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AN EVALUATION OF CANCER BIOMARKERS IN NORMAL OVARIAN EPITHELIAL CELLS AND OVARIAN CANCER CELL LINES

by

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DECLARATION

I declare that "**An Evaluation of Cancer Biomarkers in Normal Ovarian Epithelial Cells and Ovarian Cancer Cell Lines**," is my own work, that it has not been submitted for any other degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

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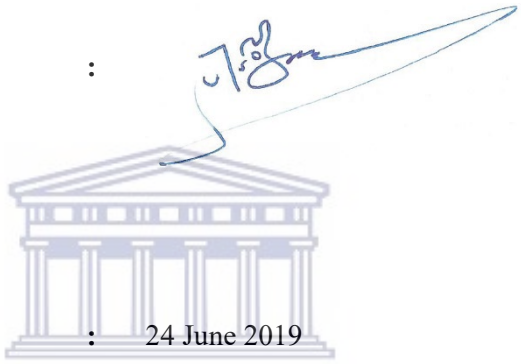

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DEDICATION

This dissertation is dedicated to the memory of my beloved father.

To my beloved mom for her endless prayers and love.

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LITERARY QUOTATION

In the name of Allah most gracious most merciful.

{Allah will raise those who have believed among you and those who were given knowledge, by degrees. And Allah is Acquainted with what you do (11)}

Quran surah Al-Mujadilah 4 (QS 58: 4)

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ABSTRACT

Introduction: Globally, there are over 190,000 new reported cases of ovarian cancers per annum. This comprises 3% to 4% of all cancers in women. Ovarian cancer is one of the leading causes of deaths in women. Ovarian cancer is the second most diagnosed gynaecological malignancy and over all the fifth cause leading to death among all types of cancer in the UK in 2004. More than 70% of epithelial ovarian cancers are diagnosed at an advanced stage. Consequently, the prognosis is poor and the mortality rate high. Thus, the survival rate is affected by how far the disease has progressed or spread. A dire need exists to identify ovarian cancer biomarkers, which could be used as good indicators of expression in ovarian cancer cells in vitro

Aim: The aim of this study was to analyse selected cancer biomarkers, which are currently under intense investigation for their suitability to diagnose epithelial ovarian cancer at an early stage. These biomarkers were analysed in terms of their *in vitro* expression in normal epithelial cells and ovarian cancer cell lines, which allows for their genomic and proteomic classification. The expression analysis of each biomarker is related to the malignancy of a tumour and, therefore, advocates its use for potential future improvement of sensitive tumour markers.

Methods: The primary human ovarian surface epithelial cell line (HOSEpiC), SKOV-3 cells and the OAW42 human epithelial ovarian tumour cell lines were used to evaluate the selected cancer biomarkers. Cells were cultured using appropriate media and supplements, and real-time quantitative polymerase chain reaction (RT-PCR) utilized to validate expression levels of the following genes: HDAC1, HDAC2, HDCA3, HDAC5, HDAC6, HDAC7, HDAC8, LPAR1, LPAR2, MUC16 and FOSL1, against normal housekeeping genes GAPDH and HPRT. In addition, immunocytochemistry was also used in the validation process of the aforementioned genes.

Significance: ovarian cancer cells express gene signatures, which pose significant challenges for cancer drug development, therapeutics, prevention and management. The present study is an effort to explore

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ovarian cancer biomarkers to provide a better diagnostic method that may offer translational therapeutic possibilities to increase five- year survival rate.

Results: HDAC5, HDAC6, LPAR1, LPAR2 and MUC16 expressed distinctively in ovarian cancers matched to other tissues or cancer types have already been identified by RT-QPCR and confirmed by immunocytochemistry and efforts to generate monoclonal antibodies to the other six genes (HDAC1, HDAC2, HDAC3, HDAC7, HDAC8 and FOSL1) encoded proteins are underway.

Conclusions: here we provide strong evidence suggesting that HDAC5, HDAC6, LPAR1, LPAR2, except MUC16 are up regulated in ovarian cancer. These data were confirmed by examining Human Protein Atlas (HPA) databases, in addition to protein expression of HDAC5, HDAC6, LPAR1, LPAR2 and MUC16 in cells cytoplasm. For future prospective, using other techniques that assess the variant expression that could explain the release of these gene candidates into the circulation with serum tumour markers, and protein expression will be strengthened

Keywords: ovarian cancer, epithelial ovarian cancers, primary human ovarian surface epithelial cell line (HOSEpiC), ascites ovarian adenocarcinoma SKOV-3 cells, diagnostic and prognostic ovarian cancer biomarkers, gene signatures, genomic and proteomic classification, expression analysis, RT-PCR, qPCR, translational therapeutic relevance of candidate genes (HDAC1, HDAC2, HDCA3, HDAC5, HDAC6, HDAC7, HDAC8, LPAR1, LPAR2, MUC16 and FOSL1)

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LIST OF ABBREVIATIONS

3-D	Three-Dimensional/Multi-Dimensional
ACS	American Cancer Society
AML	Acute Myeloid Leukemia
An-LPA	Alkenyl-Lysophosphatidic Acid
APL	Acute Promyelocytic Leukemia
ATX	Autotaxin, Ecto-Nucleotide Pyrophosphatase/Phosphodiesterase (ENPP)2
B	Brain
BRCA1	Breast Cancer1
BRCA2	Breast Cancer2
CA-125	Cancer Antigen 125
CCC	Clear Cell Carcinoma
CDK9	Cyclin-Dependent Kinase 9
cDNA	Complementary DNA
CLL	Chronic Lymphocytic Leukemia
CML	Chronic Myeloid Leukemia
CO_2	Carbon Dioxide
CS	Cytoreductive Surgery
CT	Computed Tomography
Ct	Cycles to Threshold
CTD	C-Terminal Domain
DE	Differential Expression
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
DPBS	Dulbecco's Phosphate-Buffered Saline

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E	Efficiency
EB/EtBr	Ethidium Bromide
EC	European Commission
EC	Endothelial Cell
Edg	Endothelial Differentiation Gene
EDTA	Ethylenediaminetetracetic Acid
EL	Embryonic Lethal
EOC	Epithelial Ovarian Cancer
EOT	Epithelial Ovarian Tumour
FC	Fold Change
FBS	Fetal Bovine Serum
for	Forward
FOSL1	FOS-Like 1, Ap-1 Transcription Factor Subunit
G3PDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GAPDH	Glyceraldehyde Phosphate Dehydrogenase
GPCRs	G-Protein-Coupled Receptors
H	Heart
HATs	Histone Acetyltransferases
HDACi(s)	Histone Deacetylase Inhibitor(s)
HDACs	Histone deacetylases
HGSC	High-Grade Serous Carcinoma
HGSOC	High-Grade Serous Ovarian Carcinoma
HIF-1a	Hypoxia-Inducible Factor-1a
HKG	Housekeeping Gene
HL	Hodgkin's Lymphoma
HOSEPiC	Human Ovarian Surface Epithelial Cell
HPA	Human Protein Atlas
HPRT	Human Hypoxanthine Phosphoribosyltransferase

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HRT	Hormone Replacement Therapy
Hsp90	Heat Shock Protein 90
ICC	Immunocytochemistry
K	Kidney
kb	Kilobases
L	Liver
LGSC	Low-Grade Serous Carcinomas
LPA	Lysophosphatidic Acid
LPAR1	Lysophosphatidic Acid Receptor1
LPAR2	Lysophosphatidic Acid Receptor2
LPC	Lysophosphatidylcholine
LysoPLD	Lysophospholipase D
MC	Mucinous Carcinomas
MDa	atomic mass unit or Dalton
MMP	Matrix Metalloproteinase
MMP10	Matrix Metalloproteinase 10
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic Acid
MUC16	CA125 (Cancer Antigen 125) also known as Mucin 16 or MUC16
NCBI	National Centre for Biotechnology Information
Nd	No Data
ND	NanoDrop
NTC	No Template Control
OC	Ovarian Cancer/Carcinoma
OCCP	Ovarian Cancer Cell Line Panel
OEpiCM	Ovarian Epithelial Cell Medium
OLMPT	Ovarian Tumour of Low Malignant Potential
OSE	Ovarian Surface Epithelium

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PA	Pancreas
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PET	Positron Emission Tomography-Computed
PFD	Paraformaldehyde
PL	Placenta
PLD	Phospholipase D
PLL	Poly-L-Lysine
QC	Quality Control
qPCR	Quantification Polymerase Chain Reaction
RARa	Retinoic Acid Receptor Alpha
rev	Reverse
RNA-Seq	RNA sequencing
RPM	Rounds per Minute
RT-qPCR	Real-Time Quantification Polymerase Chain Reaction
S	Spleen
siRNA	Small Interfering RNA
SM	Skeletal Muscle
Stat3	Signal Transducer and Activator of Transcription 3
T/E	Trypsin/EDTA Solution
TBE	Tris-Borate-EDTA
TCGA	The Cancer Genome Atlas
TMAs	Tissue Microarrays
TNS	Trypsin Neutralization Solution
TVS	Transvaginal Sonography

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CHAPTER 1

Introduction and Literature Review

SECTION A: OVARIAN CANCER

A1.1 Introduction

Globally, ovarian cancer (OC) is considered to be the sixth most common cancer in women [1, 2]. It accounts for 2.5% of all female cancer cases [3], and is the seventh most common source of cancer mortality[1, 2]. There are about 204,000 new cases and 125,000 deaths annually [1, 2], however, in 2018, some recent studies predict that about 22,240 new cases of ovarian cancer will be diagnosed [3]. Ovarian cancer is one of the most common malignancies in Southern Africa which accounted for 3.7% [4, 5]. Concerning, the National Cancer Registry in 2001, stated 529 ovarian cancer cases, which was classified as follow: 225 in black patients, 207 were in white patients, 69 in colour patients and 16 were accounted in the Asian community [5]. In 2012, 22.280 new cases of ovarian cancer patients recorded. There were 192.750 cases on 1 January 2012 suffering from ovarian neoplasm, with the average age of ovarian cancer diagnosis being 63 years old.

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Moreover, in 2013, 14,030 deaths due to ovarian cancer were estimated by the American Cancer Society [6, 7]. In 2018, ovarian cancer deaths will amount to 14,070 regarding ovarian cancer (OC) disease's low survival, it constitutes 5% of cancer deaths [3]. Several types of ovarian carcinoma can rise from the surface epithelium, fallopian tubes, the gonadal or sex-cord stroma, germ cells, or from metastasis resulting in a different type of tumours [8]. As a result of ovarian cancer is asymptomatic until the advanced stage; 4 out of 5 ovarian carcinomas are diagnosed in advanced stage related to abdominal spreading [3], due to, it is called a "silent killer". The opportunity of cure is pitiable, and ovarian cancer has the highest fatality-to-case ratio of all gynecological malignancies [9-11]. Around 8% of asymptomatic women aged between 25 to 40 years, were found to suffer from ovarian cancer [12].

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Identification of early symptoms may have important clinical implications according to 5-year survival for an early-stage disease is 70% to 90% compared with 20% to 30% for advanced-stage disease. The necessity to recognize ovarian cancer, in the beginning, is an important clinical concern [13]. Otherwise, ovarian cancer has indicative symptoms that interfere with another illness such as pelvic or abdominal pain bloating, eating difficulties, vaginal bleeding, feeling full quickly, and urinary symptoms (urgency or frequency). Physicians must be conscious if these symptoms are new and frequently less than 12 days per month and these signs could have related to ovarian matters. Indeed, some screening tests insufficient to detect these symptoms due to sensitivity or specify, particularly at early-stage tumours [14, 15].

A 1.2 Types of Ovarian Cancer

Ovarian cancer can be recognized by the origin of cancer cells through the heterogeneity of this disease. Ovarian carcinoma includes numerous various types originate from the ovarian surface epithelium (OSE) which has flat, featureless cells more which is closely approaching the mesothelium lining the peritoneal cavity [8]. Surface epithelial tumours are the most common in ovarian malignancies [16], accounting for 90% of all ovarian carcinoma and 2/3 of all ovarian neoplasms [17, 18]. Due to a varied collection of histological patterns of these tumour categories, many interesting studies are in progress. To have a background about the type of ovarian tumours and differentiation, it helps in controlling and management of patients in terms of appropriate treatment and follow-up [17, 19].

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Ovarian cancer has types that are not associated to epithelial characters; these types have a lower rate among the other common types of ovarian cancer that come within the ovary including the sex cord-stromal tumours (6%) of OC. These tumours develop from the ovary within supporting tissues, like, germ cell tumours, they are uncommon and initiate from different ovarian cells. Stromal ovarian cancers include granulosa-stromal tumours and Sertoli-Leydig cell tumours. Next, are germ cell tumours (3%) which grow from the reproductive cells of the ovary and are unusual and seen most commonly in teens or young women. Many altered categories include this kind of tumours such as dysgerminomas, polyembryomas, yolk sac tumours, embryonal carcinomas, non-gestational choriocarcinomas,

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immature teratomas and mixed germ cell tumours and indeterminate tumours (1%) [20]. In contrast, I-M Shih and RJ Kurman [21] reported that ovarian cancer is a collection of a heterogeneous group of tumours that are gathered from the surface epithelium of the ovary or from surface inclusions [21]. Although ovarian tumour of low malignant potential (OLMPT) is formerly included in borderline tumours which have some microscopic features of cancer; OLMPT is not able to metastasize like typical cancers and account for about 15% of EOC. Also, they are the most serious or mucinous cell types and can be treated in the same way, depending on the type of cell that causes cancer [22]. Therefore, to classify all ovarian epithelial malignancies and borderline tumours, it must follow the WHO classification as shown in Table 1.1 [23].

A2. What is Epithelial Ovarian Cancer?

Epithelial Ovarian Cancer (EOC) is the fifth most common cause of cancer mortality globally and the leading cause of death from gynaecologic cancer in the United States [24-27]. Thus, EOC accounted for 90% which comprises the majority of malignant ovarian neoplasms [23]. EOC still brings a poor prognosis although progressive diagnostic and therapeutic advances in the world are recorded by the International Cancer Study Agency—from about 200,000 new cases of EOC, more than 120,000 cases die from the disease.

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According to an age-adjusted incidence of EOC, approximately 12.8, 8.8, 7.7 and 7.6 per 100,000 women per year in the UK, the United States, France and Japan, respectively [28-32] reported that EOC is the seventh most common cancer diagnosis among women universally and the fifth most common cancer diagnosed among women in higher resource areas and calculating for 4% of the deaths from cancers in women[28, 29]. The global ratio is predicted to be 6.3 per 100,000 women and is highest in high-resource countries with about 9.3 per 100,000 women [30]. The terms epithelial ovarian tumour (EOT) and malignant are additional names for epithelial ovarian cancer (EOC) or ovarian carcinoma (OC). EOC cell lines are widely regarded as a suitable tool for the biological and molecular classification cancer of the ovary, using an in vitro multi-dimensional (3-D) culture model to recapitulate some of the growth conditions met by tumour cells in vivo [32].

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Table 1.1: Types of ovarian cancers

Epithelial tumours	
Serous tumours	
Benign	
Serous cystadenoma	8441/0
Serous adenofibroma	9014/0
Serous surface papilloma	8461/0
Borderline	
Serous borderline tumour / Atypical proliferative serous tumour	8442/1
Serous borderline tumour - micropapillary variant / Non-invasive low-grade serous carcinoma	8460/2*
Malignant	
Low-grade serous carcinoma	8460/3
High-grade serous carcinoma	8461/3
Mucinous tumours	
Benign	
Mucinous cystadenoma	8470/0
Mucinous adenofibroma	9015/0
Borderline	
Mucinous borderline tumour / Atypical proliferative mucinous tumour	8472/1
Malignant	
Mucinous carcinoma	8480/3
Endometrioid tumours	
Benign	
Endometriotic cyst	
Endometrioid cystadenoma	8380/0
Endometrioid adenofibroma	8381/0
Borderline	
Endometrioid borderline tumour / Atypical proliferative endometrioid tumour	8380/1
Malignant	
Endometrioid carcinoma	8380/3
Clear cell tumours	
Benign	
Clear cell cystadenoma	8443/0
Clear cell adenofibroma	8313/0
Borderline	
Clear cell borderline tumour / Atypical proliferative clear cell tumour	8313/1
Malignant	
Clear cell carcinoma	8310/3
Brenner tumours	
Benign	
Brenner tumour	9000/0
Borderline	
Borderline Brenner tumour / Atypical proliferative Brenner tumour	9000/1
Malignant	
Malignant Brenner tumour	9000/3
Seromucinous tumours	
Benign	
Seromucinous cystadenoma	8474/0*
Seromucinous adenofibroma	9014/0*
Borderline	
Seromucinous borderline tumour / Atypical proliferative seromucinous tumour	8474/1*
Malignant	
Seromucinous carcinoma	8474/3*
Undifferentiated carcinoma	8020/3
Mesenchymal tumours	
Low-grade endometrioid stromal sarcoma	8931/3
High-grade endometrioid stromal sarcoma	8930/3
Mixed epithelial and mesenchymal tumours	
Adenosarcoma	8933/3
Carcinosarcoma	8980/3
Sex cord-stromal tumours	
Pure stromal tumours	
Fibroma	8810/0
Cellular fibroma	8810/1
Thecoma	8600/0
Luteinized thecoma associated with sclerosing peritonitis	8601/0
Fibrosarcoma	8810/3
Sclerosing stromal tumour	8602/0
Signet-ring stromal tumour	8590/0
Microcystic stromal tumour	8590/0
Leydig cell tumour	8650/0
Steroid cell tumour	8670/0
Steroid cell tumour, malignant	8670/3
Pure sex cord tumours	
Adult granulosa cell tumour	8620/3
Juvenile granulosa cell tumour	8622/1
Sertoli cell tumour	8640/1
Sex cord tumour with annular tubules	8623/1
Mixed sex cord-stromal tumours	
Sertoli-Leydig cell tumours	
Well differentiated	8631/0
Moderately differentiated	8631/1
With heterologous elements	8634/1
Poorly differentiated	8631/3
With heterologous elements	8634/3
Retiform	8633/1
With heterologous elements	8634/1
Sex cord-stromal tumours, NOS	8590/1

Source: [23]

Continued/...

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Table 1.1: Types of ovarian cancers (continued)

Germ cell tumours		Wolffian tumour	9110/1
Dysgerminoma	9060/3	Small cell carcinoma, hypercalcaemic type	8044/3*
Yolk sac tumour	9071/3	Small cell carcinoma, pulmonary type	8041/3
Embryonal carcinoma	9070/3	Wilms tumour	8960/3
Non-gestational choriocarcinoma	9100/3	Paraganglioma	8693/1
Mature teratoma	9080/0	Solid pseudopapillary neoplasm	8452/1
Immature teratoma	9080/3		
Mixed germ cell tumour	9085/3	**Mesothelial tumours**	
		Adenomatoid tumour	9054/0
Monodermal teratoma and somatic-type tumours arising from a dermoid cyst		Mesothelioma	9050/3
Struma ovarii, benign	9090/0	**Soft tissue tumours**	
Struma ovarii, malignant	9090/3	Myxoma	8840/0
Carcinoid	8240/3	Others	
Strumal carcinoid	9091/1		
Mucinous carcinoid	8243/3	**Tumour-like lesions**	
Neuroectodermal-type tumours		Follicle cyst	
Sebaceous tumours		Corpus luteum cyst	
Sebaceous adenoma	8410/0	Large solitary luteinized follicle cyst	
Sebaceous carcinoma	8410/3	Hyperreactio luteinalis	
Other rare monodermal teratomas		Pregnancy luteoma	
Carcinomas		Stromal hyperplasia	
Squamous cell carcinoma	8070/3	Stromal hyperthecosis	
Others		Fibromatosis	
		Massive oedema	
Germ cell - sex cord-stromal tumours		Leydig cell hyperplasia	
Gonadoblastoma, including gonadoblastoma with malignant germ cell tumour	9073/1	Others	
Mixed germ cell-sex cord-stromal tumour, unclassified	8594/1*	**Lymphoid and myeloid tumours**	
		Lymphomas	
Miscellaneous tumours		Plasmacytoma	9734/3
Tumours of rete ovarii		Myeloid neoplasms	
Adenoma of rete ovarii	9110/0		
Adenocarcinoma of rete ovarii	9110/3	**Secondary tumours**	

[a] The morphology codes are from the International Classification of Diseases for Oncology (ICD-O) {575A}. Behaviour is coded /0 for benign tumours, /1 for unspecified, borderline or uncertain behaviour, /2 for carcinoma in situ and grade III intraepithelial neoplasia and /3 for malignant tumours; [b] The classification is modified from the previous WHO classification of tumours {1906A}, taking into account changes in our understanding of these lesions; *These new codes were approved by the IARC/WHO Committee for ICD-O in 2013.

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Source: [23]

Bell and Scully [33] detailed that 80% to 90% of malignant ovarian tumours is raised from the single layer of epithelial cells that are covered in the ovaries [26, 33]. Mok and his colleagues mentioned that the natural history of the EOC development remains obscure and no effective screening test exists [26]. Also, the progression from benign to an invasive tumour is quite obvious, however, for epithelial ovarian cancer, these steps still have not been approved [34]. Epithelial ovarian cancer is an effect of another disease that affects premenopausal and postmenopausal women with 80%-90% of ovarian cancers. It rises after the age of 40 and the incidence peak of aggressive EOC occurs round at age 60. Less than 1% of EOCs transpire before the age of 20, whereas 2/3 of ovarian malignancies in these young patients are germ-cell tumours [19, 31, 35]. Figure 1.1 is a simplified diagram of the origin and classification of ovarian cancer [31].

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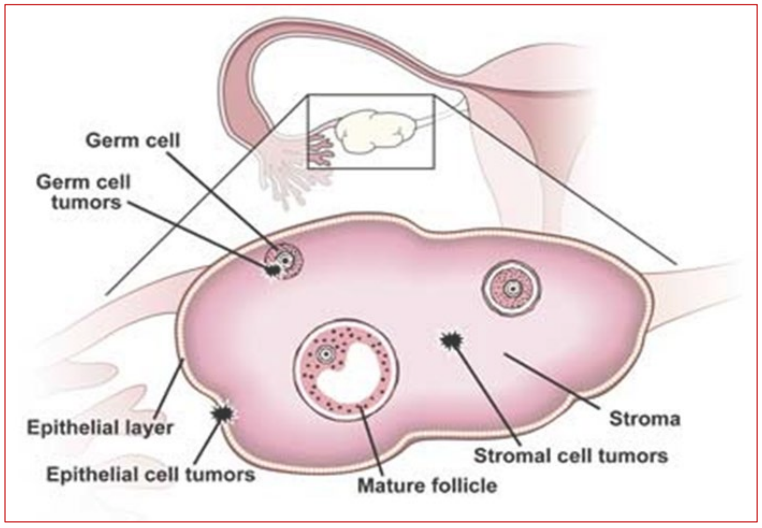

Source: [28]

Figure 1.1: Origin and classification of ovarian cancer

A2.1 Classification Histology of the Epithelial Ovarian Cancer

As many studies have stated, EOC is a heterogeneous group of neoplastic disease [29]. So, EOC can be further subdivided into various histological subtypes that fall into two main groups: Type I and Type II tumours. Type I tumour could explained as benign types, which include:

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1. **Endometrioid carcinoma** (10-20%) of epithelial ovarian cancer.

2. **Clear cell carcinoma (CCC)** is 3-10%.

3. **Mucinous carcinomas (MC)** accounting for 5–20% of primary epithelial ovarian tumours Characterized by locules lined of mucin-secreting epithelial cells and found in a single or stratified ciliated columnar cells layer rich in apical cytoplasmic mucin similar to any endocervical or colonic epithelium [26, 30, 31] and cystic tumours. In another study, MC constituted 8-10% of primary epithelial ovarian tumours [26].

4. **Low-grade serous carcinomas (LGSC)** of EOC accounted for 5% [27, 32]. LGSC profile is shown to have less karyotype instability and a lower rate of mutations [33].

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All these types accounted for 98% of ovarian carcinoma and have inherently and genetically variations from each other as reported by the epidemiological and genetically differentiations risk factor, patterns of spread, precursor lesions and molecular events during oncogenesis, prognosis response to chemotherapy. These tumours can analyse by light microscopy and can likely grow further slowly from a recognizable precursor [29, 34, 35].

Type II tumours are categorized by high-grade, rapidly progressive disease and result in high-grade serous ovarian carcinoma (HGSOC), the most common of Type II tumours accounting for 75% of all Epithelial Ovarian Cancers and are responsible for 90% of ovarian cancer deaths. It is one of the most aggressive and there are no currently precise criteria to detect HGSOC in the early stage [36], hence EOS considers as a distinct disease [8]. Krzystyniak and his colleagues have said the high-grade serous carcinoma (HGSC) account for 50-60% of all epithelial ovarian tumours and thought more originate from the fallopian tube than ovarian surface epithelium and associated with serious tubal intraepithelial carcinoma from a precursor lesion [27].

As indicated by The Cancer Genome Atlas (TCGA) project, 10% of HGSC in advanced cases is related to the incidence of multiple sites of chromosomal gain or loss containing over 30 growth-stimulatory gene amplification [27]. Expression profiling of HGSC boost the expression of the gene on chromosome instability and cell proliferation [33] and has a quite indolent behaviour correspondingly. Type I tumours could be categorized via regular somatic mutations: PTEN, CTNNB1, ARID1A, KRAS, BRAF, ERBB2, PIK3CA and mismatch repair genes that are not predictable in HGSC [37].

A2.2 Histopathology of Ovarian Epithelial Malignancy

Potential cellular origins of ovarian carcinomas are depicted in Figure 1.2. Typically, the ovarian surface epithelial tumour is known as a precursor of epithelial ovarian tumours [38]. Some histopathologic studies detailed that lining of ovaries becomes invaginated into the superficial ovarian cortex to build up cortical inclusion cyst and the mesothelial surface lining of ovaries is the most source for ovarian cancer development [39]. The original ovarian surface mesothelium is an uncommitted phenotype so it has a possibility to recite to epithelial or mesenchymal phenotypes in response to

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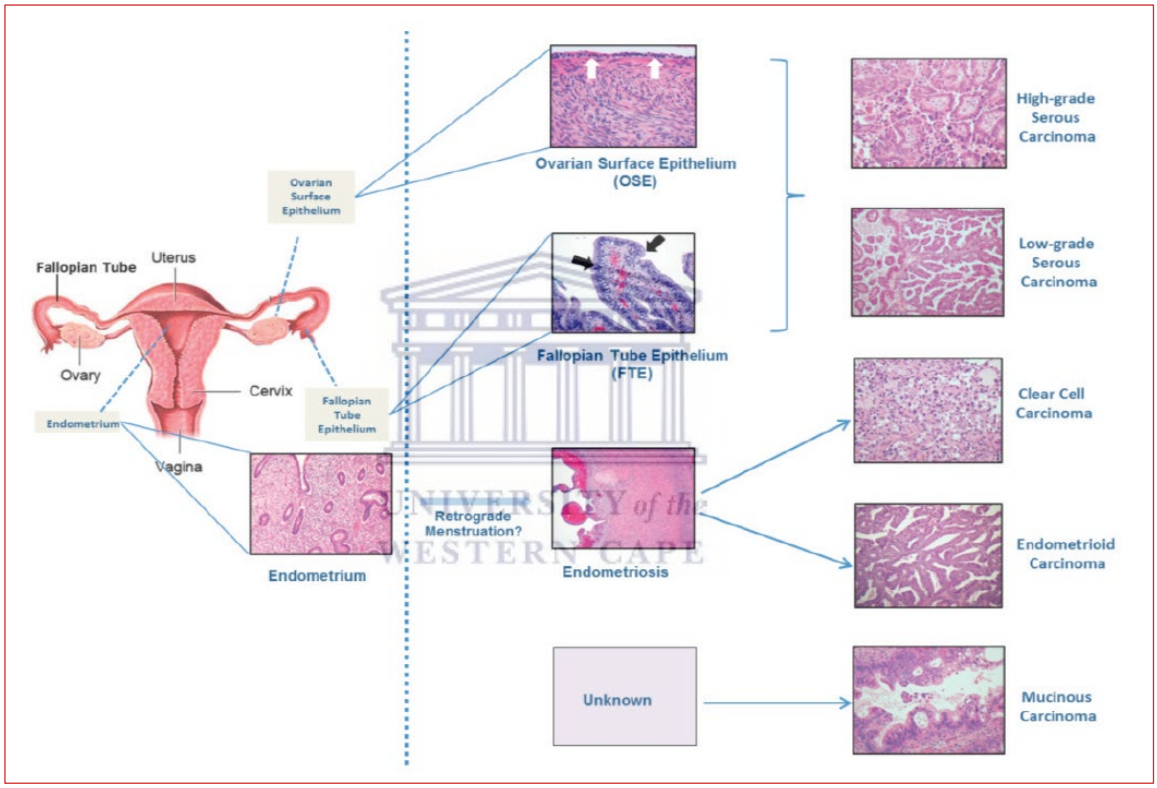
ovulation signals and phenotypic changes will happen because of mesothelial lining of an inclusion cyst, which exposes to the ovarian stromal microenvironment.

Mullerian metaplasia is usually characterized with a serious phenotype leading to undefined molecular signs and it occurs in an inclusion cyst. As a result, Mullerian metaplasia could result in neoplastic change signs in these cysts [47]. Throughout, the serous genomic, morphologic characterizations and the clinical differences noticed among epithelial ovarian cancer diseases, EOC is a definitely heterogeneous disease [49].



