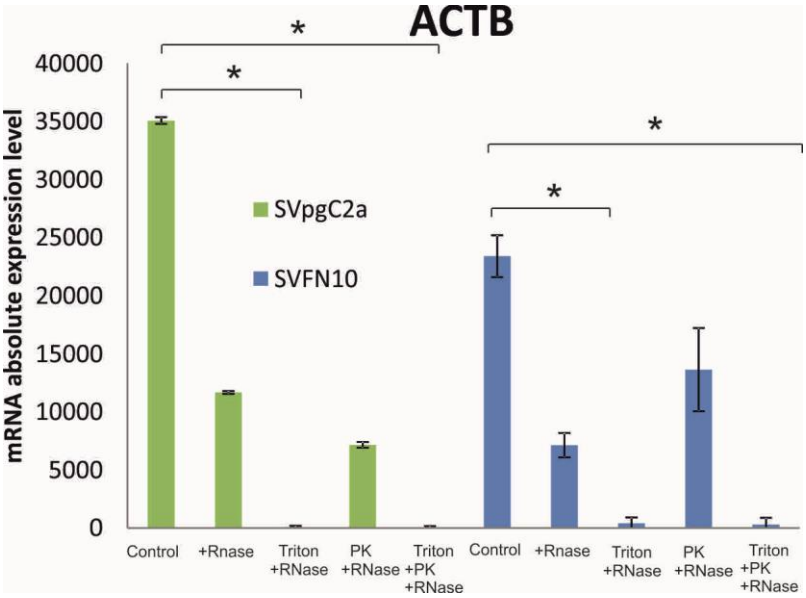


Fig 4.6: Validation of exosomal β-Actin mRNA levels. *Significantly low levels of RNA were found on treating the pellet with triton. The error bars represent the standard error of the mean of three independent experiments (n=3). Significant P values are denoted by (*P<0.05).*

Apart from ACTB, mRNA expression levels of reference gene GAPDH were also examined in treated exosomes and the expression was found similar to ACTB **(Appendix Fig 8.3, Page no. 191).**

4.4 Genes are not equally sorted into exosomes

Treated SVpgC2a and SVFN10 exosomes were also screened for other oncogenes including HOXA7, CCNB1, CENPA, DNMT3B, DNMT1, CEP55, NEK2, HELLS, and BMI1. We found low levels of mRNA packed within exosomes which were detectable after RNase treatment by RT-qPCR. Only HOXA7 mRNA expression levels were found to be upregulated (not significantly) in SVFN10 exosomes compared to SVpgC2a. Whereas MAPK8, AURKA and ITGB1 degraded with RNase treatment suggesting they are not packed with in exosomes but co purify with protein aggregates during isolation (results in **Appendix Fig 8.4, Page no. 193).**

We conclude that exosomal mRNA is protected from extracellular environment including RNase and proteinase. We also report that cancer cells package oncogenes in exosomes although a mechanism which is not fully understood but a sorting system seems to exist through which some genes appeared to be preferentially packed within exosomes.

4.5 Functional effects of exosomes on recipient cells

Many studies have reported the functional transfer and effects of miRNA from exosomes to cell (Pegtel et al., 2010, Stoorvogel, 2012) but again very few studies have focused on the effects of mRNA transfer from exosomes to cells (Valadi et al., 2007). In this study, having established the presence of mRNA within exosomes, we investigated if cancer cells utilise exosomes as a vehicle to export oncogenic mRNA to normal cells. Given that we found high levels of FOXM1 mRNA within exosomes secreted from a malignant cell line (SVFN10), we performed an exosome transfer experiment (which we subsequently termed as “transfection”). We have chosen SVpgC2a cells for transfection as they not only express low levels of FOXM1 oncogene but are also easier to grow in comparison to primary keratinocytes.

SVpgC2a cells were transfected with SVFN10 exosomes, while SVpgC2a cells transfected with self-exosomes (exosomes isolated from SVpgC2a) and exosome-depleted supernatant from ultracentrifugation were used as controls. After 24 hours of transfection, SVpgC2a cells showed observable morphological change in the SVFN10 exosome-transfected cells which became larger and more granular in appearance, similar to the morphology of SVFN10 cells. SVpgC2a cells transfected with SVpgC2a exosomes or depleted supernatant did not show much morphological change **(Fig 4.7)**. The morphological changes became pronounced at 24 hours following transfection with exosomes **(Fig 4.7)**

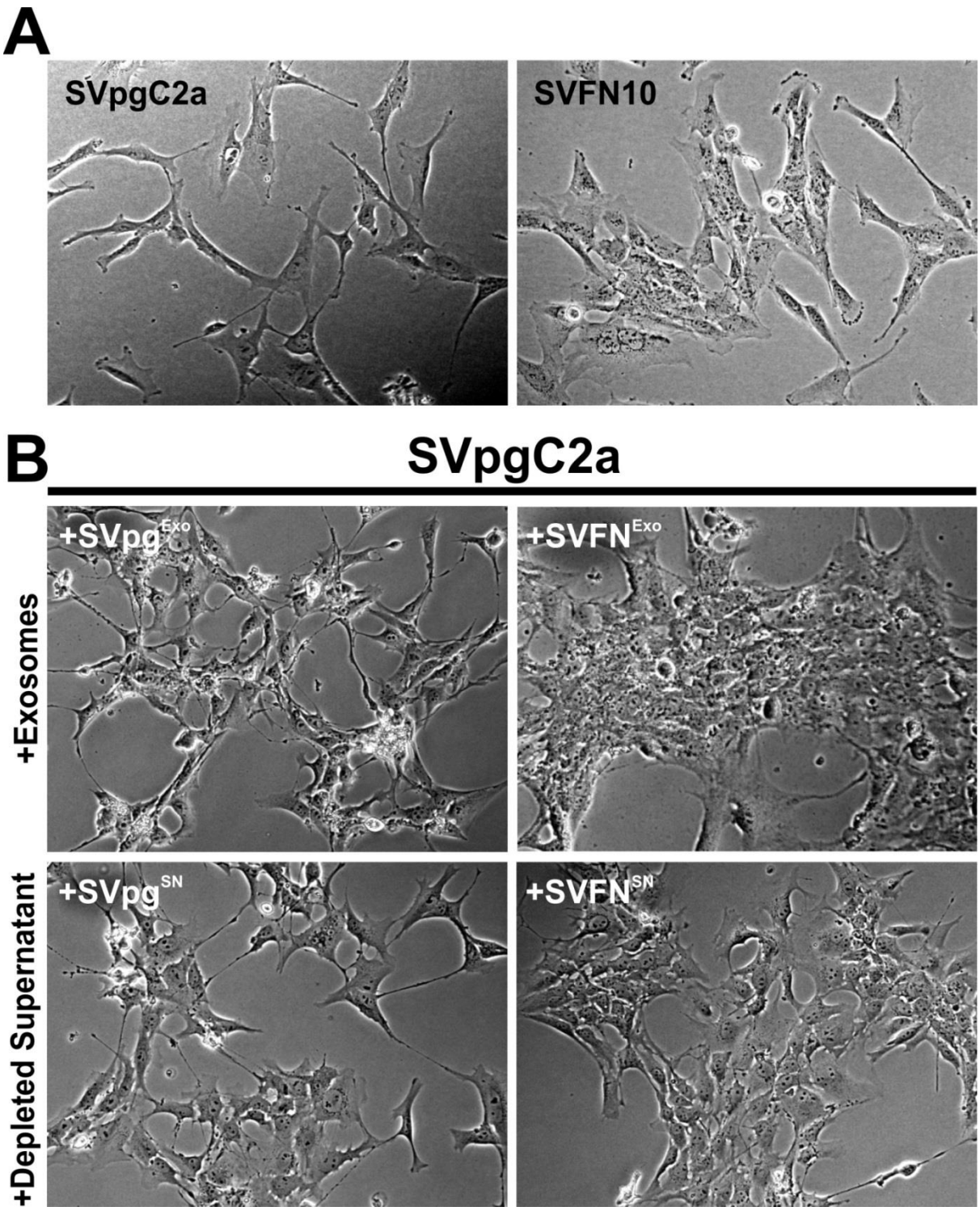


Fig 4.7: Morphological changes in SVpgC2a cells transfected with SVFN10 exosomes. ***(A)*** *Untreated SVpgC2a and SVFN10 cells in growth medium.* ***(B)*** *SVpgC2a cells at 24 hrs following transfection with SVpgC2a (+SVpg^{Exo}) and SVFN10 (+SVFN^{Exo}) exosomes and corresponding exosome-depleted supernatant (^{SN}).*

Following transfection, to investigate if the recipient cells have taken up SVFN10 exosomes (containing high levels of FOXM1 mRNA), the relative mRNA expression of FOXM1 over the reference gene ACTB was analysed through RT-qPCR. It showed slightly increased levels of FOXM1 mRNA at 48 hours of incubation, this effect was lost at 72 hours of incubation **(Fig 4.8)**. Although the relative increase in FOXM1 mRNA levels was not statistically significant, this result suggests that the SVFN10 exosomes may have delivered its cargo (FOXM1) into the recipient cell, albeit only transiently.

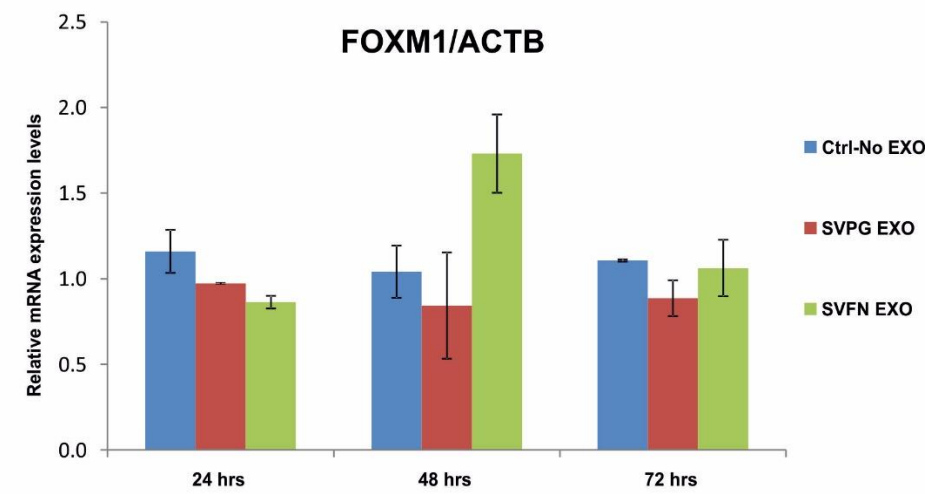


Fig 4.8: mRNA transfer from SVFN10 exosomes to SVpgC2a cells. *Relative expression of FOXM1 mRNA (normalised with ATCB reference gene) in SVpgC2a cells, transfected with SVpgC2a and SVFN10 exosomes for 24, 48 and 72 hours. Un-transfected SVpgC2a (Ctrl-No EXO) were used as controls. The error bars represent the standard error of the mean of three independent experiments (n = 3).*

4.6 Effects of Exosomes on Senescence in oral keratinocytes

Given that we noted morphological changes in recipient cells within 24h of exosomes transfection and the levels of exogenous FOXM1 mRNA did not peak until 48h, FOXM1 mRNA transfer may not be related to morphological change. We therefore further investigated the possible reason for such morphological changes in cells transfected with exosomes. Literature reported that exosomes can potentially induce senescence in cells (Urbanelli et al., 2016). Given that the morphological change did resemble senescence phenotype, we hypothesised that SVFN10 exosomes transfection may have triggered senescence in the recipient SVpgC2a cells. In order to investigate this mechanism we first checked the ability of SVpgC2a cells to senesce with drug treatment. Through a dose response experiment we found that treating SVpgC2a with a chemotherapeutic drug etoposide but not 5-Azacytindine or vehicle DMSO at the concentration of $1x10^{-7}$ M, was the minimally effective dose for activating senescence without causing toxicity to cells. These finding were verified by beta galactosidase assay **(Fig 4.9).**

Fig 4.9: Beta galactosidase assay on SVpgC2a cells. *SVpgC2a cells were treated with vehicle Dimethyl sulfoxide (DMSO; 0.05%) 5-Azacytidine (5AZA) or Etoposide (ETO) at the concentration of $1x10^{-7}$ M. Note the blue staining of SVpgC2a cells treated with etoposide (+ETO) indicating senescence. Untreated SVpgC2a cells were used as a control.*

Once senescence was established in SVpgC2a cells, they were transfected with SVpgC2a and SVFN10 exosomes for 24 and 48h hrs. No senescence was detected through Beta gal assay; hence we decided to check if senescence markers could be detected through another technique.

Through RT-qPCR, we assessed the presence of senescence markers including tumour suppressor gene (P53), cyclin dependent kinase inhibitor 2A (P16), cyclin dependent kinase inhibitor 1A (P21) and chromobox7 (CBX7). We found slight increase in mRNA expression levels of P21 and CBX7 but they were not statistically significant **(Fig 4.10)**. In control cells treated with etoposide, all 4 genes were significantly upregulated compared to untreated cells.

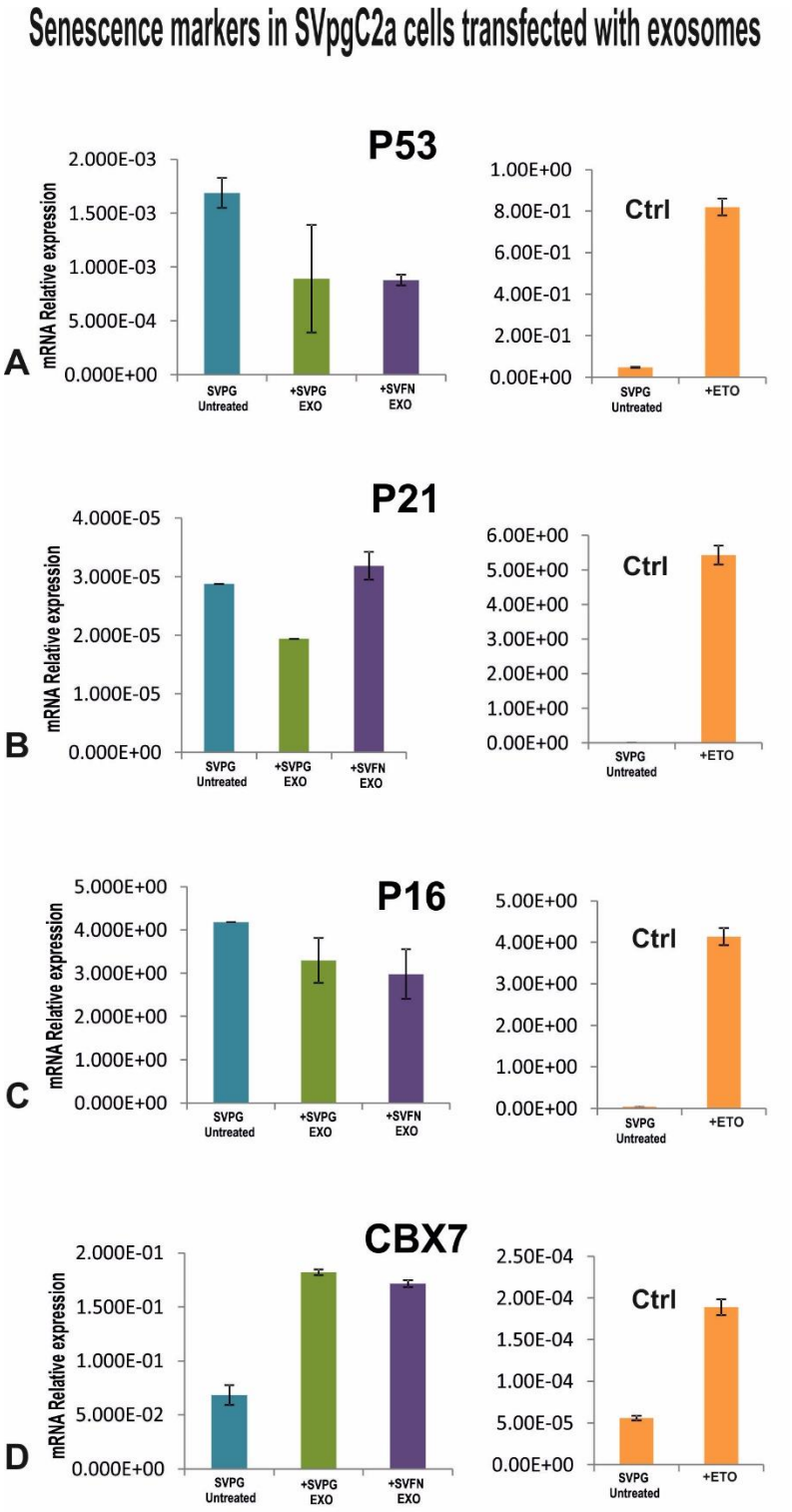


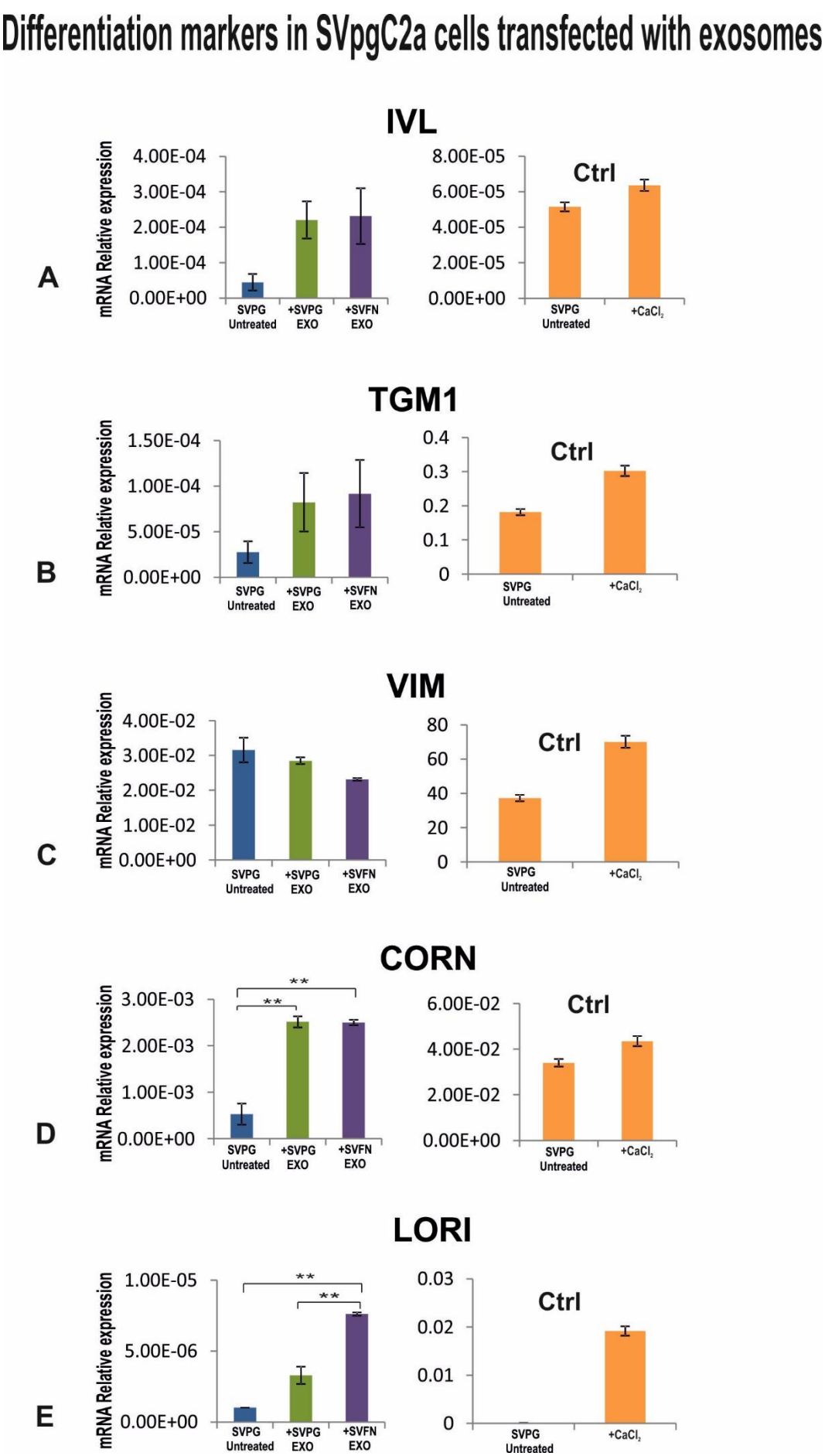
Fig 4.10: mRNA expression of senescence markers (A) *P53* ***(B)*** *P21* ***(C)*** *P16 and* ***(D)*** *CBX7 in SVpgC2a cells transfected with SVpgC2a (+SVPG EXO) and SVFN10 (+SVFN EXO) exosomes. Untreated and etoposide treated SVpgC2a cells were used as controls. The error bars represent the standard error of the mean of three independent experiments (n = 3).*

4.7 Effects of exosomes on senescence on oral fibroblasts

Since no evidence of senescence was found in oral keratinocyte (SVpgC2a) cells transfected with cancer exosomes, further research into the literature suggested that exosomes have an effect on surrounding supporting tissue (Urbanelli et al., 2016). This led us to investigate if cancer exosomes may cause senescence in normal oral fibroblast (NHOF-1) cells. NHOF-1 cells were treated with either Etoposide or transfected with SVpgC2a or SVFN10 exosomes. We were unable to establish senescence in NHOF-1 cells through Beta galactosidase assay **(Appendix Fig 8.5, Page no. 194)** and RT-qPCR. Due to the unavailability of another normal oral fibroblast cell line this experiment was not pursued.