Source: [40]

Figure 1.2: Potential cellular origins of ovarian carcinomas

A2.3 The Pattern of Tumour Cell Spreading

As demonstrated in Figures 1.3 and 1.4, the tumour cells exfoliate from the affected epithelium, the tumour cells take the passage to the peritoneal cavity throughout the peritoneal fluid. This is assisted by bowel peristalsis. By normal classic peritoneal fluid circulation, this cells more across the left

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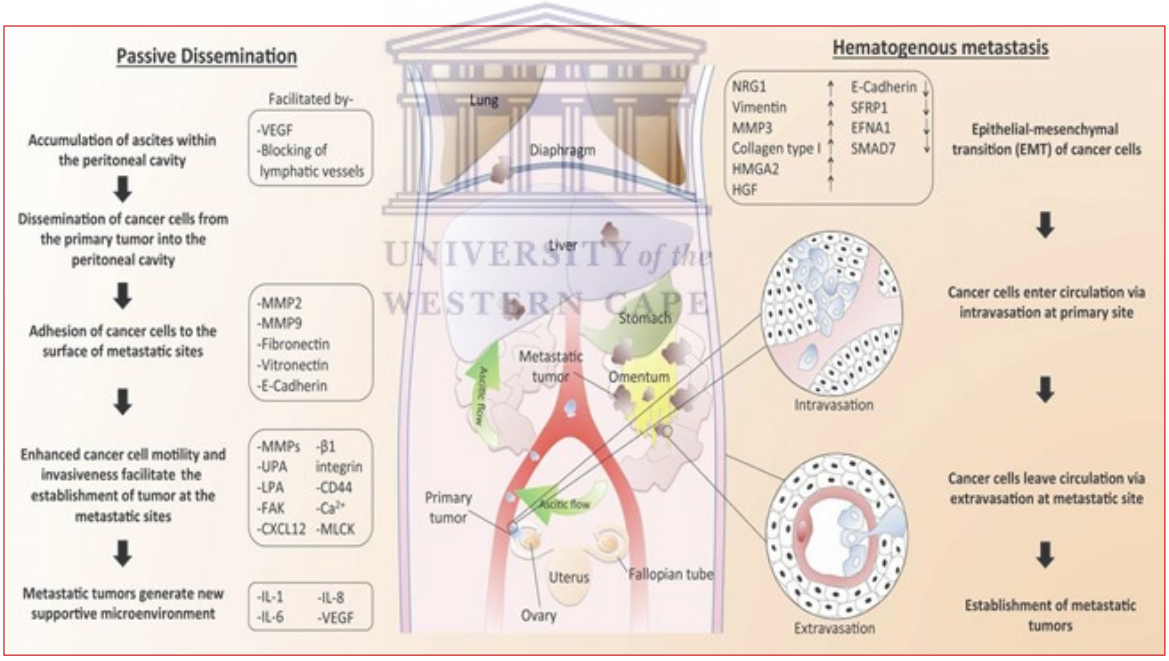
paracolic gutter and pelvis, in peritoneal reflection. The peritoneal fluid is the most frequent site to detect tumour accumulations. F Guidozzi, EW Sonnendecker and C Wright [41] reported that 6–13% of cases have local spread to the opposite ovary, whereas, up to 25% of cases spread to the uterus. Often, a tumour may spread to reproductive organs except for the vagina, cervix and vulva.

Three ovarian lymphatic passages across the ovary, are necessary for ovarian cancer surgery treatment [42], including lymphatic drainage accumulation in the ovarian blood supply right to the para-aortic nodes and drainage into the lymphatics of the broad ligament and obturator nodes. There will be the anastomotic network between the obturator, external iliac, common iliac and para-aortic basin [43]. The third drain is less significant but drainage may occur through the round ligament to the external iliac node and inguinal nodes [42]. Spreading the tumour cells via vascular is uncommon especially brain, lung and bones [5].



Source: [53]

Figure 1.3: Two mechanisms of ovarian cancer metastasis and the protein factors involved

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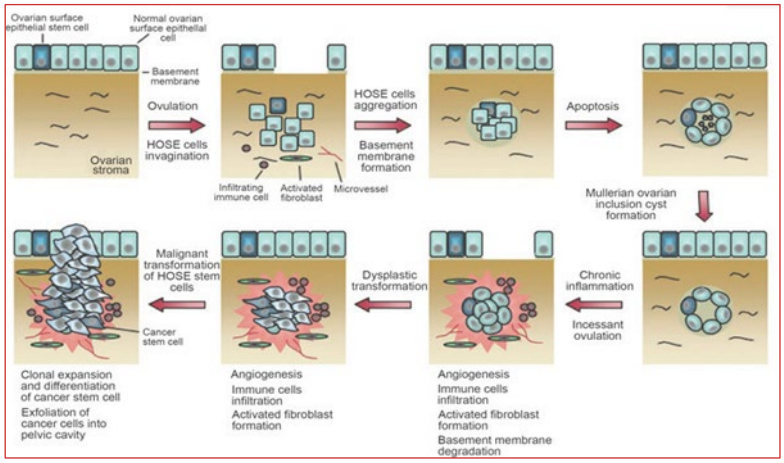

Source: [52]

Figure: 1.4: Scheme for the development of an epithelial ovarian tumour

A3. The Anatomy and Physiology of the Ovary

Obviously, the ovary has the largest account of tumour types in the body [8]. To understand ovarian cancer that originates in the ovaries must know about the ovaries and nearby structures in the body. In a female reproductive system, the ovaries are the organs nodular produce eggs (ova). Both ovaries have an almond shape and they measure about 305 cm (1.5 inches) long. They are located deep in the female's pelvis and are situated on both sides of the uterus, towards the ends of the fallopian tube. The mesovarium border is straight and is directed toward the obliterated umbilical artery and attaches to the back of the broad ligament by a short fold named the mesovarium that joins the ovary to the ovary post side of the board ligament.

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However, it is not enclosed with peritoneum and leads to the ovum, which can fall into the cavity which is a fallopian tube. The structure of the ovaries includes 3 layers which are: Epithelial cells which create the outer layer covering the ovary (called epithelium) and it is derived from the coelomic epithelium which lines the external surface of the ovaries and is a single cell layer of flat-to-cuboidal cells. Germ cells are found inside the ovary and are responsible for releasing the ova. Stromal cells make the shape of the supportive or connective tissue of the ovary. The capsule is thin of layer tissues which envelope to protect the external ovaries. On average, they measure between 3 to 5 cm in length and weigh between 2 to 4 grams. Actually, the ovary is characterized by hormonal secretion of steroidogenesis

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(Oestrogen and Progesterone) by corpus luteum. Med umbilical ligament is called the anterior of the ovary while the ureter and iliac is called the posterior of the ovary (Figure 1.5).

The infundibulum of uterine tube, end suspended via suspensory of the ovary, which is, contains the arteries and veins of the ovary at the highest point of broad ligament is called superior extremity. Also, for inferior extremity which is the angle of the body, uterine tube via proper ligament of ovary similar to gubernaculum testis [44].

The ligament of the ovary contains some non-striped, muscular fibres, which are laid within the broad ligament. The lateral surface is in contact with the parietal peritoneum, which lines the ovarian fossa; the medial surface is largely covered by the fimbriated extremity of the uterine tube. In female anatomy, the ovarian artery is exclusive, as it is a portion of a woman's reproductive system. This blood vessel transmits oxygen-rich blood to the ovaries.

The abdominal aorta remains totally located within the abdominal cavity and branches off into several different branches, the artery shoots off, including the tubal branches to the Fallopian tube, where the ovarian artery joints to the uterine artery. The artery also possesses a branch to the round ligament and from branch to the fundus. The ovarian veins convoy the arteries, once the ovaries deplete oxygen from the ovarian artery; it drains into the ovarian vein. This vein has left and right branches that are not proportionally structured. The right ovarian vein drains to the inferior vena cava, while the left flows into the left renal vein. All oxygen-depleted blood eventually returns to the lungs and the heart.

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In addition, there is internal medulla, which is characterized by lots of connective tissues, lymph, arteries and veins. Furthermore, the external cortex which the location of follicular maturation. Additionally, Cortex consists of cuboidal germinal epithelium instead of the mesothelium, due to; in the case of ovulation, there is a possibility to rupture epithelial to release oocyte. Therefore, the epithelial can grow again and refuge the rupture hole. For this reason, monthly ovulation of women leads to repeat rupture and result in the scarred look on the epithelium.

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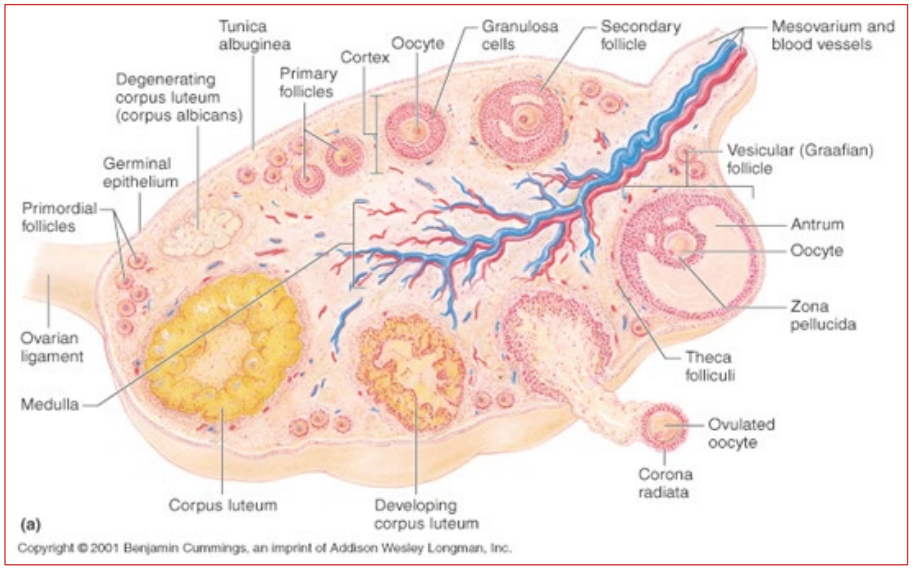

Source: [56]

Figure 1.5: Anatomy of the human ovary

Despite the fact that ovary is extra peritoneal; there is no mesothelium coverage, all the same with mesothelium of visceral peritoneum of surrounding areas. Tunica albuginea, which is compound of a layer of a thick connective tissue. Also, the spinocellular tissue where inserted in all the follicles and called stroma. In addition, it contains fibrocystic and smooth muscle cells that are included in theca extema [45].

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A4. Epidemiology

Ovarian cancer has the highest fatality-to-case ratio of all gynecologic malignancies. [4], this fact agrees with Wei Wang, et al who pointed out that ovarian cancer is one of the most common malignant tumours of female genital organs ranking third in female morbidity [46, 47]. This malignancy arises from the ovary and many gynaecological, pathological and molecular challenges in scope, among other gynaecological malignancies, ovarian cancer mortality rate increased unsteadily in women [20]. With referring Indian cancer registries, following cervical and breast cancer, ovaries are the third leading cause of cancer among women [4, 48, 49]. The National Cancer Registry in South Africa stated that there were 529 cases of ovarian cancer in 2001. of these cases, 207 were in white patients, 225 in black patients, 69 in colour patients and 16 reported in the Asian community [5].

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Ovarian cancer is the fourth leading cause of cancer mortality [50]. In Europe and the United States In 2010, ovarian carcinoma marked seventh in most cancer diagnosis among women globally. Also, the world ratio is assessed to be 6.3 per 100 000 women reaching 9.3 per 100 000 women in high resource countries [51]. Also in 2012.GLOBOCAN reported about 238,700 new ovarian cancer cases and 151,900 deaths worldwide [18, 52].

In contrast, other studies have concluded that about 240 000 women are diagnosed annually with ovarian cancer with an estimated 140,200 submitting to the disease in 2016 [36, 53, 54]. This ratio makes the case for the fertility of ovarian cancer three times compared with breast cancer mortality [54]. Some studies revealed that about 10-15% of ovarian cancer is a genetic source [55-57]. While other studies have shown that about only 5–10 % of ovarian cancer is hereditary [50, 58]. Approximately 65% to 85% of BRCA1 and BRCA2 germline mutations result in the genetic variations linked with genetic ovarian cancer [56, 59]. Ovarian cancer is mostly a monoclonal disease that originates in the ovary, as described previously [60].

A5. Aetiology and Risk Factors

Because the epithelium of the ovarian surface is a modified mesothelium [61], and it contributes to repair all the defects that happened after ovulation by the action of proteolytic enzymes and by the deposition of new matrix material [62], this criterion gives the opportunity for the development of human ovarian carcinomas [61]. Therefore, the epithelial ovarian cancer accounts for 85%-90% of all ovarian cancers [18]. Epidemiologic studies on EOC showed the majority of ovarian cancer cases occur modestly at a late age of menopause and was a weak prognosticator at menarche.

A number of studies found that around 5-15% OC cases are resulted by inherited defective genes, and these genes increase the risk of ovarian cancer [63]. Another study showed that approximately 10% of EOCs are associated with inheritance of an autosomal dominant genetic abnormality [64]. About 27% to 44% of ovarian cancers result from the inheritance of a deleterious mutation in one of the BRCA genes [65]. Inflammatory illness increases the risk of ovarian cancer as mentioned by [65].

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Obesity, smoking and an inactive lifestyle are amongst the causes that lead to ovarian cancer [66]. Table 1.2 summarizes risk factors for the development of ovarian cancer that are often overlooked [75]. Hempling and his colleagues reported that there is no significant relationship between the use of HRT and the risk of developing epithelial ovarian cancer, especially with prolonged hormonal exposure [67].

Table 1.2: Risk factors for the development of ovarian cancer

Increased Risk	Decreased Risk
Age	**Multiparty**
Null parity	**Lactation**
Early menarche or late menopause	**Hysterectomy**
Menopausal hormone replacement therapy	**Tubal ligation**
Endometriosis	**Oral contraceptive use**
Lynch syndrome	
BRCA1/2 mutation	
Source: [75]	

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A6. Staging

- I. Disease Limited to Ovaries Only

  Ia—Limited to one ovary only

  Ib—Both ovaries affected

  Ic Ia or Ib—With a tumour on the surface of one or both ovaries, ruptured capsule, cytologically positive ascites, or positive peritoneal washings

- II Disease Extending to the Pelvis

  IIa—Disease affecting tubes, uterus, or both

  IIb—Extension to other pelvic tissues

  IIc IIa or IIb—with a tumuor on the surface of one or both ovaries, ruptured capsule, cytologically positive ascites, or positive peritoneal washings

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- III Abdominal Disease or Affected Lymph Nodes or Both (Surface Liver Disease Still Stages III)

  IIIa—Microscopic involvement of abdominal peritoneal surfaces.

  IIIb—Disease up to 2 cm in diameter.

  IIIc—Disease greater than 2 cm ± regional lymph nodes.

- IV Distant Metastases

  Pleural effusions need to be positive cytologically and liver metastases should be parenchymal [5, 68].

A7. Screening

Regarding WHO criteria to screen ovarian cancer; there are steps that must be achieved, according to Wilson-Jungner [69, 70].

1. The condition being screened should be a significant health problem.
2. The natural history of the condition should understand.
3. There should be a noticeable early stage.
4. Curing at an early stage should be of more assistance than at a later stage.
5. A suitable test should devise for the early stage.
6. The test should be acceptable.
7. Intervals for repeating the test are determined.
8. Provision should make for the extra clinical workload resulting from screening.
9. The risks, both physical and psychological, should be less than the benefits.

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10. The costs should be balanced against the benefits [5, 71].

There are three screening tests presently used for ovarian cancer, serological cancer antigen (CA) 125 which is raised about 80% of ovarian cancer as a tumour-specific antigen. However, the high expression of CA125 is exclusive in 50% of women who suffer from cancer, limited to the ovarian carcinomas [65]. Besides, it can also elevate in women who suffer from benign ovarian disease and in healthy women, thereby limiting, the specificity of CA125 [65]. CA125 is costly and convenient if used in the population [5].

Even though, the bimanual pelvic evaluation is one of the applications used in screening women during gynaecologic examination and definitive if done by an experienced examiner, the sensitivity ,and specificity of this application are not satisfactory since it detects only 1 in 10,000 ovarian carcinomas in asymptomatic women [65]. Investigations of an abdominal-pelvic ultrasound insufficient to recognize and evaluate cancer [5]. Pelvic ultrasound is one of the preoperative investigations along with a chest X-ray, full blood count, serum electrolytes, urea, CA125 and liver function tests. On the other hand, to determine the extent, localization and operability of recurrent disease, advanced imaging modalities are usually required namely PET/CT and MRI.

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In general, the cost of ovarian cancer screening tests is variable. If the sensitivity of a screening test presented as 80% that would need 6000 tests on women between 45-75 years to discover one case. Or, if the sensitivity percentage is 90%, the ovarian cancer screening tests would have been 5300 tests to recognize single cancer [72]. Furthermore, if the screening is applied on a subgroup of patients who suffer a particularly high risk, the yield and cost-effectiveness will cost much more [5]

A8. Diagnoses

Currently, only 25% of Ovarian Cancers are detected in stage I [73], so, in order to improve survival rates of ovarian cancer, the diagnosis of cancer should be at an early stage. Due to the parallel symptoms of ovarian cancer with another type of cancer, symptoms at an early stage are not recognized leading to the difficulties in diagnoses[74]. Several technics are used to diagnose ovarian cancer and they differ

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globally [75]. Basically, a diagnosis should be considered in a woman who has suffered from multiple symptoms such as urinary symptoms, abdominal distension or bloating with or without abdominal pain, difficulty eating, or feeling full quickly. Transvaginal Sonography (TVS) is one of the routine diagnoses that must apply. CA125 blood serum level has most attention in these cases but it lacks in the sensitivity or specificity if used alone as a biomarker for ovarian cancer screening [73].

In women with persistent abdominal distension or feeling full and/or loss of appetite or pelvic or abdominal pain or increased urinary urgency and/or frequency, the pelvic ultrasound must do urgently. If the results of those diagnostic procedures are normal, the specialist must refer the patient to Computed tomography **(CT)** of the abdomen and, pelvis especially in cases who have a Risk of Malignancy Index scores greater than 200 [76].Both **CT** scan and Magnetic Resonance Imaging **MRI** are equivalent to assess the extent of the epithelial ovarian cancer. Using positron emission tomography-computed (**PET-CT**) to evaluate ovarian cancer at the beginning is limited and confined to non-systematic descriptive analyses [77, 78]. Both of [79, 80] have recommended that **PET-CT** acts together to characterize the primary lesion and staging of cancer. Another contrast study also described: staging is proceeded by **PET-CT** [81].On the other hand, **MRI** is not suggested for routine staging of ovarian cancer also **PET-CT** is not advised in the diagnosis or early staging of ovarian cancer [82].

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A8.1 Pathology

Actually, using pathological examination is one of the recommended choices to detect the nature of the cancer tissue including the stage of an ovarian cancer. Usually, histological sections of tissue samples and cytological assessment of fluid samples can recognize the staging of ovarian cancer. According to the Royal College of Pathologists guidelines, it must have a block of tissue for each centimetre of the maximum diameter of a tumour to define the evidence of carcinoma correctly [83].

A8.2 Intraoperative Technique

It uses to confirm the rate of all malignant disease phases by Intraoperative frozen section histopathology and to exclude the metastasis disease unless borderline disease [84-87].

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A8.3 Molecular Studies for Cancer Diagnosis

Molecular techniques have emerged as an authoritative technology to decode biological processes of normal or cancer cells. For the past 5 years, the molecular diagnostic testing has improved speedily between 10%- 20% per year. This leads to advance our basic comprehensions such as the human genome project and practical techniques. Because the speed of molecular studies improvements and the advanced procedures of molecular sample extraction, many molecular applications have advanced in protocol from manual and intensive labour to rapid automated methods [88, 89].

A8.3.1 The Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a major discovery in the 1990s, which moved from the research laboratory studies to the clinical laboratory. PCR becoming the most validated procedure in specific clinical diagnostic requests [90]. Polymerase Chain Reaction is a fast molecular diagnostic analysis and easy performance, the user can simply design assays and control reaction conditions, it satisfied clinical reversal times and is used as a universal technology that could be readily applied to multiple approaches for genetic disorders and neoplastic disease trails. In addition, PCR was an authoritative apparatus that allowed for the amplification of specific yet small size target sequences that could be analysed using several steps. The final step of the PCR procedure is **Gel electrophoresis** used for identification in the analytical process of **(i)** DNA extraction, **(ii)** PCR amplification, **(iii)** restriction endonuclease digestion, **(iv)** Gel electrophoresis [88].

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A8.3.2 The Real-Time Quantification Polymerase Chain Reaction

The Real-Time Quantification Polymerase Chain Reaction (RT-qPCR)is a highly sensitive microarray data validation method [91, 92], it is used in many scientific studies [92-94]. Although qPCR has been utilized extensively by scientists, there are many problems to analyse the massive amounts of data created [92]. It is a molecular analysis technique based on STAT sources for infectious diseases that would have a major effect on patient care [88]. Also, RT-qPCR is one of the recorded accurate and authoritative quantitative methods for gene expression analysis [95]. This technique has its application in several analyses such as pathogen quantification, cancer quantification, transgenic copy number

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determination, microarray verification and drug therapy studies [95]. qPCR has been applied in clinical oncology field to detect minimal residual disease, to investigate and quantify the translocations of chromosomes[96]. Moreover, it utilizes a standard method to detect and quantify nucleic acids from live and alive pathogens in opposite to another classical method, which only deal with live pathogens[97, 98]. Small quantities are used for infectious diseases testing. In addition, several versions of ABI platforms, the Roche Light cycler and Cepheid Smart Cycler II/Gene Expert instruments become available for real-time PCR within next the few years. In viral target tests, real-time PCR is able to perform quantitative estimation effortlessly. In this field, a STAT basis could be used for infectious disease to give a wide impact on patient care [88].

A8.3.3 Immunocytochemistry

Marrack who has initiated the first principles of Immunocytochemistry (ICC) when he manufactured chemicals against typhus and cholera microorganisms utilizing a red stain conjugated to benzidine Tetrahedron [99]**.** On the other side, Professor Albert H. Coons from Harvard School of Medicine-Boston, the U.S.A. in the early nineteen forties established the localization of some microorganisms could found through using antibodies against Streptococcus pneumonia stained with fluorescein, imagined by the ultra-violet light microscopy [100].

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Later, Coons and his assistances invented and settled the principles of ICC precisely [100-103] and introduced the fluorescein-labelled antibodies conception to detect the antigens using the ultra-violet light (fluorescence microscopy) and that concept has been improved and extended to permit the localization of antigens at the ultra-structural level [104] and utmost immunocytochemical procedures are the focus on antibodies to endogenous molecules in tissues[105].

Accordingly, ICC conducted as a reliable tool technique has been demonstrated to elucidate some scientific researches correlated with variance diagnoses that are not able to assess by conventional assays as haematoxylin and eosin staining technique [106-108]. ICC is a valuable tool to identify a tissue or cellular contents from different cell suspensions [106] or in an appropriate antigen-antibody interaction where the antibody has been tagged with a labelled tag [109] and allows one to detect a

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further detailed spatiotemporal expression of cell antigens [110].

A9. Treatment

The quantities of the post-surgery remaining tumour for patients who suffer from the advanced epithelial ovarian cancer present the greatest important prognostic factor related to survival rate and nearing to 50% for patients with a survival rate of 5 years with a null residual tumour after surgery. However, in 5% of patients who have a residual tumour whose less than 1-2 cm [111], for this reason, the surgery is administrated to surviving patients with advanced epithelial ovarian cancer. Predominately, in advanced cases of epithelial ovarian cancer with stage IV using the cytoreduction surgery (CS) is recommended when metastatic lesions exist at a distance that does not influence survival in the short time. Clearly, treatment of ovarian cancer depends on different variables resulting from the varied histopathologic entities; so the treatment is related to the specific type of tumour, tumour size, tumour position, the degree of spread and the Patient's physical condition [112]. The treatment of ovarian cancer continued to be surgery and chemotherapy.

In 2011 the European Commission (EC) declared another treatment for ovarian cancer which is a biological therapy in combination with standard chemotherapy(carboplatin and paclitaxel) as a preliminary treatment for the advanced stages (III B, III C and IV), even epithelial ovarian, fallopian tube, or primary peritoneal cancer. Indeed, this combination made a new real treatment choice for patients who suffered from advanced ovarian cancer 15 years in advance in contrast to Radiotherapy, which is not used regularly to treat ovarian cancer.

A9.1 Surgical Treatment

The first choice to solve the ovarian cancer problem is the surgical therapy to establish or confirm the suspected diagnosis, applicable surgical staging and primary cytoreductive surgery. Actually, for the patient's early stage (Stage I or II), applicable surgical staging is particularly essential and will result in the upstaging of about one-third of patients (usually to Stage III), however, advanced stage cases need surgical staging of ovarian cancer [113]. The surgically stage including debulk or cytoreductive

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the tumour to remove as much tumour as possible [15, 114, 115].

A9.2 Cytoreductive Surgery

Cytoreductive surgery (CS) is one of the techniques used originally to eliminate all metastatic and spreading lesions of an ovarian tumour through the abdominal cavity. Thus, the residual tumour should be shrinking to zero upon visual inspection. Obviously, this cytoreduction surgery has been used in progressive Cases of ovarian cancer, especially, in stage IV when metastatic lesions occur at a distance that does not influence survival in the short run. Whereas, if a residual tumour was left less 1cm, optimum surgery of cytoreduction [116], improves the prognosis of the patient [117].

For patients with involved repetition after a long disease-free interval (≥ 6 months), the secondary cytoreductive surgery would be well advised [118]. Less than 40% of women with ovarian cancer are cured [24, 25]. In cases where one ovary is affected and the patient needs to be fertile, the solution to this problem will be to remove just the affected ovary. [75]. Unfortunately, most of the ovarian cancer cases have cancer relapse, after they respond to chemotherapy, as a consequence, half of them ultimately die of cancer [119].

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A9.3 Biological Therapies or Immunotherapy

Biological therapy is a new approach to treat an advanced ovarian cancer. Sometimes, it is referred to as biotherapy, immunotherapy, or biological response modifier therapy. Also, it is known such as a relatively new addition to the family of cancer treatments. The main idea of this technique qualifies as the body's immune system directly or indirectly to inhibit the vitality and viability of cancerous cells. Biological therapies or immunotherapy produces fewer complications than other cancer treatments [120].

A9.4 Targeted Therapy

It used to treat a specific point in cancer cells with specific drugs e.g. Bevacizumab, Pazopanib and Olaparib. The main action of these drugs is to stop the molecules that support the cancer cell to grow and duplicate. In contrast to chemotherapy treatment, targeted therapy is less likely to harm normal

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cells than chemotherapy treatment, which affects the normal human cell. Both Avastin and Olaparib are ideal drugs of targeted therapy to treat ovarian cancer perfectly since they can attack the cancer cells in several ways [121].

A9.5 Hormonal Therapy

Hormonal therapy uses to manage some specific hormone release in the human body or to block the hormone's receptors to stop their viability. These techniques use to assist patients, who are suffering from cancer recurrence when they use different therapy and also to stop or block estrogen and progesterone receptors, which secrete in small amounts by ovaries, liver and other organs, or to reduce hormonal levels in the bloodstream to slow ovarian cancer growth. There are several drugs namely: tamoxifen, anastrozole, leuprolide acetate, megestrol acetate, all of which have different mechanisms to affect cancer cells behaviors [122, 123].

A10. Management of Epithelial Ovarian Cancer

Due to the heterogeneity and genetic mutations that all EOC subtypes are recognized with, the management of EOC needs more skills in surgery, chemotherapy, imaging, histopathology and palliation, Despite the efforts of the professional specialists to achieve optimum outcomes [68].

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SECTION B: OVARIAN CANCER BIOMARKERS

B1. Biomarkers in Ovarian Epithelial Cancer

As a result of the difficulties in discovering ovarian cancer at an early stage, using specific biomarkers as diagnostic tools emerged as a particular part of a cancer diagnosis [124], and this requires high sensitivity (>75%) and extremely high specificity (99.6%) to achieve an appropriate predictive value of at least 10% [73, 125].

B1.1 Mucinous Cell Surface-Associated 16 (MUC16)

MUC16 encodes the tumour marker CA-125 Cancer Antigen 125, as shown in Table 1.3 [126]. Cancer

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Antigen 125 also known as Mucin 16 (MUC16) [127, 128]. MUC16 is located on chromosome 19p13.2 [129] and is coded by sequences present inside about 179 kb of genomic DNA [130]. N Porchet and J-P Aubert [131]. Beatrice and his colleagues have said they just cloned a fractional cDNA (designated MUC16) that codes for a novel mucin that is a strong candidate for being the CA125 antigen. This project now has been confirmed by transfecting a partial MUC16 cDNA into CA125-negative cell lines and establishing the synthesis of CA125 by 3 different assays. [128].

Table 1.3: HUGO Gene Nomenclature Committee (HGNC) Symbol Report for MUC16

Approved symbol	MUC16
Approved name	mucin 16, cell surface associated
HGNC Id	HGNC:15582
Previous symbols & names	CA125
Synonyms	CA125, FLJ14303
Locus type	Gene with protein product
Chromosomal location	19p13.2
Gene family	Mucins

Source: http://www.genenames.org/cgi-bin/gene_symbol_report?hgnc_id=HGNC:15582; https://www .genenames.org/; [152]

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The three antibodies (OC125, M11 and VK-8) tested on the transfected cells, only the first two were intensely positive, indicating the differential expression of the CA125 epitopes in these cells. The cloning and expression of CA125 antigen unlocked the way to an understand the function of CA125 antigen in normal and malignant cells[128]. CA125 was first defined by Rober Bast and colleagues in 1981 [132], from 35 years ago [133] and it is an approved like an antigen that has elevated in the majority of epithelial ovarian cancer cases.

Then, in 1993, Woolas et al. believed that CA125 is a classic, “gold standard” tumour biomarker, and it occurs in high expression levels in a panel of some disease. Moreover, it is a glycoprotein protein antigen with unknown function, [128], has a high molecular mass 1 MDa glycosylated transmembrane mucin that is expressed by 80% of ovarian cancers [134] and it has many features of the membrane-bound mucin proteins [135, 136]. This antigen recognized by using OVCA433 as the antigen for

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preparing the murine monoclonal antibody OC125 in epithelial ovarian carcinoma. Further, it has a high molecular weight that displays sensitivity between 50-60% at 90% specificity in early stage postmenopausal women and 75-90% in women with advanced stage disease [126, 137, 138]. CA125 could have risen even in some benign diseases like, liver cirrhosis, endometriosis and peritonitis;, also, it has fluctuated levels in the menstrual cycle and pregnancy and also involves a number of other cell types including the peritoneum, pleura and Mullerian and coelomic epithelial and can be used in monitoring the recurrence of disease [139].

On the other hand, CA125 is not expressed in 20% of ovarian cancer adding to several factors which undermine the significance of CA-125 as an early detection biomarker [140]. In advanced epithelial ovarian cancer patients, MUC 16 concentration in serum is elevated up to 80% [141] although, it is elevated in only a few early cases [142]. Using muc16 as a separate marker is not adequately sensitive to diagnose all cases of early-stage ovarian cancer with insufficient screening techniques for this reason, in the general population, the sensitivity of CA125 will be increased if it is conjugated with other biomarkers [127, 143]. Most post-menopausal women are under increased risk of ovarian cancer so multi-screening procedures are suggested using CA125 and transvaginal sonography (TVS) [144-146].

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CA125 has its limitations and is neither sensitive nor specific enough to be used as a diagnostic biomarker for early ovarian cancer detection, as reported by Bell R et al (1998) and other prospective studies [147]. In particular, for women who suffer from advanced stage ovarian cancer, the sensitivity of CA125 is more than 90% but the sensitivity decreased to approximately 50% for stage I ovarian cancer [145]. Measurement of elevated CA125 is advised monitor for chemotherapy responses, characteristic malignant pelvic masses from benign masses, detection of relapse and improving the clinical trial design.

Also, during chemotherapy procedure, the low values in an expression of CA125 are helpful for prognostic occurrence and serial measurement of CA125 [144]. The relatively low sensitivity of CA125 relates to using it as a single screening biomarker whereas, combining it with other biomarkers, achieves the creation of a multiple biomarker panels to increase the success diagnosis and therapy [140, 148-

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150].

A combination of CA125 and TVs might increase specificity or by monitoring increases in CA125 over time [58]. CA125 (muc16) is a type I transmembrane protein consisting of an enormous N-terminal domain of more than 12,000 amino acid residues with extensive tandem repeats that are heavily glycosylated with a carbohydrate content estimated to be up to 28%, with O-linked and N-linked glycans [135]. The central domain contains up to 60 glycosylated repeated sequences constituting the tandem repeats characteristic of mucins.

The C-Terminal Domain (CTD) is composed of an extracellular unique region which contains a potential proteolytic cleavage site, a transmembrane domain and a short cytoplasmic tail with potential phosphorylation sites [135]. Muc16 has many structural matches with MUC1 and MUC4. Also, the large N-terminal region of MUC16 assists cell-cell interactions via its interaction with mesothelin and could have a role in the metastasis of EOC [151]. Thus it is plausible to assume that MUC16 may apply a number of functions in ovarian cancer similar to those other mucins [135].

Muc16 and other factors such as β. Integrins and CD44 play a role in mediating adhesion between ovarian cancer cells and the mesothelial cells that line the cavity and to the underlying stroma. All these adhesion procedures occur on the surface of ovarian cancer cells connected to mesothelin and hyaluronic acid on mesothelin cells, or to fibronectin, laminin and type IV collagen in the underlying matrix [134].

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B2. Lyso-Phosphatidic Acid

Norwegian physician, Kåre Berg, who described the human Lipo Protein A in 1963, reported that Lyso Phosphatidic Acid (LPA) is a macromolecular complex in plasma (Figure 1.6) [153]. LPA structures from LDL molecule which contains apoB-100 and one molecule of a large highly polymorphic glycoprotein named apo A [154]. Lysophosphatidic acid (LPA, 1-acyl-2-lyso-SN-glycero-3-phosphate) is bioactive which means that they are extracellular messengers or local hormones, contributing an extracellular signal [156, 157].

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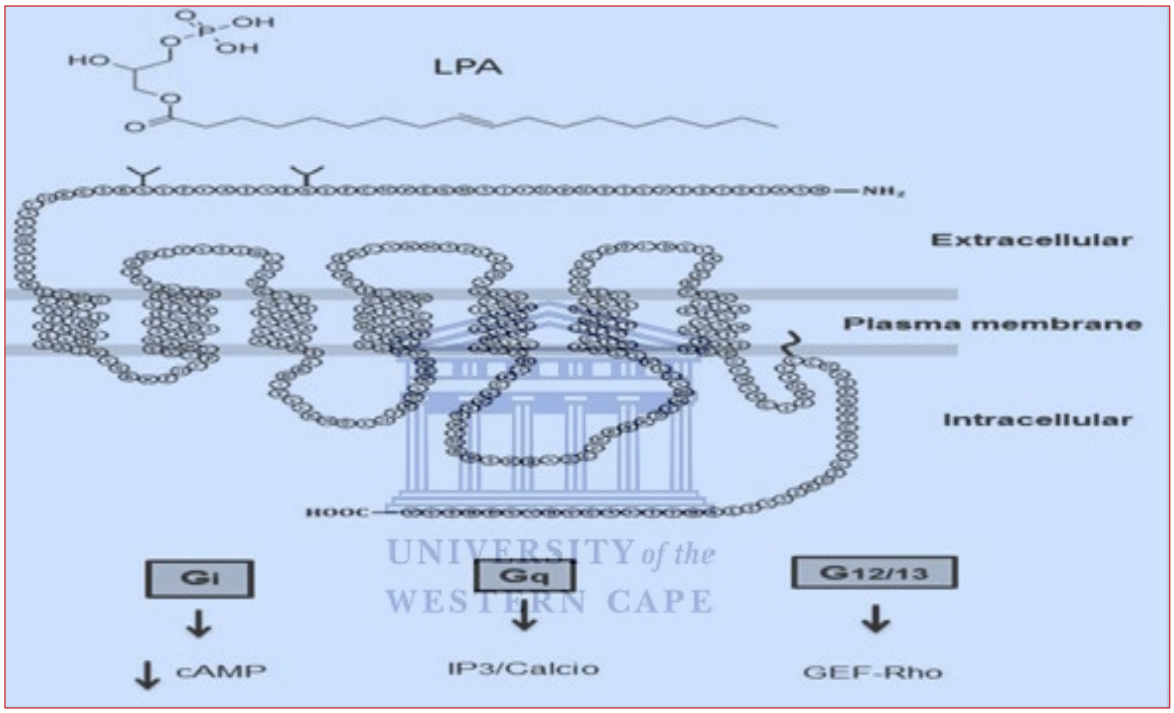
Also, the simple small glycerophospholipid (molecular weight: 430–480 Da) [170, 171], facilitates a range of biologic actions such as increase smooth muscle contraction, blood pressure and platelet aggregation discovered in the early 1980's [172], and presents in serum at concentrations of 2–20 μM and been shown to stimulate proliferation of ovarian cancer cells, surge resistance to chemotherapy, stimulation of DNA synthesis, cytoskeleton restructuring, cell survival, drug resistance, cell adhesion, migration, cytokine production and ion transport. [165, 171, 173-175]. LPA is mostly transcribed in the liver (Figure 1.7) [176].



The upper panel shows the molecular structure of a lysophosphatidic acid. The middle and lower panels display the common structure of a LPA receptor (exemplified by LPA1) and its interaction with different G proteins [166]. Also, it is known lysophospholipid [167], which is a major form of acting through G protein-coupled receptor [168].

Figure 1.6: The cellular action of lysophosphatidic acid through LPA1-3 receptor model

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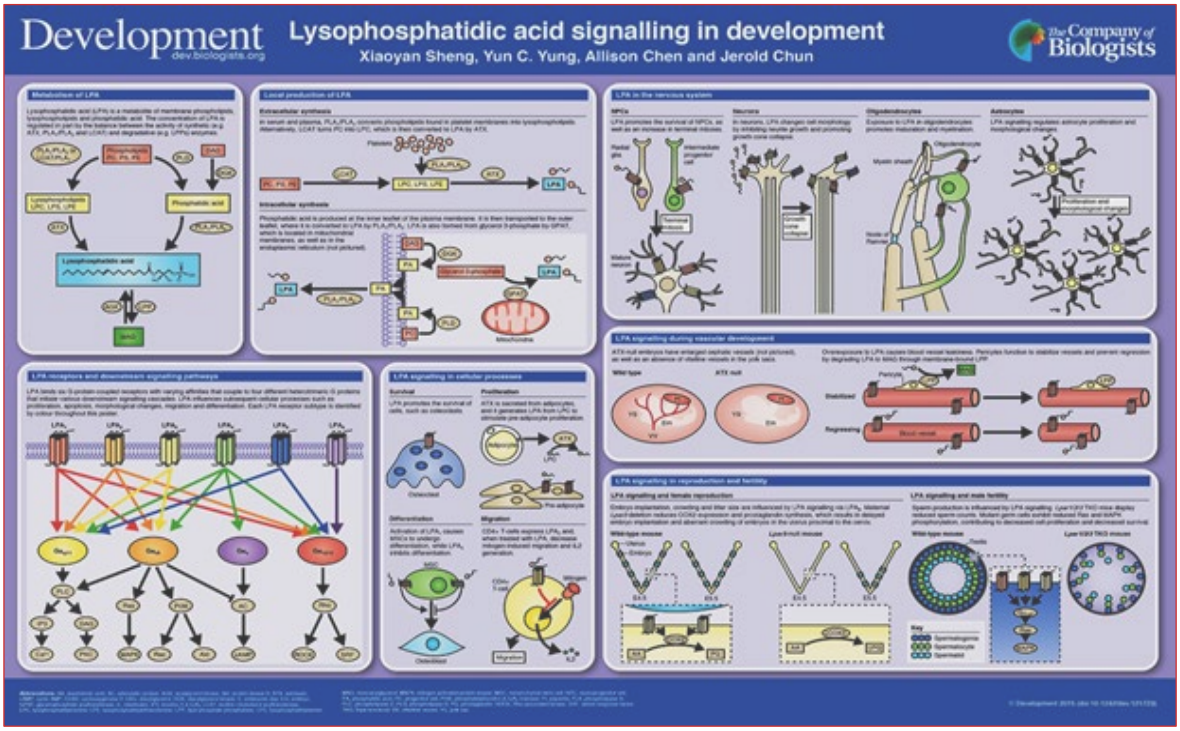

Source: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4392601/; [169]{Sheng, 2015 #573}

Figure 1.7: LPA signaling

Using LPA as a biomarker for early detection of ovarian cancer is still not approved and it is under investigation to recognize the efficacy of LPA [158, 159]. LPA implicated in human carcinogenesis firstly after seen to be elevated in ascitic fluid of ovarian cancer patients.[158, 160-163] and has many roles to control in ovarian cancer proliferation, cell survival, angiogenesis and metastasis as an autocrine factor [164, 165].Also, it can be released in plasma of ovarian cancer patients [165].

Despite coagulation process and normal constituent of serum, LPA is released by activated platelets and is usually found only at very low levels in whole blood or fresh platelet-poor plasma from healthy individuals [158, 166]. The fatty acid chain that LPA consists of could link to the glycerol backbone during various chemical linkages which are leading to create different subclasses such as [alkyl (A-LPA), acyl [167] and alkenyl (An-LPA)] [168].

LPA occurs as phospholipid and activates distinct members of the endothelial differentiation gene (Edg) which is the subfamily of G protein-coupled receptors (GPCRs) to support multiple cellular responses

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[169]. Further, LPA and other phospholipids play a role in stimulating expression of interleukin 8 in ovarian cancer which is probably produced by cancer cells and migrates from the peritoneal cavity to the circulation similar to another marker i.e. CA125 [170, 171]. Specific G-protein–coupled receptors (GPCRs) are a supervisor to mediate the biological function of extracellular LPA which includes Edg-2/ LPA1, Edg-4/LPA2 and Edg-7/LPA3, that belong to the endothelial differentiation gene (Edg) family, [172-174], so LPA interacts with specific cell surface G- protein- coupled receptors of the endothelial differentiation gen (Edg2) [175] and (Edg4),[173] subfamily which consider specific LPA receptors present on many cell types.

Various cellular responses will happen and these responses occur as follows: **(i)** stimulation of cell proliferation by increasing cell cycle progression, **(ii)** inhibiting apoptosis, inhibition of cell differentiation, (**iii**) stimulation of tumour cell invasion [176]. On the other hand, they have reported the GPCRs GPR23/p2y9 (LPA4) [177], GPR92/93 (LPA5) [178, 179] GPR87/95 (LPA6) [179, 180], p2y5 [181] and p2y10 [182] to be new, non-Edg family LPA receptors that have little sequence homology to LPA1 – 3. These new receptors still have an unknown mechanism [183-185].

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Since inventing LPA1 via Cognate cell surface receptors in 1996 [172]; That results to the identification of two extra closely related receptors (LPA2, LPA3), also, the recent identification of two more, slightly divergent, receptors (LPA4 and LPA5). In fact, all five receptors are classified as type I, rhodopsin-like G protein-coupled receptors (GPCRs) which contrast whereas, they conflict downstream signalling pathways and tissue distribution [186]. LPA6 discovered recently and has similar LPA4 properties.

The diversity and prevalent the effect of LPA to regulate many biological processes due to the heterogeneity of receptor subtypes, as shown in Figure 1.8, and expression patterns and effector pathways. Typically, from membrane phospholipids LPA is created through two main pathways: first, the sequential activity of phospholipase D (PLD) and phospholipase A2 (PLA2) and second sequential activity of PLA2 and lysophospholipase D (lysoPLD) [187]. As a consequence of the complicated metabolism of LPA, it has led to producing numerous chemically distinct species [166].

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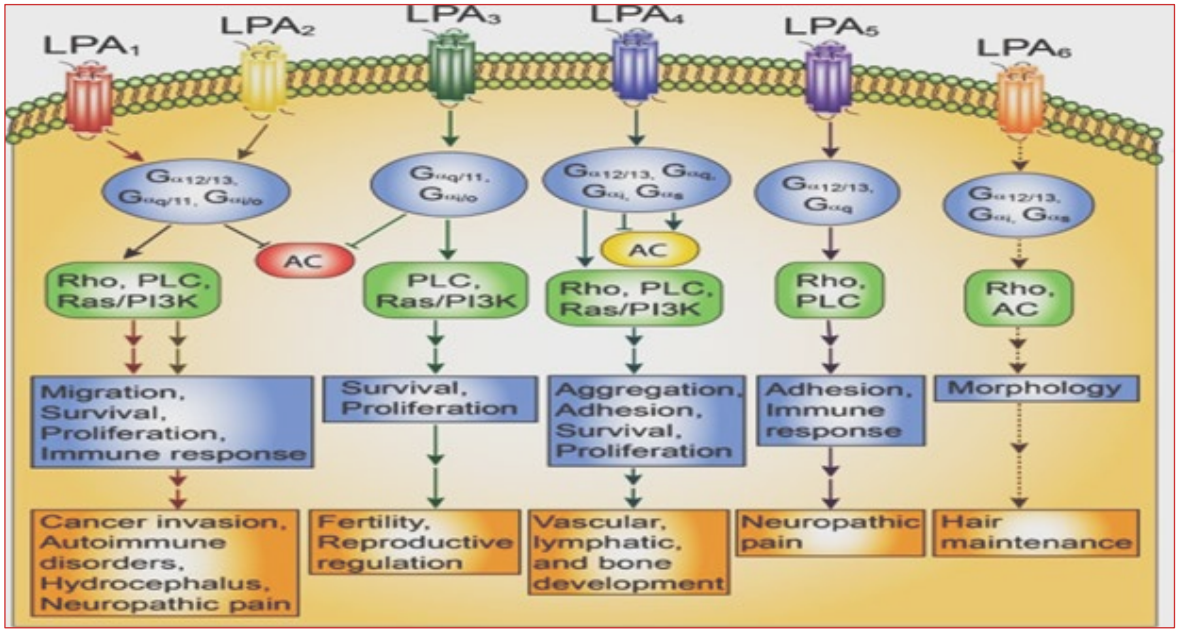

Source: [188]

Figure 1.8: Six definite LPA receptors throughout activation by its signaling pathway and functional outcomes

B2.1 LPA Receptor 1

The LPA receptor1 is the first receptor cloned and recognized from a growing number of LPA receptors that comprises the Edg-family and the purinergic receptors [189]. It is a gene found in mice and humans in many organs such as heart, brain, ovaries, intestine, testis, prostate, thymus and pancreas, among others. For LPAR1 human is located in chromosome 9 site 9q31.3 and encodes a protein of 364 amino acids with 7 putative transmembrane domains. (Molecular mass ≈ 41 kDa) (Table 1.4) [166, 172, 173, 175, 190]. As mentioned above, this receptor was the first identified in this family [172, 190].

The LPAR1 signalling occurs once this receptor connects to three G protein partners which are named and functioned as follow: Gαi/o, which discourages adenylyl cyclase, thus decreasing cyclic AMP levels, Gαq/11, which activate phospholipase C, increasing IP3 and diacylglycerol configuration and the concentration of intracellular calcium; or (3) Gα12/13, which adjust the Rho pathway [191, 192]. According to these processes, a variety of cellular responses are elicited including cell proliferation, cell shape changes, survival and cytoskeletal rearrangements, migration, rearrangements in cell-cell contacts, among many others.[166, 193, 194], also, as mentioned by an original study that the increased

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expression of LPAR1 is related to the susceptibility of hypertension [186]. The third binding between LPAR1 and Gα12/13 is critical for signalling.[194] and the main reason to locate PDZ binding domain in the carboxyl terminus of LPA1 receptors, appears to participate in signalling [116, 195].

Table 1.4: HUGO Gene Nomenclature Committee (HGNC) Symbol Report for LPAR1

Approved symbol	LPAR1
Approved name	lysophosphatidic acid receptor 1
HGNC Id	HGNC:3166
Previous symbols & names	EDG2, "endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2"
Synonyms	edg-2, Gpcr26, GPR26, LPA1, Mrec1.3, rec.1.3, vzg-1
Locus type	Gene with protein product
Chromosomal location	9q31.3
Gene family	Lysophosphatidic acid receptors
Source: http://www.genenames.org/cgi-bin/gene_symbol_report?hgnc_id=HGNC:3166	

Moreover, LPAR1 controls osteoclast differentiation and bone reabsorption [196]. Comparing to LPAr2, LPAR3 and LPAR1 expressions, MM Murph, GH Nguyen, H Radhakrishna and GB Mills [194] confirmed that LPAR1 has increased in ovarian cancer whereas LPAr2 and LPAR3 have decreased [194].

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B2.2 LPA Receptor 2

Due to the similarity between LPAR2 and LPAR1 in gene sequence search; LPAR2 did not take long to discover [197]. LPAR2 is positioned in chromosome 19 site 19p12, as shown in Table 1.5, and encodes a protein of 351 amino acids (calculated molecular mass of ≈ 39 kDa) [166, 190]. It has the same effecting pathways of LPAR1 once it binds with Gαq/11, Gαi/o and Gα12/13. By a PDZ-binding domain present in its carboxyl terminus tail LPAR2 can interact with TRIP6, a focal adhesion molecule, and with other proteins [166]. Owing to many types of research LPA2 receptor activation encourages migration in gastric and ovarian cancer cells [198, 199]. There is an association between LPAR2 and

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ovarian cancer tumorigenicity aggressiveness as concluded by Yu et al and Murph et al [185, 194]. Also, LPAR2 plays a role in endometrial cancer invasion [200]. However, the absence of LPA2 receptors decreased tumour formation [201].

Table 1.5: HUGO Gene Nomenclature Committee (HGNC) Symbol Report for LPAR2

Approved symbol	LPAR2
Approved name	lysophosphatidic acid receptor 2
HGNC Id	HGNC:3168
Previous symbols & names	EDG4, "endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 4"
Synonyms	EDG-4, LPA2
Locus type	Gene with protein product
Chromosomal location	19p12
Gene family	Lysophosphatidic acid receptors
Source: https://www.ncbi.nlm.nih.gov/nuccore/?term=LPAR2	

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B3. FOS-Like 1, AP-1 Transcription Factor Subunit

FOS like 1, AP-1 transcription factor subunit FOSL1 is a member of the FOS family and is known as a proto-oncogene, that is highly expressed in multiple solid cancers, also has regularly overexpressed in epithelial cancers and involved in invasiveness [202]. Fra-1 (Fosl-1) is a basic leucine zipper (bZIP) and transcription proteins factor of Fos family and Fra-1 (Fosl-1) protein has 271 amino acids with a predicted molecular weight of 29.4 kDa [203]. Fra-1 is included in multiple forms so it considers as mainly attributed to post-translational modification (e.g., phosphorylation) of this protein, and these forms are ranging from 30-40 kDa.

The expression of FOSL1 occurred at high levels in smooth muscle cells, bronchial epithelial cells (trachea) and the pancreas, however, its expression is low in other tissues, including the brain and prostate [204]. As seen by [205]. FRA1- gene span about 8.31 kb counting four exons and is located on chromosome 11q13, as indicated in Table 1.6. It includes two regulatory elements, an upstream 5'-

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flanking region and an intragenic sequence that known to regulate its antigen and detected in the cytoplasm under certain conditions including raised levels transcriptional induction. FRA 1 has two signals, one of them it is mainly localized in the nucleus, in conflicting, Fra-1 of oxidative stress, [206] in cancerous tissues [207]. Also, it is located in non-small-cell lung cancer [208]. In human adenomas, adenocarcinomas and neuroendocrine carcinomas, both of nuclear and cytoplasmic expression of Fra-1 are occurred [204, 209].

Table 1.6: HUGO Gene Nomenclature Committee (HGNC) Symbol Report for FOSL1

Approved symbol	FOSL1
Approved name	FOS like 1, AP-1 transcription factor subunit
HGNC Id	HGNC:13718
Previous symbols & names	"FOS like antigen 1"
Synonyms	fra-1
Locus type	gene with protein product
Chromosomal location	11q13.1
Gene family	Fos transcription factor family
Source: https://www.ncbi.nlm.nih.gov/nuccore/?term=fosl1	

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Regarding the wide variety of environmental toxicants, carcinogens and pathogens; Fra-1 transcription is strongly activated; and both of mitogens and inflammatory cytokines play roles in Fosl-1 transcription [210]. FRA-1 can control many essential processes, like inflammation, transformation, proliferation and metastasis. Further, FRA-1 is expressed highly and detected in some cancers such as breast, lung, brain, colon and prostate cancers and its knockdown affects cancer cell progression [204], ovarian cancer [211].

On the other hand, in normal tissues, the FRA expression is related to the apical surface of epithelial cells where it is inaccessible to circulating cytotoxic drugs [212]. Presently, research studies uses FRA as a diagnostic and therapeutic target in clinical and a preclinical way [212, 213]. FRA cell surface localization allows it to occurred as a soluble form when it is spread from the cell surface into the blood [212, 214].

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B4. Histones

Histones are nuclear proteins used to form nucleosome when they bind DNA, and intricate with both the packaging of DNA into chromosomes and the regulation of transcription. So, Histone acetylation or deacetylation plays a major role to regulate the structural dynamics in chromatin regulation throughout transcription [215].

B4.1 Histone Deacetylases

Histone deacetylases (HDACs) are enzymes involved in transcriptional domination [216], especially, when the acetyl groups are removed from the positive charged histone tails and this action make histones to wrap the DNA forcefully. Moreover, they play roles even in deacetylate leading to non-histone proteins degradation via the ubiquitin-proteasome pathway [217], with transcriptional_co-repressors, such as mSin3, N-CoR ,and SMRT; HDACs exist as components in multiprotein complexes [218]. HDACs have catalysed the removal of acetyl groups from lysine residues of histone and non-histone proteins [219], earning the name of 'eraser' enzymes related to cut acetate from acetylated e-amino groups of lysines in histones and other proteins [220].

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The purpose for these complexes to target exact genomic regions through interacting with DNA binding factors including nuclear receptors, methyl-binding proteins, DNA methyltransferases and histone methyltransferases [216]. In eukaryotes, histone modifications play a role in the gene expression regulation. One of these histone modifications is recognized, acetylation, which is recommended as one of the best, characterized also, it has related to active genes. So, along the direct way which is neutralized charges on the histone tail histone acetylation can affect chromatin structure. Also, it can act directly or indirectly as a binding site for proteins to control in gene transcription [221, 222].

Obviously, the best-considered histone modification is in relation with active genes called histone lysine acetylation, histone acetyltransferases (HATs) which can acetyl histones and de acetyl histone by histone deacetylases (HDACs) [223]. HATs and HDACs are controlled in the acetylation condition of a given chromatin locus through adding or eradicating acetyl groups to/from target histones,

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individually [224, 225]. Nuclear enzymes histone deacetylases (HDACs) were originally identified to regularize histone acetylation [226]. HDACs are a member of a multiprotein family, which has a precise function for each member. Also, they have roles to control in a mass (horde) of biological processes [227]. The majority role of HDACs in the human genome function to re-arrange chromatin by removing acetylation at active genes [228].

Mammalian HDACs can involve in multi-purpose, to regulate the gene transcription such as cell growth, survival and proliferation. Cancer development based on the activity of HDACs by numbers of mechanisms can control by HDACs actions, like the enrolment into different co-repressor complexes and the modulation of deacetylase activity by protein-protein interactions or by post-translational variations are principally appropriate ones [229]. As mentioned above, many biological activities are organized by HDACs organized i.e., the enzymatic activity (the ability to deacetylate histone or other non-histone protein substrates), and functional activity (the ability to regulate transcription and other biological processes).

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Actually, the biological functions of HDACs are not exactly based on their enzymatic activity in all Settings [230, 231]. On the other hand, there are no considerable variations in HDACs expression in normal tissues and cancerous tissues detected but there was evidence that the HDACs expression pattern differences in relation to tissue type. Result in HDACs inhibitors control in gene expression by encouraging specific changes in gene expression and to consequence a variety of other processes such as differentiation, growth arrest, cytotoxicity and induction of apoptosis [232]. Presently, there is a relation between HDACs and numbers of well distinguished cellular oncogenes and tumour-suppressor genes which dominate to an abnormal enrolment of HDAC activity, which in turn cause changes in gene expression [233, 234].

B4.2 Histone Deacetylase Inhibitors

HDAC inhibitors ($HDACi_s$) have roles in cancer and inflammatory diseases with therapeutic effects [235]. HDACi such as suberoylanilide hydroxamic 81 acid trichostatin-A [216] and valproic acid (VPA) inhibit the majority of HDACs isoforms [236]. Equally, HDACi boost cell cycle growth in

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normal and transformed cells [237, 238]. Although low Concentrations of HDACi mostly induce G1 arrest, high concentrations induce both G1 and G2/M arrests [238, 239]. HDACi has a mechanism involved in dropping expression of pro-angiogenesis factors including HIF-1a and VEGF *in vitro* and *in vivo* [238-240].

B4.3 Classification of HDACs

Based on HDACs homology to yeast proteins, the 18 HDACs located in humans classified into three main classes because there is cumulative proof that not all of them play an essential function. HDACs were classified as follows:

B4.3.1 Class-I

Comprise HDAC1, HDAC2, HDAC3 and HDAC8 which correlate to yeast RPD3 (*Saccharomyces cerevisiae)* and they are expressed in most cell types.

B4.3.2 Class-II

Include HDAC4, HDAC5, HDAC7 and HDAC9 have criteria of yeast HDA1, class- IIa coming up with HDAC6 and HDAC10 contain two catalytic locations. Thus, the expression pattern of this class II is more limited and could play roles in cellular differentiation and developmental processes.

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B4.3.3 Class III

HDACs include sirtuins, have homology to yeast Sir2 and have an absolute requirement for NAD+.

B4.3.4 Class IV

HDAC11 has the same conserved residues in its catalytic centre with class I and class II deacetylases.[241-243].

B4.4 Localization of HDACs

As one of the distinctive characteristics of HDACs (Figure 1.9), nucleocytoplasmic shuttling depend on different classes of HDACs structurally and functionally, HDACs play roles to determine the

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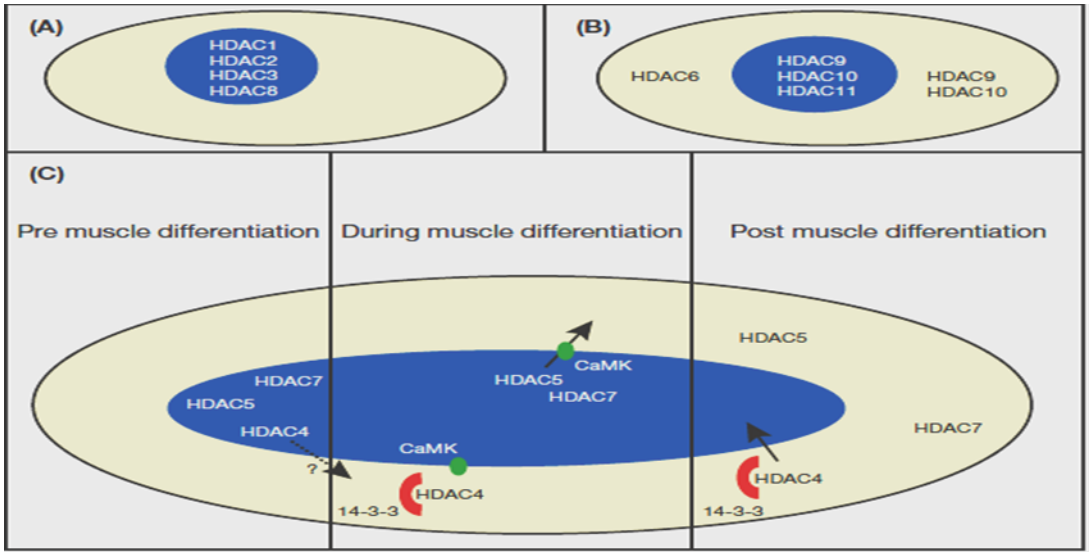
composition and the activity of the corresponding nuclear regulatory complexes [244].



Source: [232]; The nucleus filles in blue. When HDAC4/5 or HDAC7 are phosphorylated, they can retain the cytoplasm by the protein that red labeling. Also, by the calcium calmodin signaling CaMK with green labeling for nucleus export.