4.8 Effects of exosomes on differentiation in SVpgC2a oral keratinocytes

In addition to senescence, such morphological changes can also be noted in differentiating cells (Quesenberry et al., 2015). The ability of SVpgC2a cells to differentiate was verified with calcium chloride ($CaCl_2$) treatment at the concentration of 1mM and confirmed through qPCR. SVpgC2a cells were transfected with either self or SVFN10 exosomes for 48 hours. Un-treated and $CaCl_2$-treated SVpgC2a cells were used as negative and positive controls, respectively. Through RT-qPCR mRNA expression of various differentiation markers including involucrin (IVL), trandglutaminase 1 (TGM1), vimentin (VIM), cornifin (CORN), loricrin (LORI), filaggrin (FLG), keratin 1 (K1) and keratin 10 (K10) were investigated **(Fig 4.11)**. In transfected cells we found slight increase in the levels of IVL, TGM1, K1 and K10. Statistically Significant increase was noted in mRNA levels of CORN, LORI and FLG **(Fig 4.11).**


Differentiation markers in SVpgC2a cells transfected with exosomes
IVL
A
mRNA Relative expression
4.00E-04
3.00E-04
2.00E-04
1.00E-04
0.00E+00
SVPG Untreated
+SVPG EXO
+SVFN EXO
8.00E-05
6.00E-05
4.00E-05
2.00E-05
0.00E+00
Ctrl
SVPG Untreated
+CaCl2
TGM1
B
mRNA Relative expression
1.50E-04
1.00E-04
5.00E-05
0.00E+00
SVPG Untreated
+SVPG EXO
+SVFN EXO
0.4
0.3
0.2
0.1
0
Ctrl
SVPG Untreated
+CaCl2
VIM
C
mRNA Relative expression
4.00E-02
3.00E-02
2.00E-02
1.00E-02
0.00E+00
SVPG Untreated
+SVPG EXO
+SVFN EXO
80
60
40
20
0
Ctrl
SVPG Untreated
+CaCl2
CORN
D
mRNA Relative expression
**
**
3.00E-03
2.00E-03
1.00E-03
0.00E+00
SVPG Untreated
+SVPG EXO
+SVFN EXO
6.00E-02
4.00E-02
2.00E-02
0.00E+00
Ctrl
SVPG Untreated
+CaCl2
LORI
E
mRNA Relative expression
**
**
1.00E-05
5.00E-06
0.00E+00
SVPG Untreated
+SVPG EXO
+SVFN EXO
0.03
0.02
0.01
0
Ctrl
SVPG Untreated
+CaCl2

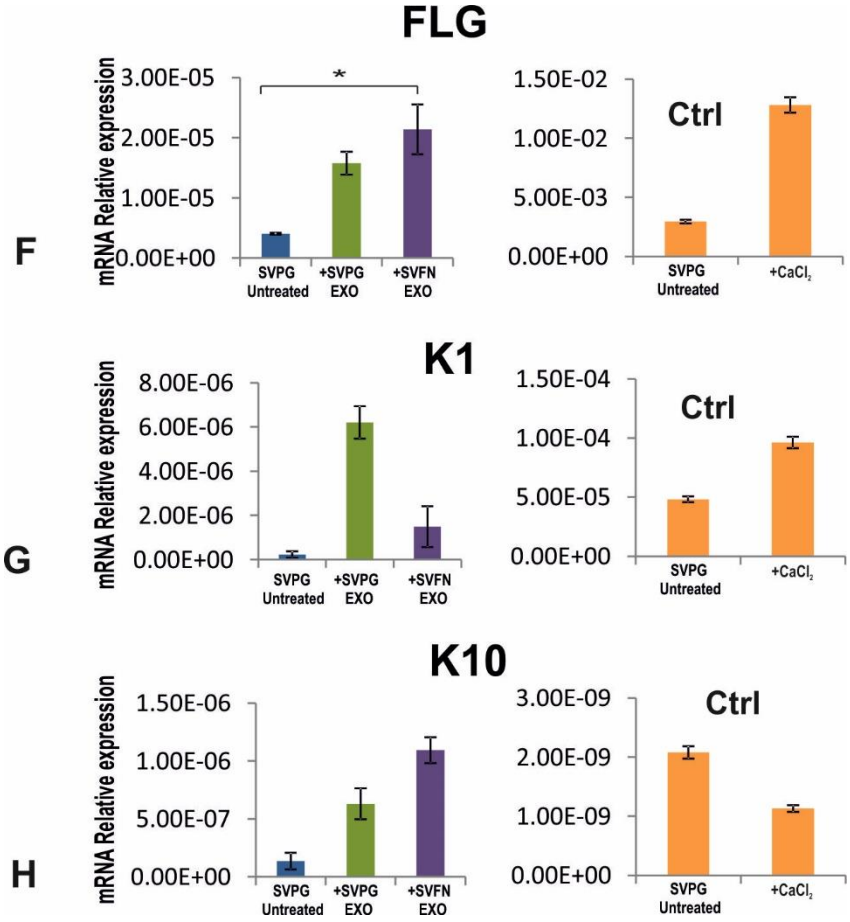


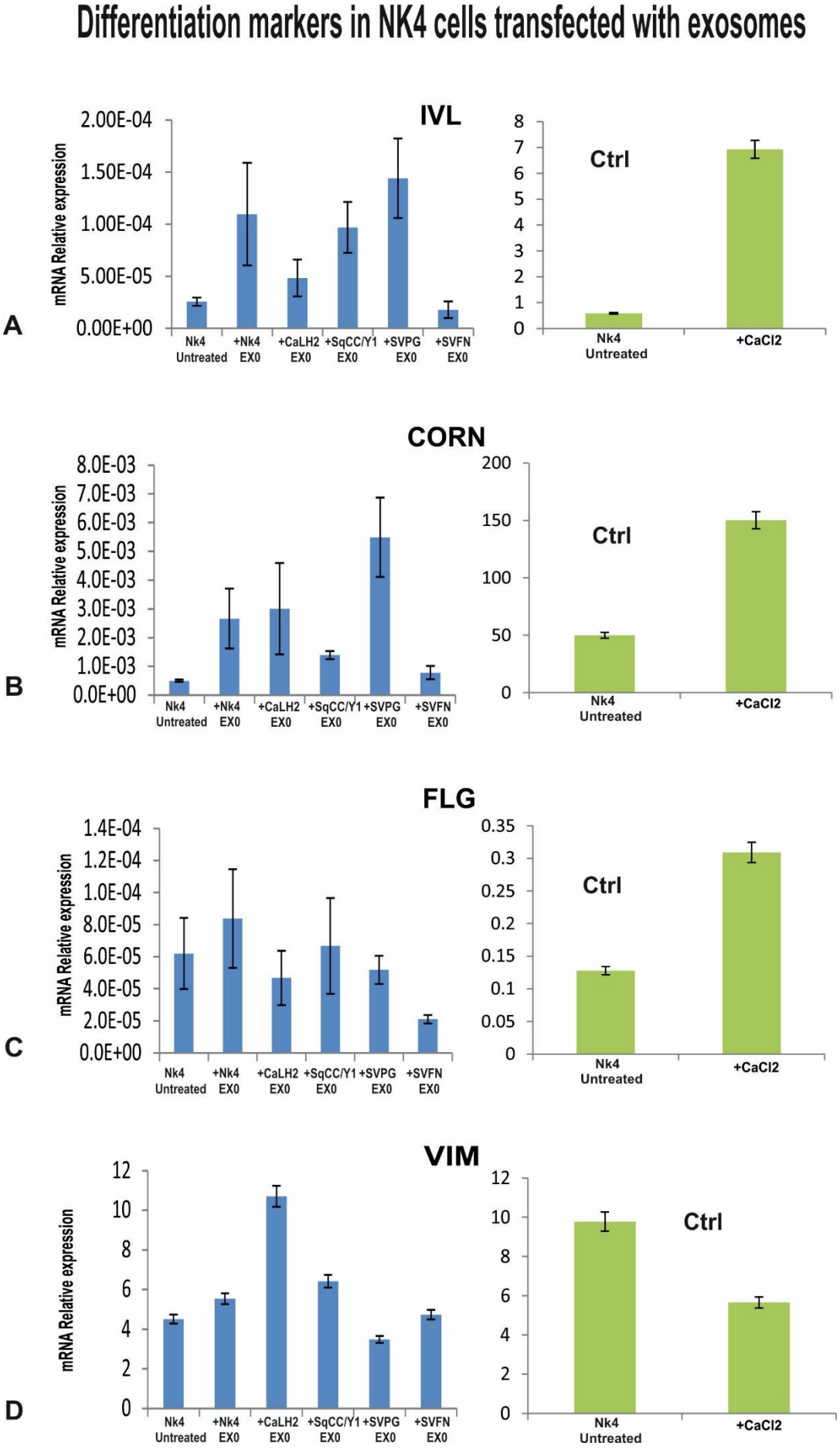
Fig 4.11: Effects of exosomes on mRNA expression of differentiation markers in recipient SVpgC2a oral keratinocytes. ***(A)*** *involucrin (IVL)* ***(B)*** *trandglutaminase 1 (TGM1)* ***(C)*** *vimentin (VIM)* ***(D)*** *cornifin (CORN)* ***(E)*** *loricrin (LORI)* ***(F)*** *filaggrin (FLG)* ***(G)*** *keratin 1 (K1) and* ***(H)*** *keratin 10 (K10) in SVpgC2a cells transfected with SVpgC2a (+SVPG EXO) and SVFN10 (+SVFN EXO) exosomes. Untreated and $CaCl_2$ treated SVpgC2a cells were used as controls. The error bars represent the standard error of the mean of three independent experiments (n = 3). Significant P values are denoted by *P<0.05 and **P<0.01.*

4.9 Effect of exosomes on differentiation in primary normal oral Keratinocytes

We further expanded our study to investigate if differentiation could be induced by exosomes in normal primary oral keratinocytes (NK4). The cells were treated with $CaCl_2$, increased expression levels of differentiation markers were verified through RT-qPCR. NK4 cells were transfected with self-exosomes; tumour derived exosomes from cell lines SqCC/Y1 and CaLH2, premalignant SVpgC2a and transformed malignant SVFN10 exosomes. Un-transfected and $CaCl_2$-treated cells were used as controls.

After 48 hours of incubation, NK4 cells transfected with exosomes did not show any morphological changes **(Fig 8.6 in Appendix, Page no. 195).** mRNA expression levels of IVL, CORN, FLG, VIM, K13, K1 and K10 were investigated through RT-qPCR **(Fig 4.12)**. Through expression levels of IVL, CORN and FLG we found that SVFN10 exosomes suppressed differentiation indicated by low expression while SVpgC2a exosomes induced differentiation indicated by increased expression. This finding is in line with mechanism of tumour development where most of the cells inside the tumour are highly proliferative and poorly differentiated.

Although it was evident that transfection with exosomes caused a reaction that was reflected by altered mRNA levels in transfected cells compared to un-transfected cells. However, no clear pattern was evident.


Differentiation markers in NK4 cells transfected with exosomes
A
IVL
mRNA Relative expression
2.00E-04
1.50E-04
1.00E-04
5.00E-05
0.00E+00
Nk4 Untreated
+Nk4 EX0
+CaLH2 EX0
+SqCC/Y1 EX0
+SVPG EX0
+SVFN EX0
Ctrl
8
7
6
5
4
3
2
1
0
Nk4 Untreated
+CaCl2
B
CORN
mRNA Relative expression
8.0E-03
7.0E-03
6.0E-03
5.0E-03
4.0E-03
3.0E-03
2.0E-03
1.0E-03
0.0E+00
Nk4 Untreated
+Nk4 EX0
+CaLH2 EX0
+SqCC/Y1 EX0
+SVPG EX0
+SVFN EX0
Ctrl
200
150
100
50
0
Nk4 Untreated
+CaCl2
C
FLG
mRNA Relative expression
1.4E-04
1.2E-04
1.0E-04
8.0E-05
6.0E-05
4.0E-05
2.0E-05
0.0E+00
Nk4 Untreated
+Nk4 EX0
+CaLH2 EX0
+SqCC/Y1 EX0
+SVPG EX0
+SVFN EX0
Ctrl
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Nk4 Untreated
+CaCl2
D
VIM
mRNA Relative expression
12
10
8
6
4
2
0
Nk4 Untreated
+Nk4 EX0
+CaLH2 EX0
+SqCC/Y1 EX0
+SVPG EX0
+SVFN EX0
Ctrl
12
10
8
6
4
2
0
Nk4 Untreated
+CaCl2

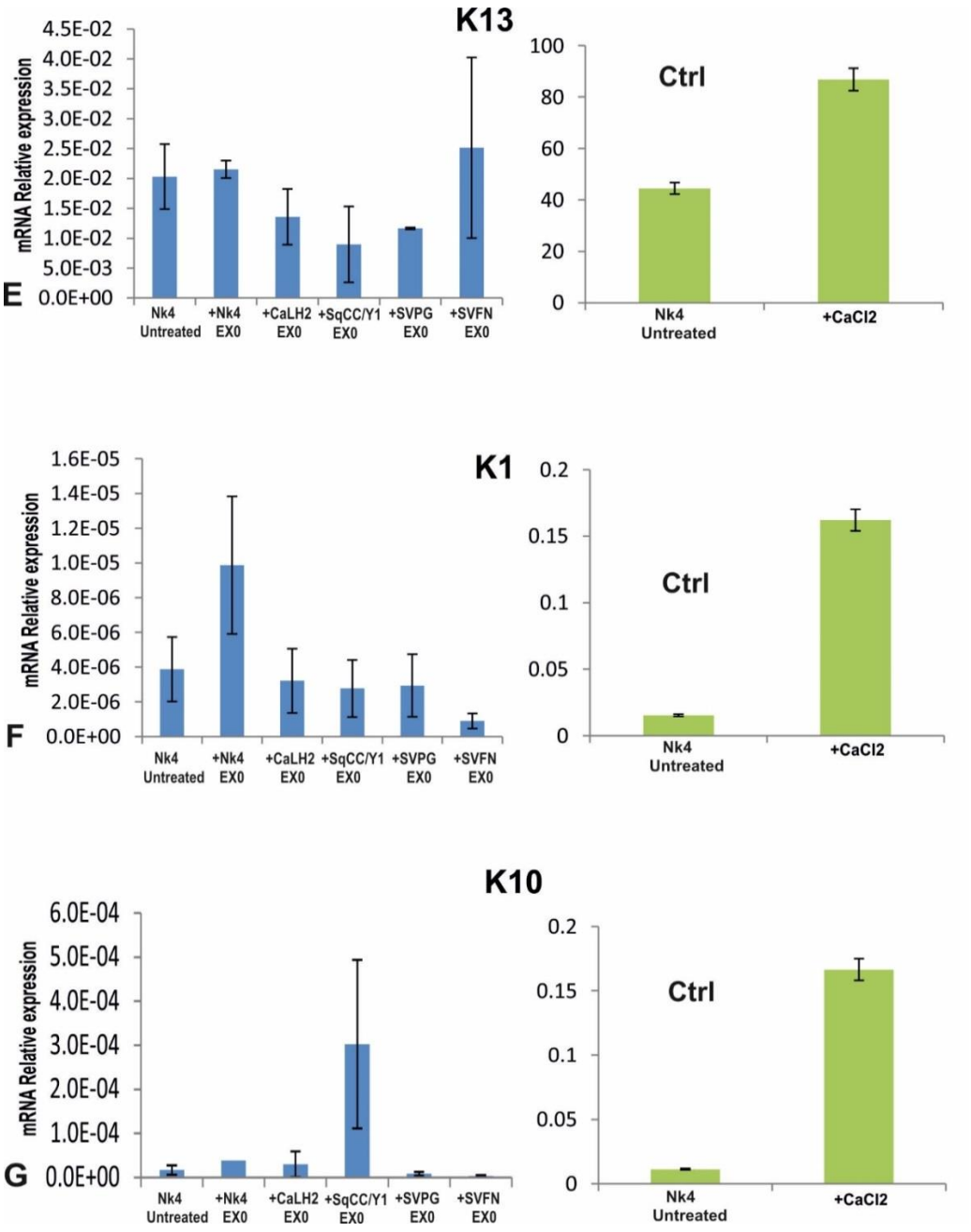


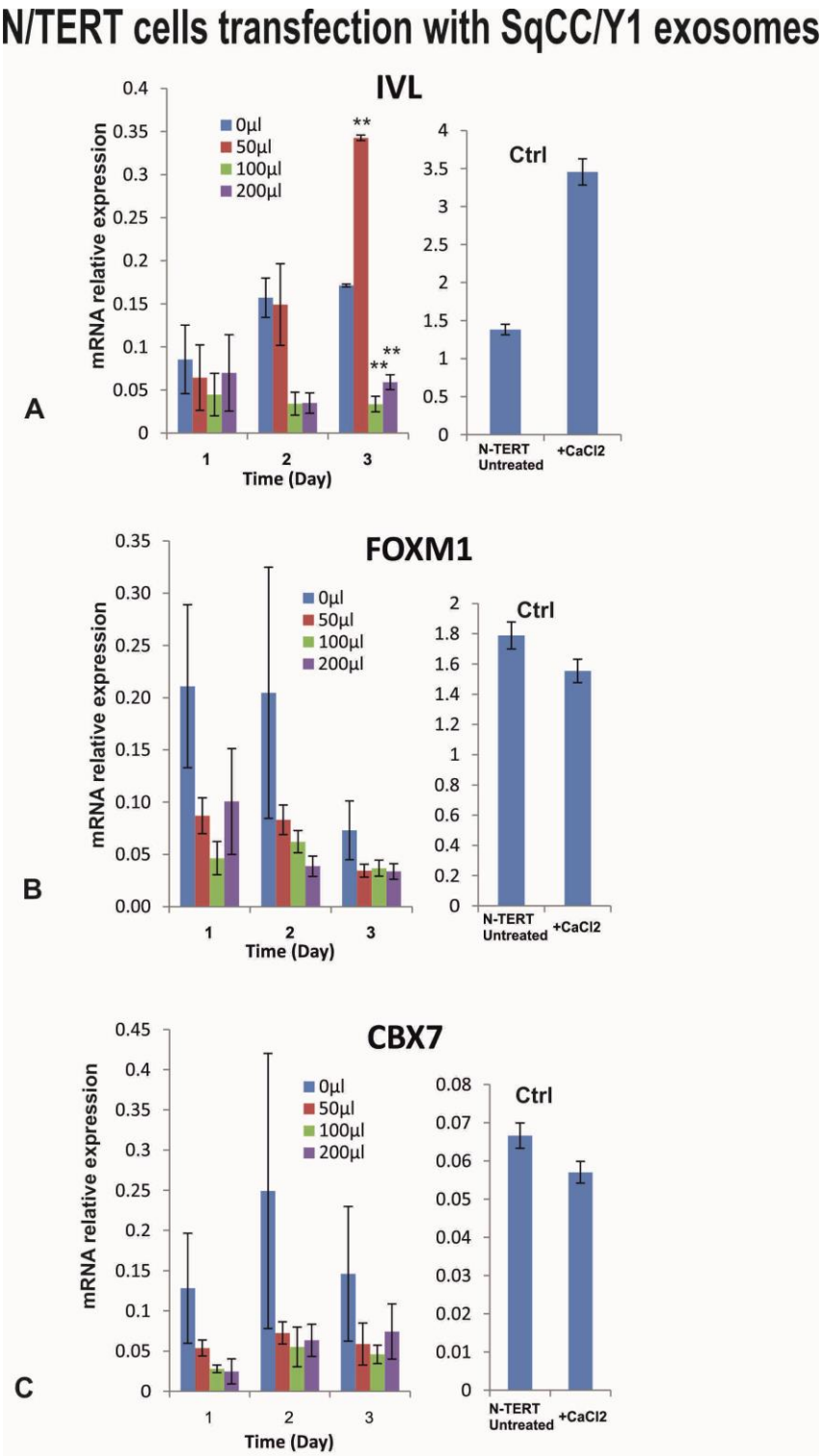
Fig 4.12: mRNA expression levels of differentiation markers in exosome transfected normal primary oral keratinocytes (NK4). (A) *involucrin (IVL),* ***(B)*** *cornifin (CORN),* ***(C)*** *filaggrin (FLG),* ***(D)*** *vimentin (VIM),* ***(E)*** *keratin 13 (K13)* ***(F)*** *keratin 1 (K1) and* ***(G)*** *keratin 10 (K10). NK4 cells were transfected with exosomes from NK4, CALH2, SqCC/Y1, SVpgC2a and SVFN10 cell lines as indicated. Untreated and $CaCl_2$-treated NK4 cells were used as controls. The error bars represent the standard error of the mean of three independent experiments (n = 3). Significant P values are denoted by *P<0.05, **P<0.01 and ***P<0.001. mRNA relative expression is calculated against reference genes YAP1 and POLR2A.*

4.10 Time and dose response of exosome transfection on differentiation in epidermal keratinocytes

Upon finding weak evidence of exosome-induced differentiation in oral keratinocytes, we re-evaluated the time and dose-response of exosomes transfection using an immortalised epidermal keratinocyte cell line (N/TERT) known to retain the potential for differentiation (Dickson et al., 2000). To date no studies have been done on the minimum required dose and time of exosome exposure. We investigated this by transfecting N/TERT with exosomes derived from a HNSCC cell line SqCC/Y1 for 24, 48 and 72 hours at the dose of 50 μl (9.35E+09 particles/ml) ,100 μl (1.87E+10 particles/ml) and 200 μl (3.74E+10 particles/ml).

mRNA expression levels of IVL, VIM, S100A16 (involved in differentiation and EMT), FOXM1 (cell cycle/oncogene), CBX7 (senescence related), MMP13 (matrix metallopeptidase 13) were explored by RT-qPCR in exosome-transfected N/TERT cells **(Fig 4.13)**. Of all these genes, VIM was the only gene significantly increased in a dose- and time-dependent fashion following exosome transfection. The rest of the genes appeared to be downregulated by exosomes although none of these effects were statistically significant. We realized that we needed to know about more relevant genes that would be affected at mRNA level by exosomal transfection.

These results were part of an MSc project performed by Christina Puspita Sari.


N/TERT cells transfection with SqCC/Y1 exosomes
A
IVL
mRNA relative expression
0µl
50µl
100µl
200µl
**
**
**
Time (Day)
Ctrl
N-TERT Untreated
+CaCl2
B
FOXM1
mRNA relative expression
Time (Day)
Ctrl
N-TERT Untreated
+CaCl2
C
CBX7
mRNA relative expression
Time (Day)
Ctrl
N-TERT Untreated
+CaCl2

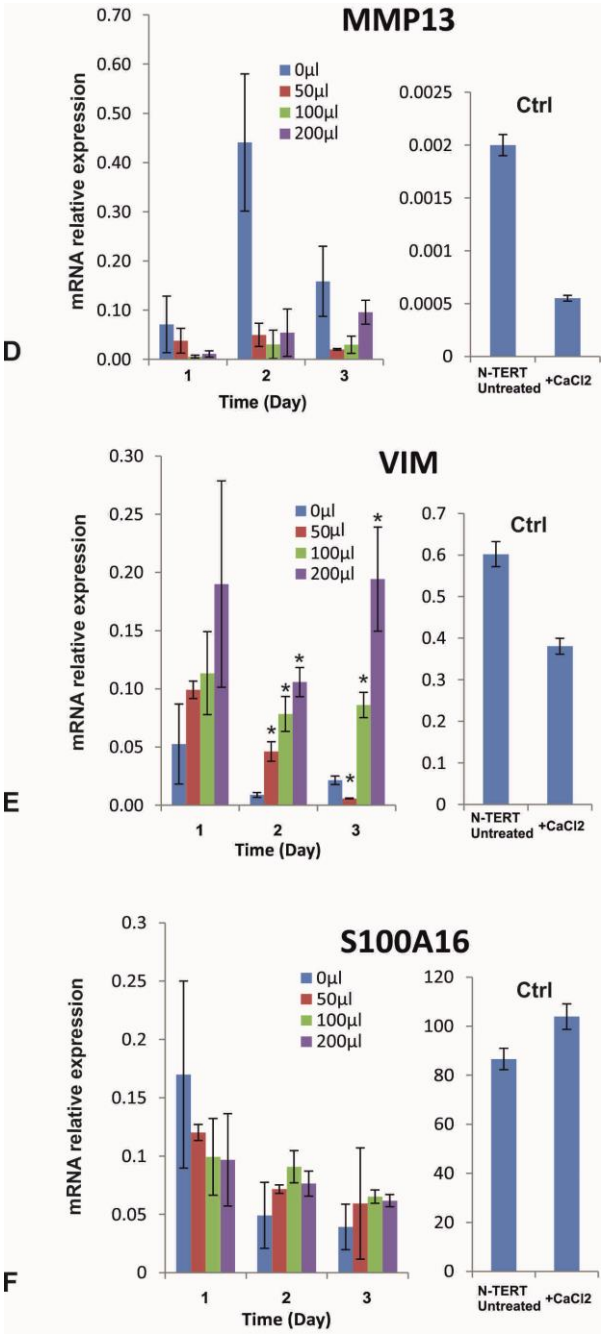


Fig 4.13: Time and dose-response effect of cancer exosomes on mRNA expression levels in recipient N/TERT cells. ***(A)*** *Involucrin (IVL)* ***(B)*** *FOXM1* ***(C)*** *CBX7* ***(D)*** *MMP13* ***(E)*** *Vimentin (VIM)* ***(F)*** *S100A16 at the dose of 0 µl (No exosomes) 50 µl (9.35E+09 particles/ml), 100 µl (1.87E+10 particles/ml) and 200 µl (3.74E+10 particles/ml). Un-treated and $CaCl_2$ treated N/TERT cells were used as controls. The error bars represent the standard error of the mean of duplicate determinations (n = 2).. Significant P values are denoted by *P<0.05, **P<0.01 and ***P<0.001.*

4.11 Microarray

To date no study has reported a total RNA microarray gene analysis of oral keratinocyte cells transfected with normal and cancer exosomes. The limitation of exploring genes through RT-qPCR is that only a limited number to genes can be explored to study the effects and mechanisms triggered by exosomes on recipient cells. By using microarray, we were able to investigate the global gene expression profile (approximately 50,000 genes) in an unbiased way to understand the functional effects in recipient cells triggered by normal vs cancer-cell derived exosomes. The microarray data has been published on Gene expression Omnibus GSE89217.

Total RNA microarray gene analysis was done for normal primary oral keratinocyte cell line (OK113) transfected with equal concentrations of normal and cancer-cells derived exosomes **(Methods Table 2.5, Page no. 65).** OK113 cells were transfected with exosomes from normal oral keratinocyte cell lines (OK113, NK4, NOK368), tumour derived cell lines (Ca1, CaLH2, SqCC/Y1), premalignant (SVpgC2a) and transformed malignant cell line (SVFN10) for 48 hours prior to harvest. No morphological changes were evident after 48 hours of incubation **(Fig 4.14)**. Un-transfected OK113 cells were used as controls.

Total RNA was extracted from transfected and un-transfected OK113 cells. Concentration and purity of RNA was verified through NanoDrop and Agilent BioAnalyzer. Microarray gene chip was run by Eva Wozniak at the genome centre, QMUL.