Figure 1.9: Localization of each HDAC in cytoplasm or nucleus

Table 1.7: HDACs class I characteristics

	Class I			
	HDAC1	HDAC2	HDAC3	HDAC8
Localization	Nucleus	Nucleus	Nucleus	Nucleus
Size (amino acids)	483	488	428	377
Chromosomal localization	1p34.1	6q21	5q31	Xq13
Catalytic sites	1	1	1	1
Tissue distribution	Ubiquitous	Ubiquitous	Ubiquitous	Ubiquitous? Smooth muscle differentiation
Substrates (partial list)	Androgen receptor, SHP, p53, MyoD, E2F-1, Stat3	Glucocorticoid receptor, YY-1, Bcl-6, Stat3	SHP, YY-1 GATA-1, RelA, Stat3, MEF2D	
Binding partners (partial list)			CDK9, SP1, PP4c	EST1B
Knockout phenotype	EL increased histone acetylation, increase in p21 and p27	Cardiac defect		

Abbreviations: H, heart; SM, skeletal muscle; B, brain; PL, placenta; PA, pancreas; L, liver; K, kidney; S, spleen. EL, embryonic lethal. Stat3, signal transducers and activators of transcription 3; CDK9, cyclin-dependent kinase 9; MMP10, matrix metalloproteinase 10; Hsp90, heat shock protein 90; HIF-1α, hypoxia-inducible factor-1α.

Source: [241]

B4.4.1 Localization of HDACs Class I

As mentioned in (Table 1.7), class I including HDAC1, 2, 3 and 8, some of them have almost 2/3 of the protein which occurred in HDAC1, 2 and 3 and the rest of remaining carboxy-terminal portion covers

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the most divergent sequences, resulting in individual characteristics among these different members [244, 245].

B4.4.1.1 HDAC1

It is a protein identified in 1996 and one of HDACs class I, which recognized in mammals firstly. HDAC1 is the prototype of HDACs family, and play a central role in transcriptional repression mediated by regulation the variety of biological processes, including cell cycle proliferation, progression and differentiation. Amusingly, HDAC1 could include in other cellular actions, such as, DNA replication and chromosome segregation, vie mechanism that does not include transcriptional repression. [246]. Moreover, it is isolated from a human T-cell line using trapoxin-based affinity matrix. It was recognized as a growth factor-an inducible enzyme with HDAC activity in mouse cell [247].

On the other hand, they noticed that HDAC1, 2 and 3 had a variety of upregulations in ovarian cancer tissues and could have roles in ovarian carcinogenesis [248]. As well as, HDAC1, 2 and3 has over-expressed in Hodgkin's Lymphoma (HL). But the decrease of HDAC1 expression in (HL) is indicated for worse prognosis [249]. High levels of HDAC1 have been announced in gastric and prostate cancers [250, 251]. In opposite, HDAC1 with a very low level in colorectal cancer [252, 253] has mentioned that HDAC1 can induce autophagy regarding a specific inhibitor or siRNA [221, 222] Since they discovered HDAC1in 1996, both of nuclear mammalian HDAC2 and HDAC3 are found, and there is a vast similarity between all of them despite HDAC1and 2 confirm more similarity than HDAC3 [254].

B4.4.1.2 HDAC2

Due to HDAC1shares 83 % amino acid identity with another class I member HDAC2 [255], both of HDAC1 and HDAC2 own matching genomic organization and this refers to both of them have arisen from relatively recent gene duplication and probably share Comparable biological functions. HDAC1, 2 have quite a prevalence in several tissues, they are found exclusively in the nucleus when there is a lack of nuclear export signals [256]. As reported HDAC1 and HDAC2 have some overlapping biological functions, But they also have dissimilar and even opposing biological functions [257].

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Separately, HDAC2 has a distinguished job and is a chief participant in HDACs class I. It plays an essential mechanism in tumour development and recurrence [258, 259]. HDAC2 often is overexpressed in several types of tumours [260-265]. In lungs patients, HDAC2 has a noticed reduced expression in protein activity especially in the patients who suffer from a chronic obstructive pulmonary disease.

More than 95% of HDAC2 has low expression in very severe lung disease [266], however, the decreasing of HDAC2 appears clearly when the tumour growth is inhibited and the apoptosis occurs [263, 264, 267]. Even though HDAC2 can play as an oncogene in some tumours, it can use as a monitor at treatment period to follow the efficacy of HDACi during therapy. HDAC2 could use as liberated prognostic marker clinically [268, 269]. Generally, both of HDAC1 and HDAC2 were recognized as components of two multiprotein complexes known as Sin3/HDAC and NuRD/Mi2/NRD [270].

B4.4.1.3 HDAC3

HDAC3 is a member of HDAC class I and located in several tissues throughout the body, including the brain which is considered the most highly expressed one in this group [271]. HDAC3 is typically expressed in neurons; however, it is also the particle of the few HDACs localized in oligodendrocytes [272, 273], and was shown to be up-regulated in lung cancers [266]. One of the essential features that HDAC3 managed is shuttling between nucleus and cytoplasm. HDAC3 is the only enzyme known to shuttle between nucleus, cytoplasm and plasma membrane this shuttling process appears to be an essential feature of its function [274, 275]. HDAC3 functions are dissimilar from HDAC1 and 2. because HDAC3 was not established in either Sin3/HDAC or NURD/Mi2/NRD [270].

B4.4.1.4 HDAC8

It is a special gene in this class I family [276], encodes a 377-amino-acid protein with a predicted molecular mass of 45 kDa [242, 277]. HDAC8 owns HDAC activity on histone H4 peptide substrates as well as on core histones [226]. In most great phosphorylation process which is organized by cyclic AMP-dependent protein kinase A [278]. The phospho-acceptor site of HDAC8 is Ser39 [279]; HDAC8 is an ideal target for posttranslational modification [219]. Although the hyperacetylation of histones H3

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and H4 results from the phosphorylation of HDAC8, the detailed biological and functional consequences of HDAC8 Phosphorylation persists to be clarified, some of the recent studies emphasize that HDAC8 has a significant role in regulating tumour cell proliferation [277]. Despite HDAC8 expressed mainly in the nucleus [241], it can be localized in the cytoplasm of human smooth muscle cell [226, 280] or specialized cellular organelles [220]. Moreover, several human tissues can express HDAC8 including; brain, liver, lung, heart, pancreas, placenta, prostate and kidney [277].

Although HDAC8 follows this group, it has the least studied and understood. At the start, HDAC8 cDNA was recognized via three independent groups using sequence homology database searches for the class I HDACs [242, 277, 281]. HDAC8 has a single structure of α/β domain, that comprises an eight-stranded parallel β sheet sandwiched between 13 α helices, also, using different HDAC inhibitors showed that HDAC8 has a restrictive inhibition pattern diverse than that of other class I HDACs. [167, 282].

B4.4.2 Localization of Class II of HDACs

Class II which identified as HDACs 4–7, has distinguished possess make it differs from the first class; the size of these proteins are larger than class I, also, in HDA4, 5 and 7 the catalytic domain is located in carboxy-terminal half of the protein (Table 1.8) [244]. Class II members regulate the expression of the gene through their enrolment by transcription factors during tissue growth and development [230]. In this study, we will focus on HDAC5, 6 and 7.

B4.4.2.1 HDAC5

HDAC5 belongs to the class II HDAC alpha family regarding wide substrates and it is a protein having two transcript variants encoding two different isoforms for this gene. Because of HDAC5 has owned many HDAC actions and it plays role in repressing transcription if bound to a promoter so it has co-immunoprecipitates with HDAC3, HDAC4 and may form multi-complex proteins [283-285]. HDAC5 has expressed in several cancers, however, it plays a certain role in cellular mechanism including cell proliferation, cell cycle progression and apoptosis [286-288]. HDAC5 has shown decreased levels of

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RNA in patient with a smoking history [289, 290] and colorectal cancer patients. Nevertheless, the real function of HDAC5 in colorectal cancer is still unknown [291-293].

Table 1.8: HDACs class IIA, IIB and class IV characteristics

Class IIA				Class IIB		Class IV
HDAC4	HDAC5	HDAC7	HDAC9	HDAC6	HDAC10	HDAC11
Nucleus/cytoplasm 1,084 2q37.2	Nucleus/cytoplasm 1,122 17q21	Nucleus/cytoplasm 855 12q13	Nucleus/cytoplasm 1,011 7p21-p15	Mostly cytoplasm 1,215 Xp11.22-23	Mostly cytoplasm 669 22q13.31-q13.33	Nucleus/cytoplasm 347 3p25.2
1 H, SM, B	1 H, SM, B	1 H, PL, PA, SM	1 B, SM	2 H, L, K, PA	1 L, S, K	2 B, H, SM, K
GCMa, GATA-1, HP-1	GCMa, Smad7, HP-1	PLAG1, PLAG2		α-Tubulin, Hsp90, SHP, Smad7		
ANKRA, RFXANK	CAMPTA, REA, estrogen receptor	FOX3P, HIF-1α, Bcl-6, endothelin receptor, α-actinin 1, α-actinin 4, androgen receptor, Tip60	FOX3P	Runx2		
Defects in chondrocyte differentiation	Cardiac defect	Maintenance of vascular integrity, increase in MMP10	Cardiac defect			

Source: [241]

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B4.4.2.2 HDAC6

HDAC6 is a unique deacetylase, possessing two internal duplications of two catalytic domains, [244, 285, 294-297] which could be made it the contrast member in this class functionally [244, 296-298]. For this reason, HDAC6 could be involved in many functions, HDAC6 [299]. It has a significant control in the misfolded protein-induced stress, and recently, HDAC6 was also found to govern the stability of the cellular pool of ubiquitinated protein via its ubiquitin-binding action [299]. In addition, it has a nuclear localization signal, a nuclear export signal and a tetradecapeptide repeat domain [300]. The ubiquitin-binding zinc finger of HDAC6 connected to mono- and polyubiquitin in addition to the ubiquitinated proteins [301, 302]. Also has an ubiquitin-binding zinc finger [301, 303]. It is important to realize, there is a relation among tumorigenesis, cell survival and overabundance HDAC6 due to, it is supposed to use as a prognostic marker.

In cancer, under those circumstances, when the expressed signal of HDAC6 is blocked as in multiple myeloma cells it result in apoptosis. On the other hand, HDAC6 has features to be a cancer drug target because of it being able to transfer normal cells to cancer cells [304]. In sequence, HDAC6 adjusts cell

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motility when interacting with cortactin [305] and HDAC6 has high affinity to bind with ubiquitinated proteins [306].

Verdal and Seigneurin-Bernyhave have proved by one of the studies that , HDAC6 can locate in cytoplasm more than in the nucleus, as well as, many HDAC6 proteins could translocate to the nucleus [298, 307]. HDAC6 is located on Xp11 chromosome and found in the brain, breast, colon, ovary, pancreas, prostate and heart, and could be excess in brain cancer, breast, ovary and pancreatic cancer [232]. Besides, HDAC6 can express in cancer cells namely; SKOV-3, SKBR3 and MCF7, and anoikis in the SKOV-3 cells [308].

B4.4.2.3 HDAC7

HDAC7 is one of HDACs class IIa that owns an important role in cancer cell proliferation and in several cancer cell lines. Knockdown of HDAC7 resulted significantly in G1/S arrest [309]. HDAC7 conducted as an significant player in tumour initiation and development, which is could be involved in tumour angiogenesis action as a vital factor for solid tumour growth and metastasis [310]. HDAC7 has a role in regulating angiogenesis and it proposed to regulate apoptosis in a cancer cell [311].

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In contrast, there is a lack of data that possess HDAC7 roles in cancer cell proliferation [312]. HDAC7 have insufficient deacetylase role that emerge the necessity of association that needs it from class I HDAC (HDAC3), and to suppress gene expression [313, 314]. HDAC7 possesses many functions to regulate the proliferate of endothelial cell (EC) [315] and to regulate the angiogenesis and vascular cell homeostasis via repressing the expression of matrix metalloproteinase (MMP) 10 in a MEF2-dependent manner during cardiovascular development and disease [316]. Under those circumstances, HDAC7 has presented a new possible mechanism of carcinogenesis regarding its regulation function [317, 318]. A partial list of protein substrates of HDACs is depicted in Table 1.9 and the implications of HDACs in cancer expression based functional studies are summarized in Table 1.10 and Figure 1.10. Thus, all members of HDAC family play pivotal roles in mediating the hallmarks of cancer cell biology. They play roles in differentiation, apoptosis, inhibition of migration, cell cycle inhibition, susceptibility to chemotherapy and anti-angiogenesis [319].

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Table 1.9: Partial list of protein substrates of HDACs

Functional Group	Protein	HDAC Implicated
Structural protein	α-Tubulin	HDAC6
Chaperone protein	Hsp90	HDAC6
DNA binding nuclear receptors	Androgen receptor	HDAC1
	Glucocorticoid receptor	HDAC2
	Estrogen receptor α	ND
	SHP	HDAC1, HDAC3, HDAC6
DNA binding transcription factors	p53	HDAC1
	p73	ND
	MEF2D	HDAC3
	GCMa	HDAC1, HDAC3, HDAC4, HDAC5
	YY1	HDAC1, HDAC2, HDAC3
	GATA-1	HDAC3, HDAC4, HDAC5
	GATA-2	HDAC3, HDAC5
	GATA-3	ND
	MyoD	HDAC1
	E2F-1	HDAC1
	E2F-2	ND
	E2F-3	ND
	RelA (in NF-κB)	HDAC3
	PLAG1, PLAG2	HDAC7
	Bcl-6	HDAC2
	c-Myc	ND
	EKLF	ND
	HIF-1α	ND
Transcription coregulators	Rb	ND
	PGC-1α	Class III
	DEK	ND
Chromatin remodeling	HMG-A1	ND
	HMG-B1	ND
	HMG-B2	ND
	HMG-N2	ND
	HMGI(Y)	ND
	SRY	HDAC3
Signaling mediators	Stat3	HDAC1, HDAC2, HDAC3
	Smad7	HDAC1, HDAC3, HDAC2, HDAC5, HDAC6
	IRS-1	ND
	β-Catenin	ND
DNA repair enzymes	Ku70	ND
	WRN	ND
Nuclear import	Importin-α7	ND

Abbreviations: ND, not determined. HMG, high mobility group.

Source: [241]

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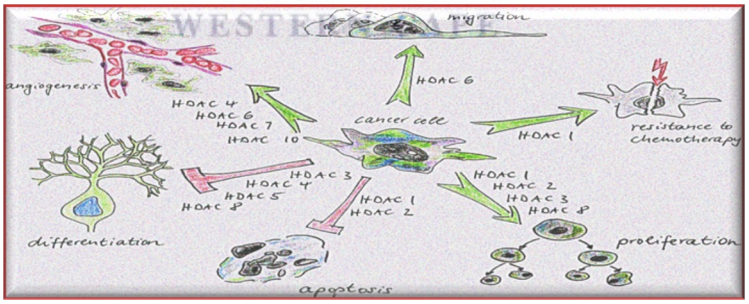

Source: [319]

Figure 1.10: The function of members of the HDAC family in cancer

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Table 1.10: HDACs in cancer: expression and functional studies

Class	Family member	Expression in tumour tissues	Function in cancer cells
Class I	**HDAC1**	Gastric cancer: elevated expression, associated with nodal spread and poor prognosis; pancreatic cancer: expression associated with de-differentiation, enhanced proliferation and poor prognosis; colorectal cancer: increased expression associated with poor prognosis; prostate cancer: increased in high grade, hormone refractory cancers; hepatocellular carcinoma: high expression associated with portal vein invasion, poor differentiation, advanced TNM stage.	Cervical cancer cells: HDAC1 knockdown results in inhibition of proliferation and induction of autophagy; osteosarcoma and breast cancer cells: knockdown causes cell cycle arrest, growth inhibition, apoptosis; colon cancer cells: knockdown suppresses growth; prostate cancer: overexpression increases proliferation and dedifferentiation; neuroblastoma cells: knockdown sensitizes for chemotherapy; CLL cells: knockdown sensitizes for TRAIL-apoptosis.
	HDAC2	Colorectal cancer : upregulation in polyps, associated with poor prognosis; cervical carcinoma: high expression in dysplasia; gastric and prostate cancer: increased Expression associated with advanced stage and poor prognosis.	Cervical cancer cells: HDAC2 knockdown results in differentiation, apoptosis and p53 independent p21 expression; breast cancer cells: increased p53 activity, inhibition of proliferation, induction of senescence, induction of apoptosis; colon cancer cells: knockdown causes growth arrest; neuroblastoma cells: knockdown induces apoptosis; genetic HDAC2 mutation reduces intestinal tumour development in APC mice in vivo; CLL cells: knockdown sensitizes for TRAIL-apoptosis
	HDAC3	Gastric, prostate, colorectal cancers : high expression associated with poor prognosis (Together with HDAC1 and 2).	APL cells: HDAC3 associated with PML-RARa fusion protein, knockdown induces differentiation genes; AML: AML-1-ETO binds HDAC3 and HDACs 1, 2, disrupts cell cycle.
	HDAC8	Childhood neuroblastoma: high HDAC8 expression significantly correlates with advanced stage disease, clinical and genetic risk factors and poor long-term survival.	Neuroblastoma cells: HDAC8 knockdown induces differentiation, cell cycle arrest and inhibits clonogenic growth; lung, colon, cervical cancer cells: knockdown of HDAC8 reduces proliferation.
Class IIa	**HDAC4**	Breast cancer: upregulation compared with renal, bladder, colorectal cancer.	APL cells: HDAC4 interacts with PLZF-RARa fusion protein, represses differentiation genes; renal carcinoma cells: knockdown inhibits expression and functional activity of HIF-1a.
	HDAC5	Colorectal cancer: upregulation compared with renal, bladder, breast cancer.	Erythroleukemia : HDAC5 shuttles from nucleus to cytoplasm upon differentiation, interacts with GATA-1.
	HDAC7	Colorectal cancer: high expression compared with bladder, renal, breast cancer tissues.	Endothelial cells: HDAC7 silencing alters morphology, migration and tube-forming capacity.
	HDAC9	ND	ND
Class IIb	**HDAC6**	Oral squamous cell cancer: high expression, increased in advanced stage; breast cancer: high expression correlates with response to endocrine treatment, inverse correlation of expression with survival and tumour size.	Targeted inhibition of HDAC6 leads to acetylation of HSP90 and disruption of its chaperone function, resulting in depletion of pro-growth and pro-survival client proteins including the Bcr-Abl oncoprotein in K562 leukemic cells; HDAC6 targeting blocks EGF induced nuclear translocation of ß-catenin and c-myc expression in colon carcinoma cells; knockdown of HDAC6 causes downregulation of HIF-1a, VEGFR1/2; HDAC6 involved in TGFb induced epithelial-mesenchymal transition of lung carcinoma cells.
	HDAC10	ND	Knockdown of HDAC10 down regulates VEGFR.
Class IV	**HDAC11**	ND	ND

CLL, chronic lymphatic leukemia; APL, acute promyelocytic leukemia; RARa, retinoic acid receptor alpha; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; ND, no data; [319, 320]

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B5. Housekeeping Genes (HKGs)

Different housekeeping gene (HKG) transcripts became used widely via lots of quantitative assays, b-actin, glyceraldehyde-3-phosphate dehydrogenase (G3PDH) or L32, Also, are known as reference genes that used basically to study mRNA expression in related to quantitive polymerase chain reaction (qPCR) which is known as the most techniques to evaluate gene expression, HKG localization, they are expected to be expressed in the tissue or cell type of interest at concentrations similar to genes of interest constitutively but RNA levels could differ on tissue or cell result in experiment conditions[321]. HKG expression is considered as a key to normalize real-time PCR data and to achieve perfect results [322] Housekeeping gene characterized with stable expression in quantification assays, therefore, it used to regulate the alterations in total RNA concentrations, data are normalized to RNA levels of internal control genes [323].

B5.1 Glyceraldehyde Phosphate Dehydrogenase

Glyceraldehyde phosphate dehydrogenase (GAPDH), also known as glyceraldehyde-3-phosphate dehydrogenase (G3PDA), is one of the housekeeping genes that are used regularly to make the assessment of gene expression data and to normalize the data of target gene expression in QPCR technique.[324].

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B5.2 Hypoxanthine Phosphoribosyl Transferase

The human hypoxanthine phosphoribosyltransferase (HPRT), also known as hypoxanthine-guanine phosphoribosyltransferase which it plays a role in recovering purines as an enzyme, and regulates the purine metabolism in man [325]. This gene was distinguished via mapping, molecular cloning and DNA sequencing approach [326].

SECTION C: RESEARCH CONTEXT

C1. Problem Statement and Research Questions

Over the past years, the frequency of ovarian cancer has continued to exist [327], although all the

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research improvements that have progressed in the field of cancer studies and all the new knowledge which related to detecting, diagnosis and prognosis. In close, Epithelial Ovarian Cancer (EOC) is still the disease that is detected at the late stage with imperfect consequence [328], It is aggressively three times more lethal and considered the fourth cause of cancer-associated death in women in the U.S [327, 329].

Women who suffered from advanced stage ovarian cancer, 30% have a 5-year survival rate [330, 331] and 70% of epithelial ovarian cancer symptoms occurred when the EOS at advanced disease stage [330]. While specific markers or several screening trials for early recognition of EOC are accessible, the diagnostic values of these biomarkers are still restricted due to lack of high costs, sensitivity and / or annoyance. As result, there is a dire necessity to detect new diagnostic and prognostic biomarkers for EOC [330, 331]. Since the lack in EOS biomarkers; there are only two biomarkers are conducted by the FDA to screen patients with EOC are HE4 and CA125, and the changes in a patients serum levels precisely reflect regression or progression of the disease.

Besides, it is almost 90% of Patients with EOC have tumours that CA125 or HE4 upregulated [144, 327, 332, 333]. However, 10% of ovarian cancer cases have not elevated serum CA125 levels related to the absence of the authentic marker for monitoring of the disease, while 90% advanced ovarian cancer can be detected with CA125 [327, 333]. To this end, dozens of biomarkers are recognized and two different procedures have been applied namely microarray and proteomic analysis which are QPCR and ICC to detect the different expression of these biomarkers, i.e. if they can be either up- or down-regulated, reliable on their target downstream genes, even though only a small fraction of them spritely or combined to increase the specificity and sensitivity of these biomarkers may actually be of clinical effectiveness as diagnostic/prognostic biomarkers or therapeutic targets.

C2. Purpose of the Study

The purpose of the study was to investigate the expression of the biomarkers (HDAC1, HDAC2, HDCA3, HDAC5, HDAC6, HDAC7, HDAC8, LPAR1, LPAR2, MUC16 and FOSL1) in three different ovarian cell lines. For this study, we have designated the HOSEPiC as a negative control

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sample and SKO-V3 and OAW42 as a target of human ovarian cancer cells *in vitro,* RNA cells expressed readily detectable levels of genes studied.

C3. Aims of the Study

The aim of the proposed study is to highlight the biomarkers, which are regarded as having the ability to diagnose Epithelial Ovarian Cancer (EOC) at an early stage. These biomarkers will be discussed in terms of their expression analysis, which allows for its genomic and proteomic classification. The expression analysis of each biomarker is related to the malignancy of the tumour and therefore advocates its use for potential future improvement of sensitive tumour markers.

Among the various procedures implemented were Genomic, proteomic and transcriptional profiling. These methods were used to recognize new tumour markers for clinical use. In this study, Real-Time Polymerase Chain Reaction, Quantitative Polymerase Chain Reaction and Immunocytochemistry will be used such technique to recognize all the genes that are mentioned above in the cell lines. In addition, to investigate a cost-effective and simple test to detect pre symptomatic ovarian cancers could facilitate earlier diagnosis and treatment. Ovarian cancer cell lines which are (SKOV-3, OAW42 and HOSEPiC) have been employed to understand the fundamental process involved in cancer cell growth, differentiation and proliferation and study the impact of biomarkers (genes) on these cells.

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C4. Objectives of the Study

In this study, the ovarian cancer biomarkers (HDAC1, HDAC2, HDCA3, HDAC5, HDAC6, HDAC7, HDAC8, LPAR1, LPAR2, MUC16 and FOSL1) were selected to:

- Analyze their gene and protein expression profiles in ovarian cell lines (SKOV-3, OAW42 and HOSEPiC)) and to further validate expression profiles via immunocytochemistry.
- Determine expression levels of the candidate genes by using QPCR techniques.
- Delineate genes responsible for the sensitivity of ovarian cancer cell lines to detect cancer at early stage, using cDNA of ovarian cell lines as a template to seek the high or low expression

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of certain genes in human ovarian cell lines (SKOV-3, OAW42 and HOSEPiC).

C5. Hypothesis

We hypothesize that the cancer gene biomarkers (HDAC1, HDAC2, HDCA3, HDAC5, HDAC6, HDAC7, HDAC8, LPAR1, LPAR2, MUC16 and FOSL1) will be differentially expressed in the selected ovarian cancer cell lines and the normal human ovarian surface epithelial cell line.

SECTION D: SUMMARY

This chapter provided the introduction and literature review on EOC and involve the epidemiology of the disease, risk factors and staging, diagnostic biomarkers, histopathologic characteristics, detection, diagnosis and prognosis, clinical presentation, signs and symptoms, molecular diagnosis, genetics and genomics and therapeutic landscape. In addition, the chapter underscored the biomarkers and the techniques used to analyse the expression of each marker in the ovarian cancer cell lines in relative to the gene expressions in human ovarian surface epithelial cell line, which is used as a control. Finally, the chapter outlined the research context of the study in terms of the problem statement, aims and objectives and hypothesis.

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CHAPTER 2

Research Methodology

2.1 Experimental Design

The focus of this chapter is to outline and describe the research methodology and experimental design that has been chosen for the study (Figure 2.1). It summarizes the materials and methods used such as chemicals required, biological kits and the maintenance of the parental human ovarian carcinoma cell line SK-OV-3 [SKOV3; SKOV-3] (ATCC® HTB-77™, purchased from American Type Culture Collection; ovarian cancer cell line, human ovarian cyst adenocarcinoma cell line (OAW-42). Further details of the experimental design are covered in the subsections that follow. The statistical methods used for data analysis are also described.

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2.2 Materials

Materials and chemicals used in this study are listed in Tables 2.1 to 2.5.

Table 2.1: General chemicals and materials suppliers

Item	Supplier
1X Phosphate Buffer Saline PBS, pH 7.2	Invitrogen
0.4 % Trypan blue stain	Invitrogen
6X Orange DNA loading dye	Fermentas
Agarose	Lonza
Dimethylsulphoxide (DMSO)	Sigma
DNA molecular weight marker ladder(100 bp)	Fermentas
Ethanol	KIMIX
Ethylenediaminetetraacetic acid (EDTA)	Merck
Methanol	Merck
Tris (hydroxymethyl) aminoethane	Merck

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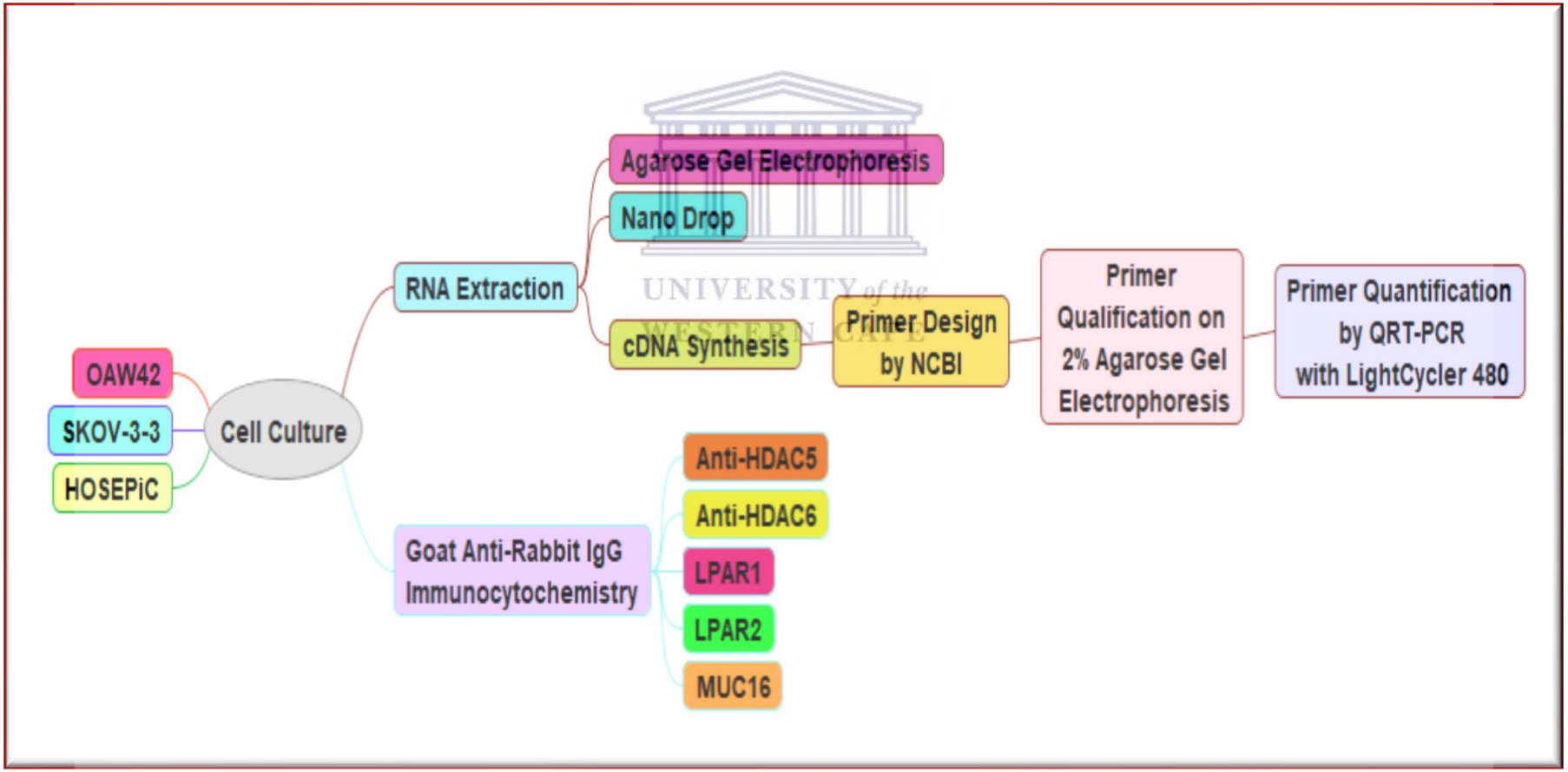

Figure 2.1: Experimental design

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Table 2.2: Cell culture materials and suppliers

Item	Supplier
Dulbecco Modified Eagle's Medium (DMEM)	Lonza
Foetal Bovine Serum (FBS)	Biochrom
Penicillin/Streptomycin (10.000 U/ml)	Lonza
Trypsin (2.5 %)	Gibco®
Ovarian Epithelial Cell Medium (OEpiCM) Media Cat. #7311)	Inqapa
Trypsin/EDTA Solution (T/E, Cat. #0103)	Inqapa
T/E Neutralization Solution(TNS, cat# 0113)	Inqapa
DPBS (Ca^{2+} - and Mg^{2+} - Free cat. #0303)	Inqapa
Poly- L-Lysin(PLL) 2 µg/cm2 Kit	Inqapa
PCR Master Mix (2X)	ThermoScientific
KAPA SYBER® FAST qPCR Kit for LightCycler®480	KAPABiosystems
NucleoSpin® RNA/Protein Extraction Kit	Macherey-Nagel GmbH
Transcriptor First Strand cDNA Synthesis Kit	Roche

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Table 2.3: Stock solutions and buffers

Stock Solution Buffers	Composition
1X Tris-EDTA (TE)	10 mM Tris-HCl (pH 8.0), 1 mM EDTA
10 X Tris, Boric Acid and EDTA (TBE)	0.9 M Tris, 0.9 M Boric Acid and 25 mM EDTA, pH 8.3
1 X Tris, Boric Acid and EDTA (TBE)	10 X TBE was diluted 1:10 times
Paraformaldehyde [334] 4 % (w/v)	Prepared in half the final volume distilled water, which was heated to 60 °C, the pH was adjusted to 7.4, the solution was made up to final volume, filter sterilized and stored at 4 °C

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Table 2.4: Equipment are used and supplier

Instrument	Supplier
Axioplan 2 Imaging Fluorescence Microscope	Zeiss
Basic 20 Ph-Meter	LASEC
Biosepectrum® Imaging System	UVP
Automated Cell Counter TC20™	Bio-Rad
Eppendorf 5417 R Microcentrifuge with Rotor F45-30-11	Eppendorf
GeneAmp PCR System 2700	Applied Biosystems
Inverted Light Microscope	Nikon TMS-F
Leica EC3 Digital Camera	Leica Microsystems Ltd
NanoDrop ND1000	ThermoScientific
Qubit®2.0 Fluorometer	Invitrogen
Sorvall TC6 Centrifuge H400	American Instrument Exchange, Inc.
Thermomixer Comfort 1.5 ml	Eppendorf
Vortex Mixer	Labnet International Inc.
Water Jacketed CO2 incubator	LASEC
Waterbath	Labcon

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Table 2.5: Cell lines and the media

Cell line	Organism	Tissue	Media
OAW42	Human	Epithelial ovarian cancer	DMEM: supplemented with 10% FBS, 1% antibiotic / antimycotic, 0.5% gentamycin and 1% glutamax
SKOV-3	Human	Epithelial ovarian cancer	DMEM: supplemented with 10% FBS, 1% antibiotic / antimycotic, 0.5% gentamycin and 1% glutamax
HOSEPiC	Human	Epithelial surface immortalized cells	OEpiCM consists of 500 ml of basal medium, 5 ml of Ovarian Epithelial Cell Growth Supplement (OEpiCGS, Cat. No. 7352) and 5 ml of penicillin/streptomycin solution

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Table 2.6: Antibodies used in imunocytochemistry

Marker	Manufacturer	Clone	Dilution	Type of Antibody
HDAC5	ThermoFisher	ab55403	1/100	Polyclonal
HDAC6	Biocom	ab56926	Use a concentration of 10 µg/ml	Polyclonal
LPAR1	Biocom	ab84788	Use a concentration of 5 µg/ml	Polyclonal
LPAR2	Biocom	ab130280		Polyclonal
MUC16	ThermoFisher	ab134093	1/100 - 1/250.	Monoclonal
Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488)	Biocom	AB150077		

2. 3 Methodology

2.3.1 SKOV-3 and OAW42 Cell Culture

SKOV-3 (also variously abbreviated as SK-OV-3; SKOV-3] was derived from ascites of a patient with ovarian adenocarcinoma, was generously obtained from Prof Hiss' laboratory at passage 889 as well as OAW42 is from an ascites of a patient with ovarian cystadenocarcinoma. It was generously obtained from Prof Hiss lab at passage 645. Both of cell lines sorted at -80°C. Both SKOV-3 and OAW42 were grown in T-25 flask which included media Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS (Hyclone), 1% antibiotic/antimycotic (Sigma), 0.5% gentamicin (Sigma) and 1% glutamax (Gibco) at 37°C with 5% CO2.

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2.3.1.1 Thawing of Cells

After the cryovials were placed in water bath at 37 C° for 1-2 min for thawing. Once the ice was melted the 1.5 ml contents of cryovial were transferred to a 15-ml tube which contained 5 ml of pre-warmed DMEM, the 15-ml tube was put directly in the centrifuge for 3 min at 3000 rpm. The cells pellet was re-suspended in 4 ml fresh pre-warmed DMEM after discarding the supernatant.

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2.3.1.2 Cell Culture

After thawing, the cells were seeded in in 25 cm^2 cell culture flasks and incubated in an atmosphere of 5 % CO_2 at 37 °C in humidified incubator water-jacketed CO_2 incubator. These cells spent almost two weeks to be confluent and reach to 80-90% of cell density and the medium must be changed every 3-4 days when the cells were checked for normal morphology, density and absence of bacterial and fungal contaminations continuously.

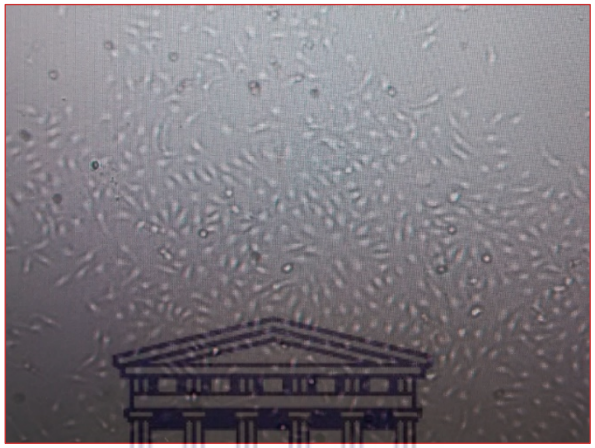

Figure 2.2: Morphological appearance of SKOV-3 in monolayer culture as viewed with an inverted microscope

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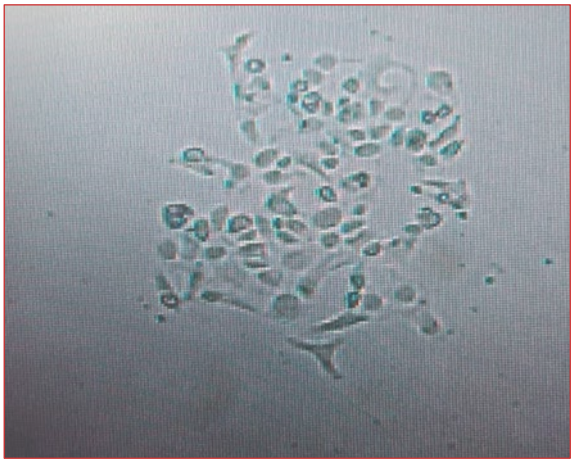

Figure 2.3: Morphological appearance of OAW42 in monolayer culture as viewed with an inverted microscope

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2.3.1.3 Trypsinization of Cells

Once the cells reached 80-90% confluence, the media was discarded and the cells were washed twice with 2.5 ml PBS for each wash and 2.5 ml of 0.25 % trypsin solution was added to the flask and the flask placed immediately in the incubator for 1-5 min with checking the cells during incubation period by PrimoVert phase contrast microscope (Zeiss). Once the cells detached, about 2.5 ml of pre-warmed media was added to stop the trypsinization reaction. Later, all the flask contents was transferred to 15-ml tubes and then centrifuged for 3 min at 3000 rpm. The supernatant was discarded and the pellet re-suspended in pre-warmed fresh medium.

2.3.1.4 Cell Counting with the Countess Automated Cell Counter

Automated cell counting with the Countess Automated Cell Counter: about 10-20 µl was taken from the cell suspension tube and mixed with an equal volume of 0.4% trypan blue stain, and then the counter slide was loaded with 10 µl of the sample.

2.3.1.5 Cryopreservation of Cells

The cells were suspended in incomplete medium containing 10% dimethylsulphoxide (DMSO) after trypsinization as mentioned in 2.3.1.3 and the cell number determination was done as indicated in 2.3.1.4. The cryovials were prepared properly with complete information such as date of freezing, cell type, the passage number of the cells, the names of the users (initials) and the media for the cell line. 1 ml of the cells (at a concentration of 2-4 x 10^6 cells per ml) was aliquoted into the cryovials, labelled and sealed and were immediately placed at -150°C for long-term storage.

2.3.2 HOSEPiC Cell Line

The HOSEPiC cell line obtained from ScienCell Research Laboratories. HOSEpiC was isolated from a human ovary at passage one and delivered frozen. Each vial contains >5 x 10^5 cells in 1 ml. HOSEpiC is categorized by immunofluorescence with antibodies specific to cytokeratin-14, -18 and -19. HOSEpiC is negative for HIV-1, HBV, HCV, mycoplasma, bacteria, yeast and fungi.

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HOSEpiC is guaranteed to further expand for 5 population doublings under the conditions provided by ScienCell Research Laboratories. Before initiating cell cultures, a non-treated T-75 flask was coated with Poly-L-Lysine (PLL, 10 mg/ml; ScinceCell) to promote cell attachment and growth as follows: 10 ml redistilled water was added to a T-75 flask followed by 15 µl of PLL stock solution and then rapidly mixed. The flask was left in the incubator overnight at 37°C. The next day, the contents of the flask was discarded and the flask washed twice with redistilled water. About 10-20 ml of complete Ovarian Epithelial Cell Medium (OEpiCM; Catalogue #7311) was added to a T-75 flask. The HOSEpiC cryovial was thawed and the contents (1 ml/vial) was re-suspended and poured carefully into the equilibrated PLL-coated culture vessels. A seeding density of more than 5 x 10^5 cells/cm^2 was achieved and the cells incubated in a humidified atmosphere (relative humidity~ 80%) of 5% CO_2 and 37°C. After 2-3 days, the medium was aspirated to remove any remaining DMSO and unattached cells. When the cells reached 70-80% confluence, medium was replenished every other day, until the cell culture reached about 90% confluence.

Once the cells were confluent, the cells were trypsinized and prepared for seeding into another T-75 flask coated with PLL (2 µg/cm^2) done on the day before. All reagents: OEpiCM, trypsin/EDTA solution, trypsin neutralization solution and DPBS (Ca^{2+}- and Mg^{2+}-free) were equilibrated to room temperature. The cells were rinsed firstly with 5 ml DPBS, then 5 ml of trypsin/EDTA solution was added to the T-75 flask and gently agitated to make sure all the cells were covered by the trypsin/EDTA solution. The cells were incubated at 37°C for 5 min until the cells detached. While the cells incubated, 5 ml FBS was poured into a 50-ml tube to transfer the cell suspension from the flask and re-incubated for 1 or 2 min more. After the incubation, both sides of the flask were tapped softly to dislocate cells from the surface and they were checked under PrimoVert phase-contrast microscope (Zeiss). To make sure all the cells detached, the flask was rinsed twice with 5 ml of TNS solution and the residual cells transferred to the 50-ml centrifuge tube which contained the cells. The cells were spun in the 50-ml tube at 3000 rpm for 3 min. Cells were seeded as described above or cryopreserved for future use.

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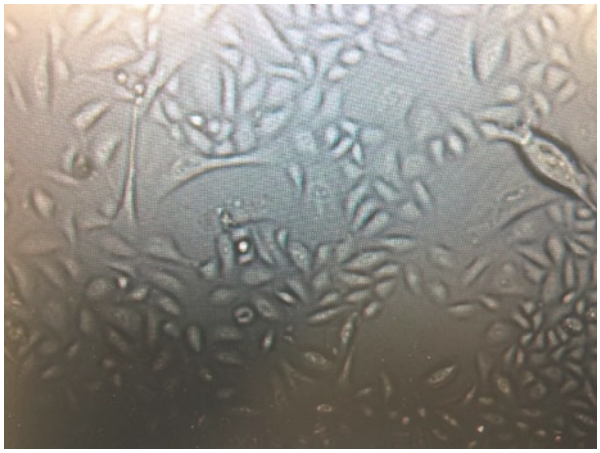

Figure 2.4: Morphological appearance of HOSEpiC ovarian surface epithelium cells in monolayer culture as viewed with an inverted microscope

2.3.2.1 Poly-L-Lysine Stock Solution Product Overview

As described previously [335], PLL is a synthetic compound that has a highly positive charge due to the amino acid side chains which support and increase cell adhesion by shifting surface charging onto the culture substrate. Also, it supports the nutrient outgrowth. PLL is available as a stock solution that includes polymers in the 70 – 150 kDa range. The commercial vial contained 10 mg/ml (Cat. 30413 ScienCell) filtered and sterile, and the toxicity of PLL is ≤ 20 by LAL assay [335].

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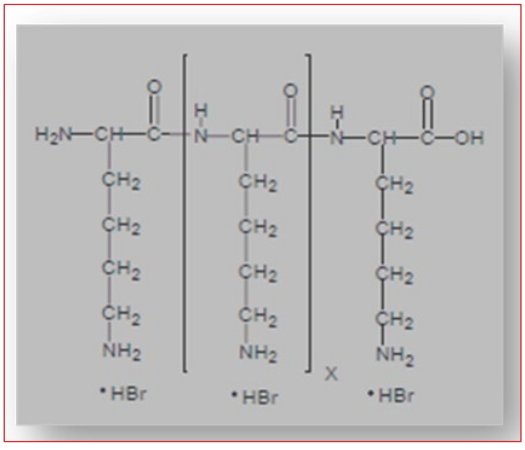

Source: [336]

Figure 2.5: Poly-L-lysine structure as a charge enhancer

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2.3.2 RNA Extraction

According to Roche, the kit is suited for total RNA to be isolated from 1 x 10^6 cultured cells. The cultured cells were suspended in 200 µl PBS. From green capped-vial containing lysis/binding buffer, about 400 µl was added and vortexed for 15 s. The sample was transferred to a High Filter Tube by inserting one High Filter w1 Tube in one Collection Tube; then, the entire sample was pipetted into the upper reservoir of the Filter Tube (max. 700 µl) and it was directly inserted into a standard table top centrifuge and spun for 15 s at 8 000 x g.

After centrifugation, the filter tube was removed from the Collection Tube and the flow-through was discarded and recombined with the Filter Tube and then used in Collection tube. After re-insertion into the Filter Tube: from DNase incubation buffer (white cap), 90 µl was pipetted per sample into a sterile reaction tube followed by the addition of 10 µl DNase I.

After that, the sample was mixed and incubated, the solution pipetted onto the glass filter fleece in the upper reservoir of the Filter Tube. After standing for 15 min at 15 - 25°C, 500 µl wash buffer I was added to the upper reservoir of the Filter Tube assembly and spun for 15 s at 8 000 x g. Again the flow-through was discarded and recombined with the Filter Tube and used with the Collection Tube, i.e., 500 µl wash buffer II from the blue capped tube was added to the upper reservoir of the Filter Tube assembly, the spun at 15 s at 8 000 x g; the flow-through was discarded and combined with the Filter Tube and used with the Collection Tube. About 200 µl wash buffer II was added to the upper reservoir of the Filter Tube assembly and spun for 2 min at maximum speed (nearly 13 000 x g) to remove any residual wash buffer.

After discarding the Collection Tube, the Filter Tube was inserted into a clean, sterile 1.5-ml microcentrifuge tube. Finally, to elute the RNA, 50 µl from the Elution Buffer was added to the upper reservoir of the Filter Tube and centrifuged for 1 min at 8 000 x g to yield the extracted RNA.

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2.3.2.1 Preparation of 1% Agarose Gel for RNA Electrophoresis

To prepare a 1 % (w/v) agarose gel, the agarose powder was dissolved in 1X TBE buffer then heated for 2-3 min in a microwave oven until the mixture reached transparency. Once the mixture have cooled, 2-3 µl red gel dye (Biotium) was added which is an alternative dye to Ethidium Bromide (EB) and is more sensitive and less toxic than EB.

2.3.2.2 Agarose Gel Electrophoresis of RNA

Assessment of the integrity of the electrophoresed RNA on 1% agarose gel was performed (Figures 2.6 – 2.8). The RNA sample was prepared by adding 3 µg to 1 µl of 6X loading buffer (Fermentas) and then the mixture was heated for 4 min at 65°C by using a Thermomixer before loading the sample into gel wells. To estimate the size of RNA fragments, Gene Ruler 1 kb DNA ladder (Fermentas) was loaded as standard. The 1% agarose gel was buffered with 1X TBE Buffer (pH ~ 8.3) for the sample to load on. The sample was electrophoresed at 90 V constant for 60 min using a Power Pac Basic HC 300W system (Bio-Rad). The gel was imaged with an UVP Transilluminator and photographed with the BioImaging Systems Chemi HR 410 Camera using a Syber-Gold filter at 302 nm.

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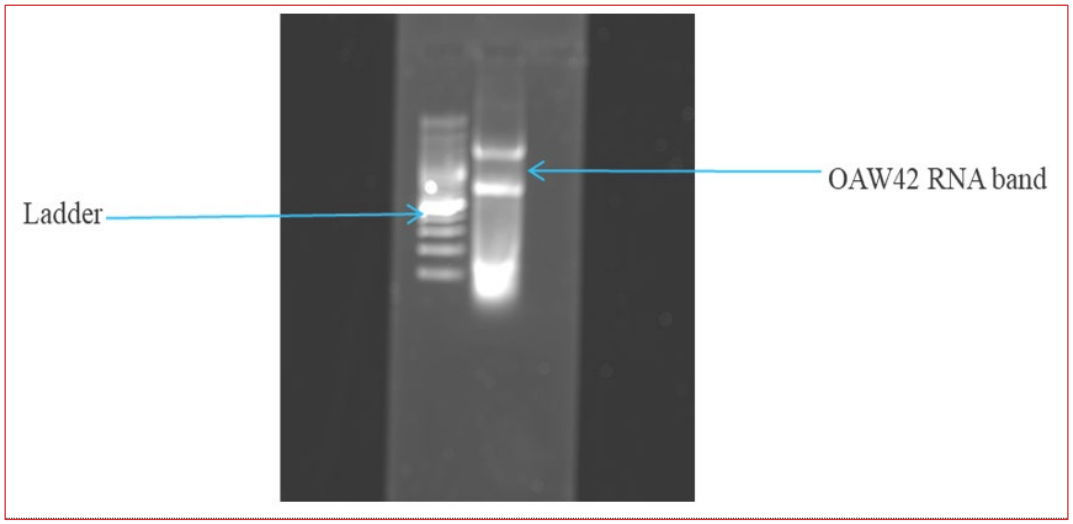

Figure 2.6: OAW42 RNA bands run on the agarose gel and DNA ladder

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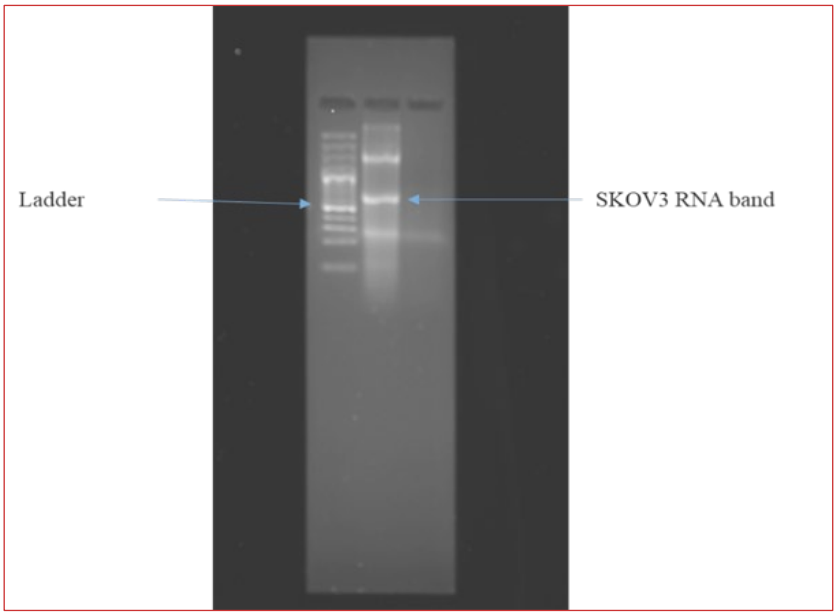

Figure 2.7: SKOV-3 RNA band run on the agarose gel

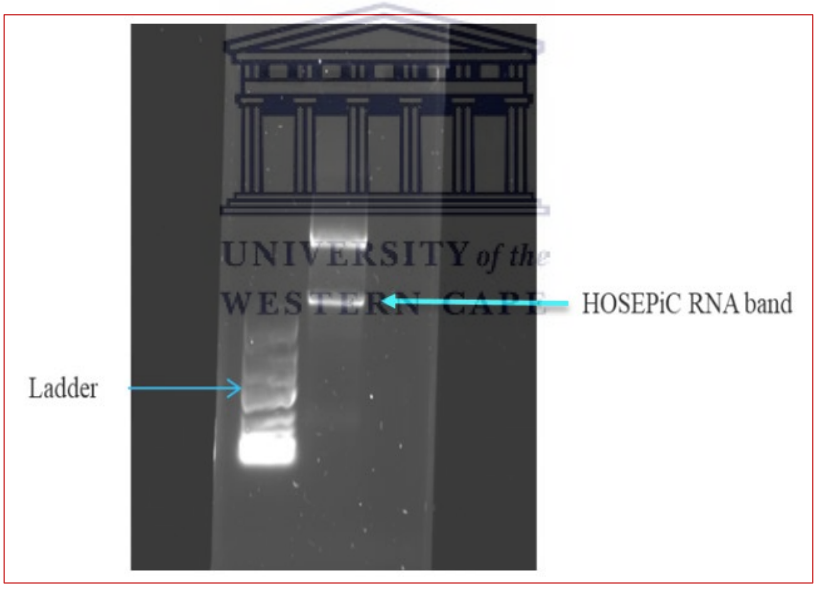

Figure 2.8: HOSEPiC RNA run on the agarose gel

2.3.3 cDNA Synthesis

According to the Transcript First Strand cDNA Synthesis kit by Roche, the cDNA synthesis protocol is as follows (Table 2.7): A sterile, nuclease thin-walled PCR tube which contained 5.5 µg of total extracted RNA from the respective cell line (SKOV-3, OAW42 and HOSEPiC) was

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placed on ice and the following additions made: 1 µl Anchored-oligo [dT] 18 primer, 2 µl random hexamer primer and 6.5 µl water (PCR) grade to yield a total volume of 13 µl.

Table 2.7: Exact volumes of cDNA synthesis procedure

Component	Mass / Volume	Final Concentration
Total RNA	5.5 µg	1µg total RNA
Anchored-oligo[dT]18 primer	1 µl	2.5 µM
Random hexamer primer	2 µl	60 µM
Water, PCR-grade	Variable (6.5 µl) to make total volume=13 µl	
Total volume	13 µl	

The template-primer mixture was denatured by heating the tube at 65°C in a thermal block cycler with heated lid (to minimize evaporation) for 10 min. Once the cycle stopped, the tube was cooled immediately in ice. Later, about 4 µl 5X Transcriptor Reverse Transcriptase Reaction Buffer, 0.5 µl Protector RNase Inhibitor (40 U/µl), 2µl Deoxynucleotide mix, 10 mM each and 0.5 µl Transcriptor Reverse Transcriptase, 20 U/µl were added to achieve a final volume 20 µl.

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Table 2.8: Optimum volumes for the cDNA synthesis procedure

Component	Volume	Final concentration
Transcriptor Reverse Transcriptase Reaction Buffer, 5X concentration	4 µl	1X[8 mM $MgCl_2$]
Protector RNAse Inhibitor, 40 U/µl	0.5 µl	20 U
Deoxynucleotide mix, 10 mM each	2 µl	1 mM each
Transcriptor Reverse Transcriptase, 20 U/µl	0.5 µl	10 U
Final volume	20 µl	

All the reagents in the tube were mixed (**NOT** vortexed) cautiously followed by brief centrifugation and the sample at the bottom of the tube was collected. cDNA was quantified as explained in (sections 2.3.4 and 2.3.5).

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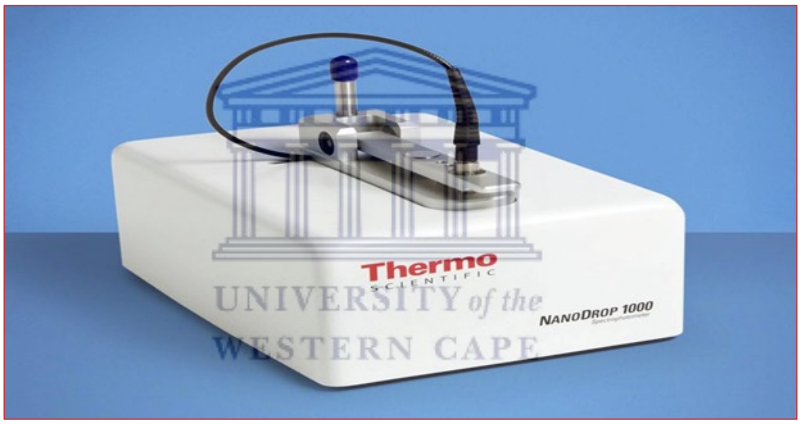
2.3.4 Nanodrop Determination of cDNA and RNA Concentration

The concentrations of RNA and cDNA were determined with a NanoDrop 2000C spectrophotometer system (Figure 2.9). Once the system was turned on and Nucleotide icon was selected called blank, 2 µl of deionized water or elution buffer was added to the optical surface then the optical arm was closed and the blank icon was set to zero. The arm was opened and the optical surface wiped. Once the zero set has finished about 1-2 µl of eluted RNA was pipetted and pressed on the optical surface and the arm closed; thus, click RNA measure icon to quantify. To estimate the purity of RNA or cDNA, sample was measured at 260 nm / 280 nm absorbance. The ratio of RNA was ~ 2.0 and for cDNA was ~1.8. The measurement of each sample was repeated three times.



https://www.biocompare.com/Product-Reviews/41292-NanoDrop-ND-1000-From-Thermo-Scientific/

Figure 2.9: Nanodrop apparatus

2.3.5 Determination of cDNA and RNA Concentration by IQuant™ NGS-BR dsDNA Assay

This assay is used to identify the quality of cDNA or RNA or protein (Figure 2.10). Preparing the master mix was the first step which was as follows: the kit included: Buffer solution, Activator, Std1 and Std2. The master mix solution was constituted in a 1.5-ml tube with about 1000 µl buffer and 5 µl from activator then in PCR tubes with about 200 µl from the working solution added to Std1 tube, Std2 tube and sample tube. After that, all the tubes were spun down and then left at room

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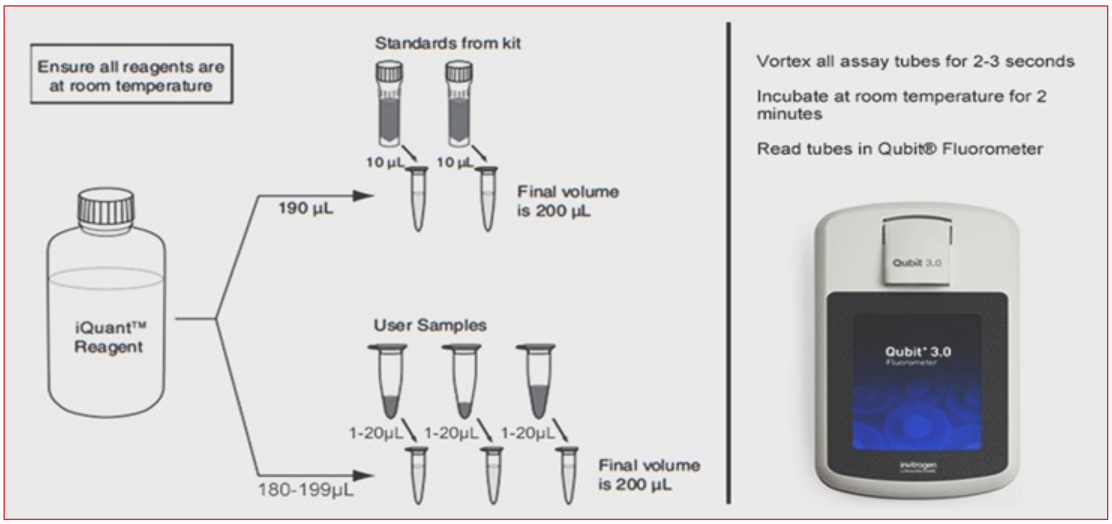
for 2 min. Finally, a fluorescence microplate reader or fluorimeter was used to assess the samples.



Assay to determine the concentration of cDNA and RNA.
https://www.thermofisher.com/za/en/home/industrial/spectroscopy-elemental-isotope-analysis/molecular-spectroscopy/fluorometers/qubit/qubit-assays.html

Figure 2.10: Procedure of the RNAIQuant™ NGS-BR dsDNA assay

2.3.6 Primer Design for qRT-PCR

The primers in this project were designed for the candidate genes that are listed in Table 2.9. The reason for these designs was to amplify a region of the coding sequence of each marker gene using default parameters with the exception of PCR product size parameter, which was set to select primers to amplify the product in the range 100-233 bp. The online Primer3 Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) was used to design these primers. Thus, the mRNA sequences for all the genes were obtained from the National Centre for Biotechnology Information (NCBI). The primers were purchased from Inqaba Biotech (Pretoria, South Africa).

2.3.7 Using Polymerase Chain Reaction (PCR) to Optimize the Proper Conditions for Each Primer

The PCR mixture consisted of 12.5 µl of 2X master mix (Universal Mastermix, catalogue GMO-UN-A100, Europe Diagnosed SA, Liège, Belgium), 1 µl of each primer (forward and reverse), 6

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µl nuclease-free water and 2 µl of template cDNA and 2.5 µl 25 mM $MgCl_2$ to get the total volume which is 25 µl. After thawing, PCR Master Mix (2X) was gently vortexed and briefly centrifuged. In a thin-walled PCR tube on ice, the following components were added for each 25 µl reaction mixture (Table 2.10). The samples were gently vortexed and spun down. PCR was amplified by 7500 Fast Real-Time PCR System (Applied Biosystems, catalogue 4351107, Foster City, CA 94404 USA) using the suggested thermal cycling conditions outlined in Table 2.11.