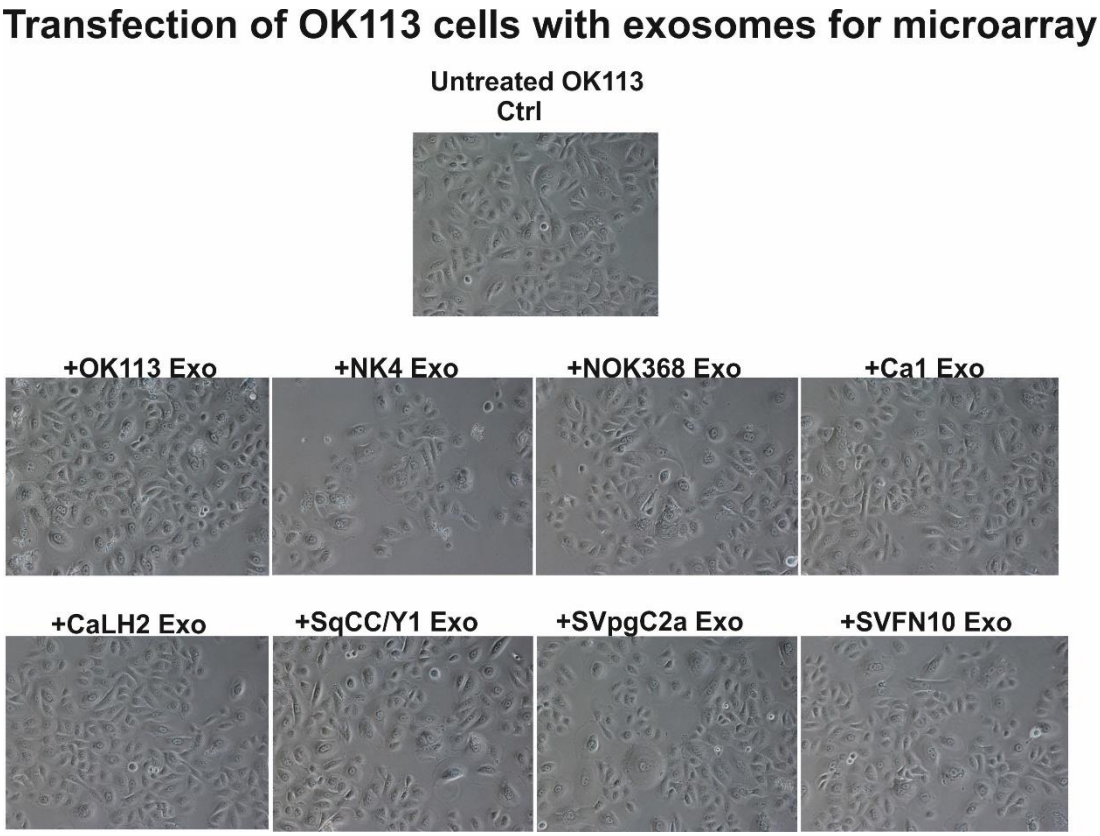


Fig 4.14: Morphology of OK113 with and without transfection at 48hrs

Images (x200) showing morphology of un-transfected OK113 cells in comparison to transfected OK113 with self-exosomes and exosomes isolated from NK4, NOK368 (normal oral keratinocyte) Ca1, CaLH2, SqCC/y1 (tumour derived oral keratinocyte) SVpgC2a (premalignant oral keratinocyte) and SVFN10 (transformed malignant oral keratinocyte) . Cells were transfected for 48 hours.

4.11(1) Gene expression profile in exosome-transfected normal oral keratinocytes

Microarray data was analysed in two categories. Firstly, comparison was made between un-transfected and exosomes-transfected OK113 to analyse genes that are up- and down-regulated by exosomes transfection irrespective of their origin **(Fig 4.15)**.

Comparing between transfected and un-transfected OK113 cells revealed approximately 38.4% of the genes were upregulated while majority of the genes i.e. 61.6% were down regulated **(Fig 4.15 A)**. This pattern of expressing matched with our qPCR data as we noted a global decrease in the mRNA levels in most of the genes studied.

The top 50 of the most upregulated and downregulated genes were shortlisted **(Fig 4.15 B),** from which 20 were chosen for validation on a biological replicates through RT-qPCR **(Fig 4.17 A)**. Selection of the genes was based on whether they were coding or non-coding (this study focused on coding genes) and the availability of specific primers sequence. Upon validation using RT-qPCR, we found that MMP9, TUBB6, FEZ1, OAS1, PGAM1 (upregulated) and TSC22D3, OTUD1, BBOX1 (down regulated) were the most reproducible. These genes are found to be involved in important cellular functions such as regulation of extracellular matrix, cytoskeleton, intracellular transport, immune response, regulation of P53 and glycolysis. The genes along with their cellular functions are listed in **Table 4.1.**

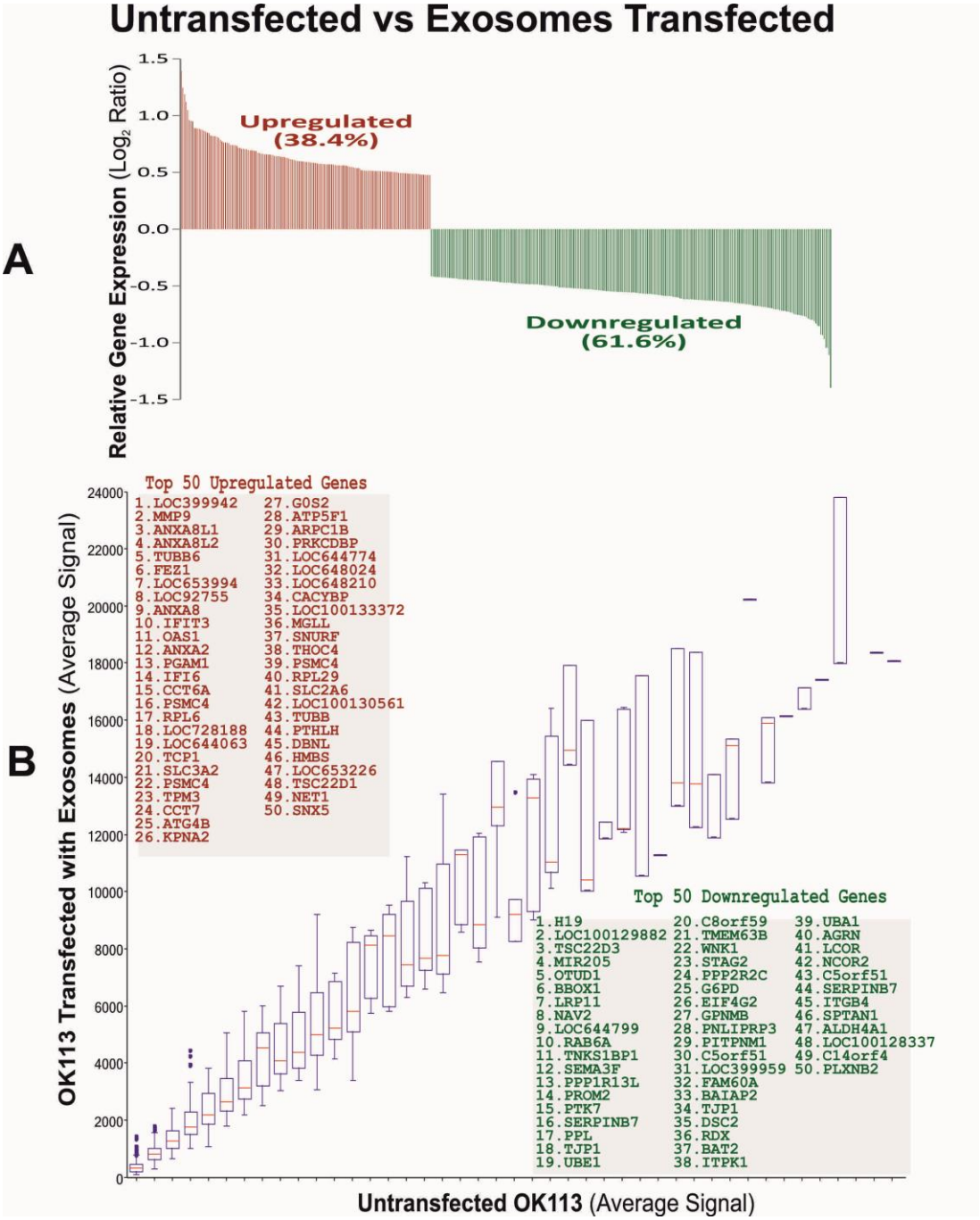


Fig 4.15: Total RNA Microarray gene analysis between un-transfected vs exosome transfected oral keratinocytes. *Gene analysis of untransfected OK113 cells compared to transfected OK113 cells by normal (OK113, NK4, NOK368) and cancer (SqCC/Y1, CaLH2, Ca1, SVpgC2a, SVFN10) exosomes. Data was pooled from eight transfected samples.* ***(A)*** *Relative gene expression showing 38.4% of upregulated and 61.6% of down regulation of genes* ***(B)*** *List of top 50 upregulated (red) and down-regulated (green) genes obtained by Log2 ratio of gene expression between transfected and un-transfected. The diagram was created by Dr Muy-Teck Teh.*

Table 4.1: Genes affected by exosomes and their cellular functions.

These genes were found to be up **(red)** and down **(green)** regulated by normal and cancer exosomes in normal oral keratinocyte cell line OK113.

GENES	FUNCTION
MMP9 - Matrix matallopeptidase 9	Breakdown of matrix
TUBB6-Tubulin Beta 6	Constituent of cytoskeleton
FEZ1-Fasciculation And Elongation Protein Zeta 1	Resposnsible for intracellular transport
OAS1-2'-5'-Oligoadenylate Synthetase 1	Immune regulator
PGAM1 - Phosphoglycerate mutase 1	Catalyst in glycolosis
TSC22D3- TSC22 Domain family member 3	Inflammatory and immune effects
OTUD1- OTU deubiquitinase 1	Regulates tumour supressor gene P53
BBOX1 - Gamma butyrobetain hydroxylase 1	Regulates metabolic pathway

4.11(2) Differential gene expression profile between normal and cancer exosomes transfection

The second category of microarray gene data analysis was done by comparing normal and cancer exosome transfected OK113 cells to identify which genes are differentially up and down-regulated by cancer exosomes **(Fig 4.16)**.

Comparison between OK113 cells transfected with normal oral keratinocytes exosomes (NK4, NOK368 and OK113) and with cancer exosomes (Ca1, CaLH2, SqCC/Y1, SVpgC2a and SVFN10), revealed almost equal percentage of the genes were up (50.3%) and down (49.7%) regulated. Similarly, we shortlisted 50 top most up and down-regulated genes in an attempt to understand if recipient cells respond differently to cancer exosomes. Out of these 50 genes 20 were shortlisted based on the same criteria as mentioned earlier, for validation using RT-qPCR.

We found EFEMP1, EEF2K, DKK3, LAMP2, SRPX, SPARC, and ADAM9 among the up regulated genes and SPRR2E, IGFBP3, RRP12, KLF6 among the downregulated genes were the most reproducible genes through RT-qPCR **(Fig 4.17 B)**. In agreement with our experiments on N/TERT **(Fig 4.13 E, Page no. 128)**, vimentin (VIM) was also found to be upregulated by cancer exosomes **(Fig 4.16 B #32).**

These genes are found to be involved in vital cellular functions including cell growth and adhesion, regulation of cellular morphology, protein synthesis, Wnt signalling pathway, autophagy, deposition of extracellular matrix, regulation of cornified cell envelop , maturation of ribosomal subunits and regulation of signal transduction pathways. Genes and their functions are listed in **Table 4.2.**

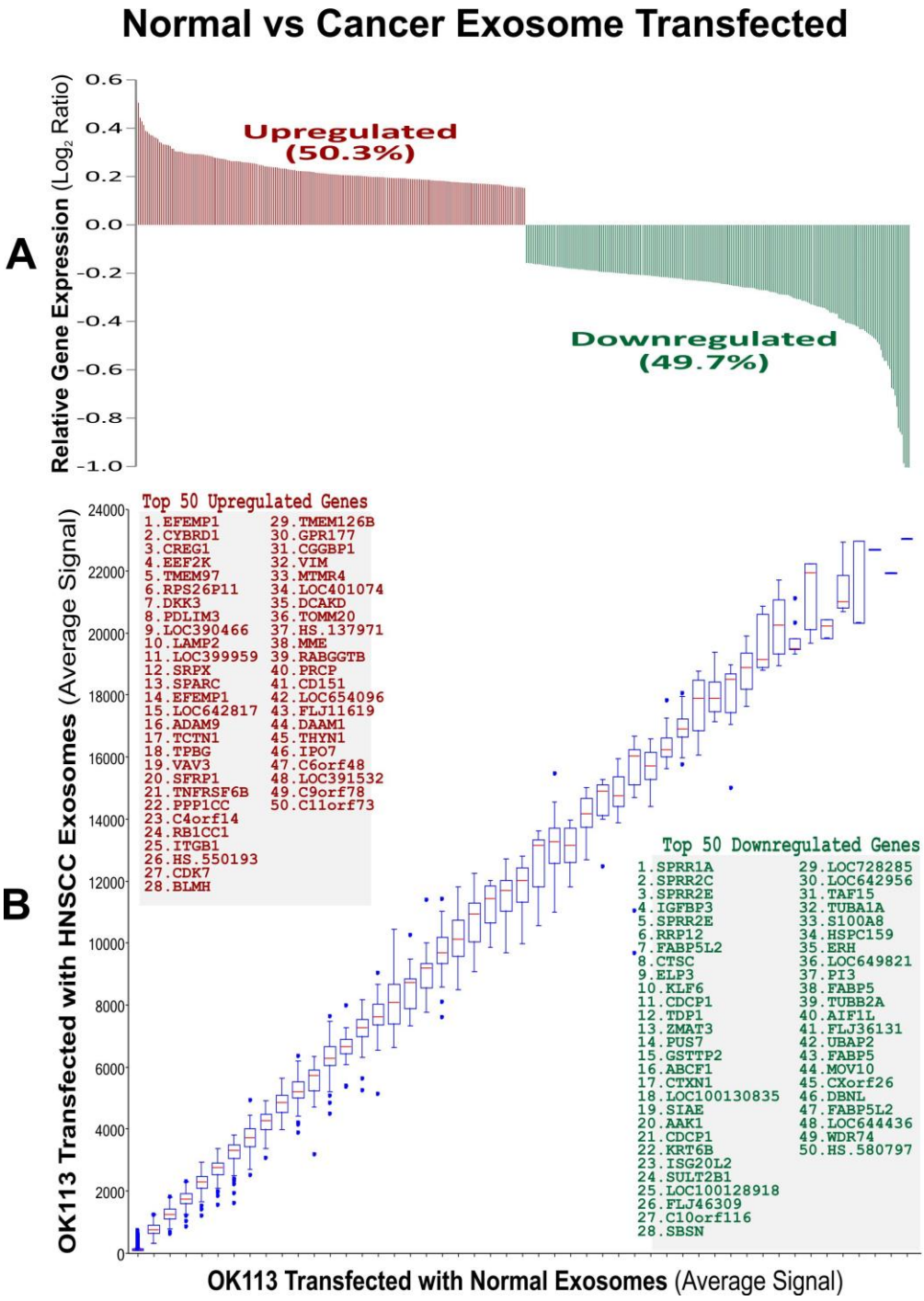


Fig 4.16: Total RNA Microarray gene analysis between normal and cancer exosome transfected oral keratinocytes. *Gene analysis of pooled data from OK113 cells transfected by normal exosomes (OK113, NK4, NOK368) compared with OK113 cells transfected by cancer exosomes (SqCC/Y1, CaLH2, Ca1, SVpgC2a, SVFN10).* ***(A)*** *Relative gene expression showing 50.3% of upregulated and 49.7% of down regulated genes* ***(B)*** *List of top 50 upregulated (red) and down-regulated (green) genes obtained by Log2 ratio of gene expression between cancer and normal transfected cells. The diagram was created by Dr Muy-Teck Teh.*

Table 4.2: Genes affected by cancer exosomes and their cellular functions. These genes were found to be up **(red)** and down **(green)** regulated by cancer exosomes in normal oral keratinocyte cell line OK113.

GENES	FUNCTION
EFEMP1-EGF containing fibulin like extracellular matrix protein 1	Regulates cell morphology, adhesion, growth and motility
EEF2K- Eukaryotic elongation factor 2 kinase	Regulation of protein synthesis
DKK3- Dickkopf WNT signaling pathway inhibitor 3	Involved in Wnt signaling pathway
LAMP2- Lysosomal associated membrane protein 2	Participates as a chaperone in autophagy pathway
SRPX- Sushi Repeat Containing Protein, X-Linked	Involved in cell growth, cytoskeletal organisation and cellular spreading
SPARC- Secreted Protein Acidic And Cysteine Rich	Regulates the deposition of extracellular matrix
ADAM9- ADAM Metallopeptidase Domain 9	Involved in cell adhesion, proliferation, migration and proteolysis
SPRR2E- Small protein rich protein 2E	Maintains cornified cell envelop
IGFBP3- Insuline like growth factor binding protein 3	Carries growth factors
RRP12- Ribosomal RNA processing 12 homolog	Involved in ribosomal subunit maturation and export
KLF6 - Kruppel Like Factor 6	Regulates growth-related signal transduction pathways

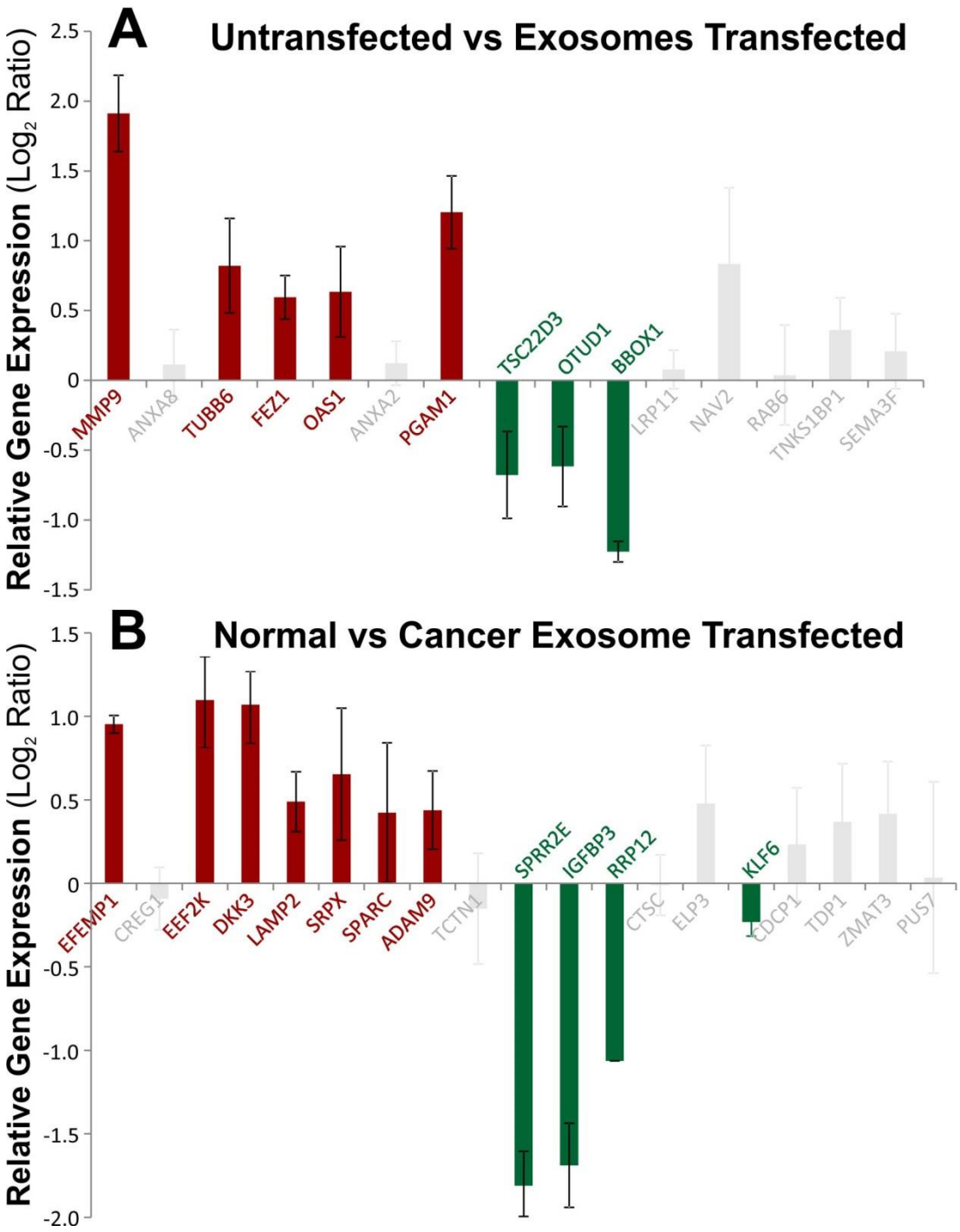


Fig 4.17: Validation of microarray results through RT-qPCR. ***(A)*** *mRNA expression of OK113 cells transfected with exosomes compared to un-transfected.* ***(B)*** *mRNA expression of OK113 cells transfected with cancer exosomes compared to normal exosomes. Red and green represents up and down regulated genes respectively. Grey indicates the genes which were not reproducible via RT-qPCR. The error bars represent the standard error of the mean of three independent experiments (n = 3).*

Through microarray gene analysis we were able to have an insight into the genes expression profile triggered by exosome transfection and by cancer exosomes exclusively. We had planned to study the expression of these genes identified through microarray on another normal oral keratinocyte cell line transfected with exosomes, but due to time and financial restrictions we were unable to carry out such an experiment. To take full advantage to these gene markers their function and pathway connections are being researched and understood.

4.12 Effective dose and exposure time of exosome

Isolation of exosomes through ultracentrifugation is very labour some and time consuming. From seeding cells to getting one exosome pellet (enough for 1 experiment) can take up to 2 to 3 weeks. Till now no guidelines are available in the literature on the most effective exosome dose and exposure time to cause a response in recipient cells. Because of this gap in our knowledge we aimed to further investigate using RT-qPCR on the time and dose responses of exosomes in recipient cells by measuring the validated genes from the above microarray study.

Through microarray gene analysis we were able to recognize the pattern of expression of a number of genes affected by exosome transfection. We used this information to determine the lowest dose and shortest time period required by exosomes to trigger a response in recipient cells.

Normal oral keratinocyte cell line OK113 seeded in a 6 well plate at the confluency of $0.2x10^6$ cells/well. Once attached they were transfected with tumour derived SqCC/Y1 exosome at the dose of 50µl, 100µl and 200µl with the particle/ml concentration of 1.87E+11 for 24 and 48 hrs prior to harvest for RT-qPCR. According to RT-qPCR gene validation results above, we chose genes MMP9 and BBOX1 as they were the most up- and down-regulated genes in recipient cells, respectively. Un-treated OK113 cells were used as controls.

MMP9 mRNA expression was found to be highly dependent on exosome dose. The trend of increase was similar at 24 and 48h hrs but the gene activation was greater at 24 hrs compared to 48 hrs **(Fig 4.18 A)**. Conversely, BBOX1 was dose-dependently down–regulated. The trend of downregulation was more pronounced at 48 hrs compared to 24hrs **(Fig 4.18 B)**.

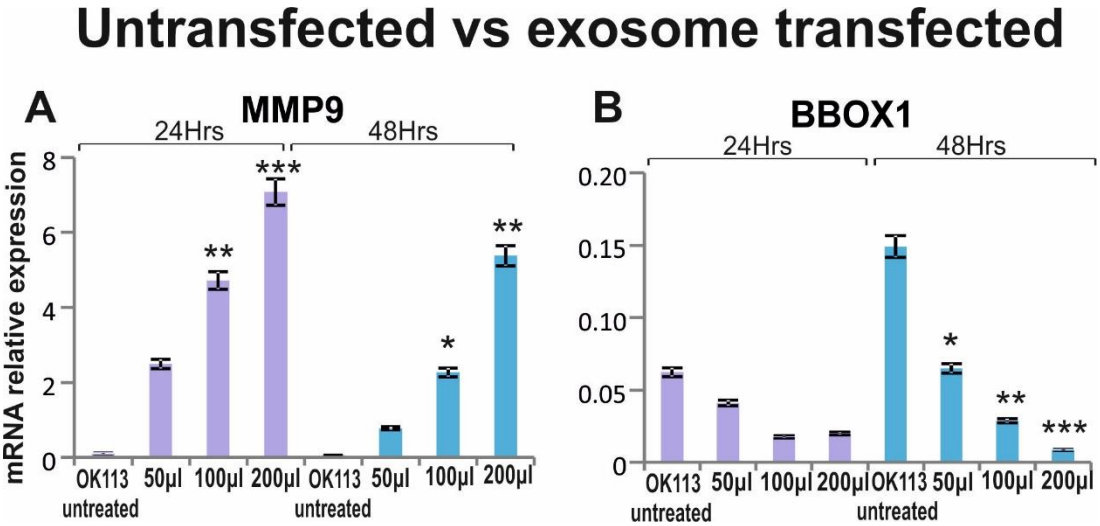


Fig 4.18: Time and dose-response of exosome transfection on gene expression in recipient cells. *mRNA levels of MMP9 (up-regulated) and BBOX1 (down-regulated) at 24 and 48 hours in OK113 cells transfected with SqCC/Y1 exosomes at the dose of 50,100 and 200µl (1.87E+10 particle/ml concentration). The error bars represent the standard error of the mean of two independent experiments (n = 3). Significant P values are denoted by *P<0.05, **P<0.01 and ***P<0.001*

A similar experiment was done to measure MMP9 and BBOX1 in OK113 cells transfected with self-exosomes and the findings were highly comparable to that of SqCC/Y1 exosome transfection (result not shown). This indicates that regardless of the origin of exosomes (whether it was normal or cancer-derived), exposure to exosomes triggers specific genes in recipient cells.