Table 2.9: Control genes evaluated and oligonucleotide sequences used as primers for amplification

Gene	Ensemble Accession Number	Primer Name	Primer Sequence 5'-3'	Product Size (bp)	TA (°C)
HDAC1	Accession: NM_004964.2	For P HDAC1	GGAAATCTATCGCCCTCA	1687bp	59
		Rev P HDAC1	AATCATAAGCTACCGGACAA		
HDAC2	Accession: NM_001527.3	For P HDAC2	ATAAAGCCACTGCCGAAGAA	245 bp	59
		Rev P HDAC2	TCCTCCAGCCCAATTAACAG		
HDAC3	Accession: NM_003883.3	For P HDAC3	ACGTGGGCAACTTCCACTAC	219bp	59
		Rev P HDAC3	GACTCTTGGTGAAGCCTTGC		
HDAC5	Accession: NM_005474.4	For P HDAC5	GTGACACCGTGTGGAATG AG	243 bp	59
		Rev P HDAC5	AGTCCACGATGAGGACCTTG		
HDAC6	Accession: NM_001321225.1	For P HDAC6	AAGTAGGGAGAACCCCCAGT	203 bp	59
		Rev P HDAC6	GTGCTTCAGCCTCAAGGTTC		
HDAC7	Accession: NM_015401.4	For P HDAC7	CCCAGCAAACCTT CTACCAA	192 bp	59
		Rev P HDAC7	AAGCAGCCAGGTACTCAGGA		
HDAC8	Accession: NM_018486.2	For P HDAC8	GGTGACG TGTCTGATGTTGG	165 bp	59
		Rev P HDAC8	AGCTCCCAGCTGTAAGACCA		
FOSL1	Accession: NM_005438.4	For P FOSL1	AGCTGCAGAAGCAGGAAGGAG	226 bp	61
		Rev P FOSL1	GGAGTTAGGGAGGGTGTGGT		
LPAR1	Accession: NM_001351398.1	For P LPAR1	ATTTCACAGCCCCCAGTTCAC	194 bp	61
		Rev P LPAR1	TAGATTGCCACCATGACCAA		
LPAR2	Accession: NM_004720.5	For P LPAR2	CCAATCTGCTGGTCATAGCA	167 bp	61
		Rev P LPAR2	CCAGCCCTCAAGTGAAAGTC		
MUC16	Accession: NM_024690.2	For P MUC16	AGCATCCTGGACGTAACCAC	173 bp	62
		Rev P MUC16	CAGGTGGAAGGGTGTTCTGT		
GAPDG	Accession: NM_002046.6	For P GAPDH	ACCCACTCCTCCACCTTTG	178 bp	59
		RevP GAPDH	CTCTTGTGCTCTTGCTGGG		
HPRT	NM_000194	For P HPRT	TGACACTGGCAAAACAATGCA	308	59
		Rev P HPRT	GGTCCTTTTCACCAGCAAGCT		

Forward indicated by "for" and reverse primers indicated by "rev", respectively, following the gene symbol. The primers were reconstituted in 1 X TE Buffer and stored at -20°C until required. In addition, the expected product size and calculated annealing temperature for the qRT-PCR reaction are pointed.

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Table 2.10: Reaction volumes for each PCR component

Reagent	Test	Control
PCR Master Mix	12.5 µl	12.5 µl
Forward primer	1.0 µl	1.0 µl
Reverse primer	1.0 µl	1.0 µl
Template DNA	2 µl	
Water, nuclease-free	6 µl	6 µl
$MgCl_2$	2.5 µl	2.5 µl
Total volume	25 µl	25 µl

Table 2.11: Suggested thermal cycling conditions for each PCR steps

Step	Temperature (°C)	Time	Number of Cycles
Initial denaturation	95	5 min	1
Denaturation	94	30 s	25-40
Annealing	Tm±5	30 s	
Extension	72	0.45 min/kb	
Final Extension	72	10 min	1

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2.3.8 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

As mentioned in section 2.3.3, cDNA of the HOSEPiC, OAW42 and SKOV-3 cell lines were synthesized to serve as templates for amplification of genes using the primers listed in Table 2.8. Owing to qPCR analysis, a Roche LightCycler 480 (software Version 1.2.9.11) system using a KAPA SYBR® FAST for ABI Prism® PCR Mix (2X) (Kapa Biosystems), according to manufacturer's datasheet and was optimized for all primer combinations to perform qRT-PCR reactions followed by a dissociation (melt) curve analysis. From cDNA stock solutions, serial dilutions were made for concentrations ranging from 200 ng to 0.02 ng which were used as templates for standard curve analysis which might be used to calculate PCR efficiency and to determine template concentrations; 200 nM forward primer and 200 nM reverse primer were used for each reaction in a final reaction volume of 20 µl as indicated in Table 2.12.

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Water was included as a No Template Control (NTC) to detect contamination. In more detail, to triplicate wells were added 4 µl cDNA as a template and 16 µl of working solution which included 10 µl of KAPA SYBR® FAST for ABI Prism® PCR Mix (2X), 0.4 µl primer forward, 0.4 µl primer reverse, and 5.2 µl nuclease-free water for each gene. In this case, the qPCR experiment estimated the expression intensity for each target gene from template-definite biological conditions, and the expression of each gene was expressed in Cycles to Threshold (Ct) of qPCR, a relative value that typifies the cycle number at which the quantity of amplified DNA achieved the threshold level.

As result of the technical variability between experiments, the Ct needs to be normalized. Also, the differential expression is done gene-by-gene by matching the normalized Ct values (Delta Ct or ΔCt) of all the replicates between three groups of samples, control group (HOSEPiC) and target group (SKOV-3 and OAW42). The expression of mRNA levels for each target gene was detected, as mentioned, with the LightCycler 480 II machine, by using KAPA SYBR® FAST for ABI Prism® PCR Mix (2X), for each target gene.

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Next, the standard curves were created to achieve the relative quantification of each gene expression by debating at least 3 points in triplicate of 10-fold serial dilutions of cDNA in nuclease-free water, starting from 1:10 of a volume of undiluted cDNA recorded from 1.0 µg of total RNA. In addition, the slopes of standard curves ranged from -3.17 to -3.87. Blank and standard controls (GAPDH: glyceraldehyde phosphate dehydrogenase and HPRT: human hypoxanthine phosphoribosyltransferase) as endogenous controls were run in parallel to confirm amplification efficiency in each experiment and to normalize differences in the amount of total cDNA added to each reaction.

HOSEPiC was used as a calibrator sample / reference sample for relative quantification. To obtain the cycle threshold (Ct) values, a threshold of 0.1 was recognized and all reactions were tested in triplicate at 4°C.

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Table 2.12: The exact volumes of qPCR reagents

Reagent	Final concentration
KAPA qPCR Mix (2X)	10 µl
Forward Primers	0.4 µl
Reverse Primers	0.4 µl
Template (cDNA)	0.4 µl
PCR-grade deionized H_2O	Variable
Final Volume	20 µl

This protocol included three steps for QRT-PCR (denaturation, annealing and extension) followed by 35 cycles consistently. Regarding the variability on nucleotide sequences of the gene of interest, these steps had some variations in annealing temperatures. The housekeeping genes HPRT and GAPDH were used in QRT-PCR reaction for all interested genes, as well as for all HOSEPiC, OAW42 and SKOV-3 cell lines.

A 96-well PCR plate on a Roche LightCycler® 480 Gene Scanning Software, Version 1.5 was used to perform the RT-QPCR reaction, as mentioned previously, which describes the standard parameters used for this reaction. In total, the products were kept at -20°C for further analysis such as a 1% agarose gel electrophoresis.

The comparative Ct method which was first stated in 2001 [337] and mentioned as $2^{-\Delta\Delta C}$T method was used to calculate the relative fold changes in gene expression, normalized to housekeeping genes. Quantification of the relative changes in gene expression using real-time PCR requires definite equations, assumptions and the testing of these assumptions to analyze the data correctly. This method will not determine the absolute transcript copy number, rather a fold change. Normalized equation simplifies to expression gene ratio [337].

Equation 1: $R=2^{-}[\Delta ct\ target - \Delta ct\ control]$

Equation 2: $R=2^{-}\Delta\Delta Ct$

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2.3.9 Immunocytochemistry

2.3.9.1 Cell Culture

Immunocytochemistry (ICC) was achieved in four serial steps. First, the cells cultured as described in section 2.3.1. When the cells reached 80-90% confluence, the cells seeded on autoclaved positively-charged glass slides (76 mm x 26 mm; 1.0 mm to 1.2 mm thick). In the second step, the cells were immunostained: cells were fixed to keep the proteins in the cells and conserve their chemical and structural state by washing with 1X 1% PBS for three min then fixed by immersion in 4% paraformaldehyde (PFD) for 20 min at room temperature. Consequently, the cells were washed 3 times for 5 min each single time in PBS, then cells were permeabilized to remove lipids from membranes and to allow the antibodies to cross the cell membranes. Actually, without the permeabilization step the antibodies would be restricted to the outside of the cell due to their size; also, the permeabilization needs fixation and, hence, limits the procedure to revising dead cells. The reason for the washing steps after immunostaining was realistic to eradicate any unbound antibodies. In the third step, the cells visualized by a microscope and images assessments.

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2.3.9.2 Antibodies

The studied antibodies were used at the indicated dilutions as mentioned in Table 2.6. The cell staining and washing—as mentioned in Table 2.12 and Figure 2.13—and antibody incubations were performed at the Histology Laboratory at Tygerberg Hospital in a Leica Bond Autostainer (Figure 2.11).

Figure 2.11: Leica Bond Autostainer (Leica)

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Table 2.12 represents a flowchart of the ICC assay protocol and Figure 2.12 summarizes the four serial steps of the ICC.

Table 2.12: Flowchart of immunocytochemistry (ICC) assay protocol

1
Rinse the fixed cells in PBS.

2
Heat slide in Antigen Retrieval buffer (0.1 M Tris, pH 9.5, 5% urea) in a water bath at 95ºC for 10 min.

3
Rinse the slide 3 times in PBS.

4
Permeabilize the cells in 0.1% Triton X-100 for 10 min at room temperature.

5
Rinse the slide 3 times in PBS.

6
Block the cells in 10% goat serum for 1 hour at room temperature.

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7
Incubate the cells in the primary monoclonal antibodies diluted in 10% goat serum, overnight at 4ºC

8
Rinse the slide 3 times in 1% goat serum

9
Incubate the cells in the fluorophore-conjugated secondary antibodies diluted in 10% goat serum, for 2 hrs at room temperature, away from light.

10
Rinse the slide 3 times in 1% goat serum, away from light.

11
Add DAPI (300 ng/mL) in the final wash (10 min) to stain nuclei

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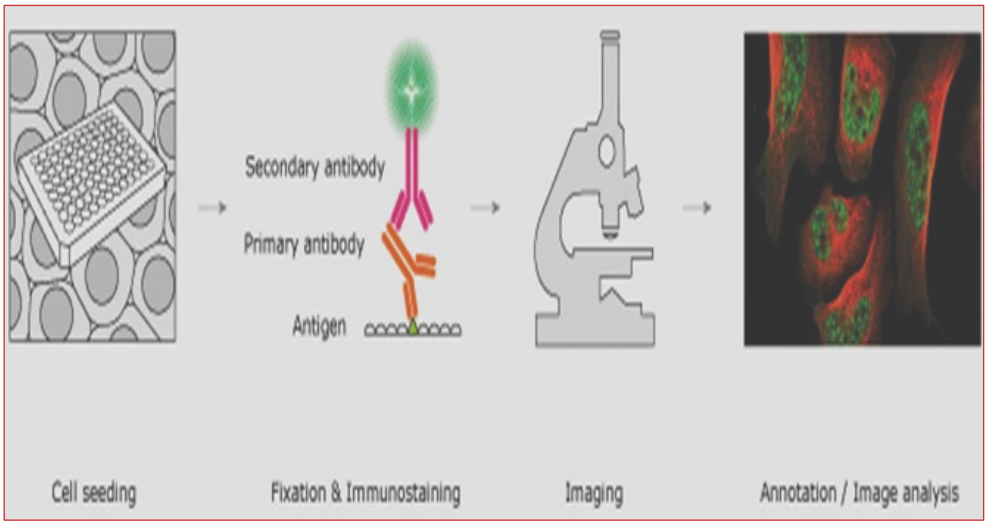

(**i**) Cell seeding, (**ii**) Immunostaining, (**iii**) Imaging, and (**iv**) Image analysis.
https://www.proteinatlas.org/learn/method/immunocytochemistry

Figure 2.12: The four steps of immunocytochemistry (ICC)

2.4 Statistical Analysis

The data were conveyed as the mean±SE. Statistical analysis was achieved with 2-way ANOVA with non-repetitive measurement using GraphPad Prism version 6.0 (GraphPad™, San Diego, US; www.graphpad.com). A value of $p<0.05$ was assumed to be statistically significant. The Pair-Wise Fixed Reallocation Randomisation Test© software version was used to perform the RT-qPCR data analysis.

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CHAPTER 3

Analysis of Gene Expression of Target Candidate Genes in SKOV-3 and OAW42 Ovarian Cancer Cell Lines

3.1 Analysis of Genes Expression Via RT-qPCR

Quantitative PCR (qPCR) or real-time PCR (RT-PCR) is a modification of the traditional end-point PCR technique which allows for the simultaneous detection and quantification of genes in real time [334], and it is confidently established as a major research technology and is the standard for revealing and quantification of RNA targets [338, 339]. Since the first report on the application of this technology in 1993, qPCR has become the preferred method for measuring gene expression [94, 340].

Other applications of qPCR includes the validation of gene expression microarrays, copy number determination for genomic or viral DNA, allelic discrimination assays and mutation detection [341, 342]. Due to the high quality-throughput, synergistically with regular introduction of boosted or original chemistries, more trusty instrumentation and upgraded protocols have also contributed to the improvement of qRT-PCR-based clinical diagnostic assays [339, 342-344].

Despite the extensive utilization qRT-PCR in the diagnosis and the detection of disease-specific prognostic markers in leukaemia and the detection of disease-related mRNA expressed by circulating cancerous cells in patients with solid malignancies, the clinical value of qPCR-related tests in other disease states remains unclear [339]. One of the major issues is the possible technical deficiencies that affect qPCR assay performance and which can lead to erroneous results. Another concern is the limit of detection [288] and analytical sensitivity of these kits which needs extensive development to perform to diagnostic industry standards.

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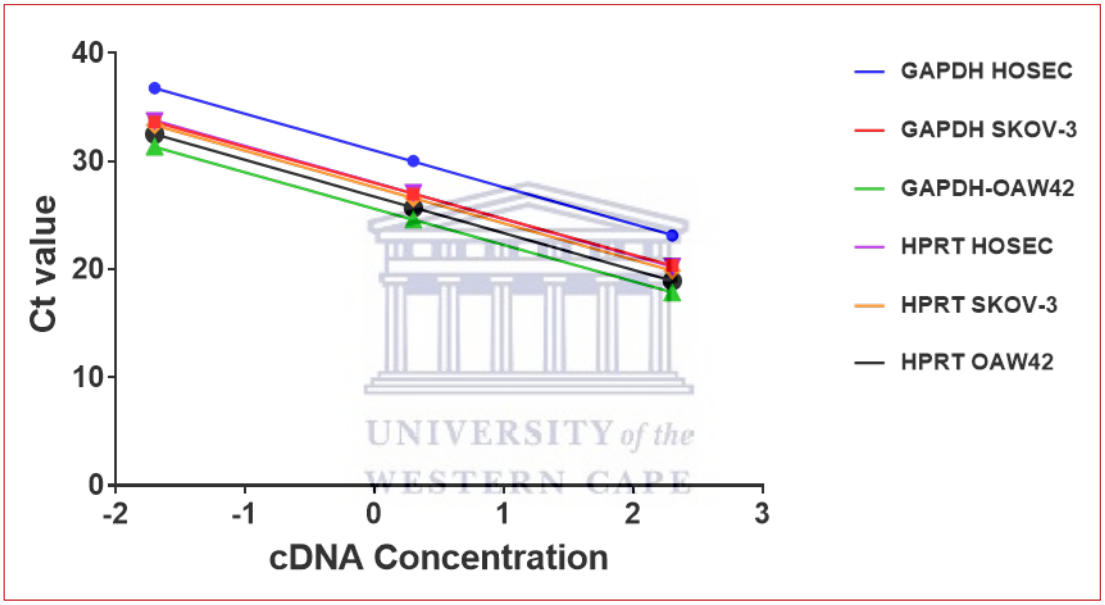
3.2 Presentation and Interpretation of the Results

In this part of the study, the aim was to determine the relative expression of 11 candidate genes in two ovarian cancer cell lines, viz., SKOV-3 and AOW42, compared to the matching control ovarian cell line (HOSEpiC). This was done to aid in the characterization of these genes and to determine whether they are differentially regulated in ovarian cancer. Using a serial dilution of pooled cDNA samples, primer efficiency QC was performed on the primer pairs. The slope of the standard curve should be between -3.2 to -3.5 (Figure 3.1), and illustrate an R^2 value of >0.98 as presented in Table 3.1.



Housekeeping genes (HKGs): Glyceraldehyde phosphate dehydrogenase (GAPDH) and human hypoxanthine phosphoribosyltransferase (HPRT)

Figure 3.1: Standard curve representation of both HKGs in all 3-cell lines evaluated

The dissociation curve should display one peak indicative of the target product and no peaks in the nuclease-free water (no template control, NTC) to indicate no contamination. Standard and disassociation curves were analysed using the Pair-Wise Fixed Reallocation Randomisation Test© for each primer pair (Table 2.9). Primer pairs which conceded HDAC1, 2, 3, 5, 6, 7, 8, FOSL1, LPAR1, LPAR2, MUC16, HPRT and GAPDH, were used for qPCR sample analysis.

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The maximum allowable variability on technical replicates was set at 0.24 cycles, which means that Ct values that differed by more than 0.24 cycles were excluded from the analysis.

Table 3.1: Primer efficiency (E), slope and R^2 value for each primer pair as calculated using Pair-Wise Fixed Reallocation Randomization Test© software

Gene	HOSEPiC			SKOV-3			OAW42		
	Slope	Efficiency (%)	R^2	Slope	Efficiency (%)	R^2	Slope	Efficiency (%)	R^2
GAPDH	-3.4	96.84	0.99	-3.34	99.25	1	-3.37	98.03	0.99
HPRT	-3.38	97.63	1	-3.36	98.44	0.99	-3.39	97.24	1
HDAC1	-3.42	96.06	0.99	-3.38	97.63	0.99	-3.41	96.45	0.99
HDAC2	-3.44	95.3	0.99	-3.41	96.45	0.99	-3.37	98.03	0.99
HDAC3	-3.45	94.92	0.99	-3.36	98.44	1	-3.39	97.24	0.99
HDAC5	-3.41	96.45	0.99	-3.42	96.06	0.99	-3.38	97.63	1
HDAC6	-3.39	97.24	1	-3.37	98.03	0.99	-3.36	98.44	0.99
HDAC7	-3.4	96.84	0.99	-3.36	98.44	1	-3.43	95.68	0.99
HDAC8	-3.42	96.06	1	-3.44	95.3	1	-3.39	97.24	1
FOSL1	-3.39	97.24	1	-3.38	97.63	0.99	-3.42	96.06	1
LPAR1	-3.34	99.25	0.99	-3.37	98.03	1	-3.4	96.84	0.99
LPAR2	-3.46	94.54	0.99	-3.44	95.3	0.99	-3.39	97.24	1
MUC16	-3.36	98.44	1	-3.42	96.06	1	-3.34	99.25	1

An E value of two indicates 100% primer efficiency and an R^2 value of 0.99 indicates a good correlation between Ct and sample concentration in HOSEpiC, SKOV-3 and OAW42 cells.

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Differential gene expression (DE) values were also quantified by the Pfaffl model and this method requires the use of the Relative Expression Software Tool Pair-Wise Fixed Reallocation Randomisation Test© software (REST®). To explain for biological variability between the three cell isolates, Pair-Wise Fixed Reallocation Randomisation Test© analyses were done on triplicates with GraphPad Prism version 6.0 (GraphPad™, San Diego, US; www.graphpad.com). Differentially expressed genes were ranked by fold-change relating sample to its corresponding control.

A fold change (FC, i.e., the ratio of expression values between a sample and respective control) cut-off for 2-fold expression difference and a $p \geq 0.05$ (as indicated by red-dotted lines in graphs) was set to define a gene target as significantly up- or down-regulated. The relative expression levels

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of each gene were normalized to the geometric mean of the two reference genes and scaled to the control group (Figures 3.4, 3.5, 3.6 and 3.7). GraphPad Prism and Pair-Wise Fixed Reallocation Randomisation Test© statistical analyses were performed for all samples [252].

The mRNA expression levels of HDAC1, HDAC2, HDAC3, HDAC5, HDAC6, HDAC7, HDAC8, FOSL1, LPAR1, LPAR2 and MUC16 in the two ovarian cancer cell lines, adenocarcinoma (SKOV-3) and cystadenocarcinoma (OAW42) were investigated by normalization against the HKGs (housekeeping genes) GAPDH and HPRT in the normal human ovarian surface epithelial cell line (HOSEPiC) by qRT-PCR.

The Ct values of the HKGs (GAPDH and HPRT) as measured in both the control cell line (HOSEPiC) and the cancer cell lines (SKOV-3 and OAW42) were for the Ct value of HPRT1 expression which was found to be relatively more stable for the 3 cell lines evaluated compared with GAPDH which was a highly unstable gene expressed in the three different cell lines (Figure 3.2). For this reason, the HPRT p-values were used to interpret the expression of genes.

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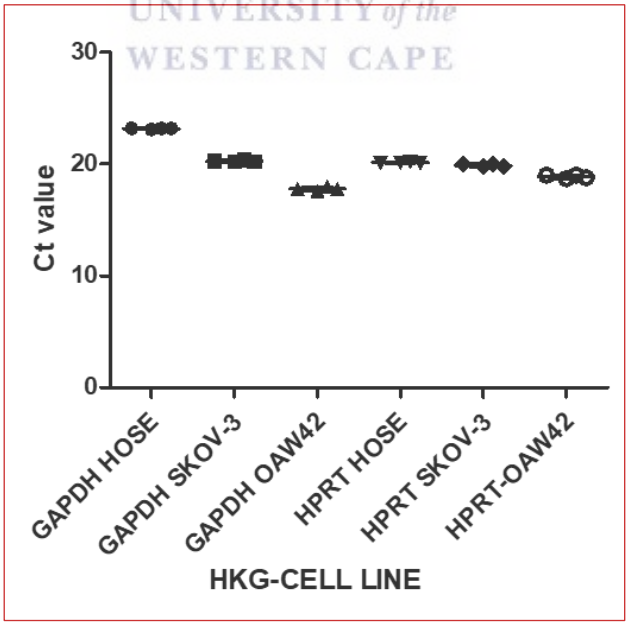

HKGs evaluated were GAPDH and HPRT.

Figure 3.2: QPCR Ct values for different HKGs in different cell lines

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HPRT fulfilled most criteria as a suitable HKG in that it was stably expressed and it did display the least fluctuation as shown in Figure 3.3. Thus, HPRT was the better HKG gene to use in the normalization of the target genes expressed. However, there were no significant differences in the relative expression of the candidate genes when a comparison was made between the normalization against HPRT vs GAPDH.

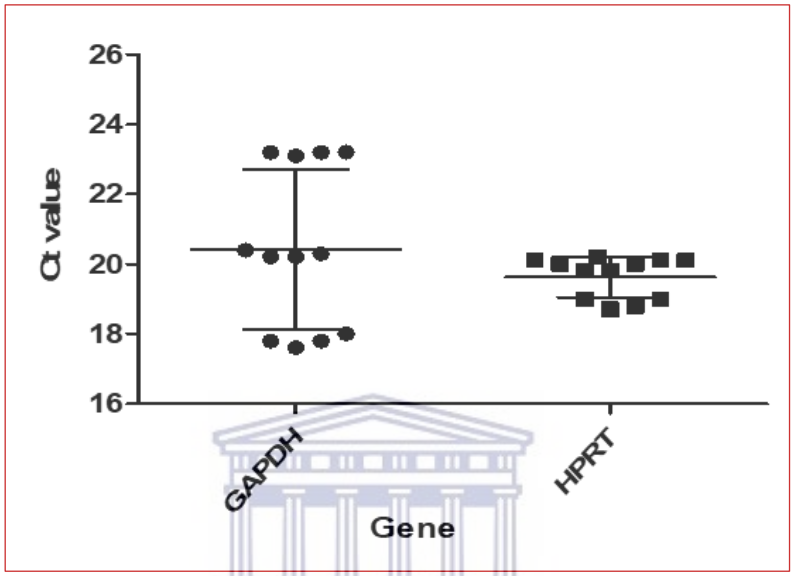

Figure 3.3: Combined Ct values for GAPDH and HPRT across all the cell lines.

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Melting curve analysis was performed on all 11 genes to validate the specific amplification of the putative genes. Figures 3.4 and 3.5 show the melting curves of PCR products produced for *GAPDH* and *HPRT.* The presence of only one melting peak in the range of 75°C to 90°C indicates specific amplification of the respective genes. Additional peaks will refer to the amplification of non-specific PCR fragments, which could occur as a result of contamination, mis-priming (referring to the annealing of primers to cDNA sequences other than the target cDNA) or primer-dimers (primers annealing to themselves).

The melting curves for *HDAC1, 2, 3, 5, 6, 7, 8, LPAR1, 2, FOSL1* and *MUC16* (hereinafter indicated in non-italic) also yielded a single peak (data not shown), implying the specific amplification of these genes. Figures 3.4 and 3.5 show the melting curves and amplification plots

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for the reference gene in the SKOV-3 cell line. Supplementary data for the other cell lines are presented in the Appendix.

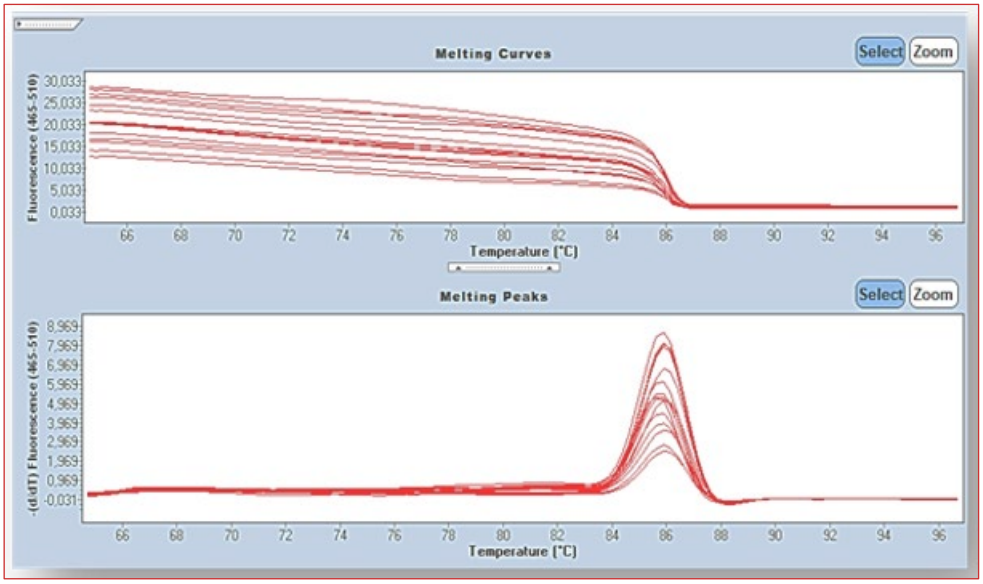

Figure 3.4: GAPDH melting curves and melting peaks in SKOV-3 cells

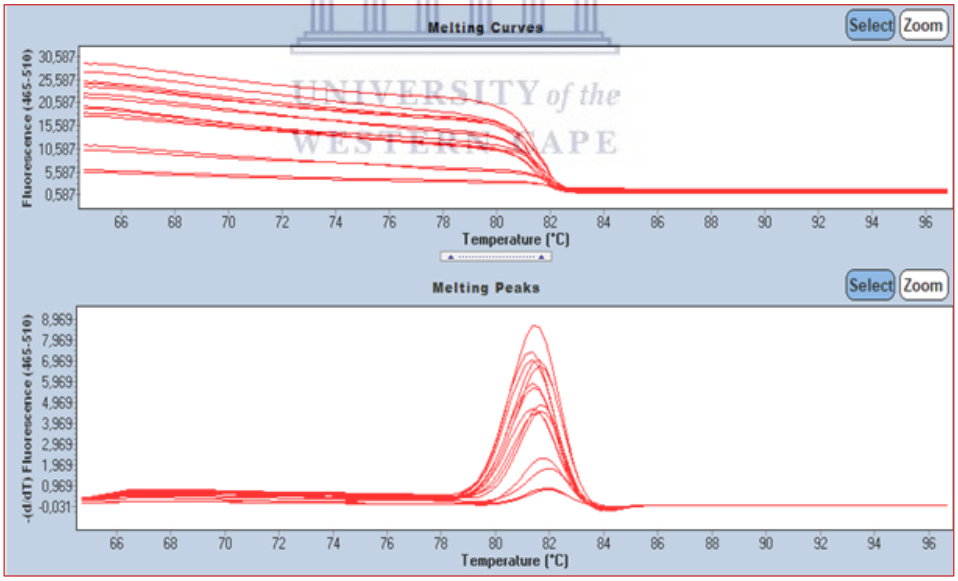

Figure 3.5: HPRT melting curves and melting peaks in SKOV-3 cells

The gene expression patterns between the two cancer cell lines for these HKGs as identified by real-time PCR were in concordance with established microarray data [345] and the gene expression

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intensities were quantified as fold change for each gene fragment and the computed ratio was described in terms of the fold change up or down (Tables 3.2 – 3.5; Figures 3.6 - 3.10). Confidence Intervals (CI) and p-values for the fold change were also calculated using a GraphPad Prism and REST384© statistical analysis software. Variances were considered significant if $p \geq 0.05$. Real-time quantitative PCR (qRT-PCR) data demonstrated a 1.7-fold upregulated HDAC1 mRNA expression in the SKOV-3 (Figure 3.6) compared to the OAW42 cell line which was 0.5-fold upregulated when normalized against GAPDH in both cell lines (Figure 3.8).