To further investigate specific genes triggered by cancer exosome, an identical transfection experiment was set up to understand that effect of exosome dose and exposure time on genes EEF2K (up-regulated) and SPRR2E (down-regulated) by cancer exosomes. OK113 cells were transfected with self-exosomes at the dose of 50µl, 100µl, 200µl and SqCC/Y1 exosome with the same range of doses and particle/ml concentration as mentioned previously. Untreated OK113 cells were used as controls. A LOG_2 ratio was used to calculate the difference between the normal and cancer exosome transfection.

We found that in the first 24 hrs of transfection, EEF2K was up regulated with increasing number of exosomes while the expression was down regulated at 48 hrs **(Fig 4.19 A)**. The levels of SPRR2E were down-regulated with increasing exosome particles which was more pronounced at 48 hrs **(Fig 4.19 B)**.

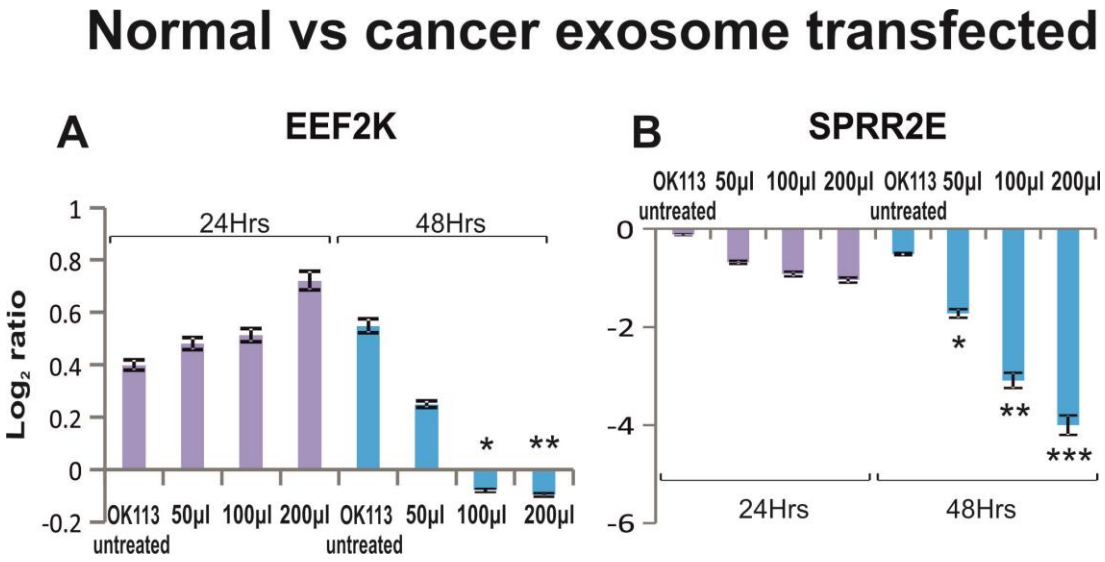


Fig 4.19: Time and dose-response of normal vs cancer exosome transfection on gene expression in recipient cells. *Log_2 ratio of mRNA relative expression of OK113 cells transfected with self and SqCC/Y1 exosomes.* ***(A)*** *mRNA expression of EEF2K (up-regulated) and* ***(B)*** *SPRR2E (down-regulated) at 24 and 48 hours. Different doses of OK113 and SqCC/Y1 exosomes were used 50µl ,100µl and 200µl while the particle/ml concentration was 1.87+E10. The error bars represent the standard error of the mean of two independent experiments (n = 2). Significant P values are denoted by *P<0.05, **P<0.01 and ***P<0.001*

We conclude that a concentration of exosomes as low as 3E+09 particle/ml was enough to elicit statistically significant gene expression responses. The most noticeable change in mRNA expression were noted at around 3E+10 particles/ml which in our experiment coincided with 200µl of exosome stock volume. Up-regulated mRNA levels of MMP9 and EEF2K, and down-regulated mRNA expression levels of BBOX1 and SPRR2E were consistent with the microarray data. We found that 24 hours of exosome exposure was enough to elicit altered gene expression patterns.

4.13 Discussion

Initial research in the field of exosomes had proposed their role in maintaining cellular homeostasis by removing undesired molecules from cells (Raposo and Stoorvogel, 2013, Harding et al., 2013) including harmful cytoplasmic DNA (Takahashi et al., 2017). However, emerging evidence has revealed that exosomes play an important role in cell to cell communication by activating various signalling pathways in recipient cells (Schorey et al., 2015, Cheung et al., 2016).

In the field of OSCC the functional significance of oral cancer exosomes on normal recipient oral keratinocyte cells is unknown. Hence, we took the opportunity to explore the effects of exosomal mRNA on recipient cells to better understand the mechanism of cell to cell communication within OSCC development and progression.

In this study, successful isolation and characterisation of exosomal RNA was verified by Agilent bio-analyzer and RT-qPCR. Through treatment with RNAse, proteinase K and Triton-X it was shown that exosomal RNA is well protected within the surrounding lipid bi-layer and only degrades with Triton-X breaking down the lipid bi-layer **(Fig 4.2, 4.4, 4.6, Page no. 109,111,112)**. Our data also shows that not all genes are equally expressed within exosomes **(Fig 4.3,4.5, Page no. 110,112)**, indicating a mechanism of selective packaging.

Further, the functional importance of cancer exosomes was studies by transfecting non-cancerous cells with cancerous exosomes expressing high levels of FOXM1 oncogene, morphological changes within recipient cells were noted within 24 hours of incubation **(Fig 4.7, Page no. 115)**. In addition mRNA expression of FOXM1 oncogene also increased within recipient cells at 48 hours but only transiently **(Fig 4.8, Page no. 116)**. This indicates that mRNA content of tumour derived exosomes was transferred to normal recipient cells. Further research needs to be done in order to find if long term exposure to cancerous exosomes would result in normal cells changing phenotype to cancerous cells permanently.

As suggested by literature such morphological changes might be a result of exosomes inducing senescence (Urbanelli et al., 2016). We screened for senescence phenotype through beta-gal assay. Through RT-qPCR expression of senescence markers including P16, P53, P21 and CBX7 were explored **(Fig 4.10, Page no. 119)**. Upon finding no evidence of recipient cells senescing, further literature review was done, where studies have reported that exosomes are capable of inducing differentiation into recipient cells (Cheung et al., 2016). Expression of differentiation

markers including IVL, TGM1, VIM, CORN, LORI, FLG, K1, K10 and K13 in exosome transfected cells was explored through RT-qPCR but no specific trend could be established **(Fig 4.11, 4.12, Page no. 122, 125)**.

In order to determine dose and time required by exosomes to elicit a response in recipient cells, N/TERT (normal immortalised epidermal keratinocytes) cells were transfected with cancer exosomes with different doses at three time points (24h, 48h, 72h). Through RT-qPCR, differential gene expression of IVL, FOXM1, CBX7, MMP13, VIM and S100A16 were observed. Interestingly, except for VIM all the other genes were found to be downregulated by exosome transfection **(Fig 4.13, Page no. 128)**. We hypothesize that this downregulation might be a self-protecting mechanism within cells such as autophagy (Shintani and Klionsky, 2004) or RNA interference (Tijsterman and Plasterk, 2004) that shuts down the expression of important genes upon exposure to foreign exosomes. Evidence of such mechanism can be found in RNA interference involving dicer effect (Tijsterman and Plasterk, 2004).

In order to explore specific genes altered by exosomes, gene expression microarray profiling was done on normal oral keratinocyte cells (OK113) transfected with self, normal (NK4, NOK368) and cancer exosomes (SqCC/Y1, CaLH2, Ca1, SVpgC2A, SVFN10) for 48h.

Through microarray profiling we found that exosomes irrespective of their origin (normal or cancer) altered gene expression in recipient cells. Amongst the top differentially altered gene list, we have validated using RT-qPCR that MMP9, TUBB6, FEZ1, OAS1 and PGAM1 genes were upregulated in recipient cells compared to untreated cells, while TSC22D3, OTUD1 and BBOX1 were found to be downregulated **(Fig 4.17, Page no. 137)**.

Cancer exosomes were found to exclusively increase the expression of EFEMP1, EEF2K, DKK3, LAMP2, SRPX, SPARC and ADAM9 while the expression of SPRR2E, IGFBP3, RRP12 and KLF6 was down regulated **(Fig 4.17, Page no. 137)**.

We look into literature to understand the physiological and pathological involvement of these markers in cellular functions.

4.13(1) Genes up regulated by exosome transfection

MMP9 Matrix metallopeptidase 9 is a class of zinc dependent proteinases (Nagase and Woessner, 1999) involved in the breakdown of extracellular matrix in normal physiological processes such as embryonic development (Gu et al., 2015) and wound healing (Amar et al., 2017) and pathological processes such as cancer metastasis (Deryugina and Quigley, 2006).

In OSCC salivary MMP9 has been found to be upregulated in patient sample compared to normal healthy saliva suggesting its potential use as an early diagnostic tool (Peisker et al., 2017). In another study upregulated expression of MMP9 was observed in patients with metastatic oral cancer compared to patients with non-metastatic oral cancer (Qin et al., 2017). To our knowledge, we are the first to report increased expression of MMP9 as a result of oral keratinocyte exosome transfection.

MMP9 may play an important role in angiogenesis and neovascularization (Bergers et al., 2000). For example, MMP9 appears to be involved in the remodelling associated with malignant glioma neovascularization (Wang et al., 2003).

MMP9 is greatly upregulated during human respiratory epithelial healing (Buisson et al., 1996). Using a MMP9 deficient mouse model, it was seen that MMP9 coordinated epithelial wound repair while deficient mice were unable to remove the fibrinogen matrix during wound healing (Mohan et al., 2002).

Limited studies have looked into the effects of exosomes on MMP9. It has been reported that hepatocellular carcinoma-derived exosomes enhance migratory and invasive ability of non-cancerous recipient cells by triggering PI3K/AKT and MAPK signalling pathway with increased secretion of active MMP9 (He et al., 2015).

TUBB6 Tubulin beta 6 class V has been found to be preferentially expressed in cells with secretory function such as pancreatic, salivary ductal cells (Chao et al., 2012). Abnormal distribution and expression of β- tubulin isotypes have been reported in numerous malignancies (Orr et al., 2003), indicating a more aggressive and drug resistant tumour phenotype (Kavallaris, 2010). Hence it has been proposed that altered expression of TUBB6 may be associated with tumorigenesis and may be a useful diagnostic tool and a potential prognostic marker (Chao et al., 2012). Any influence of TUBB6 on OSCC development is yet to be established.

FEZ1 Fasciculation and elongation protein zeta 1 has been known for its role in onset and progression of neurodegenerative disorders (ND) such as Alzheimer's and schizophrenia (Chua et al., 2013). Defects in intracellular transport and autophagy are the causative factors of ND.

In normal physiology the FEZ1 binds to neuronal SNARE protein Syntaxin 1A and control the synaptic vesicle exocytosis (Chua et al., 2012). FEZ1 disruption results in intracellular transport defects (Maturana et al., 2010).

Through microarray, presence of FEZ1 in exosomes has been reported in a rat model of pancreatic adenocarcinoma where it is proposed to induce vascular endothelial growth factor (VEGF) (Nazarenko et al., 2010) GSE18812. The biological mechanism behind the activation of VEGF by FEZ1 is largely unknown. This is the first study to report aberrant expression of FEZ1 due to exosome transfection.

OAS1 2′-5′ oligoadenylate synthase (OAS) is well known for its function as an immune regulator (Choi et al., 2015). Research has also shown that OAS1 is involved in other cellular functions such as apoptosis (Domingo-Gil and Esteban, 2006) required for elimination of viral-infected cells and inhibiting tumorigenesis (Castelli et al., 1998). OAS1 is also known for its tumour suppression in breast and ovarian cancer by activating apoptosis of cancer cells and countering tumour progression (Latham et al., 1996). In addition, OAS1 is also found to regulate nuclear events, including pre-mRNA splicing (Sperling et al., 1991). To date no study has reported increased expression of OAS1 due to exosome transfection.

PGAM1 Phosphoglycerate mutase 1 is an important enzyme in the glycolytic pathway of glucose metabolism where it catalyses the conversion of 3-phosphoglycerate into 2-phosphoglycerate (Jiang et al., 2014). PGAM1 has been found to be up regulated in human colorectal, liver, lung (Durany et al., 1997) and breast (Durany et al., 2000) cancer tissue and hence has been extensively studied as the potential candidate for developing cancer therapeutics (Sheng and Tang, 2016). A recent study has shown that PGAM1 inactivation leads to nucleotide depletion, which causes defective DNA repair, suggesting that targeting PGAM1 increases cancer cell susceptibility to DNA damaging agents (van Vugt, 2017).

4.13(2) Genes downregulated by exosome transfection

TSC22D3 TSC22 Domain family member 3 also known as Glucocorticoid-induced leucine zipper (GILZ) is a potent anti-inflammatory protein. GILZ regulates signal transduction pathways of inflammation and plays a role in cell survival (Espinasse et al., 2016) by exerting pro- or anti-apoptotic effects, depending on the cell type and the stimuli used (Asselin-Labat et al., 2004). In a study on apoptosis of myocardial cells during myocardial infarction, has shown that miR-140-3p regulates the expression of TSC22D3 in mouse model (Liu et al., 2015).

OTUD1 OTU deubiquitinase 1 is a cysteine protease that hydrolyzes the cleavage of ubiquitin in vitro (Mevissen et al., 2013). Cozier and colleagues were the first to report that OTUD1 is located in chromosome 10p12 (Cozier et al., 2012). OTUD1 regulates the stability of tumour suppressor gene P53. Not much is known about its substrates and its role in cellular functions is largely unknown. OTUD1 overexpression increased p53 stability, whereas OTUD1 knockdown decreased p53 stability. OTUD1 has also been known to inhibit cell growth and increases apoptosis by controlling S and G2/M phase checkpoints (Piao et al., 2017).

BBOX1 gamma-butyrobetaine hydroxylase 1 catalyses the formation of L-carnitine, essential for transport of fatty acids across the mitochondrial membrane (Rigault et al., 2006). Not much is known about other cellular functions of BBOX1. Through meta-analysis of microarray data from 13 different types of cancers, BBOX1 has been proposed to have an important role in cancer development (Dawany et al., 2011).

4.13(3) Genes up-regulated exclusively by cancer exosomes

EFEMP1 Epidermal growth factor containing fibulin like extracellular matrix protein 1 (EFEMP1) also known as Fibulin 3 is a member of the fibulin family of secreted glycoprotein (Shen et al., 2017). Fibulin family is known to regulate cell morphology, adhesion, growth and motility (Timpl et al., 2003). Initially, EFEMP1 was identified as a senescence protein (Lecka-Czernik et al., 1995) which takes part in regulation of body weight and behavioural control (Weedon et al., 2008). EFEMP1 is linked to the primary precursor of elastin and hence is widely distributed throughout the body (de Vega et al., 2009). It is also detected in ensheathing olfactory cells, promoting their proliferation (Vukovic et al., 2009). Studies have reported that EFEMP1 regulates matrix metalloproteinase (MMPs) and tissue inhibitors of matrix metalloproteinase (Rahn et al., 2009, Wen and Kesari, 2008)

The role of EFEMP1 is controversial in tumorigenesis. It has been found to play tumour suppressive functions in tumours origination from lungs, endothelial cells and Kaposi sarcoma and elicit oncogenic activities in tumours of brain and pancreas (Obaya et al., 2012).

In recent studies EFEMP1 has been found to play a more pro tumour development role. In a study on bladder cancer high expression of EFEMP1 has been found in T2 vs T1 tumour stage, correlating with increased tumour invasiveness, while knockdown restored the invasive and migratory potential (Han et al., 2017). High expression of MiR-338-5p has been shown to supress to the expression of EFEMP1 reducing migration, invasion and promote apoptosis in brain cells (glioma) (Lei et al., 2017). In gastric cancer through meta-analysis of multiple micro array studies, EFEMP1 has been identified as a potential prognostic biomarker (Min et al., 2017).

EEF2K Eukaryotic elongation factor-2 kinase is a structurally and functionally unique protein kinase in the calmodulin mediated signalling pathway. It is involved in the regulation of translation and protein synthesis (Zhu et al., 2015, Kaul et al., 2011).

Previous studies have reported increased expression of EEF2K in breast cancer (Meric-Bernstam et al., 2012) and glioma (Zhang et al., 2011a), where it plays a

critical role in cell cycle, autophagy and apoptosis (Tekedereli et al., 2012, Leprivier et al., 2013) making it a potential target for cancer therapy.

DKK3 is a member of dickkopf WNT signalling pathway inhibitor family. Wnt signalling plays an important role in embryogenesis (Komiya and Habas, 2008) and cancer development (Zhan et al., 2017). The expression of DKK3 has been found to be down regulated in cancer of many origins including glioma (Gotze et al., 2010), oesophageal SCC (Liu et al., 2011), gastric adenocarcinoma (Yu et al., 2009), colorectal adenocarcinoma (Wang et al., 2012), breast cancer (Veeck et al., 2009) and cervical squamous cell carcinoma (Lee et al., 2009). This loss of expression was mainly due to CpG island methylation (Gotze et al., 2010, Yu et al., 2009, Ding et al., 2009). Hence, DKK3 was considered a tumour suppressor gene.

However studies on head and neck squamous cell carcinoma have reported overexpression of DKK3 in HNSCC cell lines associated with higher rate of metastasis and shorter disease-free survival rate (Katase et al., 2012). In oral squamous cell carcinoma increased expression of DKK3 has been correlated with cancer progression where its expression changes from cell membrane in normal mucosa to cytoplasm in dysplasia suggesting its involvement in carcinogenesis (Fujii et al., 2011).

In a recent study overexpression of DKK3 was found to increase cellular proliferation, invasion, and migration in HNSCC and resulted in increased expression of cyclin D1 and c-myc mRNA expression (Katase et al., 2017).

LAMP2 lysosomal associated membrane protein 2 is one of the major protein components of lysosome. It is an important protein in the chaperone mediated autophagy pathway, responsible for the lysosomal degradation of approximately 30% of modified and oxidatively damaged cytosolic proteins (Saha, 2012).

In a study on Oesophageal squamous cell carcinoma (ESCC) LAMP2 expression levels correlated with tumour histological differentiation and TNM stages. High expression of LAMP2 predicted poor prognosis in patients with ESCC (Li et al., 2017). In salivary adenoid cystic carcinoma (SACC) over expression of LAMP2 was observed in patient samples compared with normal tissue. It was found to be

associated with carcinogenesis and progression of SACC, suggesting its usefulness as a molecular target (Huang et al., 2016)

Similar to exosomes, LAMP2 is endocytic in origin hence some studies on plasma and serum derived exosomes have reported its presence within exosomes suggesting its use as an exosomal specific marker (Caby et al., 2005, Caradec et al., 2014).

SRPX sushi repeat containing protein, X-linked is involved in cell growth, cytoskeletal organisation and cellular spreading (Burnicka-Turek et al., 2010). In invasive hepatocellular carcinoma cells high expression levels of SRPX was found to promote cellular proliferation (Lin and Chuang, 2012). Through meta-analysis of cancer microarray studies SRPX was identified to be up-regulated in cancers of five different origins, although its specific role and mechanism has not been investigated (Ma et al., 2009). To date no association has been published between exosomes and SRPX.

SPARC Secreted protein acidic and rich in cysteine also known as osteonectin is a matricellular glycoprotein that mediates interaction between cells and their surrounding microenvironment (Framson and Sage, 2004). SPARC is highly expressed during embryogenesis but the expression is reduced in adult tissue. However, increased expression is noted in response to inflammation, tissue injury, tumour invasion and metastasis (Arnold and Brekken, 2009, Chlenski and Cohn, 2010).

In breast cancer increased expression of SPARC was found, associated with increased levels of MMP2 and MMP9 which resulted in degradation of extracellular matrix leading to increased tumour invasion and metastasis (Kim et al., 2017).

In oral squamous cell carcinoma SPARC aberrant expression was detected both in stromal and tumour cells collected from saliva, biopsy material, and fresh cell scrapings of patients with OSCC, where it correlated with presence of metastasis, patient survival and tumour grade (Aquino et al., 2013) suggesting its potential as a prognostic marker. To date no association has been made between exosomes and cellular up regulation of SPACR.

ADAM9 ADAM metallopeptidase domain 9 is a transmembrane protein involved in cell adhesion, proliferation, migration and proteolysis (Ambatipudi et al., 2011, Duffy et al., 2011). Through gene profiling, ADAM9 has been implicated as a potential oncogene and therapeutic target in breast cancer (Fry and Toker, 2010) and NCI-60 human tumour cell lines (Kohn et al., 2012).

Increased expression of ADAM9 has been identified in high risk oral premalignant lesions, where it possibly plays a role in transformation into OSCC (Tsui et al., 2009). In another study on OSCC, genome wide analysis of copy number alteration showed high amplification frequency of ADAM9 suggesting it has a potential oncogenic role (Vincent-Chong et al., 2013). Currently, no study has reported increased expression of ADAM9 due to exposure to cancer exosomes.

4.13(4) Genes down regulated exclusively by cancer exosomes

SPRR2E Small Proline Rich Protein 2E is part of the human epidermal differentiation complex on chromosome 1q21 and code for precursor proteins of the cornified cell envelop, a structure which is characteristic for terminally differentiated keratinocytes (Gibbs et al., 1993). This envelope serves as a protective barrier against extracellular and environmental factors.

In a study on epidermal squamous cell carcinoma low expression of SPRR2 was noted in malignant keratinocyte cell lines compared to normal suggesting defective terminal differentiation, a characteristic of carcinogenic transformation (Lohman et al., 1997). Similar expression of SPRR2 has been observed in neoplastic keratinocytes of the anal track (Zucchini et al., 2001).

To the best of our knowledge, expression of SPRR2E has not been studied in the development of oral squamous cell carcinoma and its down-regulation due to exosome transfection has not been reported.

IGFBP3 insulin like growth factor binding protein 3, a major carrier protein for insulin-like growth factors (IGF)-I and IGF-II in circulating blood, is known to regulate the

bioavailability of insulin and insulin-like growth factors by modulating their interactions with signalling receptors in insulin like growth factor (IGF) signalling pathway (Jogie-Brahim et al., 2005).

Historically IGFBP3 was considered as a tumour suppresser gene as it's over expression was found to induce apoptosis in breast cancer (Butt and Williams, 2001, Gill et al., 1997).

In recent studies the prognostic potential of IGFBP3 has been reported. In hepatocellular carcinoma and oesophageal squamous cell carcinoma, low levels of IGFBP-3 expression correlated with clinicopathological features of the tumour stage and poor overall patient survival rate (Yan et al., 2017, Zhao et al., 2012).

In OSCC studies, up-regulation of IGFBP3 has been reported in cell lines derived from cervical lymph nodes where mRNA expression was significantly associated with lymph node metastasis through an IGF-independent mechanism (Yen et al., 2015). Due to high levels of IGFBP3 and its positive correlation to disease progression and metastasis, it has been proposed as a potential biomarker in OSCC (Zhong et al., 2008). More research needs to be done to determine the expression of IGFBP3 in oral keratinocytes and its association with exosome exposure.

RRP12 Ribosomal RNA processing 12 homolog encodes a nuclear protein involved in ribosomal subunit maturation and export (Oeffinger et al., 2004, Vanrobays et al., 2008). RRP12 was found to play an important role in cell cycle progression and response to DNA damage, where loss of RRP12 caused defects in S-phase entry and progression, in efficient DNA damage response, and a delay in M/G1 transition (Dosil, 2011).

The effect of RRP12 in mammalian cells was studied in nuclear stress conditions in osteosarcoma cells. It was found that the overexpression of RRP12 stabilized P53 expression promoting resistance to cytotoxic stress while down regulation enhanced susceptibility to cytotoxic stress (Choi et al., 2016).

KLF6 Kruppel Like Factor 6 is involved in differentiation and development. Its role in growth-related signal transduction pathways, cell proliferation, apoptosis and angiogenesis drew attention to its possible role in tumorigenesis (Bieker, 2001). KLF6 has been extensively studied for it tumour suppressor function in prostate (Narla et al., 2001), colorectal (Reeves et al., 2004), hepatocellular carcinoma (Kremer-Tal et al., 2004)and glioblastoma (Camacho-Vanegas et al., 2007). It performs its tumour suppression function by promoting G1 cell cycle arrest mainly through cyclin-dependent kinase inhibitor 1A promoter transactivation (Kimmelman et al., 2004). In a recent study down regulation of KLF6 was found to promote NF-$_k$B oncogene signalling which suppresses programmed cell death and promotes tumour growth and invasion in glioblastoma (Masilamani et al., 2017).