Against HPRT, HDAC1 levels presented a 1.8-fold in SKOV-3 with a p=0.04 (Figure 3.7) which were significantly upregulated when compared to low HDAC1expression, i.e., 0.24-fold in the OAW42 cell line with p=0.08 (Figure 3.9).

Table 3.2: Analysis of candidate gene expression in SKOV-3 cells compared to HOSEpiC cells normalized to GAPDH

SKOV-3 Relative to GAPDH	Gene	Absolute regulation ratio	Log2 ratio
HOSEpiC/SKOV-3	HDAC1	2.86	-1.52
HOSEPiC/SKOV-3	HDAC2	1.62	-0.7
HOSEPiC/SKOV-3	HDAC3	1.624	-3.55
HOSEPiC/SKOV-3	HDAC5	0.07	-3.73
HOSEPiC/SKOV-3	HDAC6	25.28	-4.66
HOSEPiC/SKOV-3	HDAC7	3.07	-1.62
HOSEPiC/SKOV-3	HDAC8	1.45	-0.54
HOSEPiC/SKOV-3	LPAR1	7.26	2.86
HOSEPiC/SKOV-3	LPAR2	34.77	-5.12
HOSEPiC/SKOV-3	FOSL1	0.002	-8.94
HOSEPiC/SKOV-3	MUC16	188.70	-7.56

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The levels of HDAC2 mRNA in SKOV-3 normalized to GAPDH expression was decreased 0.7-fold (Figure 3.6). Also, it represented a 1.04-fold change decrease in OAW42 (Figure 3.8). Normalizing to HPRT, HDAC2 showed downregulated mRNA expression in SKOV-3 equivalent

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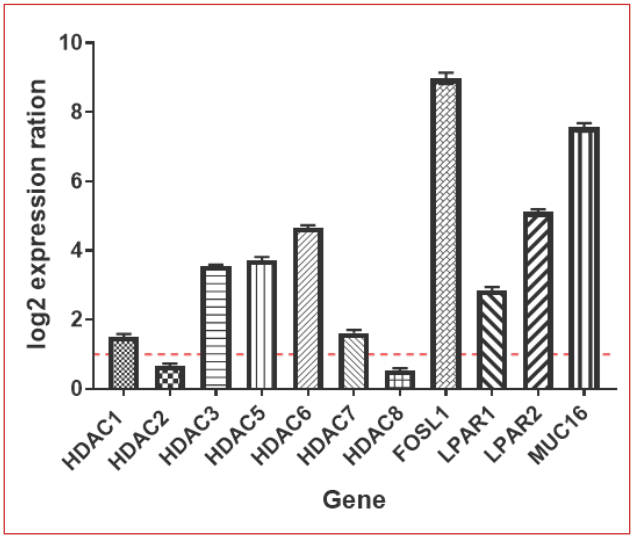
to a 0.94-fold change (p=0.07; Figure 3.7), matching to OAW42 which expressed HDAC2 at a significantly lower level, i.e., 1.79-fold change (p=0.13; Figure 3.9).



Expression relative to the control cell line HOSEPiC normalized against GAPDH. Red dotted lines indicate the cut-off for a 2-fold expression difference.

Figure 3.6: Expression of target genes relative to GAPDH in SKOV-3 cells

Table 3.3: Analysis of candidate genes expression in SKOV-3 cells compared to HOSEpiC normalized to HPRT

SKOV-3 Relative to HPRT	Gene	Absolute regulation ratio	Log2 ratio
HOSEpiC/SKOV-3	HDAC1	3.38	-1.76
HOSEPiC/SKOV-3	HDAC2	1.918	-0.94
HOSEPiC/SKOV-3	HDAC3	13.83	-3.79
HOSEPiC/SKOV-3	HDAC5	15.67	-3.97
HOSEPiC/SKOV-3	HDAC6	29.85	-4.9
HOSEPiC/SKOV-3	HDAC7	3.63	-1.86
HOSEPiC/SKOV-3	HDAC8	1.71	-0.78
HOSEPiC/SKOV-3	LPAR1	8.32	3.05
HOSEPiC/SKOV-3	LPAR2	41.06	-5.36
HOSEPiC/SKOV-3	FOSL1	580.03	-9.18
HOSEPiC/SKOV-3	MUC16	0.42	-7.81

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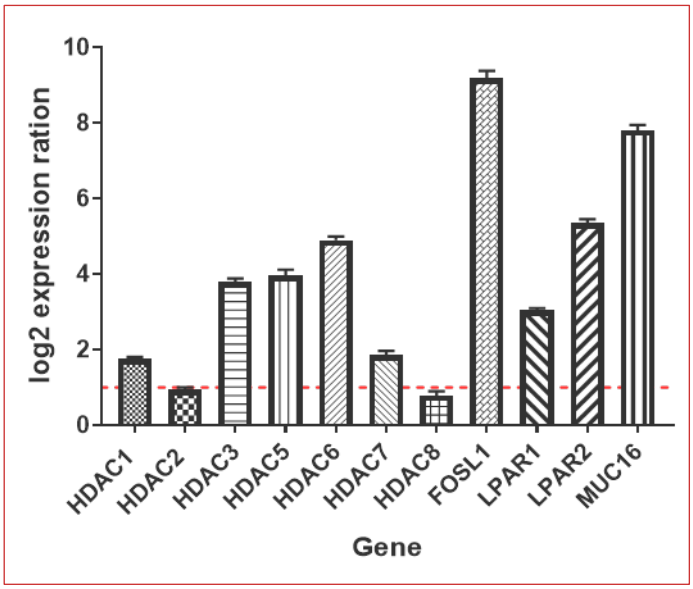

Expression relative to the control cell line HOSEPiC normalized against HPRT. Red dotted lines indicate the cut-off for a 2-fold expression difference.

Figure 3.7: Expression of target genes relative to HPRT in SKOV-3 cells

Table 3.4: Analysis of candidate gene expression in QAW42 cells compared to HOSEpiC cells normalized to GAPDH

OAW42 Relative to GAPDH	Gene	Absolute regulation ratio	Log2 ratio
HOSEPiC/OAW42	HDAC1	1.42	-0.51
HOSEPiC/OAW42	HDAC2	0.48	1.04
HOSEPiC/OAW42	HDAC3	0.54	0.88
HOSEPiC/OAW42	HDAC5	0.07	-3.73
HOSEPiC/OAW42	HDAC6	3.94	-1.98
HOSEPiC/OAW42	HDAC7	3.94	-1.17
HOSEPiC/OAW42	HDAC8	1.40	-0.49
HOSEPiC/OAW42	LPAR1	0.03	4.67
HOSEPiC/OAW42	LPAR2	1.06	-0.09
HOSEPiC/OAW42	FOSL1	-26.35	-4.72
HOSEPiC/OAW42	MUC16	0.71	0.48

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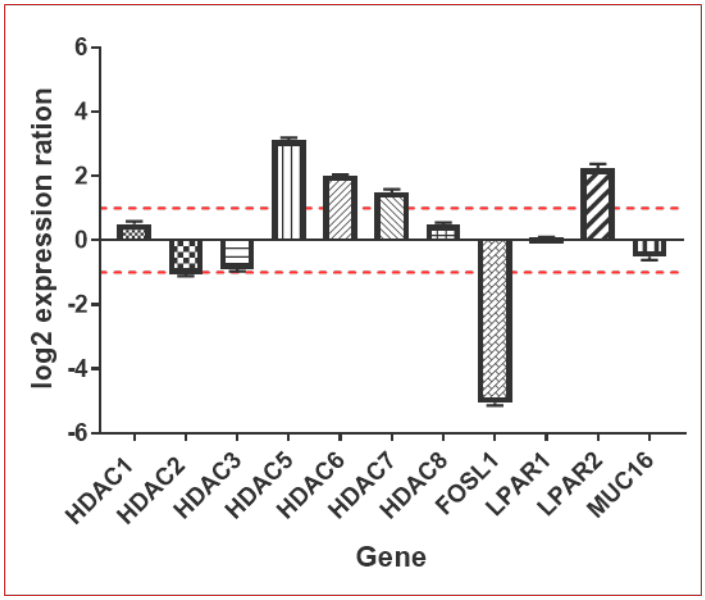

Expression relative to the control cell line HOSEPiC normalized against GAPDH. Red dotted lines indicate the cut-off for a 2-fold expression difference. Values below the zero line represent down-regulation of genes.

Figure 3.8: Expression of target genes relative to GAPDH in OAW42 cells

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Table 3.5: Analysis of candidate gene expression in QAW42 cells compared to HOSEpiC cells normalized to HPRT

OAW42 in relative with HPRT	GENE	Absolute regulation ratio	Log2 ratio
HOSEPiC/OAW42	HDAC1	0.84	0.24
HOSEPiC/OAW42	HDAC2	0.28	1.79
HOSEPiC/OAW42	HDAC3	0.32	1.63
HOSEPiC/OAW42	HDAC5	5.18	-2.37
HOSEPiC/OAW42	HDAC6	2.33	-1.22
HOSEPiC/OAW42	HDAC7	1.33	-0.41
HOSEPiC/OAW42	HDAC8	0.83	0.26
HOSEPiC/OAW42	LPAR1	0.02	5.42
HOSEPiC/OAW42	LPAR2	0.63	0.66
HOSEPiC/OAW42	FOSL1	2.79	-1.48
HOSEPiC/OAW42	MUC16	0.42	1.23

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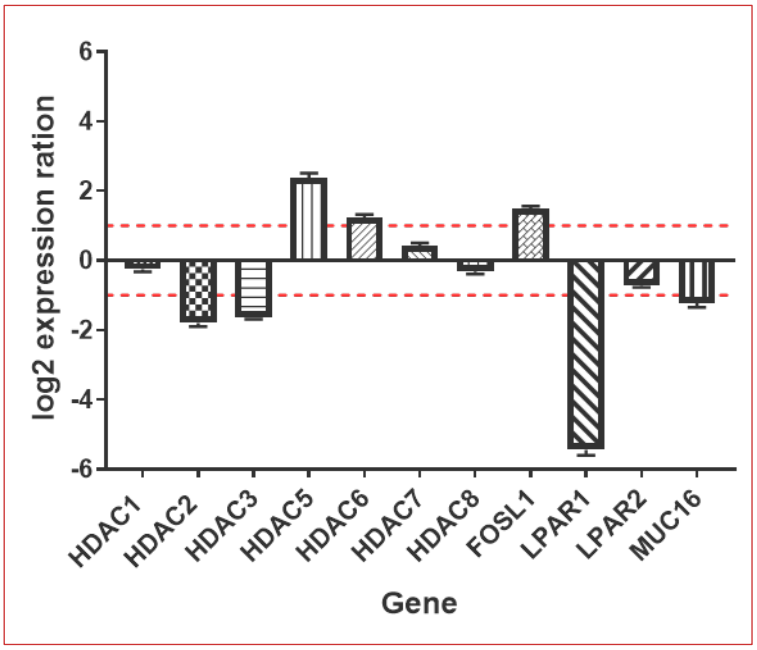

Expression relative to the control cell line HOSEPiC normalized against HPRT. Red dotted lines indicate the cut-off for a 2-fold expression difference. Values below the zero line represent down-regulation of genes.

Figure 3.9: Expression of target genes relative to HPRT in OAW42 cells

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HDAC3 mRNA was expressed in higher levels, i.e., fold change of -3.55 in SKOV-3 to GAPDH normalization (Figure 3.6), but HDAC3 showed lower expression in OAW42, i.e., a 0.88-fold change (Figure 3.8). In contrast, HDAC3 mRNA expression was upregulated as indicated by a 3.79-fold change in SKOV-3 (Figure 3.7) against HPRT, with p=0.02, and HDAC3 showed downregulated expression in QAW42 cells as judged by a 1.63-fold change which was not significant (p=0.08; Figure 3.9).

HDAC5 mRNA was expressed in relatively lower levels, i.e., 3.7-fold in SKOV-3 cells relative to GAPDH as shown in Figure 3.6 while Figure 3.8 depicts an increased HDAC5 mRNA expression level in OAW42, i.e., a 3.17-fold change. In Figure 3.7, HDAC5 mRNA in SKOV-3 displayed an upregulated expression relative to HPRT, which was a 3.97-fold change with p=0.02. Also, HDAC5 mRNA expression was increased in OAW42, i.e., 2.37-fold change (p=0.05; Figure 3.9). As shown in Figure 3.6, the relative density of HDAC6 mRNA was upregulated in SKOV-3 equivalent to 4.66-fold change and its expression was also high in OAW42 as indicated by a 1.98-

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fold change (Figure 3.8). Although HDAC6 expression was increased significantly relative to HPRT (4.9-fold change; p=0.02) in SKOV-3 cells (Figure 3.7), its expression in OAW42 was less (1.22-fold change; p=0.05) as shown in Figure 3.9. Relative to GAPDH, HDAC7 mRNA was upregulated in SKOV-3 by 1.6-fold change (Figure 3.6), similar to that observed for OAW42 cells (Figure 3.8). In QAW42 cells, HDAC2 mRNA expression was downregulated by 1.17-fold (Figure 3.8). Against HPRT, HDAC7 mRNA expression showed a 1.86-fold increase in SKOV-3 cells (p=0.12; Figure 3.7) and it did not decrease significantly (0.4-fold change; p=0.31) in OAW42 cells (Figure 3.9).

Analysis of HDAC8 mRNA expression revealed low levels in all samples tested. Notably, there was a 0.54-fold increase in SKOV-3 cells (Figure 3.6), which was relatively similar to HDAC8 mRNA decreased in OAW42 cells as indicated by a 0.49-fold change (Figure 3.8). Thus, HDAC8 mRNA expression was not significantly downregulated in SKOV-3 cells (0.78-fold change; p=0.30; Figure 3.7). Likewise, in OAW42 cells, HDAC8 mRNA expression was decreased (-0.26-fold change; p=0.32; Figure 3.9).

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In examining FOSL1 mRNA, relative to GAPDH, it was observed to be highly expressed in SKOV-3 cells (8.94-fold change; Figure 3.6). However, in OAW42 cells, FOSL1 mRNA expression is downregulated (-4.72-fold change; Figure 3.8). Against HPRT HKG, as shown in Figure 3.5, FOSL1 mRNA was significantly upregulated 9.18-fold in SKOV-3 cells (Figure 3.7) exceeding FOSL1 expression in OAW42 cells which was significantly upregulated by 1.48-fold (p=0.01; Figure 3.9).

Relative to GAPDH, the expression of LPAR1 mRNA was high in SKOV-3 cells (2.86-fold change; Figure 3.6), but it was very low in OAW42 cells (0.03-fold change; Figure 3.8). However, relative to HPRT, there was a 3.05-fold increased expression of LPAR1 mRNA in SKOV-3 cells (Figure 3.7) and a -5.4-fold change (downregulated expression) in OAW42 (Figure 3.9) with parallel p=0.01 in both cell types. Figure 3.6 shows a 5-fold increased LPAR2 mRNA expression

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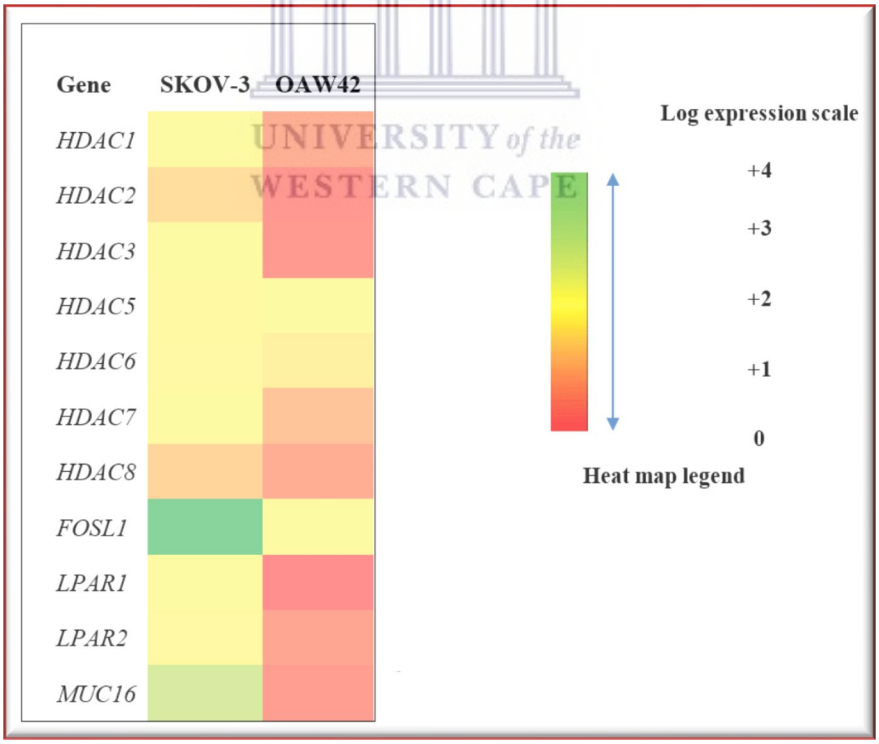
in SKOV-3 cells, relative to HPRT, whereas expression of this candidate gene in OAW42 cells is upregulated 2.3-fold (Figure 3.8).

Despite the significant increase in relative density of LPAR2 (5-fold change; p=0.01) in SKOV-3 cells (Figure 3.7), the relative density of LPAR2 in OAW42 cells was significantly decreased by 0.6-fold (p=0.02; Figure 3.9). Lastly, relative to GAPDH, MUC16 mRNA was upregulated 8.1-fold in SKOV-3 cells (Figure 3.6), but it was 0.5-fold downregulated in OAW42 cells (Figure 3.8). Compared with HPRT, MUC16 was expressed significantly higher in SKOV-3 cells (8.2-fold change, p=0.008; Figure 3.7), whereas in OAW42 cells, it was significantly downregulated 1.2-fold (p=0.01; Figure 3.9).

Figure 3.10 shows mRNA expression of candidate genes displayed as a heat map. Expression ratios signify fold changes in expression of stimulated cells over control cells.



Red corresponds to reduced gene expression, whereas green increased illustrates increased expression (p≤0.05).

Figure 3.10: Comparison of mRNA expression of candidate genes in SKOV-3 and QAW42 cells

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3.3 Contextual Discussion and Perspective

3.3.1 Introduction

Specific ovarian cancer biomarkers (Table 2.9, Chapter 2) were purposely selected for this project. The relative expression ratios of these candidate genes in the ovarian cancer cell lines SKOV-3 and OAW42 were estimated cells relative to HOSEpiC ovarian surface epithelium cells as a control. The expression levels of all the target genes were determined after normalization to GAPDH and HPRT as housekeeping genes (HKGs).

3.3.2 Biomarker Validation

Bioinformatics software tools which analyse gene and protein expression data and gene regulatory networks, have contributed immensely to the discovery of candidate biomarker discovery for different disease states. The Human Protein Atlas (HPA) is a comprehensive repository of protein expression profiles of normal and cancer tissues, as well as cell lines [346]. It also allows researchers to evaluate specific target genes as biomarkers for cancers due to the availability of whole transcriptomes created from sequencing of RNA/transcripts (RNA-Seq) of tissue samples [347, 348]. The atlas has a huge sample selection, which includes a large proportion of the common tissues in the human body. This allows for the tissue-specific RNA expression analysis of all the human protein-coding genes simultaneously. A new version (www.proteinatlas.org) showcases RNA and protein expression data conforming to 91 and 80%, respectively, of the putative protein-coded genes [348].

Significantly, the main idea for HPA is to allow for a systematic investigation of the human proteome, by linking high-throughput generation of authorized antibodies with protein profiling in human tissues and cells. Another feature is the utilization of tissue microarrays (TMAs), dependent on mRNA transcript sequences from several databases, such as sequences from RefSeq at the NCBI (www.ncbi.nih.nlm.gov) and Ensemble genes at ENSEMBL (ww.ensembl.org) and protein coding transcripts. For the current study the expression levels of all 11 prospective biomarkers

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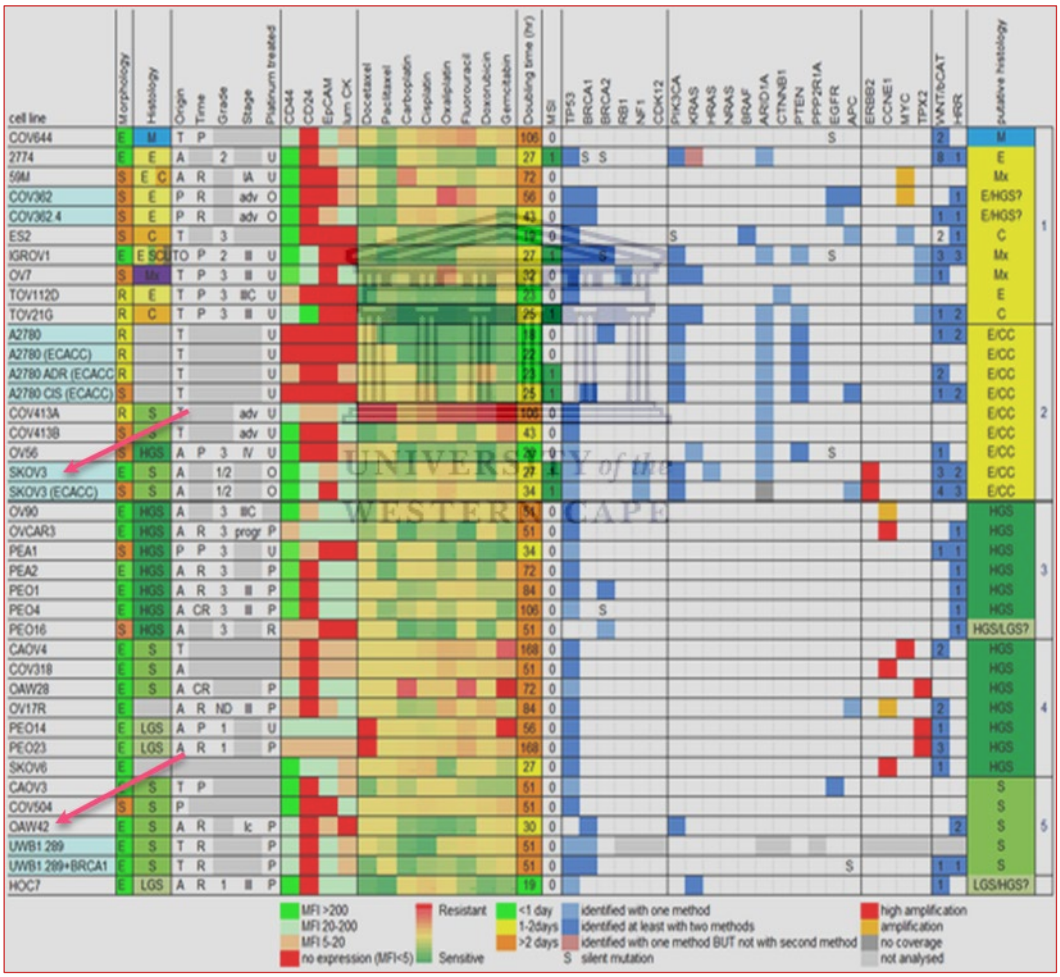
(listed in Table 2.9) were detected in various cancer and their respective control cell lines or tissue cells utilizing the HPA navigator [346].

In addition to the target genes evaluated in this study, there were other genes that were differently expressed in SKOV-3 and OAW42 cells (Figure 3.11) such as *PTEN*, *ARID 1A* and *TP*53. These genes are mutated in a some ovarian cell lines, as well as, numerous other different cancers and are thus not specific to SKOV-3 and OAW42. Therefore, these genes will be good general biomarkers for cancers, but will be good candidate biomarkers for specific cancers like ovarian cancer.



Clinical Importance of in Vitro Morphological Subtypes, including the putative cell lines SKOV-3 and OAW42 [349, 350].

Figure 3.11: Ovarian cancer cell line panel (OCCP)

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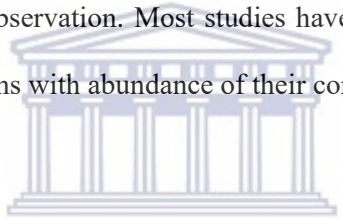
The 11 target genes were subjected to cross-cancer tissue-specific analysis in the HPA. Figures 3.12 – 4.16 give a graphical display of 5 genes and their expression in selected cancers, including ovarian carcinomas for both databases. Data on the remaining six genes are found in the Appendix. The HPA database compares expression of the genes in human cancer tissue types. The expression outlines of all 11 prospective biomarkers (listed in Table 2.9) were investigated in altered cancer tissues using the HPA.

As shown in Figure 3.11 (Appendix), HDAC8 was significantly upregulated selectively in ovarian cancer as shown by HPA analysis. This does not agree with the qRT-PCR data generated in the current study since the qRT-PCR results suggest that the expression levels of the protein encoded by this gene is downregulated in SKOV-3 and OAW42 relative to control HOSEpiC cells. It is a known fact that gene expression and qRT-PCR data do not need to be the same as there are many factors that contribute to this observation. Most studies have shown a 40% correlation between cellular concentrations of proteins with abundance of their corresponding mRNA, in both bacteria and eukaryote [351].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There are factors that contribute to the 60% variation observed between gene and protein expression levels, including the different lifetimes of both mRNA and proteins [352]. Other important factors contributing to this variation are regulation post-transcriptionally and during translation and protein degradation. Thus, one would expect the protein results (from the SDS-PAGE analysis) to be more representative of the actual abundance of the proteins and therefore affect the permeability of the outer membrane.

Based on the qRT-PCR data, HDAC1, HDAC3, HDAC5, HDAC6 and HDAC7 were expressed highly in SKOV-3 relative to HOSEpiC cells, which correlate with HPA proteomics results in the Appendix, with the exception of HDAC2 low gene expression in SKOV-3 cells compared to its high expression level in HPA. Unlike in OAW42 expression of HDAC1 and HDAC7 genes were downregulated, which disagrees with the HPA data. In this respect, the qRT-PCR results show that

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HDAC2, HDAC3, HDAC5 and HDAC6 are expressed in higher levels in OAW42 relative to HOSEpiC, which is in agreement with HPA data.

QRT-PCR results for FOSL1, LPAR1, LPAR2 and MUC16 in SKOV-3 cells were upregulated, supporting the HPA data (Appendix)—similar: LPAR1, FOSL1 and MUC16 were upregulated in OAW42 which in agreement with HPA data; dissimilar: LPAR2 was downregulated which does not agree with HPA data.