In HNSCC KLF6 loss of heterozygosity correlated with disease progression, recurrence and poor patient survival in oral and oropharyngeal squamous cell carcinoma patient samples, while silencing KLF6 in HNSCC cell lines increased cellular proliferation and decreased P21 expression (Teixeira et al., 2007). To date down-regulation of KLF6 in cancer has not been associated as a result of exosome transfection in recipient cells.

4.14 Limitations

Isolating enough exosomes for RNA isolation can be challenging as it is easy to miss the collection of the final exosome pallet. Hence the yield of one isolation cycle can vary from another.

The expression of the effected genes by exosome transfection on recipient cell can vary depending on the source of exosomes and the recipient cells.

Due to gaps in our knowledge we are not sure if exosomes contain full length RNA or short segments. Although, in our study we have used EGFP primers and other full-length FOXM1 primers with similar results, indicating that the full-length mRNA is inside the exosomes. However, this does not prove if it is an intact full-length mRNA unless we sequence it through and through using RNAseq.

There is a problem with traceability of cancer exosomes whereby the tumour location may not be found. This limits the clinical benefit of detecting cancer without a treatment option. Exosomes should be exploited for developing cancer vaccines or targeted immunotherapeutics to enable diagnostic test to be coupled with an effective intervention.

Chapter 5

Early Oral Cancer Diagnostic System

5.1 Introduction

Currently oral squamous cell carcinoma (OSCC) is diagnosed by histopathological examination of biopsy tissue, which is very subjective to pathologist opinion. Secondly, histopathology is unable to identify lesions which would potentially develop into OSCC (Warnakulasuriya et al., 2007). By the time these changes become evident to the pathologists, the disease is well established with enduring consequences.

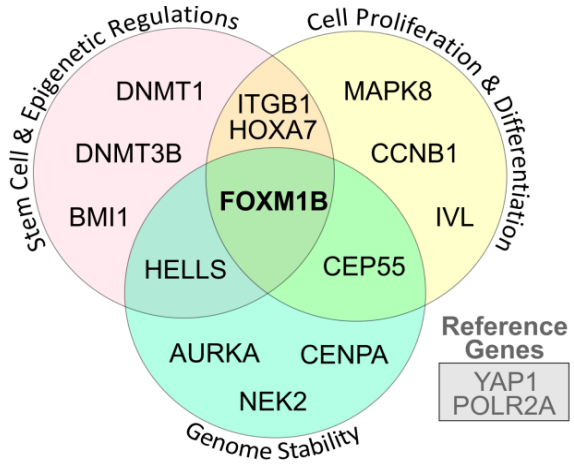
Hence an early detection system is much needed that would aid in diagnosing alongside pathological findings. Given that molecular alterations precede phenotypic change in many cancers, identifying molecular markers associated with early phase of oncogenesis may be the key to early cancer diagnosis.

We have previously identified an oncogene FOXM1 involved in initiation and progression of head and neck squamous cell carcinomas (Gemenetzidis et al., 2009). FOXM1 belongs to FOX family which consist of 55 distinct mammalian members grouped in 17 subfamilies according to sequence homology within the DNA binding domain (Kalin et al., 2011). In Humans there are 3 FOXM1 isoforms. FOXM1A is found to be transcriptionally inactive due to the presence of inhibitory exon while FOXM1B and FOXM1C are transcriptionally active and play an important role in cell cycle and proliferation (Korver et al., 1997). It is expressed in embryonic tissue but in healthy adult tissue the expression is limited to highly proliferative tissues such as intestines, testis, thymus and colon (Ye et al., 1997).

The active role of FOXM1 in cell cycle is indicative of its subsequent role in tumourigenesis. Elevated expression of FOXM1 are found in cancers of different tissues and organs such as basal cell carcinoma, liver, breast, prostrate, lung, brain, colon, pancreas, testis, kidney, ovary and oral cancers and has been shown to be related to poor prognosis (Teh, 2012b). Both FOXM1B and FOXM1C isoforms were found to be upregulated in cancers. However, it has been found that FOXM1B was more potent in inducing malignant transformation than FOXM1C (Lam et al., 2013).

We have developed a diagnostic system based on FOXM1 called quantitative malignancy index diagnostic system (qMIDS) consisting of 14 molecular biomarkers associated with FOXM1 (isoform B). Functionally they are involved in regulation of the cell cycle, differentiation, ageing, genomic stability, epigenetic and stem cell renewal along with two reference genes. Their mRNA expression was translated into a metric scoring system using an algorithm **(Fig 5.1).**

In a study involving 299 patients, qMIDS was validated on fresh biopsy samples from normal, dysplastic and head and neck squamous cell carcinoma respectively. We tested the diagnostic precision of this system compared to conventional histopathological examination of the biopsy samples, as it quantifies the biomarkers and indicates the aggressiveness of the disease. This method of diagnosing cancer is being exploited for early disease detection with an aim to improve disease prognosis and reduce the cost of health care (Teh et al., 2013).



$$MI = \sum_{i=1}^{n} \left| Log_2 \left[\frac{T(T_{nm})}{T_n(T_m)} \right] \right|_i \bullet Log_2 [Q_4 (Q_3 - Q_1)]$$

Fig 5.1: Fourteen biomarkers associated to FOXM1*. With the exception of IVL and ITGB1 which are downregulated the rest are upregulated with an increased expression of FOXM1. The algorithm translated the gene expression signature into a malignancy index values, calculated to fit a linear scale. In the formula T is the target gene normalized against two reference genes. T_n is the sum of n number of target gene mRNA transcripts measured in each cDNA samples. Tm represents a median value. T_{nm} is the sum of the n number of T_m values. Q_1, Q_3 and Q_4 represents the first (25%), third (75%) and fourth (100%) rank quartile of the x number of target gene absolute fraction Log_2 ratio ($\left| Log_2[T(T_{nm})/T_m(T_n)] \right|$) distribution values within each cDNA sample (Teh et al., 2013).*

Small cohorts of FFPE samples were tested in the original study but they were taken from different patients at different stages of the disease. In contrast, the FFPE samples in this study were collected from the same patient at different time points during the progression of the disease. This gave an advantage of observing the trend and accuracy of qMIDS index in progressive OSCC as it transforms from dysplasia to lymph node metastasis. This study aims to test qMIDS on retrospectively collected archival formalin fixed paraffin embedded (FFPE) tissue samples from normal healthy oral mucosa, oral dysplasia, OSCC and lymph node metastasized tumour and its correlation to disease stage.

The samples were collected at The Gade Institute, Bergen, Norway. The use of human tissue was approved by research ethics committee from Norway REK Vest: 2010/481-7. The samples were collected from four cancer patients over a period of time, at different stages of oral squamous cell carcinoma including dysplasia, tumour and lymph node metastasis.

Gene expression levels in biopsy specimens were quantified using RT-qPCR. The mRNA levels of 14 biomarkers and two reference genes were translated into qMIDS indexes to indicate disease aggressiveness of each sample.

Results

5.2 Validation of qMIDS on FFPE samples

Formalin fixation followed by dehydration and paraffin embedding (FFPE) is the most commonly used method to preserve clinical tissue samples for histological studies and to establish archival tissue banks. Extracting high quality RNA from FFPE tissues remains a challenge for molecular studies (Evers et al., 2011). RNA can degrade due to dehydration caused by sample treatment before and during fixation (Hewitt et al., 2008) or long term storage in paraffin (von Ahlfen et al., 2007). In addition, crosslinking of formaldehyde to other macromolecules reduces the yield of RNA (Feldman, 1973).

To overcome these problems we used a commercially available kit from Qiagen which overcomes formalin crosslinking and maintains the integrity of RNA (von Ahlfen et al., 2007).

Preliminary results showed that moderate to good yield of RNA could be extracted from FFPE tissue, which was verified by nanodrop. In addition, the gene signature within FFPE tissue was sufficiently preserved that enabled quantification of qMIDS assay. The qMIDS assay was successfully validated on 15 FFPE samples consisting of 3 normal healthy oral mucosa and triplets of dysplasia, OSCC tumour and lymph node metastatic tumour of disease tissues from four cancer patients.

Normal samples gave qMIDS index values between 1 and 4 **(Fig 5.2 A)**. In patient 1 there was a gradual and statistically significant increase in the qMIDS index value from dysplasia to lymph node metastatic tumour **(Fig 5.2 B)**. In patient 2 statistically significant increase in qMIDS index value was observed from OSCC to lymph node metastatic tumour **(Fig 5.2 C)**. In patient 3 and 4 increase in qMIDS index was noted between dysplasia and OSCC tumour although not statistically significant **(Fig 5.2 D and E)**. A combined analysis of qMIDS score from all four patients did not show any statistically significant trend between normal, dysplastic, tumour and lymph node metastatic tumour.

Through our findings, we report that there is a possible correlation between patient's histopathological diagnosis and the acquired qMIDS score from FFPE samples. This finding needs to be further verified on a larger sample size.

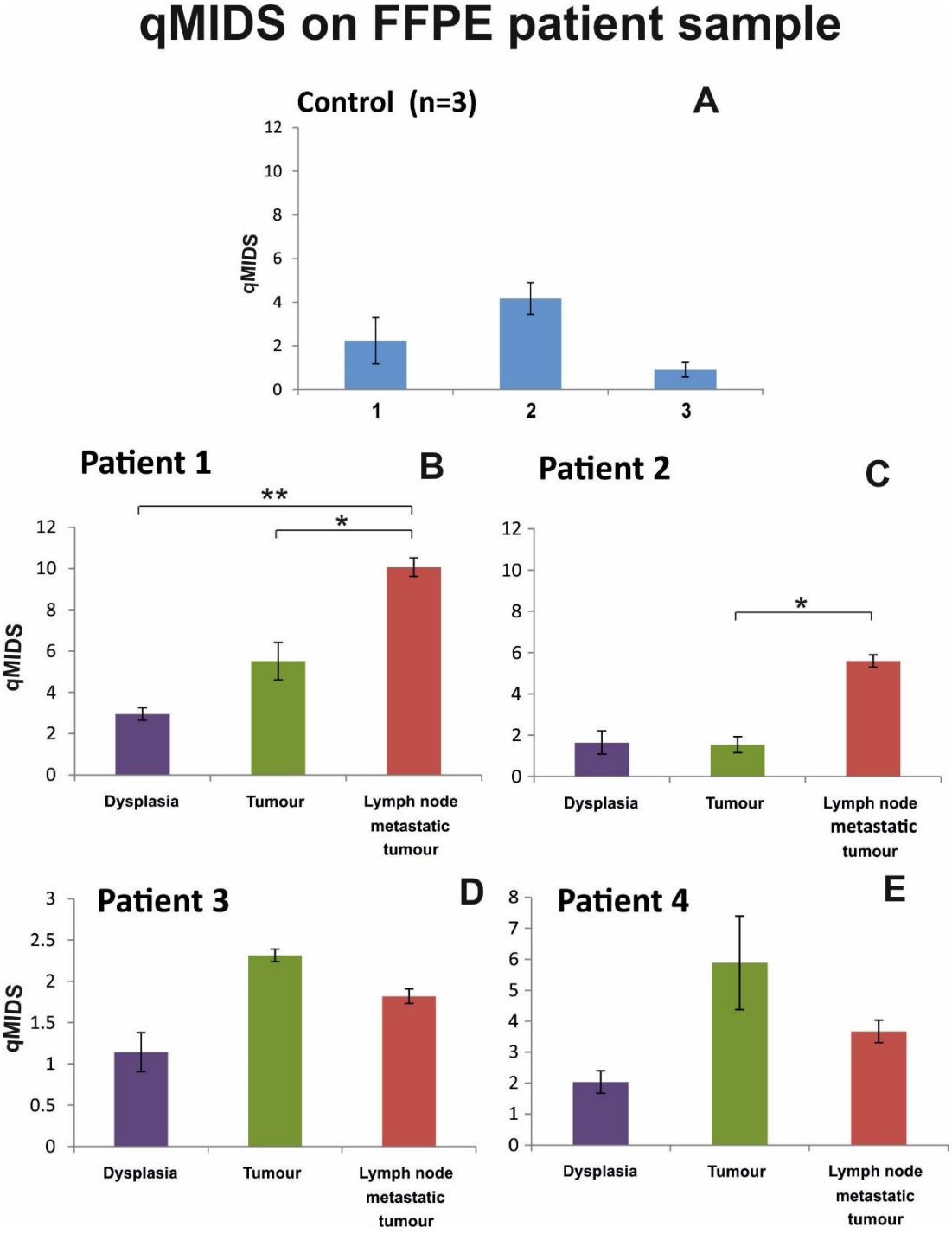


Fig 5.2: *qMIDS index values of (A) normal healthy oral mucosa (B) Patient 1 (C) Patient 2 (D) Patient 3 (E) Patient 4. Each sample was processed in duplicates; mean of malignancy index values was calculated. The error bars represent the standard error of the mean where n=3. T test was performed using Graph Pad software, significant p values are denoted by (*).*

5.3 Discussion

In diagnosing OSCC the importance of identifying pre-cancerous lesions and early detection of the disease cannot be under emphasized. These factors are of immense importance for early intervention leading to decreased mortality and morbidity.

Currently the most reliable method for OSCC diagnosis is histopathology. Key limitations of histopathology are that it is extensively time consuming and subjective to pathologist interpretation. In addition presence of dysplasia can be missed as the molecular changes responsible for malignant transformation do not produce histopathologically detectable changes (Braakhuis et al., 2003). Hence there is a clinical need for a quick, quantitative and conclusive molecular test that would complement histopathology.

With this aim in mind qMIDS was developed. It is based on FOXM1 (isoform B) oncogene and its downstream targets. It is a rapid method in comparison to histopathology as biopsy tissue does not require fixation and embedding. It is also quantitative based on RT-qPCR which is widely available in diagnostic laboratories (Teh et al., 2013).

In comparison to the developed world the prevalence of OSCC is much higher in the underprivileged parts of the world. High incidence of the disease is found in South East Asian population including India, Pakistan and Bangladesh (Joshi et al., 2014). This is mostly due to lack of awareness regarding risk factors such as smoking and chewing tobacco in rural population. In addition lack of finances leads to poor healthcare facilities resulting in late diagnosis which further increases the burden on healthcare systems. Setting up qMIDS in high incidence regions can be challenging due to lack of infrastructure and equipment required for storage and processing of fresh biopsy tissues. Hence, validating qMIDS on FFPE samples is of prime importance for such populations as fixing samples in formalin is a cost effective method of preserving samples, it has no special storage requirements and can be easily shipped for analysis.

Although our study is limited to a small patient sample size, we were fortunate to get retrospectively collected FFPE samples from OSCC patients with known diagnostic outcomes. The samples were collected at different stages of the disease including dysplasia, OSCC tumour and metastasis to lymph nodes. Such archival tissues are very rare to find as they require long term patient follow up. Our data shows significant correlation between progressions of the disease at different stages with an increasing

qMIDS index. Due to lack of more FFPE samples we were unable to validate our findings on a larger sample size.

5.4 Limitations

Although qMIDS is efficient in segregating high risk individuals from low risk, it has certain limitations. It is an invasive test, requiring biopsy samples leading to patient discomfort.

In addition, cancer development involves change of expression of multiple genes leading to disruption of multiple cellular pathways. qMIDS only focuses on the expression levels of oncogene FOXM1B and its associated genes. Other biomarkers, although some may be feeding into FOXM1 pathway, such as Ras (Murugan et al., 2012), c-myc (Perez-Sayans et al., 2011), EGFR (Bernardes et al., 2013) and cyclin D1 (Das et al., 2011) that play key roles in OSCC development and progression could be overlooked using qMIDS diagnostic system.

Despite subjective opinion, histopathology (on frozen section) has an advantage that it is less time consuming in reporting than qMIDS as the latter requires purification of mRNA from tissues and RT-qPCR requiring at least two hours using the current protocol. The pathologist can visualize abnormal changes in the tissue while the patient is being operated for tumour removal. This guides the surgeon in obtaining clear disease free margins, reducing the chances for recurrence.

Chapter 6

Meta-analysis of head and neck cancer genetic biomarkers

6.1 Introduction

Head and neck squamous cell carcinoma (HNSCC) is the commonest form of head and neck cancer. It is a multifactorial disease predisposed by many risk factors such as smoked or chewed tobacco, excessive alcohol consumption, betel quid, areca nut and human papilloma virus. It is also a result of abnormal genetic alterations such as point mutations, amplifications, rearrangements and deletions of genes, making way for tumour progression (Williams, 2000). In order to detect genetic changes a molecular diagnostic test is much needed, that would aid in early diagnosis, resulting in better prognosis.

Much progress has been made in cancers of other origins such as breast and lung where reliable diagnostic markers HER2 and EGFR mutant have been identified respectively (Pao and Chmielecki, 2010, Pinto et al., 2013). Apart from assisting in diagnosis these markers also aid in directing a more customised therapy resulting in a more favourable outcome.

Efforts are being made in the field of HNSCC to find reliable biomarkers that would reflect the molecular make-up of the tumour. Through systemic reviews and meta-analysis studies, EGFR and cyclic D1 have been reported as potential serum diagnostic marker (Guerra et al., 2016), furthermore ANO1 and FADD have been reported as possible prognostic markers (Reddy et al., 2016). Via meta-analysis genomic changes such as hyper methylation of RAS association domain family protein 1a (RASSF1A), a tumour suppressor gene has been associated to high risk of developing HNSCC (Meng et al., 2016).

Previously developed diagnostic system (qMIDS) heavily relies on the expression levels of FOXM1B oncogene and its associated genes within a biopsy sample. It overlooks numerous other genetic biomarkers that are independent from FOXM1B and play key roles in carcinogenesis. In order to overcome this limitation of qMIDS, in this study we aimed to explore unbiased HNSCC biomarkers, which would be independent of FOXM1B expression.

Meta-analysis of eight independent HNSCC microarray studies was done to obtain most significantly up and down regulated genes in studies comparing HNSCC samples with normal samples. The expression of selected genes was established in approximately hundred OSCC biopsy samples in pairs of margin and core. Molecular findings from each sample were correlated with patient's clinical report.

Results

6.2 Gene selections from 8 microarray datasets

A cancer microarray database called Oncomine (Rhodes et al., 2004) (www.oncomine.org) was used to select eight head and neck cancer studies. All these studies analysed HNSCC cancer samples versus normal samples **(Table 6.1)**. The bioinformatics gene selection was initially part of previous intercalated BSc project (performed by Sungjae Hwang).

Table 6.1: Selected microarray studies *along with PubMed ID, GEO accession number, number of tumour, normal and lymph node metastasis samples. *microarray data submitted only to Oncomine database.*

Study type	PMID	GEO	Tumour	Normal	LNM
Hypopharyngeal Squamous Cell Carcinoma vs. Normal	14676830	GSE2379	34	4	
Tongue Squamous Cell Carcinoma vs. Normal	19138406	GSE13601	37	20	
Head and Neck Squamous Cell Carcinoma vs. Normal	14729608	Ginos Head-Neck*	41	13	
Tongue Squamous Cell Carcinoma vs. Normal	15170515	GSE6631	22	22	
Hypopharyngeal Squamous Cell Carcinoma vs. Normal	16205657	GSE1722	6	4	2
Nasopharyngeal Carcinoma vs. Normal	16912175	GSE12452	31	10	
Oral Cavity Squamous Cell Carcinoma Epithelia vs. Normal	15381369	GSE3524	16	4	
Tongue Squamous Cell Carcinoma vs. Normal	18254958	GSE9844	53	22	

From the above mentioned eight studies 20 differentially expressed genes were selected based on their P-value. Out of which 10 were significantly up regulated and10 were significantly down regulated **(Table 6.2)**.

Table 6.2: Over expressed and under expressed genes selected from 8 microarray studies on head and neck squamous cell carcinoma.

Over expressed genes	Under expressed genes
COL5A1	CRNN
SERPINH1	CLEC3B
PLAU	ABCA8
FNDC3B	SPINK5
MFAP2	TTC9
COL1A1	EMP1
FN1	SORBS2
LPCAT1	HLF
CDCA5	KLK12
COL4A2	DUOX1

6.3 Functional validation of candidate markers on cancer cell lines

Primers for each gene were designed using the Roche Applied Science Universal Probe Library Assay Design Centre for RT-qPCR. Initial testing of primer specificity and expression of selected biomarkers was established on cDNA from normal and HNSCC cell lines **(Fig 6.1B).**

Based on primer specificity and good reproducibility three differentially expressed biomarkers were identified, of which, PLAU, FN1 and CDCA5 were found to be upregulated; whereas CRNN, CLEC3B and DUOX1 were found to be downregulated when comparing cancer to normal cell lines. For ease we called them q6 (quantification of selected six biomarkers). The q6 biomarkers were found to be involved in important cellular function, listed in Table 6.3

Table 6.3: Shortlisted over expressed (Red) and under expressed (Green) genes and there function.

Genes	Function
PLAU	This gene encodes a secreted serine protease that converts plasminogen to plasmin.
	It plays an important role in degrating extracellular matrix.
FN1	Fibronectin is involved in cell adhesion and migration processes including embryogenesis,
	wound healing, blood coagulation, host defence and metastasis.
CDCA5	Cell division cycle associated 5 or Human Sororin gene is involved in sister chromatid cohesion,
	separation and tumour genesis.
CRNN	Cornulin belongs to the "fused gene" family and is involved in mucosal/epithelial immune
	response and epidermal differentiation.
CLEC3B	Encodes tetranectin protein which is found to be a potential biomarker for metastatic oral cancer.
DUOX1	Dual oxidase 1 encodes a glycoprotein and is a member of the NADPH oxidase family.
	It is important for antimicrobial defense at mucosal surfaces.

6.4 Validation of candidate markers on clinical samples

RT-qPCR assays was carried out to produce quantitative data on expression of these selected genes (relative to two reference genes) on paired margin and core fresh biopsy samples from OSCC patients.

Individual relative expression between core and margin tissue was calculated for each gene. In order to obtain a clinically meaningful index value from 6 genes, we derived an equation to summarise the degree of differential gene expression.

q6 Value / Differential ratio = (Sum of Log2 Ratios of the 3 upregulated genes) – (Sum of Log2 Ratios of the 3 downregulated genes).

Differential ration (q6) value of each sample is listed in **Table 8.1 in appendix section, Page no. 196.**

We found that within the 100 patient samples, two distinct groups of patient population could be identified **(Fig 6.1 A)**. The group of patients with positive q6 values expressed the predictable expression of q6 markers which was noted in oral cancer cell lines with PLAU, FN1 and CDCA5 being upregulated and CRNN, CLEC3B and DUOX1 being downregulated. Majority of the patients fell into this group **(Fig 6.1 C)**. While the other group of patients had lesser number of patients, exhibiting negative

q6 values with inverse expression of q6 markers with PLAU, FN1 and CDCA5 being down regulated and CRNN, CLEC3B and DUOX1 were up regulated **(Fig 6.1 E)**.

The patient group in the middle had a mixed expression of q6 markers with most of the q6 markers following the expected trend. Approximately ten patients were found to be in this category **(Fig 6.1 D)**.

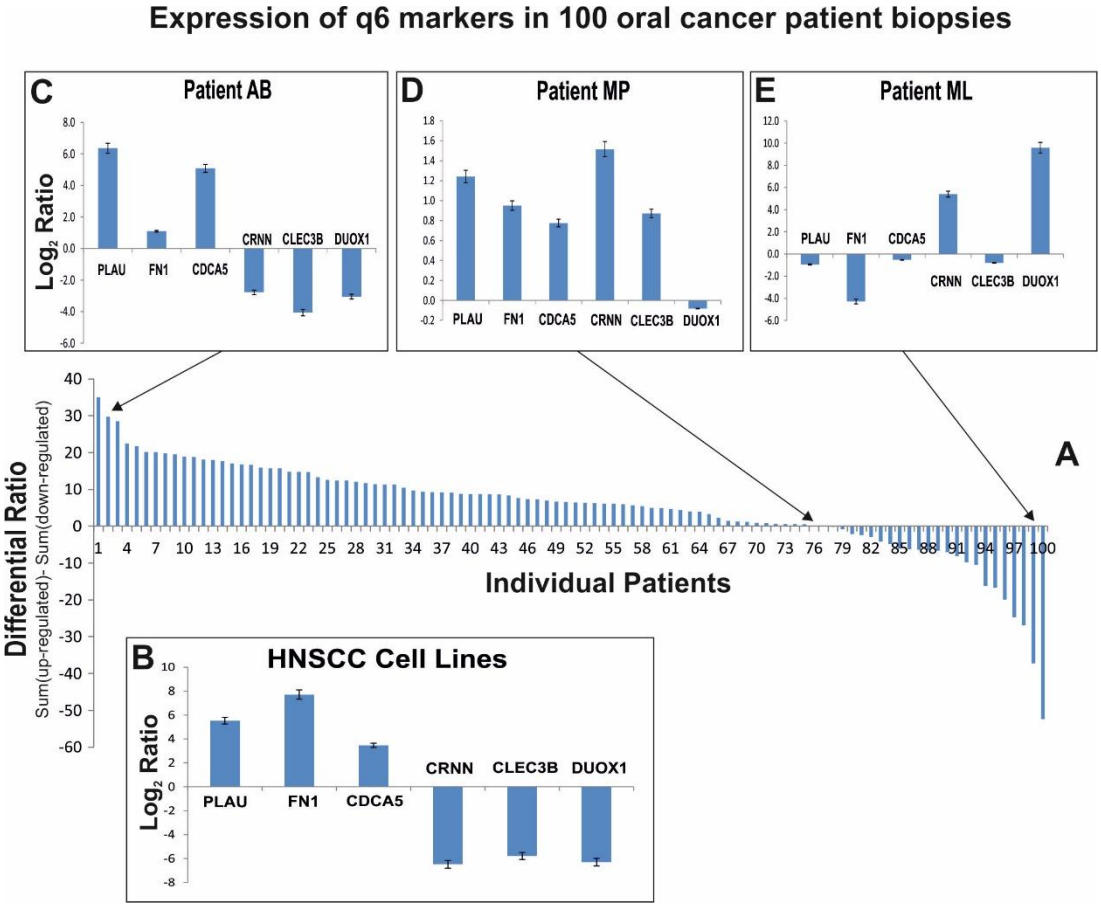


Fig 6.1: Log 2 ratio of q6 biomarkers *between margin and core biopsy tissue samples.* ***(A)*** *Differential ratio of biopsy samples from 100 OSCC patients* ***(B)*** *Pattern of expression of the six biomarkers in HNSCC cell lines SCC4 and SqCC/Y1* ***(C)*** *Patient AB* ***(D)*** *Patient MP* ***(E)*** *Patient ML.*

6.5 Clinicaopathological analysis of two patient groups with opposing q6 gene expression

In order to understand the different pattern of q6 biomarkers, our collaborating clinician Dr. Anand Lalli (Dept. of Oral Surgery, Barts and the London School of medicine and dentistry) helped in correlating patient's clinical reports with our molecular findings.

Table 6.4: Summary of patient's sociodemographic and clinicopathological report.

			+ q6 values	- q6 values	
Patient Details	Age	Mean (years)	63.2	56.9	p=0.047*
		Range (years)	52-71	52-63	
	Gender	male:female	9:4	1:7	p=0.0179*
	Ethnicity	British	9	5	p=0.051
		Bangladeshi	2	2	p=0.041*
		Others	2	1	p=0.472
	Risk Factor Habits (n)	Smoking	8	4	p=0.345
		Alcohol	6	0	p=0.045*
		Paan Usage	4	6	p=0.027*
Tumour Details (n)	Primary		13	5	p=0.840
	Recurrence at 2 years		0	3	p=0.168
	Differentiation on Grade	Well	0	1	p=0.668
		Moderate/Poor	13	7	
	Site	Buccal	3	2	p=0.968
		Floor of Mouth/ Tongue	5	3	p=1.000
		Other	4	3	p=0.413
	Size	T1	5	0	p=0.735
		T2	3	2	
		T3	3	5	
		T4	2	1	

Mann Whitney U Test; *p<0.05 indicating a statistically significant difference between subgroup A and B.

Patient and tumour details were collated retrospectively for the 20 most positive and 20 most negative q6 values. In each group, data collection was incomplete for a number of patients (for example due to an inability to trace the clinical records or the clinical records being incomplete for all the data required in this study) and therefore these individuals were removed from the analysis.

Clinical data from 13 patients with +q6 values was compared to 8 patients with –q6 values. We found statistically significant difference in age, sex, ethnicity, alcohol consumption and paan usage among the two groups. In the +q6 group the mean age was 63 with more males than females, while in -q6 group the mean age was 56 with more females than males (P=0.04). Additionally, more patients of Bangladeshi descend were found to be in -q6 group (P=0.04) **(Table 6.4)**.

Statistically significant difference was found in the two groups with regards to associated risk factors. High levels of alcohol consumption was found in +q6 group (P=0.04), while patients in the -q6 group were mostly paan chewers (P=0.02) **(Table 6.4)**. No difference was found in the smoking habits, tumour site and size among the two groups. Recurrent lesions were only found in -q6 group although not statistically significant, which might be due to small sample size **(Table 6.4)**.

6.6 Discussion

Even with increasing advancement in the field of oral cancer diagnosis and treatment. The five year survival rate has remained unchanged. Currently the mainstays of treatment for advanced tumours are surgery combined with post-operative radiotherapy. All HNSCC patients are subjected to the same treatment irrespective of the genetic makeup of the presenting tumour. This is due to the gap in our knowledge regarding relevant prognostic biomarkers that can be used to distinguish between sub populations of HNSCC patients and to inform the most suitable intervention based on individual tumour molecular profile.

Molecularly targeted therapy is expected to be more selective towards cancer cells minimizing damage to normal cells; however targeted therapy involves genome based individualized treatment of oral cancer patients. Importantly, the determination of genetic signatures and potential biomarkers for aggressiveness of HNSCC would allow predicting the chances of survival and recurrence of the disease. Developing potential important biomarkers would allow more precise therapeutic approach for each individual case during the treatment.

Previously we have developed a quantitative malignancy index diagnosis system called qMIDS. It was based on FOXM1 oncogene and its related genes. In this study we aim to explore new potential biomarkers which are not restricted to FOXM1B expression but also differentially expressed in cancer vs normal tissue. Using the online database Oncomine, a list of differentially expressed genes were shortlisted from 8 independent microarray studies. These genes include PLAU, FN1, and CDCA5 which were found to be significantly upregulated in HNSCC and CRNN, CLEC3B and DUOX1 which were significantly downregulated.

Tissue samples for this study were collected at the time of tumour resection. The decision to treat surgically was made on standard clinic-pathological factors by a multi-disciplinary team of surgeons, oncologists and allied health professionals in conjunction with the patient's wishes as per NHS best-practice policy. This would suggest the study sample represents a proportion of all head and neck tumours diagnosed in the study period with other tumours being either inoperable (e.g. due to size, position, metastatic spread, patient's general health or patient wishes) or managed without a tissue sample being generated (e.g. chemo-radiotherapy or laser ablation).

Gene expression level was measured using absolute RT-qPCR in paired margin and core OSCC specimens. Data was collected from 100 patients with the purpose to validate if these genes could be used as OSCC biomarkers.

We found that within this group of patients, existed two distinct subgroups one which expressed the predictable expression of q6 biomarkers with PLAU, FN1, CDCA5 being upregulated and CRNN, CLEC3B and DUOX1 being downregulated. Patients in this group gave a positive q6 value. Majority of the patients were found to be in this group. From the clinical reports of 13 patients from this group we found that this group consisted of mostly older men who consumed more than the recommended units of alcohol per week along with smoking. Both of these are known high risk factors for developing OSCC. This finding is consistent with the majority of oral cancers found in the UK population as a whole.

The other group consisting of twenty three patients who showed an inverse expression of q6 biomarkers with PLAU, FN1, CDCA5 being downregulated while CRNN, CLEC3B and DUOX1 being upregulated. This study utilised tissue samples collected from the Royal London Hospital locality in Tower Hamlets, East London which comprises the highest concentration of Bangladeshi individuals in the Western World with specific cultural risk factors for OSCC that are usually only prevalent on that subcontinent, such as ‘paan’ (areca nut) usage. This population is known to be at higher risk of developing OSCC than the UK population as a whole and in particular at a younger age and in women due to these cultural influences. Therefore, the finding that patients with negative q6 values (8 patients) in this study are markedly different from the positive group in age (significantly younger) and gender (more females) as well as paan users is extremely interesting as it suggests that the biomarker response confirms the epidemiological data indicating these are two distinct groups of OSCC.

This is further supported by the statistically lower reported alcohol consumption in the negative group as alcohol usage is low in Islamic cultures but a significant issue in the UK population as a whole.

All 3 patients with a recurrence of their primary tumour were in the –q6 group, although no statistically significant difference between the two groups was noted. This may be an important finding as it suggests that the negative q6 group are more prone to recurrence. In addition to the small sample size it must be noted that to be included in this study the recurrence must have been treated by surgical excision and therefore operable so this finding must be interpreted with extreme caution but warrants further investigation.

Patients in both the groups suffered from the same disease and were offered the same treatment. The genetic analysis of the biopsy tissue indicates that molecularly the tumours in each group were different and showed different expressions of q6 biomarkers.

A further study conducted in our lab by an MSc student Huma Habib Dar on chemotherapeutic drug and gamma irradiated resistant oral keratinocyte cell lines demonstrated that the downregulation of PLAU biomarker might be an indicator of tumour being resistant to radiation therapy with high chances of recurrence **(Fig 6.2)**. This finding needs to be verified and validated through further studies.

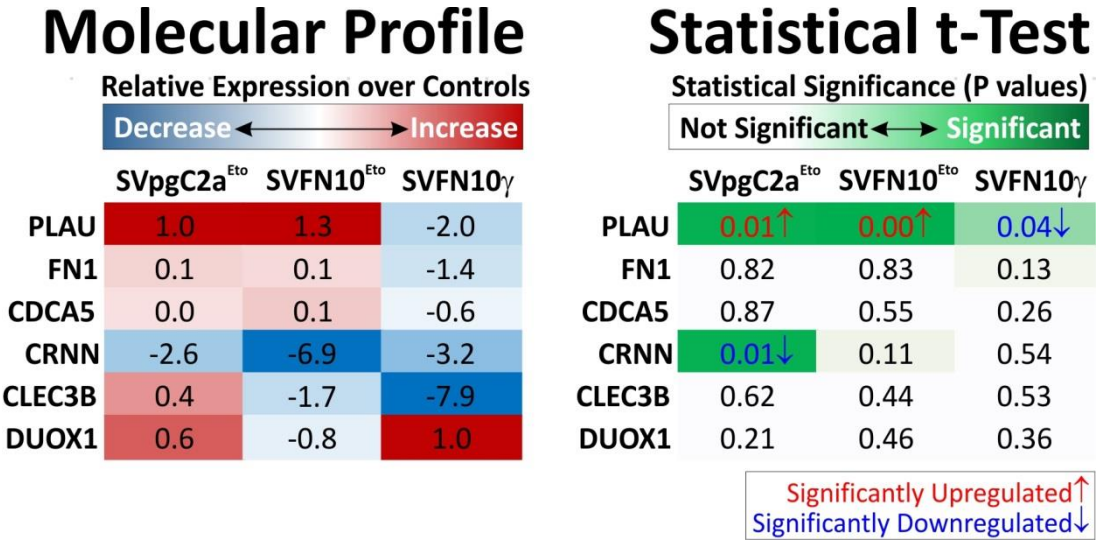


Fig 6.2: *Relative expression of cells treated with Etoposide ($SVpgC2a^{ETO}$, $SVFN10^{ETO}$) and gamma irradiated (SVFN10γ) compared to untreated controls showing data of q6 genes and corresponding statistical t-test (p-value). The diagram was created by Dr Muy-Teck Teh.*

In view of the biomarkers used in this study representing tumours that are resistant or responsive to chemo and radiotherapy (RT) this is potentially an extremely important clinical finding as it identifies patients who, for example, may not respond to post-operative RT and therefore could benefit from a more aggressive surgical approach.

Smoking and tobacco usage is difficult to interpret as the clinical records often recorded cigarette smoking and pack per year etc. but did not record whether paan users incorporated tobacco in their mixture. Additionally, cigarette smoking is

reported as a predominantly male practise in the Bangaldeshi population and therefore it is unsurprising that there was no difference between the two groups. However, it may have been expected that the negative group would present with more buccal sulcus tumours as this is the predominant site of paan placement and larger tumours as the Bangladeshi population are known to be poor users of healthcare facilities but this was not evident in the study perhaps due to the small sample size.

All patients were followed up for at least 2 years following surgical resection of their primary tumours and all tumours were histologically confirmed oral squamous cell carcinomas (OSCC) .The 18 primary OSCC were excised and subsequently treated with post-operative RT with the tissue samples for this study being taken prior to RT whilst the recurrences had originally been resected and all had post-op radiotherapy at that time.

Interpretation of results from a retrospective study of this type must be done with caution particularly when assessing patient treatment modalities, which cannot ethically be influenced by the study design. The various forms of selection bias was a significant concern in this study as was the small sample size augmented by the inability to collect patient details on a proportion of each group. However, this data indicates the need for a prospective observational study of the correlation between patient factors and OSCC treatment response.

We further look into literature to understand the role of q6 bio markers in OSCC development and progression.

PLAU (plasminogen activator, urokinase) encodes a serine protease involved in degradation of the extracellular matrix and possibly tumour cell migration and proliferation. PLAU has been shown to be a novel biomarker with high tumour expression levels in head and neck squamous cell carcinoma and is linked to inferior disease free survival rate with increased disease progression and relapse (Sepiashvili et al., 2012). In breast cancer cells, PLAU was indicated to have a marked role in transformation by increasing invasiveness (Iliopoulos et al., 2011).

In prostate cancer and laryngeal SQCC PLAU gene amplification was preferentially found in advanced stage, but not detected in benign lesions, suggesting PLAU may have a tumour stage-dependent expression pattern (Bloch et al., 2007, Hui et al., 2015). To date no study has related altered PLAU expression to drug or radiotherapy resistance.

FN1 (Fibronectin 1) encodes fibronectin, a glycoprotein present in a soluble dimeric form in plasma, and in a dimeric or multimeric form at the cell surface and in extracellular matrix. Fibronectin is involved in cell adhesion and migration processes including embryogenesis, wound healing, blood coagulation, host defence and metastasis. A study on salivary gland carcinoma has verified using cDNA array and immunohistochemistry that FN1 is overexpressed in mucodermoid, acinic cell and salivary ductal carcinoma (Zhang et al., 2011b).

FN1 is also a downstream target of SATB1 oncogene. It is found to be up regulated in oesophageal squamous cell carcinoma resulting in enhanced cell proliferation and migration ability (Song et al., 2017)

In other cancers FN1 induced specific metalloproteinases, such as MMP9/MMP2 to promote invasion and metastasis (Qian et al., 2011, Shibata et al., 1997, Moroz et al., 2013).

CDCA5 (Cell division cycle associated 5 or Human Sororin gene) is involved in sister chromatid cohesion, separation and tumour genesis. The cellular localization and the levels of Sororin proteins differ in stages of cell cycle. A higher level of protein is seen from S to G_2 phase and a decline following mitotic exit (Zhang and Pati, 2012). The role of CDCA5 has not been extensively studied in tumour initiation but a study on lung cancers has shown high levels of CDCA5 and its association with poor prognosis (Nguyen et al., 2010).

In a study on OSCC, CDCA5 was found to be upregulated in 4 human OSCC cell lines by RT-qPCR. Knockdown of CDCA5 inhibited the growth of OSCC cells while the expression levels of CDCA5 proteins were found to be associated with overall survival (Tokuzen et al., 2016).

Evidence from the literature suggests that CDCA5 is likely to play a significant role in OSCC progression, so that targeting CDCA5 may be a potentially useful diagnostic and therapeutic approach for OSCC patients.

CRNN (Cornulin) also known as squamous epithelial heat shock protein 53 belong to the “fused gene” family and is involved in mucosal/epithelial immune response and epidermal differentiation (Contzler et al., 2005). CRNN is an important molecule in normal oesophageal tissue; it is localized to cytoplasm and perinuclear region of the prickle and functional cell layers. In oesophageal squamous cell carcinoma it is 5 fold down regulated in 89% of cases indicating the loss of CRNN during the transformation

from normal to neoplastic (Pawar et al., 2013). Significant loss of CRNN expression is associated with advanced stage, invasive behaviour of the tumour, lymph node metastasis and poor survival (Chen et al., 2013, Hsu et al., 2014, Pawar et al., 2013).

Similar to oesophageal squamous cell carcinoma CRNN expression is found to be down regulated in OSCC (Imai et al., 2005a). Direct sequencing of CRNN gene elucidated loss of heterozygosity and microsatellite instability at chromosome 1q21.3 region, as possible mechanisms of CRNN down regulation in OSCC (Salahshourifar et al., 2015).

These findings provide evidence on the role of CRNN in tumour progression of OSCC as a prognostic marker to predict disease outcome.

CLEC3B (C-type lectin domain family 3, member B) encodes tetranectin protein which is found to be a potential biomarker for metastatic oral cancer. In serum and saliva of metastatic OSCC compared to primary OSCC, tetranectin was found to be significantly under expressed. Decreased levels of tetranectin have been assoiciated with cancer progression. Though the exact mechanism is not known but it is believed this down regulation aids in the activation of plasminogen cascade causing increased proteolytic processes (Arellano-Garcia et al., 2010).

In ovarian and breast cancer, decreased serum levels of CLEC3B has been associated with adverse prognosis due to poor treatment response (Hogdall et al., 1993b, Hogdall et al., 1993a)

These findings support that CLEC3B may be used as a markers for metastasis.

DUOX1 (dual oxidase 1) encodes a glycoprotein and is a member of the NADPH oxidase family. Named as dual oxidase because it has both a peroxidase homology domain and a gp91phox domain. This protein generates hydrogen peroxide and thereby plays a role in the activity of thyroid peroxidase, lactoperoxidase, and in lactoperoxidase-mediated antimicrobial defense at mucosal surfaces.

It has been found that in 50% of lung cancers NADPH oxidase DUOX1 and DUOX2 go under epigenetic silencing via hyper methylation of CpG-rich promoter regions and can be restored after treatment with 5-aza 2'-deoxycytidine. Introducing normal levels of DUOX1 into lung cancer cell lines increased cell migration and wound repair without affecting cell growth (Luxen et al., 2008).

In liver cancer low levels of DUOX1 expression were found, while normal levels were indicative of disease free survival. Hence DUOX1 is a potential prognostic tool (Chen et al., 2016). To date the potential of DUOX1 as a diagnostic or prognostic tool has not been explored in HNSCC.

Through literature review it is much evident that q6 biomarkers are responsible for much of the molecular changes required for the transformation of normal tissue into disease tissue. Through multiple microarray studies the expression of these six biomarkers have been established in HNSCC with PLAU, FN1, CDCA5 being upregulated and CRNN, CLEC3B, DUOX1 being downregulated. Although through our study it seems that there is a possibility that the inverse expression of these six markers might not necessary mean that the individual is disease free but suggests that the individual might be presenting a tumour that is genetically different which may possibly linked to therapeutic treatment resistance. The expression of q6 biomarkers can be different depending on the carcinogen or the risk factors that the patient is exposed to. More research is needed to understand the mechanisms altering the expressions of q6 biomarkers.

6.7 Limitations

Similar to previously developed qMIDS, acquiring q6 values also required biopsy samples, which makes it an invasive methods leading to patient discomfort.

Obtaining q6 values required RNA extraction followed by conversion into cDNA and establishing relative expression of each gene through RT-qPCR. This process can be time consuming requiring at least two hours.

In ability of finding clinical records retrospectively led to analysing a very small sample size which might not be a true representation of a group. Further request has been sent to collaborating clinician to provide additional clinical data.

Within the clinical findings tobacco usage in Paan users was not established as they were not asked if they added tobacco to Paan. Subsequently Paan also contain areca nut a known OSCC carcinogen. It is important to distinguish if the molecular finding in –q6 patients (paan users) are an effect of chewed tobacco within Paan or areca nut. It can possibly be a combined effect of both carcinogens.

Since Paan usage is habitual in South Asian population determining the patient's ethnicity is essential. We were informed by the collaborating clinician that patients of South Asian origin, born in the UK identified themselves as British and were not further questioned regarding Paan practice, although it is possible for these patients to be paan chewers.

Chapter 7

Discussion

OSCC is the commonest tumour in the oro-facial region with increasing incidence in the recent years. Curing the disease is challenging, as it depicts a heterogeneous tumour with high morbidity and mortality rate. Clinico-pathological data, tumour site, lymphnode metastasis (TNM) staging, histological grade and invasion have been evaluated in great depth in relation to OSCC. Co-morbidity factors like the use of tobacco, alcohol consumption and various other factors including genetic pre-disposition have been looked at for finding a suitable treatment protocol. The problem in understanding the complexity of oral cancer lies in the biological heterogeneity of the tumour. Similar heterogeneity has been noted in clinical presentation, histopathology, and molecular changes at cellular levels.

In spite of the disease being diagnosed, a prediction of behaviour and response to treatment remains elusive. Early screening of asymptomatic high risk patients using non-invasive techniques will provide an opportunity to closely monitor early signs of disease aiding in lower mortality and morbidity. In this study we look beyond the histopathological features of OSCC, increasingly into the molecular and genetic changes driving the disease which will help in segregating a high risk population from low risk. Such a method will also aid in designing and implementing of a targeted drug therapy (Bavle et al., 2016).

Currently histopathology is the gold standard technique used for diagnosing OSCC lesions. Histopathology has certain limitations such as by the time these histological changes become evident the disease is at a much advanced stage. Since the oral cavity is surrounded by multiple sets of lymph nodes the disease spreads at a very fast rate causing much destruction of the surrounding tissue and bone leading to a low overall survival rate. In addition, histopathology is not a quantitative technique and is very subjective to pathologist interpretation which might vary from one clinician to another. The clinical appearance and histopathological features of OSCC lesions might vary among patients. Some lesions are followed by pre-cancerous lesions and dysplasia while others might not (Carnelio et al., 2011). Secondly, not all dysplastic lesions transform to OSCC at least 50% of the lesions arise from clinically normal mucosa (Speight, 2007). Hence it is difficult to predict as to what causes the transformation of a benign lesion into malignant lesion.

Since OSCC is a heterogeneous disease, a single site tissue biopsy taken at one time point does not reflect the ongoing changes occurring in the tumour and the surrounding tissues.

7.1 Non-invasive exosome based liquid biopsy

Liquid biopsy from body fluids such as blood and saliva offers an alternative to the limitation of heterogeneity in OSCC tissue biopsy as the liquid biopsy would not be site specific and can be taken multiple times causing less patient discomfort due to its less invasive nature. These biopsies will not only reflect the heterogeneous make-up of the disease but can also be used to follow treatment response, reveal the presence of residual disease and alert the oncologist in case of re-emerging disease. Ultimately, if clinicians can match results from liquid biopsies with a cocktail of targeted anticancer therapies, the promise is that advanced cancers could become manageable chronic diseases, much like HIV is today (Webb, 2016).

Researchers have known for decades that tumours shed cells in body fluids (circulating tumour cells – CTCs) and that apoptotic cancer cells shed DNA fragments into the blood stream (circulating tumour DNA – ctDNA). Living cancer cells also shed extracellular vesicles containing cellular material including DNA, RNA and proteins, known as exosomes and microvesicles (Ko et al., 2016).

In this study we propose the use to exosomes based liquid biopsy for OSCC early diagnosis. Compared to CTCs and ctDNA exosomes are much more stable and resistant to destruction due to the protective lipid bi-layer outer membrane. In addition exosomes are more abundant and hence easier to detect than CTCs and ctDNA (Lang et al., 2012).

Exosomes are considered superior as diagnostic and prognostic tools than microvesicles as they are endosomal in origin and enriched for proteins related to transport and fusion (*e.g.* flotillin, caveolin-1) (Logozzi et al., 2009), heat shock proteins (*e.g.* Hsp90), and lipid-related proteins (Zeringer et al., 2015). In addition they contain nucleic acids such as miRNA and mRNA (Valadi et al., 2007) as well as double stranded DNA (Thakur et al., 2014). Microvesicles are produced by the budding of plasma membrane and are more reflective to cellular cytoplasm. Historically, due to different methods of EVs isolation studies have used microvesicles and exosomes interchangeably and hence it is difficult to differentiate them on the basis of their contents. In order to overcome this issue Professor Suresh Mathivanan from the La Trobe Institute for Molecular Science in Australia has created a valuable online resource that catalogues the cargo of exosomes and microvesicles from various origins, with the database including lipids, proteins, and RNAs (http://www.exocarta.org).

In this study we successfully isolated exosomes from oral keratinocyte cell culture supernatant and body fluids including whole blood, plasma and saliva. Validation of effective isolation was done using particle size (zetasizer, NTA) and imaging techniques (SEM, TEM and NTA) **(Fig 3.3, 3.4, 3.5, 3.6, 3.7 Page no. 88-92)**. Through western blotting samples were tested for the presence of exosome specific proteins such as ALIX, CD63 and CD9. We found that oral keratinocyte exosomes express more of ALIX protein (part of the ESCRT III complex), than tetraspanin proteins (e.g CD63 CD9 and CD81) which are much talked about in exosome literature (Rana and Zöller, 2013) **(Fig 3.8, Page no. 95)**. Tetraspanin proteins are cell surface proteins regulating cell morphology, motility, invasion, fusion and signalling in the brain, immune system and on tumours (Hemler, 2005).

In addition, CEP55 a centrosomal protein and a partner of ALIX in ESCRT machinery, was found to be exclusively expressed in cancer exosomes **(Fig 3.8, Page no. 95)**. We suggest a potential use of CEP55 as a biomarker to selectively isolate OSCC cancer exosome from body fluids. We have attempted to validate the specificity of CEP55 as a cancer exosome marker through gold label immuno TEM **(Fig 3.10, Page no. 98)**, whereby we found that CEP55 protein was indeed localised on exosomal membranes. Further validation through a quantitative technique such as nano flow cytometry would be required to confirm if CEP55 protein is enriched in OSCC derived exosomes.

Our further characterisation study on exosomes confirmed that RNA contents of exosomes are well protected within the lipid bi-layer membrane and is resistant to degradation by RNase. We further questioned whether RNA cargo could be passed from one cell to another via exosomes. This led us to perform exosome transfection study. We found that cancer exosomes containing oncogene FOXM1B mRNA were able to transfer its mRNA content into recipient cells (normal oral keratinocytes with very low level endogenous FOXM1B expression), although only transiently as the elevated levels of FOXM1B mRNA was lost after 72 hours **(Fig 4.8, Page no. 116)**. This finding is not surprising as other studies have found the same phenomenon either by the presence of a cellular protective mechanism such as autophagy (Danzer et al., 2012, Baixauli et al., 2014) or the RNA interference through dicer complex as part of the RNA-induced silencing complex (RISC) (Jayachandran et al., 2012) which halters the undesired foreign invasion of exosomal contents. This protective mechanism against exosomes within recipient cells is yet to be explored.

In order to further understand the functional effects of normal and cancer exosomes on recipient cells, we performed microarray expression profiling in recipient cells transfected with either normal or cancer exosomes. We found that exosomes, irrespective of their origin (normal and cancer), altered gene expression patterns of certain genes in recipient cells. Upregulation of MMP9, TUBB6, FEZ1, OAS1 and PGAM1 were noted. These genes are found to be responsible for essential cellular functions such as breakdown of extracellular matrix, regulation of cytoskeleton, intra cellular transport, immune regulation and cellular metabolism (Peisker et al., 2017, Chao et al., 2012, Chua et al., 2013, van Vugt, 2017). While the expressions of TSC22D3, OTUD1 and BBOX1 involved in signal transduction cell survival pathway, regulation of P53 and cellular transport respectively were found to be downregulated (Espinasse et al., 2016, Piao et al., 2017, Dawany et al., 2011) **(Fig 4.17A, Page no. 137) (Table 4.1, Page no. 133)** . Perturbation of these genes appears to suggest that exosomes may trigger matrix reorganisation and cellular motility in recipient cells.

When comparing gene expression profiles of recipient cells transfected with normal and OSCC exosomes, we found that EFEMP1, EEF2K, DKK3, LMP2, SRPX, SPARC and ADAM9 were differentially upregulated in normal oral keratinocyte recipient cells. These genes are known to be involved in regulation of cellular morphology, protein synthesis, embryonic development, autophagy pathway, tissue remodelling, deposition of extracellular matrix and cellular adhesion (Han et al., 2017, Sugai et al., 2017, Li et al., 2017, Lin and Chuang, 2012, Kim et al., 2017, Duffy et al., 2011). While genes including SPRR2E, IGFBP3, RRP12 and KLF6 involved in maintaining cornified cell envelop, regulating growth factors and nuclear proteins were downregulated (Zucchini et al., 2001, Yen et al., 2015, Choi et al., 2016, Masilamani et al., 2017) **(Fig 4.17B ,Page no. 137) (Table 4.2, Page no. 136)**. Perturbation of these genes appears to indicate that cancer exosomes may trigger an oncogenic programme in recipient cells. Further studies are required to confirm the role of cancer exosomes on surrounding cells and tissues.

Similar to other techniques liquid biopsy based on exosomes also have some limitations such as challenging and time consuming isolation, fluctuating yield as the final pellet can be easily missed while collection, simple techniques such as western blotting and RT-qPCR can be challenging due to lower protein and RNA yield. Due to gaps in our knowledge it is still unknown as to how long it will take to detect cancer exosomes in body fluids during the course of disease progression. More research is required to understand the dose and time needed by exosomes to exert an effect in the recipient cells. Through our dose and time experiment we know that some genes

are affected within 24 hours while others show a more drastic change at 48 hours **(Fig 4.18, 4.19, Page no. 140, 141)**.

7.1(A) Recent technological advancements for exosome detection

The alluring idea of diagnosing cancer through a blood test has attracted much attention in recent research which has driven the technological advancements for easier detection of exosomes in body fluids. On-chip nano holographic imaging which is a handheld platform can detect nanoscale objects in a mobile format. It can be used in a practical clinical setting (McLeod et al., 2015). Similarly, nanopore ion occlusion based sensing measures single nanoparticles as they are driven through a nano-pore creating an ionic current flow. The change in the current is proportional to the size of the particle, enabling quantitative particle sizing with appropriate calibration (Maas et al., 2014).

Recently developed miniaturized nuclear magnetic resonance has been able to sensitively measure exosomes (Issadore et al., 2011). Surface Plasmon resonance (SPR) based nanosensors have gained much attention because of their ability to sensitively detect the binding of exceeding small numbers of molecules as few as 1 molecule (Kravets et al., 2013). Weissleder/Lee group had developed an SPR based nano-plasmonic exosome (nPLEX) detector. It consists of a series of nanohole array coated with affinity ligands to capture a specific type of exosome. It can measure up to 10^5 independent nanopore arrays. The utility of nPLEX was demonstrated on samples from ovarian cancer patients which could be readily differentiated from normal controls. Each measurement required a small volume of 0.3μl of sample (Im et al., 2014).

The use of micro and nano technology designed to overcome the technical challenges of exosome isolation and detection are ongoing. These techniques will allow rapid sample preparation and molecular analysis from small sample volumes in order to fully utilize the clinical potential of these circulating biomarkers. Such newly developed technological methods are yet to be tested on large cohort of OSCC patients.

7.2 Significance of genetic biomarkers for OSCC diagnosis

Today we know that genetic changes precede the occurrence of OSCC lesions. Hence the importance to testing genetic bio-markers for early detection of the disease can not be underestimated. This would significantly improve the 5 year survival rate of OSCC patients. In addition lower the cost of treatment for healthcare systems.

Such efforts have greatly benefitted patients suffering from cancers of other origins. In breast cancer patients, screening for BRCA1/2 gene mutation has led to early detection and optimized treatment leading to improved patient survival (Grindedal et al., 2017). Similarly in prostate cancer elevated levels of serum prostate-specific antigen is used for segregating high risk individuals from low risk (Helfand et al., 2017).

Ongoing efforts are being made to find an appropriate set of genetic biomarkers that would aid in early OSCC detection. Salivary IL-8, IL-6 and TNF-α have been proposed as potential diagnostic bio-marker (Sahibzada et al., 2017). Although testing their reliability on large cohort of patient samples is still pending. Through gene profiling array studies on OSCC samples aberrant expression of genes essential for normal cellular functions has been reported. These genes include TGF-β (Quan et al., 2013) , TP53 and EGFR (Bundela et al., 2014).

In this study we validated a previously developed OSCC diagnostic system called qMIDS (quantitative malignancy index diagnostic system) on retrospectively collected FFPE samples from OSCC patients. qMIDS is based on the expression of oncogene FOXM1 isoform B and its downstream targets (Teh et al., 2013). FOXM1B is a transcription factor essential for cell cycle (Myatt and Lam, 2007). Increased expression of FOXM1B has been noted in cancers of many origins including oral, oesophageal, breast, lungs, kidney, bladder, prostate, colon, liver, pancreas, testis, ovary and uterus. This indicates that upregulation of FOXM1 may be an early molecular signal required for aberrant cell cycle and cancer initiation (Teh et al., 2013).

During the development of qMIDS, its efficiency was tested on a small set of FFPE samples. Fortunately for this study we were able to get archival FFPE samples collected from the same patient at different stages of the disease including dysplasia, tumour and lymph node metastasis. The samples were provided by our collaborators

from Norway. These samples gave us the opportunity to correlate qMIDS index to disease stages.

Moderate to good yield of RNA could be isolated from FFPE samples for RT-qPCR. It was found that qMIDS index successfully correlated with disease stage with higher index values indicating advanced disease stage and vice versa.

Validating qMIDS on FFPE samples has a greater impact on high risk communities of the impoverished world where limitations of infrastructure and storage facilities for fresh biopsy samples might not allow testing of genetic diagnostic adjuncts such as qMIDS.

7.3 Non- bias OSCC bio-markers

Multiple co-factors such as smoking, alcohol, betel quid chewing, HPV and genetic predisposition are involved in the development of OSCC. This gives rise to tumours that are molecularly different from each other depending on the exposure to the type of carcinogen (Yeh et al., 2016). Due to this heterogeneity among tumour types some patients responded better to treatment while others developed resistance resulting in poor prognosis, recurrence and a less than 5 year survival rate.

Classifying tumours based on their genetic makeup will aid in providing targeted therapy. Hence it is essential to study large cohort of OSCC patient samples and determine genetic biomarkers that can differentiate one group of OSCC tumour sub-type from another.

In order to identify new OSCC biomarkers, we performed a non-bias meta-analysis of 8 OSCC microarray studies, where gene expression profiles were compared between OSCC and normal mucosa samples. Six bio-markers were selected for further validation on 100 OSCC biopsy tissues. These genes included PLAU, FN1, CDCA5 which are upregulated in OSCC while CRNN, CLEC3B and DUOX1 are found to be downregulated. We called this set of bio-markers q6. These genes are found to be involved in breakdown of extracellular matrix, cell adhesion and division, epidermal differentiation and mucosal defence (Zhang et al., 2015, Yen et al., 2013, Tokuzen et al., 2016, Salahshourifar et al., 2015, Arellano-Garcia et al., 2010, Ito et al., 2016) **(Table 6.3 , Page no. 165).** When we analysed the expression of q6 across the 100 OSCC samples, a subgroup of OSCC samples were found to have inverse gene expression pattern (we called this group negative group) compared to the rest (positive group) **(Fig 6.1 A, Page no. 167).**

This led us to retrospectively correlate molecular findings with socio-clinical records of two extreme ends of the q6 expression spectrum. We compared twenty patients from each group and found that the group of patient showing positive q6 values were mostly men of older age group associated to heavy smoking and alcohol consumption while the group with negative q6 values were mostly younger female who chewed paan **(Table 6.4, Page no. 168)**. Although our patient sample size was small, recurrence of OSCC lesions was mostly noted in q6 negative group (Paan chewers). The q6 panel of genes may potentially be exploited as prognostic biomarkers for segregating high and low risk of tumour recurrence due to radioresistance in OSCC patients. These finding needs to be further verified on a larger cohort of OSCC patients. Ideally the molecular findings should be correlated prospectively to patient's socio-clinical records. Due to retrospective correlation in this study patient records were difficult to find due to death, relocation, and mismatch among patients.

7.4 Final Conclusion

In conclusion our work will assist in translating the use of genetic biomarkers for early detection of OSCC as an adjunct to histopathology resulting in development of personalized therapy for better prognosis and improved survival rate. We recommend the use of non-invasive screening methods such as exosome based liquid biopsy for segregating high risk individuals from low risk, enabling resources to be focused on high risk groups thereby lowering the burden on the health care system as funds can better be utilized to improve OSCC patient outcome by treating them earlier.

We propose the presence of a biological mechanism through which cancer cells under stress conditions such as signalling dysregulation a hallmark of cancer alter the composition, content and number of exosomes released. These cancer exosomes further aid in passing on the oncogenic message to the neighbouring cells and distant cells by traveling through body fluids. Although to date the mechanism is not fully understood we believe that cancer exosomes play important roles in providing a fertile ground for local invasion and distant metastasis (Melo et al., 2015).

Since exosomes reflect parental cells they offer a great advantage in diagnosing OSCC through non-invasive biopsy. They can also prove beneficial in cases where biopsies are very invasive and sometimes even life threatening such as brain and lungs cancers. In pancreatic cancer biopsies, the acquired diagnostic tissue is mostly so small that it fails to reflect the extend of the disease and the heterogeneity of the

tumour (Panagiotara et al., 2017). In such circumstances a non-invasive liquid biopsy based on exosomes might offer a solution.

In addition to diagnostics, exosomes can be utilized to monitor patient progress throughout treatment. Failure and resistance to treatment can be identified earlier and can provide an opportunity to develop tailored therapeutics. It has also been shown that exosomes guide the metastatic process. In a study on pancreatic cancer metastasis to liver was found to be directed by tumour exosomes (Melo et al., 2015).

Exosomes have also been proposed for use as therapeutic drug delivery vehicles. In comparison to other nano drug delivery vehicles they offer the advantage of being non-immunogenic as they can be isolated from the patient's body fluids (Ha et al., 2016). Clinical trials for melanoma and non-small cell lung cancer has shown that exosomes can be safely used to deliver immunotherapeutic regimens. They induced both innate and adaptive immune response, disease stabilisation and long term survival for patients (Delcayre and Le Pecq, 2006).

In the field of cancer diagnostics, prognostics and therapeutics; exosomes offer a possible solution to current limitations. In order to tap their full potential for clinical translation a uniform and efficient method of isolation and verification needs to be adopted by researchers. A strong collaboration of engineers, biologist, clinicians and policy makers can further pave the way for future implementation of exosome technology for patient benefit.

7.5 Future directions

This study is the stepping stone to establish the use of non-invasive exosome based biopsy for OSCC patients. Validation of biomarker surface proteins, CEP55 through nano FACS analysis will facilitate its use as a fishing protein to segregate OSCC exosome from a mixed population of exosomes. We aim to uncover more robust protein-biomarkers through mass-spectrometry that can be identified in patient saliva or serum.

In this study through microarray we reported unique genes altered by normal and cancer exosomes. Some of these genes such as BBOX1, FEZ1 and SRPX has not been studied with regards to OSCC development and little is known about their biological functions. We further need to understand the cellular functions of these genes and the pathways they are involved in, in order to understand the biology through which exosomes effect surrounding cells.

We have shown through RT-qPCR that mRNA of oncogenes can be transferred to recipient cells through exosomes. We further aim to perform gene sequencing of OSCC derived exosomes to determine the sequence of oncogenes packed within exosomes.

In the capacity of this study we have only been able to study the diagnostic potential and functional significance of exosomes. In future we aim to study microvesicles which also classify as extracellular exosome and are released by cells through budding of cell membrane. They are bigger in size compared to exosomes measuring 200 nm and hence will offer better workability.

Future study aims to validate the 6 novel genes mentioned above for cancer prognosis to compliment the diagnostic test qMIDS. The expression of q6-biomarkers can be further validated by protein level quantification via western blotting in normal and cancer cell lines, along with immunostaining that would add information on protein localization in association to its function.

Appendix

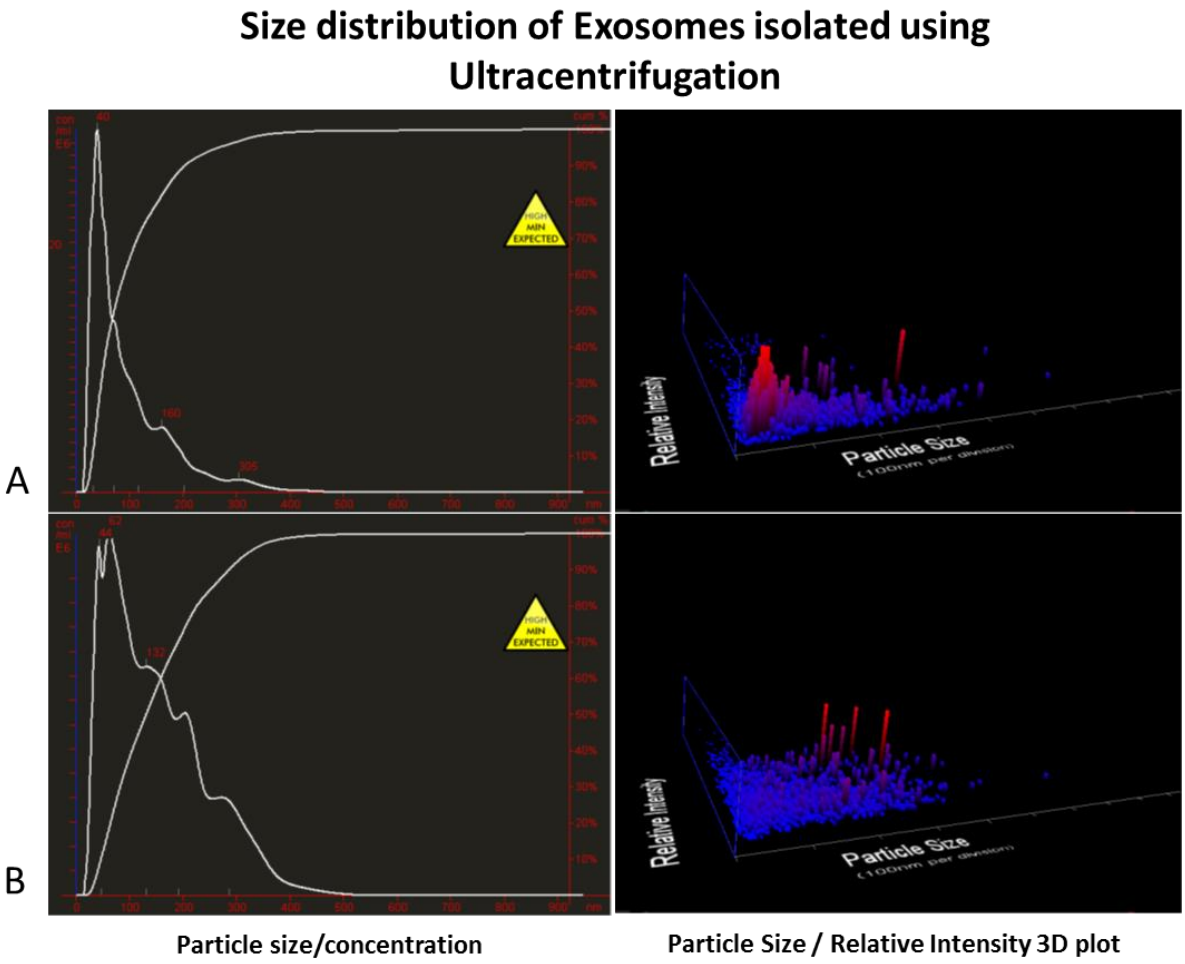


Fig 8.1: ***(A) SVpgC2A*** *exosomes with a Mean: 101nm, Mode: 40nm, size distribution (SD): 81nm. Total Concentration: 136.88 particles / frame, 21.65E8 particles / ml* ***(B) SVFN10*** *exosomes with Mean: 151nm, Mode: 62nm, SD: 92nm. Total Concentration: 162.46 particles / frame, 25.16E8 particles / ml. N=3. NanoSight LM20 was used.*

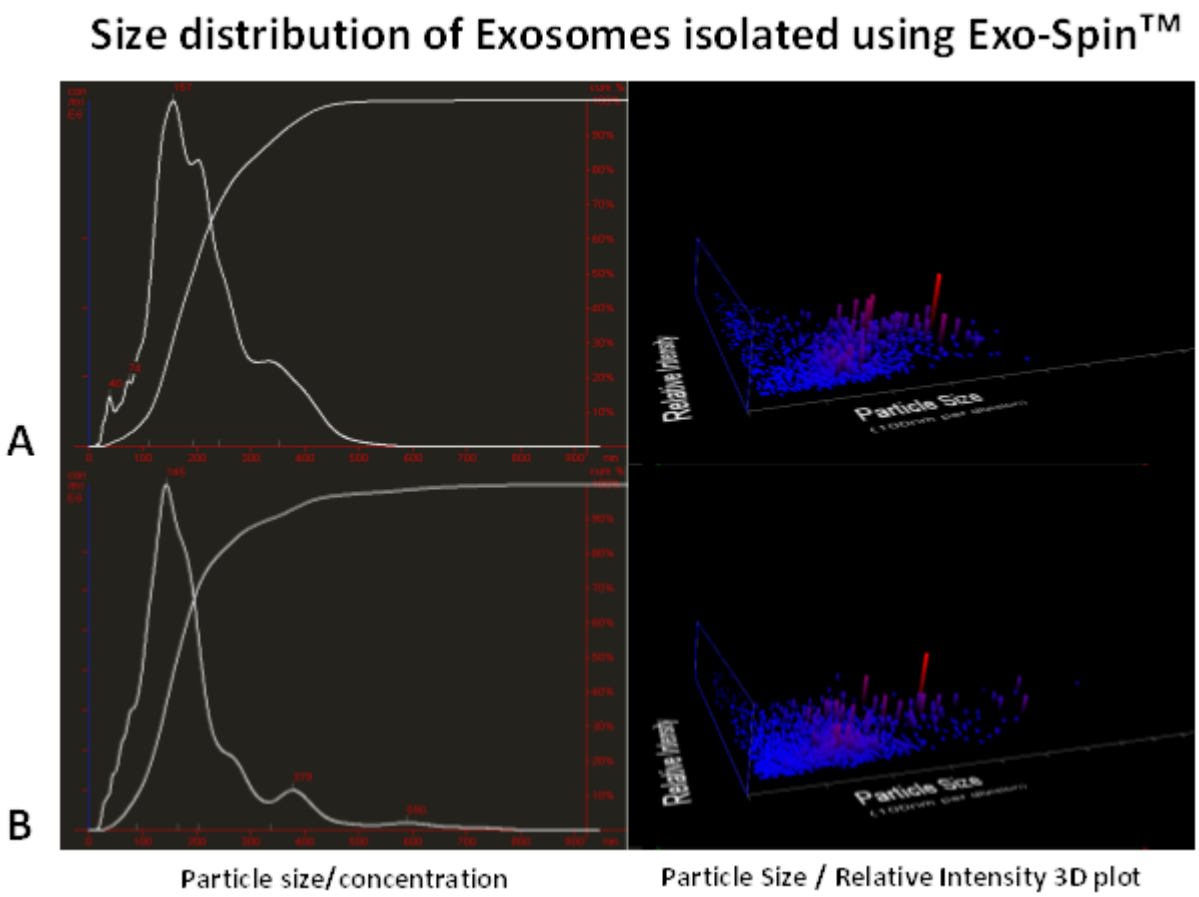


***Fig 8.2**: **(A)** Exosomes isolated from **SVpgC2A** with a Mean: 213nm, Mode: 157nm, Size Distribution (SD): 91nm. Total Concentration: 55.80 particles / frame, 8.58E8 particles / ml **(B) SVFN10** exosomes with Mean: 194nm, Mode: 145nm, SD: 113nm. Total Concentration: 81.76 particles / frame, 12.54E8 particles / ml. N=3. NanoSight LM20 was used.*

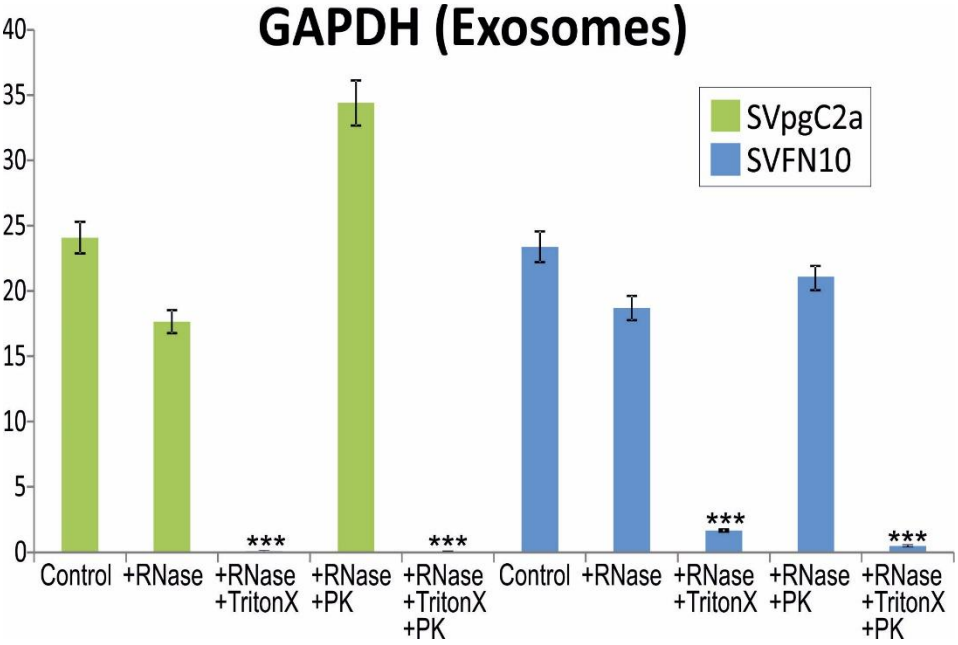
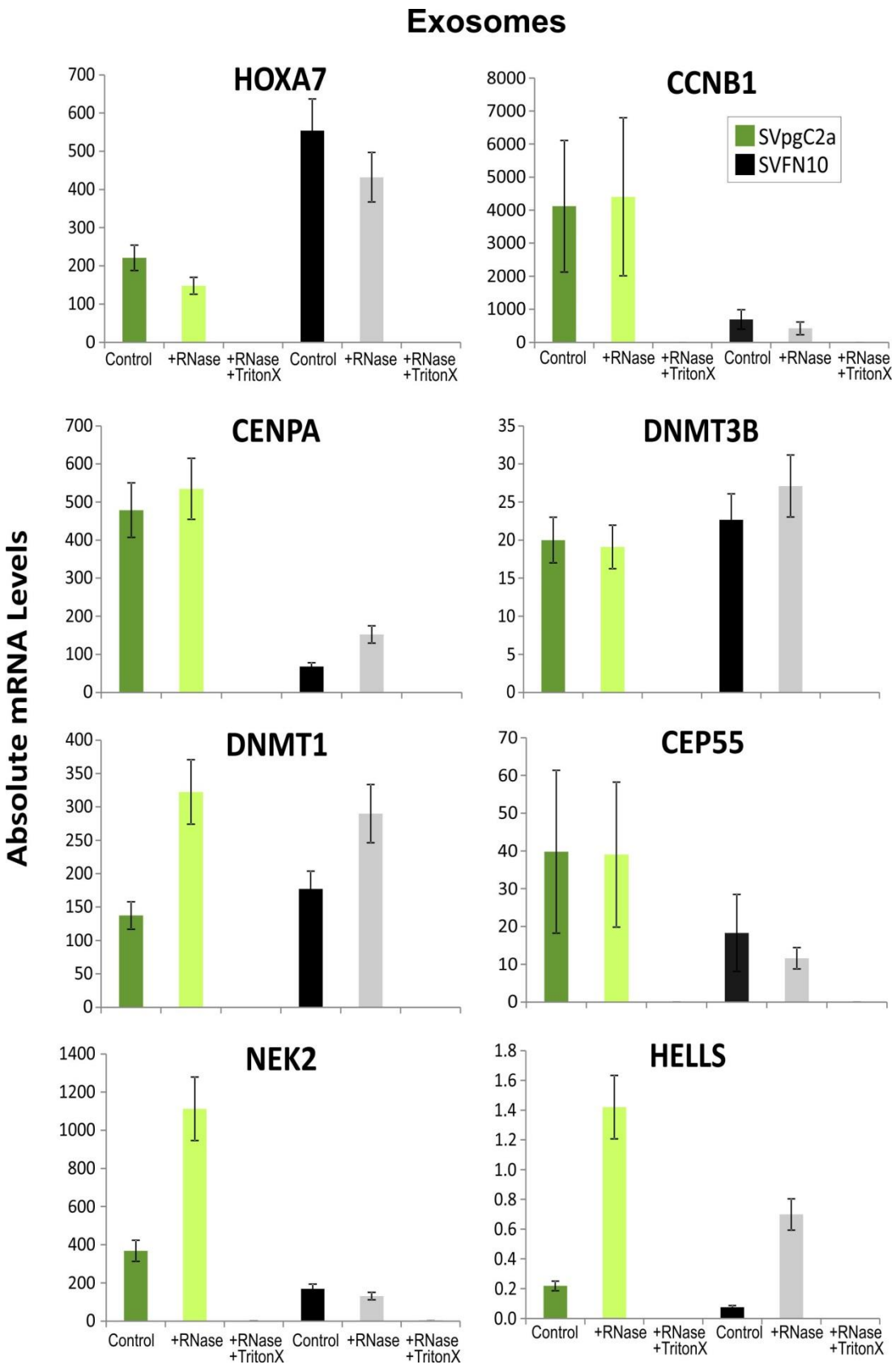


Fig 8.3: Validation of exosomal GAPDH mRNA levels*. Significantly low levels of RNA were found on treating the pellet with triton. The error bars represent the standard error of the mean of three independent experiments (n=3). Significant P values are denoted by (*P<0.05).****


Exosomes
Absolute mRNA Levels
HOXA7
CCNB1
SVpgC2a
SVFN10
CENPA
DNMT3B
DNMT1
CEP55
NEK2
HELLS
Control
+RNase
+RNase +TritonX

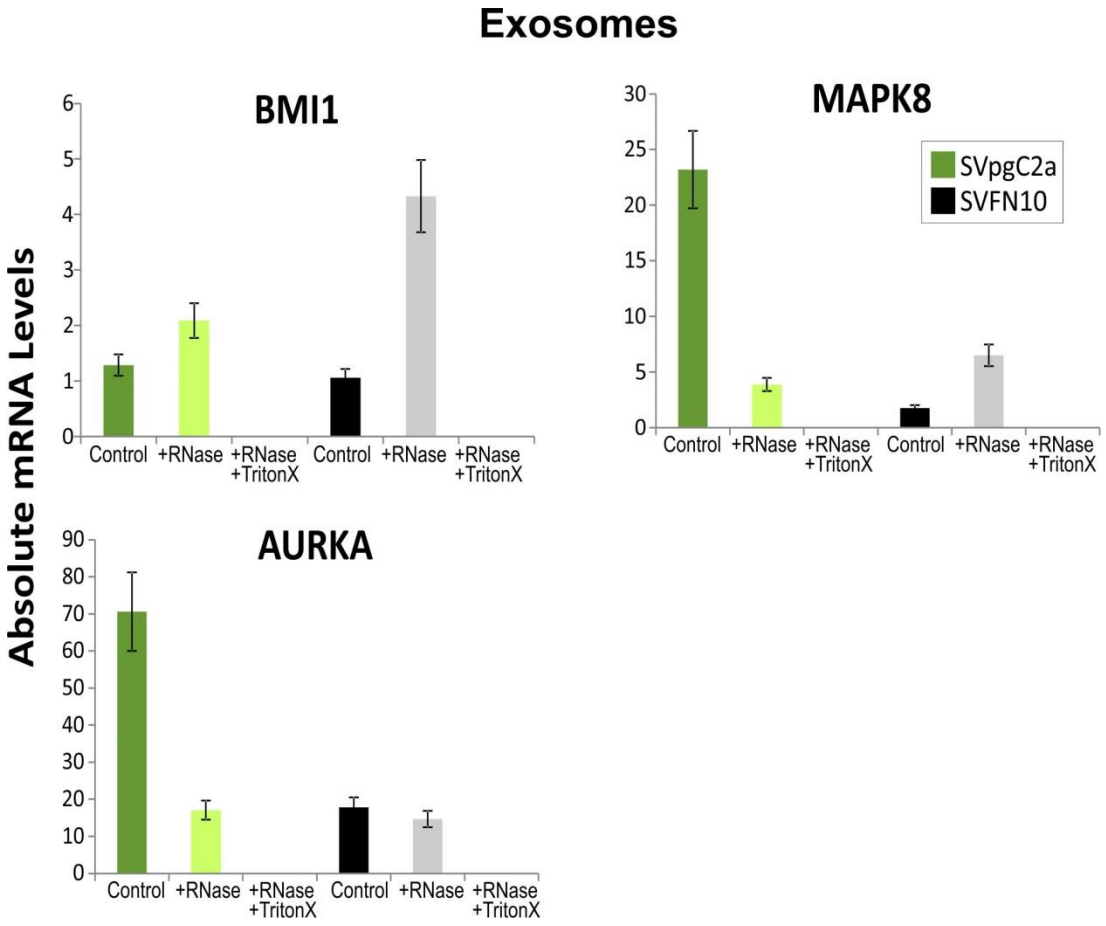


Fig 8.4: *Exosomal RNA quantification and mRNA detection of genes including HOXA7, CCNB1, CENPA, DNMT3B, DNMT1, CEP55, NEK2, HELLS, BMI1, MAPK8 AND AURKA by Rt-qPCR. Exosome pellets were treated with RNase A and Triton-X. Data was produced by MSc student Mohammad Arshad Aziz.*

This data showed selective packaging of genes within exosomes. mRNA levels of oncogene HOXA7 was three fold increased in SVFN10 derived exosomes compared to SVpgC2a exosomes.

However, oncogenes MAPK8 and AURKA mRNA were not reisitant to RNAse A treatment suggesting that some mRNA could be co-precipated with the exosome pellet and protected by protein complex outside the exosomes.

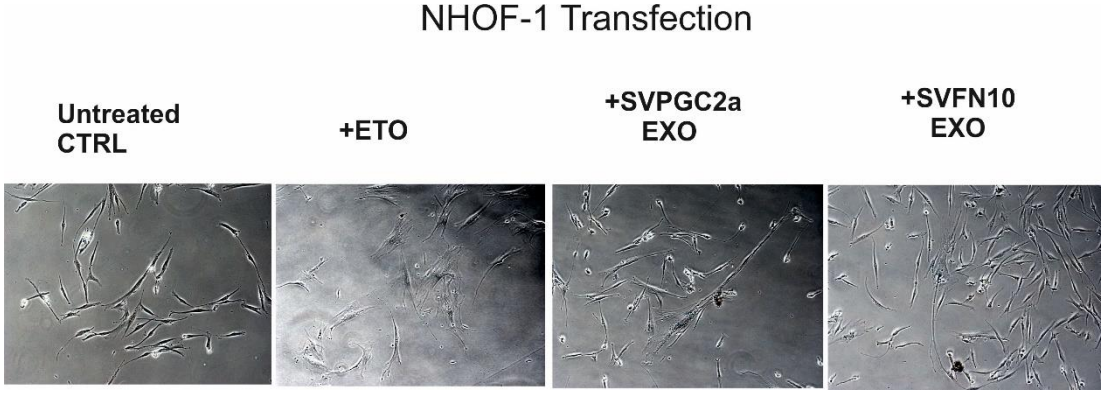


Fig 8.5: *Beta galactosidase assay on NHOF-1 fibroblast cells treated with Etoposide (ETO) at the concentration of $1x10^{-7}$, SVpgC2a and SVFN10 exosomes. Untreated SVpgC2a cells were used as a control.*

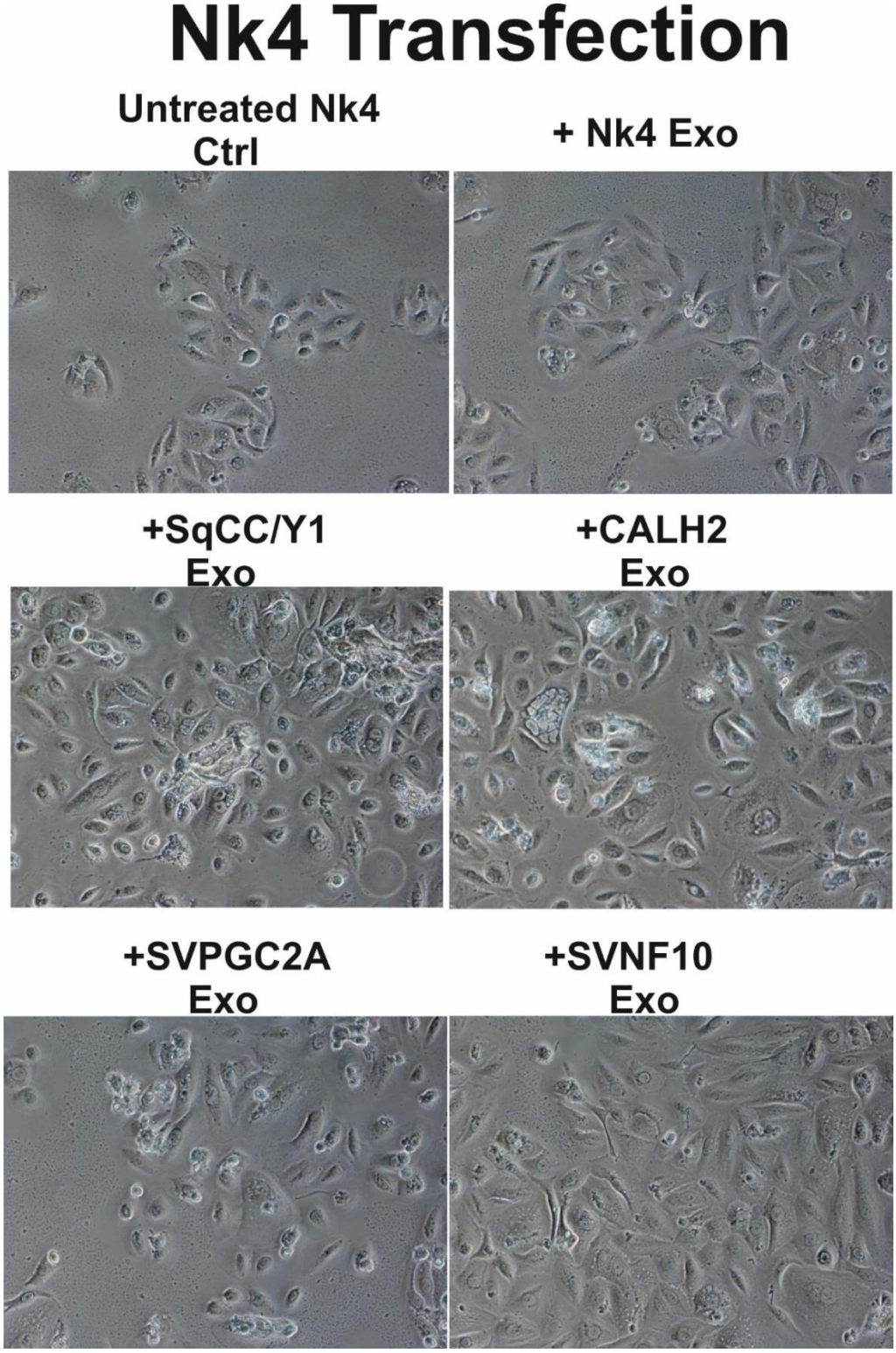


***Fig 8.6**: NK4 cells transfected with self exsomes (+NK4 EXO), exosomes isolated from tumour derived cell lines (+SqCC/Y1 EXO and +CaLH2 EXO), exosomes from premalignant cell line (+SVpgC2a EXO) and transformed malignant cell line (+SVFN10 EXO). Images at the magnification 10x taken after 48 hours of incubation.*

***Table 8.1**: Differential expression (q6 Values) between up and down regulated q6 genes.*

Patient	q6 values	Patient	q6 values
1	35.1	51	6.5
2	29.7	52	6.4
3	28.5	53	6.2
4	22.4	54	6.2
5	21.7	55	6.1
6	20.2	56	6.0
7	20.1	57	5.7
8	19.8	58	5.5
9	19.6	59	5.0
10	19.0	60	4.9
11	18.8	61	4.7
12	18.1	62	4.4
13	18.0	63	4.0
14	17.7	64	3.9
15	17.1	65	3.3
16	16.7	66	2.3
17	16.6	67	1.5
18	15.9	68	1.3
19	15.7	69	1.2
20	15.7	70	0.9
21	14.8	71	0.8
22	14.7	72	0.7
23	14.7	73	0.6
24	13.3	74	0.6
25	12.6	75	0.5
26	12.4	76	0.1
27	12.4	77	-0.2
28	12.1	78	-0.2
29	11.7	79	-0.8
30	11.4	80	-2.2
31	11.3	81	-2.4
32	11.3	82	-2.9
33	10.5	83	-4.2
34	9.7	84	-4.9
35	9.3	85	-6.1
36	9.3	86	-6.3
37	9.2	87	-6.4
38	9.1	88	-6.5
39	8.8	89	-6.7
40	8.7	90	-7.1
41	8.7	91	-8.1
42	8.7	92	-9.8
43	8.7	93	-10.5
44	8.4	94	-16.2
45	7.6	95	-16.7
46	7.4	96	-20.0
47	7.3	97	-24.7
48	7.0	98	-26.9
49	6.7	99	-37.3
50	6.6	100	-52.4

Presentations and prizes

This study has been presented at the following events

1. PhD day – Institute of Dentistry QMUL 2013
   Poster presentation “Early Oral Cancer Diagnostic System “
   1st prize winner

2. British Society Of Dental Research- BSODR 2015
   Oral presentation “ Functional significance and biomarker potential of oral cancer exosomes “

3. William Harvey day, Barts and The London School of Medicine and Dentistry – 2015
   Oral presentation “ Functional significance and biomarker potential of oral cancer exosomes “

4. PhD day – Institute of Dentistry QMUL 2016
   Oral presentation “ Developing liquid biopsy for OSCC patients “

5. PhD day – Institute of Dentistry QMUL 2017
   Poster presentation “ OSCC prognostic biomarkers “

6. British Association of Head & Neck Oncologists – BAHNO 2017
   Poster presentation “ Can genetic profiling aid clinical decision making in head and neck cancer “
   1st prize winner

Queen Mary University of London

Barts and The London School of Medicine and Dentistry

Early Oral Cancer Diagnostic System

Fatima Qadir, Dr Ahmad Waseem, Dr Muy-Teck Teh

Centre for Clinical and Diagnostic Oral Sciences, Institute of Dentistry

Introduction

- FOXM1 has been shown to be involved in cancer initiation and progression (Gemenetzidis et al., 2010).
- An early cancer diagnostic system (qMIDS) has been created based on 14 FOXM1 associated biomarkers.

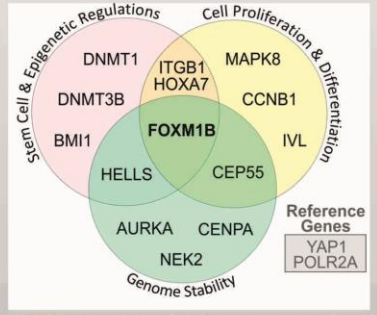


Fig 1: 14 FOXM1 biomarkers and 2 reference genes translates the gene signature into a malignancy index value from biopsy sample.

- These biomarkers are involved in the regulation of cell cycle, differentiation, ageing, genomic stability, epigenetic and stem cell renewal.
- The objective of this study is to validate this molecular system on formalin-fixed paraffin embedded (FFPE) tissue samples

Methods and Materials

Gene expression levels in biopsy specimens were quantified using absolute quantitative reverse transcription PCR (qPCR).

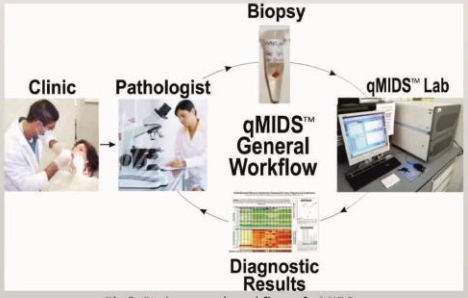


Fig 2: Basic general workflow of qMIDS.

- By means of an algorithm, the mRNA expression levels of the panel of 14 biomarkers and 2 reference genes were translated into a quantitative malignancy index diagnostic system (qMIDS).
- The qMIDS assay has been previously verified using fresh frozen biopsy specimens (Teh et al. 2013).
- In the current study, we investigate if FFPE tissue sections could be used for the qMIDS assay.
- A total of 20 FFPE samples (5 normal, 5 dysplastic, 5 oral squamous cell carcinoma samples and 5 lymph node metastatic tumours) were tested on qMIDS.

Results

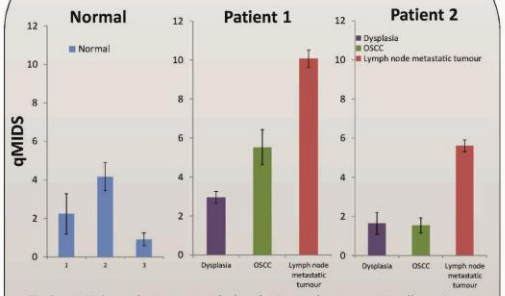


Fig 3: qMIDS results on normal, dysplasia, oral squamous cell carcinoma (OSCC) and lymph node metastatic tumours.

- A moderate to good yield of mRNA could be extracted from the FFPE tissue. The gene signature appear sufficiently preserved that enabled quantification by qMIDS assay.
- Notable data variations were found in the qMIDS data. This could be due to suboptimal RNA quality purified from FFPE tissues. Alternative explanation could be tissue heterogeneity which contributed to the data variation.

Conclusion:

Further optimisation is required on a larger number of FFPE samples to validate the use of qMIDS assay on FFPE samples.

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Teh, MT, et al., 2013, 'Exploiting FOXM1-orchestrated molecular network for early squamous cell carcinoma diagnosis and prognosis', *Int J Cancer*, vol. 132, no. 9, pp. 2095-106.

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Can genetic profiling aid clinical decision making in head and neck cancer?

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INTRODUCTION:

The concept of cancers having unique molecular signatures was first shown experimentally in 2000[1] but relating this to clinical presentation and disease behaviour is essential to delivering a patient benefit. For breast cancer, molecular profiling has identified multiple biologically distinct tumours which are almost indistinguishable by conventional histology. These biomarkers were subsequently found to also be accurate prognostic indicators of disease progression such that they can be used to determine the most effective treatment for individual breast cancer patients. Head and neck cancers also have identifiable molecular subtypes[2,3] but their clinical significance remains uncertain.

AIMS:

To identify genetic biomarkers that differentiate individual (sub)populations of head and neck cancer patients and to correlate these genetic differences with clinical presentation.

METHODS:

Eight independent oral cancer microarray studies published on Oncomine were meta-analysed. Top ranked, up- and down-regulated genes were identified and six differentially expressed genes were shortlisted, of which, PLAU, FN1 and CDCA5 were up regulated; whereas CRNN, CLEC3B and DUOX1 were down regulated. These are the 'q6' biomarkers which are reported to be involved in essential cellular functions including proliferation, migration, cell adhesion, immune system evasion and epithelial differentiation[4]
Fresh biopsy samples from 100 oral cancer patients were collected in pairs of margin and core tumour tissue. An algorithm was developed to quantify expression of q6 biomarker levels determined by RT-qPCR. Patient specific details were retrospectively collated from their clinical records. All tissues and patient details were collected and stored in accordance with local regulatory policy.

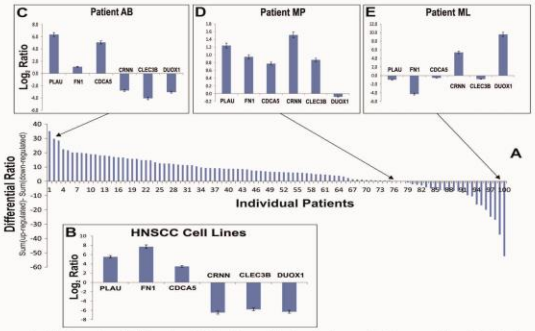

Fig 1: *(A) Expression of q6 biomarkers in 100 oral cancer biopsies showing two distinct groups with positive (C) and negative (E) q6 values. For comparison (B) shows expression of q6 biomarkers in head and neck cancer cell lines.*

			+ q6 values	- q6 values	
Patient Details	Age	Mean (years)	63.2	56.9	p=0.047*
		Range (years)	52-71	52-63	
	Gender	male:female	9:4	1:7	p=0.179*
	Ethnicity	British	9	5	p=0.051
		Bangladeshi	2	2	p=0.041*
		Others	2	1	p=0.472
	Risk Factor Habits (n)	Smoking	8	4	p=0.345
		Alcohol	6	0	p=0.045*
		Paan Usage	4	6	p=0.027*
Tumour Details (n)	Primary		13	5	p=0.840
	Recurrence at 2 years		0	3	p=0.168
	Differentiation Grade	Well	0	1	p=0.668
		Moderate/Poor	13	7	
	Site	Buccal	3	2	p=0.968
		Floor of Mouth/ Tongue	5	3	p=1.000
		Other	4	3	p=0.413
	Size	T1	5	0	p=0.735
		T2	3	2	
		T3	3	5	
		T4	2	1	

Mann Whitney U Test; *p<0.05 indicating a statistically significant difference

Table1: *Socio-demographic and clinico-pathological details of patients in the q6 positive and q6 negative sub-groups showing statistically significant differences representing two known high-risk groups for head and neck cancer cancer – q6 positive are older, male alcohol users whilst q6 negative are younger, female, Bangladeshi paan users.*

RESULTS:

From 100 patient samples, two subgroups were identified with opposing expression of q6 biomarkers, positive and negative (Figure 1). Correlation with clinical information revealed that the subgroup with the most positive q6 values were predominantly older males who consumed excessive alcohol whilst more negative q6 values were representative of younger, Bangladeshi, female paan users (Figure 2). Therefore the positive and negative q6 subgroups represent two recognised high-risk populations for head and neck cancer in the UK. Interestingly, all patients who developed a recurrence of their primary tumour were in the negative subgroup.

CONCLUSION:

This data represents the first reported correlation of distinct molecular signatures in head and neck cancer with the clinical presentation of the disease. Larger scale longitudinal studies are now warranted to establish the linkage between these different genotypes and disease progression or treatment response. This is the first step towards the ultimate goal of personalised molecular-signature-guided treatment for head and neck cancer patients.

Acknowledgements:
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