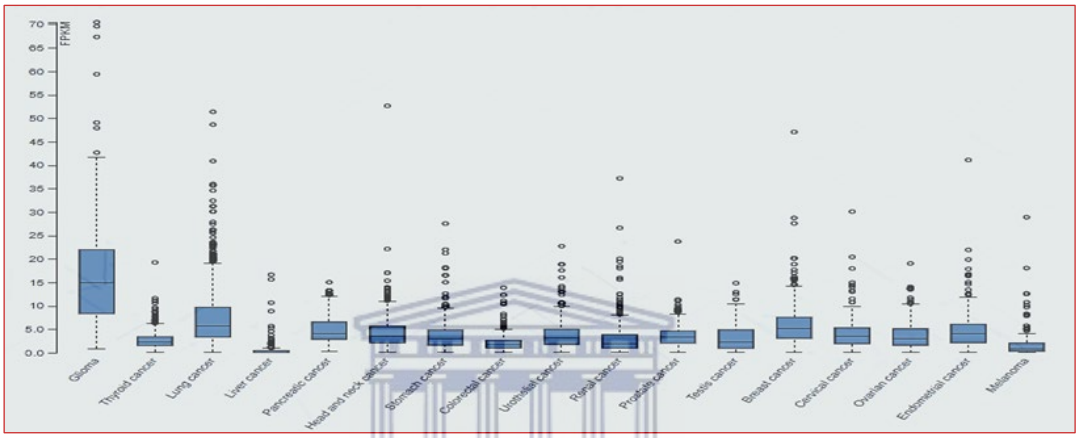

Figure 3.12: LPAR1expression in selected cancer types

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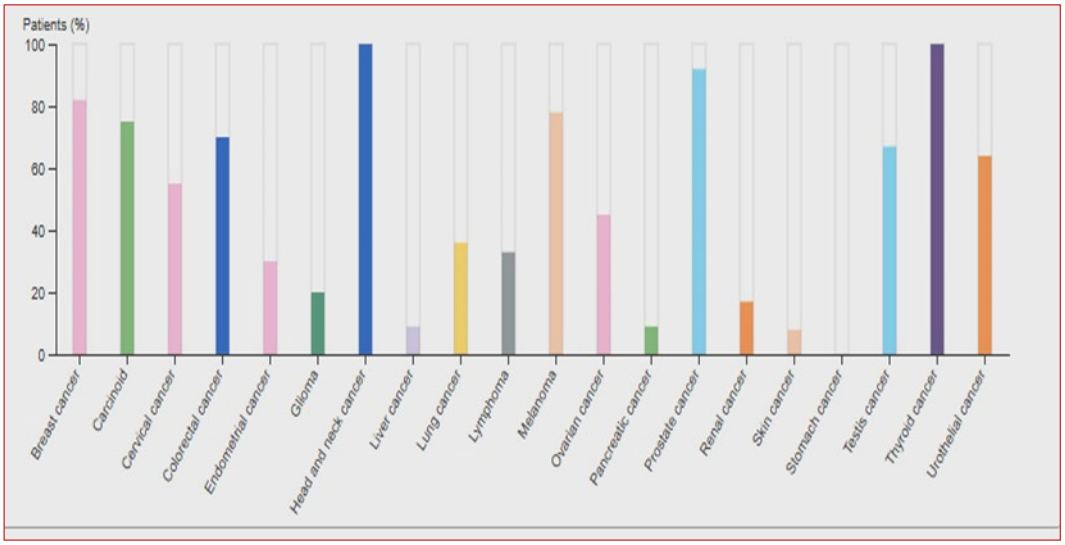

Figure 3.13: LPAR2 expression in selected cancer types

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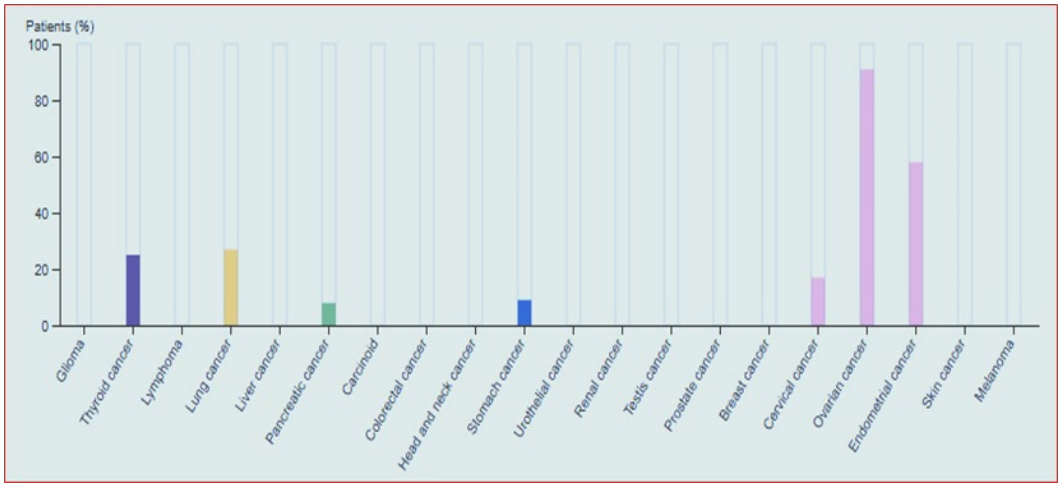

Figure 3.14: MUC16 expression in selected cancer tissues

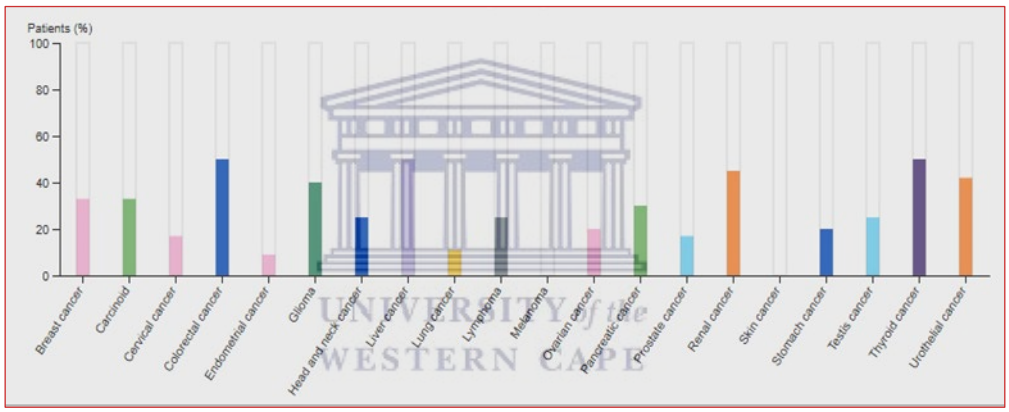

Figure 3.15: HDAC5 expression in selected cancer tissues

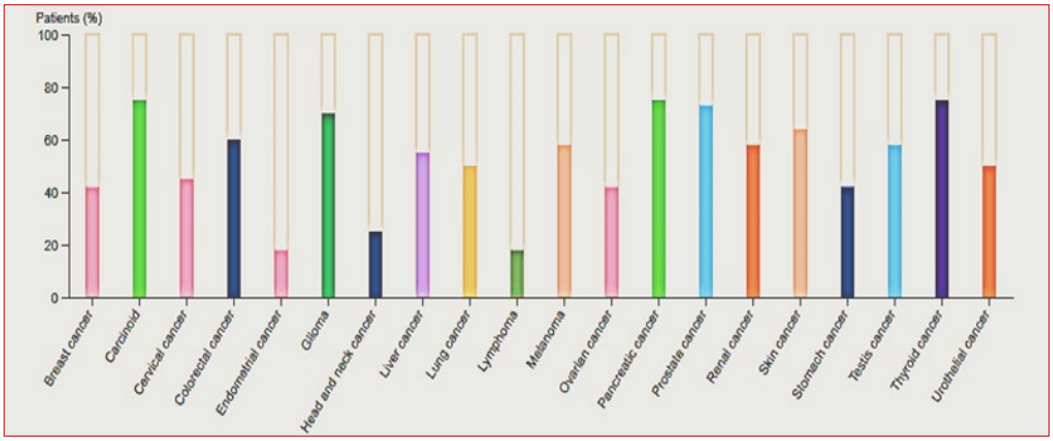

Figure 3.16: HDAC6 expression in selected cancer tissues

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3.3.3 Network Analysis

STRING analysis done on all 11 target genes indicate a definitive link between the HDAC gene members and FOSL1, thus suggesting that these genes interact both genetically and biologically and will have a direct effect on one another if the expression level of one gene is affected. However, this is not always true in all cases. MUC16 does not appear to share any interactions with any of the other target genes, whereas LPAR1 and LPAR2 only interact with one another (Figure 3.17).

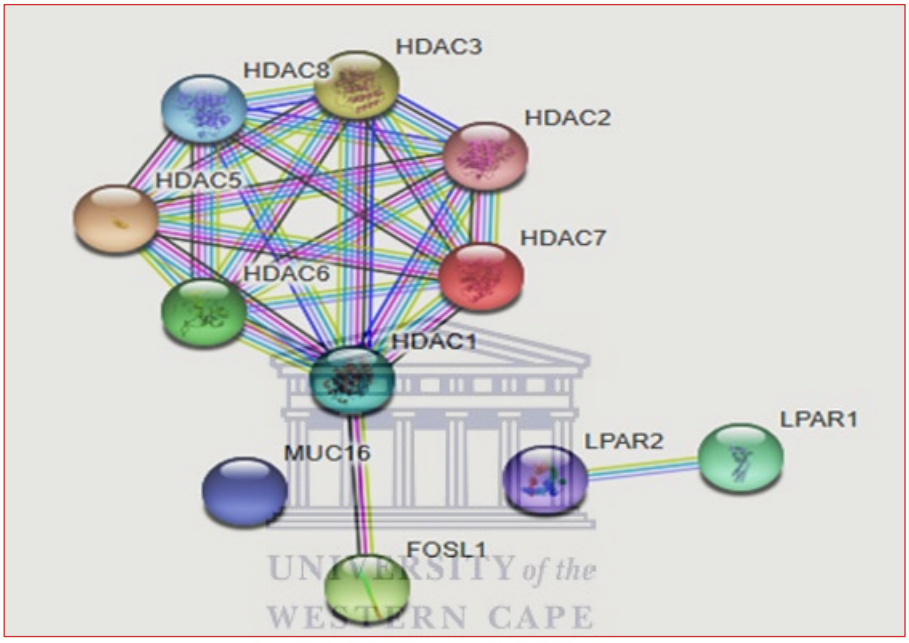

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Figure 3.17: Definitive links between all objective genes

3.3.4 QRT-PCR Conclusion

Throughout the last three decades, the progress of science has improved our lives in ways that would have been unpredictable [353]. Molecular technologies have been used widely to discover disease-causing genes and in the diagnosis of congenital disorders and cancer. These discoveries have enabled scientists to gain insight into the molecular mechanisms of the development of malignant cancer cells. Understanding the mechanisms that contribute to the development of cancers are crucial in developing tailor-made treatments for all of the different cancer types. However, there are still numerous limitations of the current molecular technologies in the diagnosis

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and treatment of certain cancers. Moreover, genetic technologies that range from cytogenetic analysis and standard PCR-based methods to the more informative quantitative PCR are still not sensitive and specific enough for the development of diagnostic tools for all types of cancers. Quantitative PCR was considered a necessary tool to help in the improvement of human health and human life and is it classified as a valuable and confirmatory technique with high sensitivity and specificity concerning its ability to investigate varied infections with ease in many studies and monitor the expression of genes concurrently.

There is, however, still a problem with the availability of cancer diagnostic markers for the early detection of cancers. This investigation demonstrated the potential value of the qRT-PCR analysis to identify genes overexpressed in ovarian cancer and their subsequent link to genes measurable in OAW42 and SKOV-3 relative to HOSEpiC cell line. The findings provide evidence for an association between the expression levels of the studied genes and ovarian cancer pointing to a rationale for further research assessing their potential clinical efficacy.

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CHAPTER 4

Immunocytochemical Expression Analysis of the Selected Genes in OAW42, SKOV-3 and HOSEPiC Cells

4.1 Background

Immunocytochemistry (ICC) is a procedure whereby the researcher can anatomically visualize the localization of a specific antigen (protein) under a fluorescence microscope by using a specific primary antibody. ICC as a diagnostic technique is a valuable tool to identify a tissue or cellular contents from different cell suspensions [113-115]. In an appropriate antigen–antibody interaction where the antibody has been tagged with an labeled tag [116], it allows one to detect a further detailed spatiotemporal expression of cell antigens[117].

H. Coons, who was the first presenter of immunofluorescence in 1941, used precise antibodies characterized with a fluorescent dye to recognize the localization of t molecules in tissues. Initial efforts by other researchers to label antibodies with usual dyes yielded inadequate details as the labels were not sufficiently visible under the microscope in tissues [107]. Certain criterion was modified and developed by A. H. Coons and his associates to underlay principles of the immunofluorescence method [368] based on:

1. Specific binding of antibodies to molecules [369], which could be visualized with colored stains,

2. Antibodies probable binding with fluorescent dyes (fluorescent antibodies) and used as immunospecific stains for the histochemical recognition of antigens [109],

3. Direct method which is used the unlabeled antigen directly as immunospecific reagents for

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the histochemical detection of antibodies [108, 110, 370].

4. The quality of interaction between the antibody-antigen controlling in the authority and accuracy of immunocystochemical procedure was based on the quality of the reagents that were used in the dye assay which minimized the effects of cross-reactivity of the immunoglobulin solution which could have undesirable or nonspecific staining [110].

4.2 Presentation and Interpretation of the Results

In this part of the project, we selected the five most important genes, which showed differential expression among three cell lines (OAW42, SKOV-3 and HOSEPiC to perform ICC. The rest of the potential candidate genes have been excluded due to the lack of additional funding, and according to the network analysis string software as showed in Figure 3.17. To determine the subcellular distribution of HDAC5, HDAC6, LPAR1, LPAR2 and MUC16 proteins in human epithelial ovarian cancer, ICC was performed and slides viewed with a Carl Zeiss ZEN 2.3 SP1 Black 64-bit microscope. Analysis was done on ovarian cancer cell lines OAW42 and SKOV-3, as well as the human ovarian surface epithelial cell line, HOSEpiC, using specific antibodies for HDAC5, HDAC6, LPAR1, LPAR2, MUC16 as shown in Table 2.6. SKOV-3 was selected based on the fact that it is the most common cell line used in high-grade surface ovarian carcinoma (HGSOC) preclinical research studies [364, 371, 372].

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However, the efficacy of this cell line is now open to query, without the defining genomic variations seen in most patient samples [373]. Regarding this, studies have been identified, where various ovarian carcinoma can develop from the human ovarian surface epithelial (OSE) cells during the ovulatory repair mechanism [374, 375], and about 85% of human ovarian carcinomas could arise from ovarian surface epithelium [376]. According to the significance of OSE nature in ovarian cancer studies, there is paucity in experimental systems. Based on some studies that suggest that ovarian cancers cannot arise from OSE in animals, there is a suggestion to drop this cell line in animal models that represent human ovarian epithelial cancer studies [71, 377].

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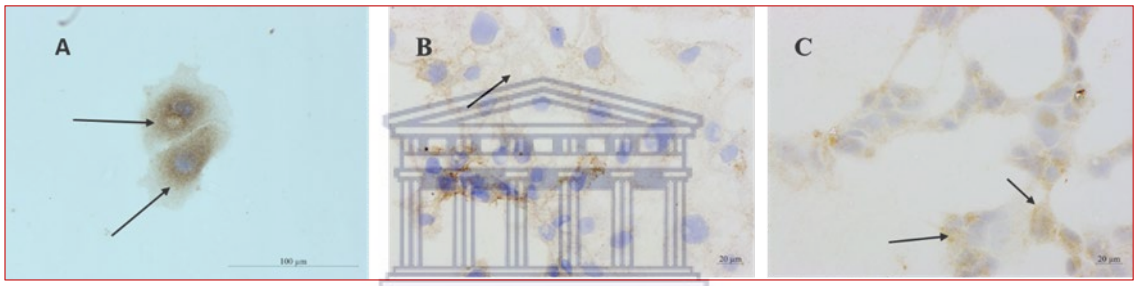
As mentioned by CLS Cell Lines Service GmbH - Dr. Eckener-Straße 8 - 69214 Eppelheim - Germany (http://www.clsgmbh.de/oaw-42.pdf), OAW42 is a valuable cell line for biological studies on human ovarian cancer, epithelial monolayer and adherent cell lines, and, therefore, could be the model cell line of choice for research on ovarian tissues. In the present study, we evaluated the 5 different candidate genes as potential biomarkers for ovarian cancer diagnosis.

In our studies, LPAR1 show low cytoplasmic expression in SKOV-3 and OAW42 cells, which is indicated by light brown colour intensity in their cytoplasm. High expression of LPAR1 was observed in HOSEpiC nucleus, as indicated by the dark strong intensity stain of the HOSEpiC cytoplasm (Figure 4.1).



Cells were stained with haematoxylin and eosin (H&E).). A, B and C show HOSEpiC-, SKOV-3- and OAW42-cell cytoplasm, respectively, stained with H&E and the LPAR1 antibody. The dark purple stain represents the nuclei, while the brown stain denotes LPAR2. The black arrows point to the localization of LPAR2. Images were taken at 20X magnification.

Figure 4.1: ICC analysis of LPAR1 expression in HOSEpiC, SKOV-3 and OAW42 cells

LPAR2 protein expression was upregulated in HOSEpiC cell cytoplasm and downregulated in OAW42 and SKOV-3 cells as observed by the light intensity of their cytoplasm stain (Figure 4.2). Figure 4.3 shows that the expression levels of HDAC5 was high in the SKOV-3 cell cytoplasm and moderate to strong in the HOSEpiC cell cytoplasm. The expression of this gene was much lower in OAW42 cell cytoplasm, when compared to the level of intensity in the SKOV-3 and HOSEpiC cells. The staining intensity of HDAC6 proteins was stronger in OAW42 and HOSEpiC cell cytoplasm when compared to the expression in the cytoplasm of SKOV-3, which showed moderate intensity Figure 4.4.

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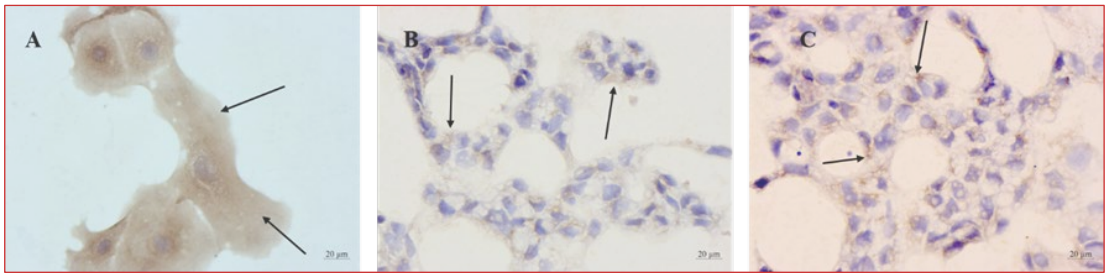
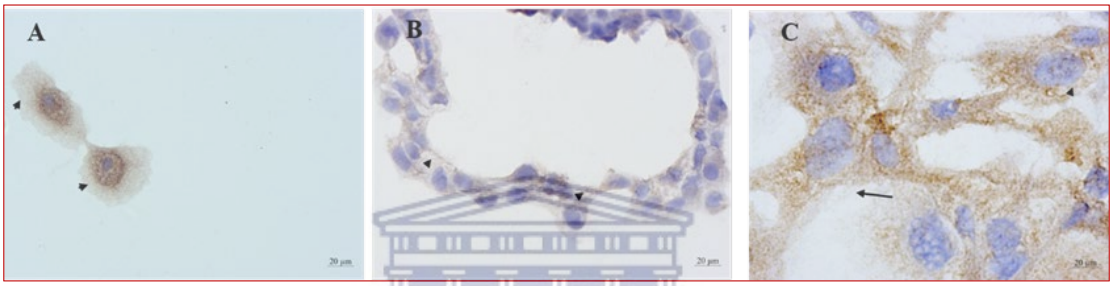


Cells were stained with haematoxylin and eosin (H&E). A, B and C show HOSEpiC-, OAW42- and SKOV-3-cell cytoplasm, respectively, stained with H&E and the LPAR2 antibody. The dark purple stain signifies the nuclei, while the brown stain represents LPAR2. The black arrows point to the localization of LPAR2. Images were taken at 20X magnification.

Figure 4.2: ICC analysis of LPAR2 expression in HOSEpiC, OAW42 and SKOV-3 cells



Cells were stained with haematoxylin and eosin (H&E). A, B and C show HOSEpiC-, OAW42- and SKOV-3-cell cytoplasm, respectively, and stained with H&E and the HDAC5 antibody. The dark purple dye means the nuclei, whereas the brown stain represents HDAC5. The black arrows point to the localization of HDAC5. Images were taken at 20X magnification

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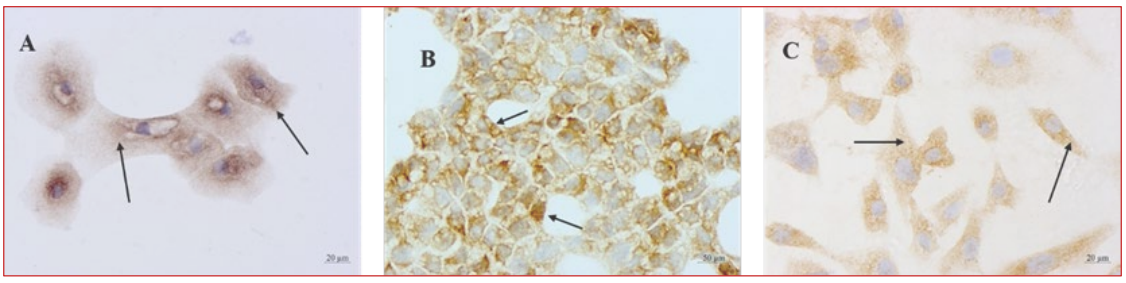
Figure 4.3: ICC analysis of HDAC5 expression in HOSEpiC, OAW42 and SKOV-3 cells



Cells were stained with haematoxylin and eosin (H&E). A, B and C show HOSEpiC-, OAW42- and SKOV-3-cell cytoplasm, respectively, stained with H&E and the HDAC6 antibody. The dark purple dye means the nuclei, whereas the brown stain represents HDAC6. The black arrows point to the localization of HDAC6. Images were taken at 20X magnification.

Figure 4.4: ICC analysis of HDAC6 expression in HOSEpiC, OAW42 and SKOV-3 cells

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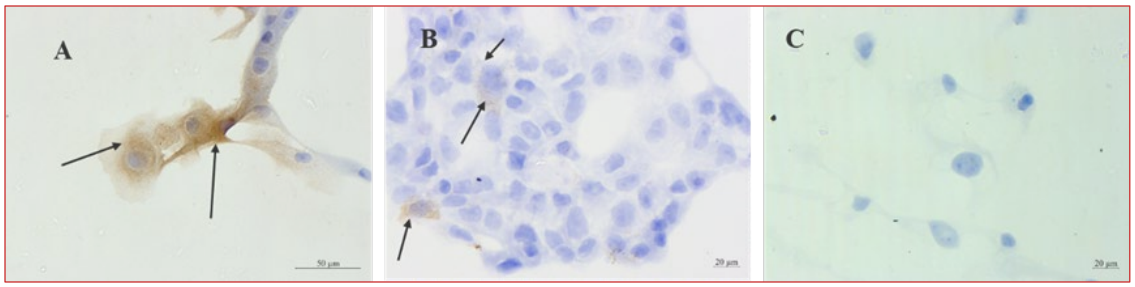
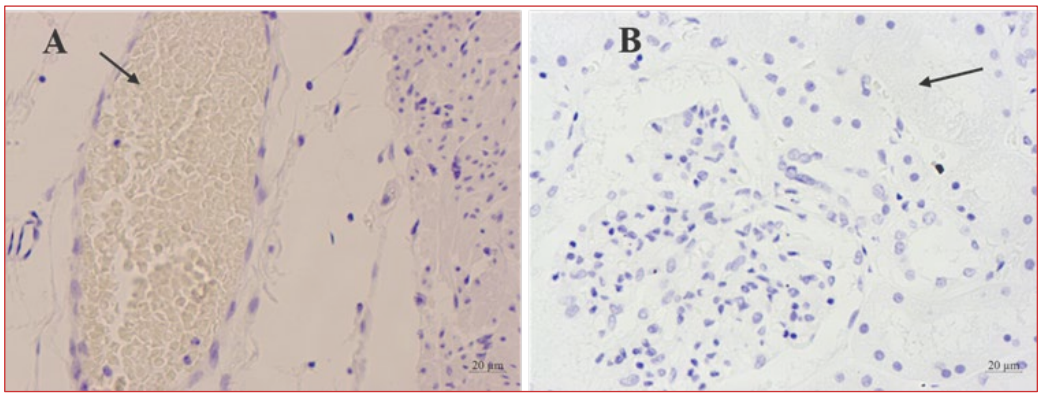
The SKOV-3, OAW42 and HOSEpiC cell lines were also evaluated with for their expression of CA125 (M11) with the mouse monoclonal antibody for CA125 (Product # MA5-12425). The levels of CA125, also known as MUC16 as mentioned in section B1.1, revealed that this biomarker is expressed in HOSEPiC cells (Figure 4.5A) and OAW42 cells (Figure 4.5B), but not in SKOV-3 cells (Figure 4.5C).



Cells were stained with haematoxylin and eosin (H&E). A and B show MUC16 expression in HOSEpiC and OAW42 cells, respectively, and C show no expression of MUC16 in SKOV-3 cell cytoplasm. The dark purple dye denotes the nuclei, whereas the brown stain represents MUC16. The black arrows point to the localization of MUC16. Images were taken at 20X magnification.

Figure 4.5: ICC analysis of MUC16 (CA125) expression in HOSEpiC, OAW42 and SKOV-3 cells

Figure 4.6A shows the expression of M11 in stomach as a positive control and the negative expression of M11 in kidney as a negative control, indicating that the conditions were indeed optimized for CA125 expression.



A: M11 monoclonal antibody-positive expression as a positive control in stomach; **B:** M11 monoclonal antibody-negative expression as a negative control in kidney

Figure 4.6: M11 monoclonal antibody-positive expression in stomach and -negative expression in kidney

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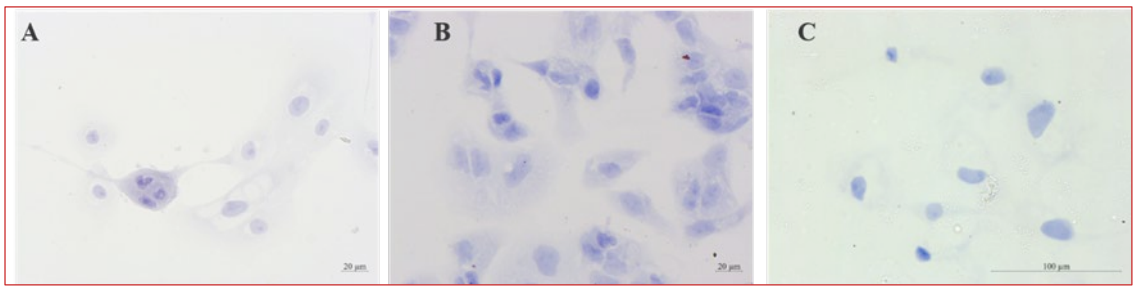
In addition, Figure 4.7 shows the negative control for the HOSEpiC, OAW42 and SKOV-3 cell lines with no binding to the antibodies evaluated, i.e., antibodies directed against HDAC5, HDAC6, LPAR1, LPAR2 and MUC16.



A, B and C respectively show the morphology of HOSEpiC, OAW42 and SKOV-3 cells stained with haematoxylin and eosin (H&E) before stain them with specific antibodies to antibodies directed against HDAC5, HDAC6, LPAR1, LPAR2 and MUC16

Figure 4.7: Morphology of HOSEpiC, OAW42 and SKOV-3 cells stained with specific antibodies

4.3 Contextual Discussion and Perspective

4.3.1 Introduction

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As mentioned in section 3.3.2, the Human Protein Atlas (HPA) is a large, comprehensive database based on experimental studies detailing the expression of numerous proteins in a variety of different cell lines [359], and for that reason we used HPA to evaluate the protein expression of (*HDAC5, HDAC6, LPAR1, LPAR2* and *MUC16*). In addition, Gene Cards, which is a combined human gene collection database about all human genes, and their corresponding protein product, gives information of the expression levels of these genes in normal cells, the genetic variants in these genes and their links to human diseases, and the biological pathways in which they function. [362, 378-380]. It also gives information of the location of gene expression in the normal human cell.

The ICC data in this project have permitted us to build a new supply with integration of RNA and protein expression data, i.e., the RNA expression levels as measured by RNA sequencing (RNA-Seq) and the protein levels detected by staining with immunohistochemistry. Due to

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immunohistochemistry based on enzymatic amplification, technology is not quantitative so it will not be surprising when the correlation between the RNA-Seq and the protein levels results show low correspondence for many genes; also, sometimes the target yields information implicating binding to unrelated proteins [361, 381].

4.3.2 HPA Analysis, Results and Discussion

The five target gene proteins (HDAC5, HDAC6, LPAR1, LPAR2 and MUC16) were subjected to cross-cancer tissue-specific analyses in the HPA. HPA analysis found the mentioned genes were expressed at low levels in ovarian cancer tissue, which agree with some of our ICC results (Figures (4.1-4.5).

Although LPAR1 showed low intensity cytoplasmic staining in OAW42 and SKOV-3, we could not find HPA data to confirm that because the LPAR1 protein expression in cancer tissues analysis is pending: https://www.proteinatlas.org/ENSG00000198121-LPAR1/pathology.

LPAR2 showed low intensity cytoplasmic staining in OAW42 and SKOV-3 cells, similar to HDAC5 which gave low protein expression in OAW42 and which agreed with the HPA low scores as indicated in Figures 4.8 and 4.9, respectively.

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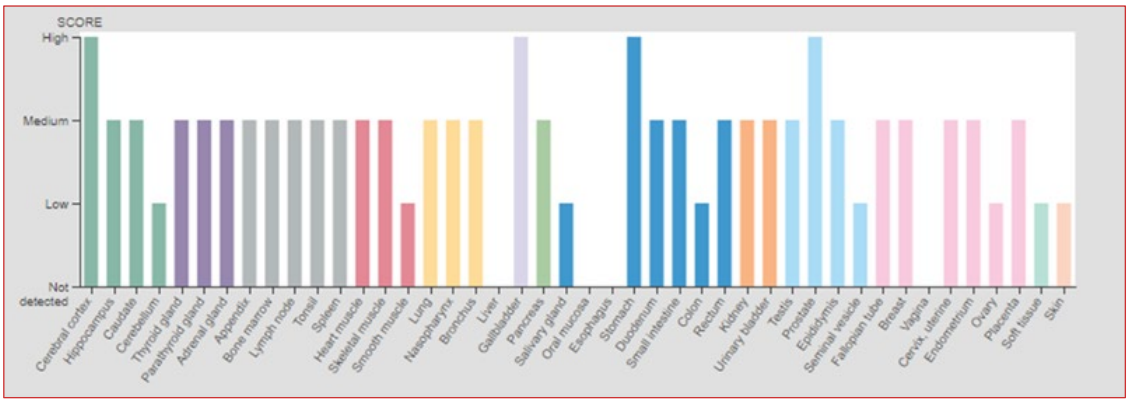

Figure 4.8: Protein expression overview of LPAR2 in various tissues according to HPA database score

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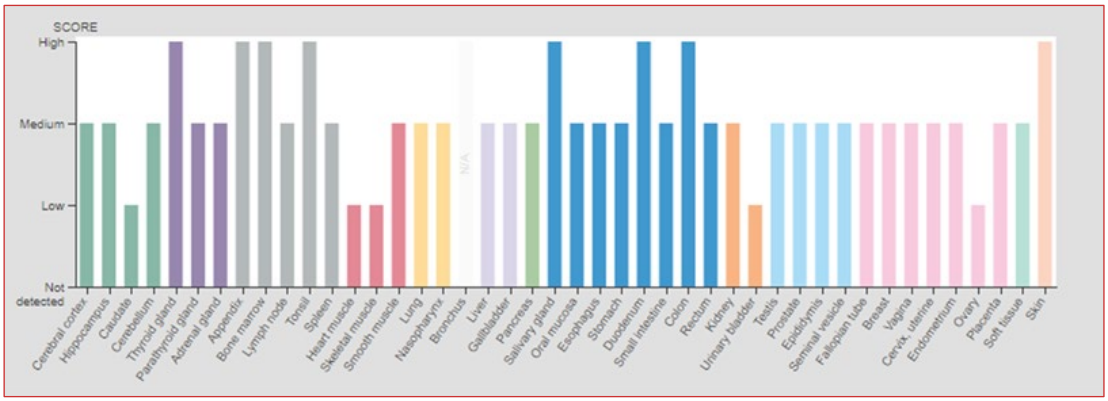

Figure 4.9: Protein expression overview of HDAC5 in various tissues according to HPA database score

However, HDAC5 showed high intensity staining in SKOV-3 cells, which is in disagreement with the HPA (Figure 4.9). Moreover, HDAC6 gave high cytoplasmic staining in OAW42 and moderate protein expression in SKOV-3, which does not corroborate with the HPA database investigation (Figure 4.10).

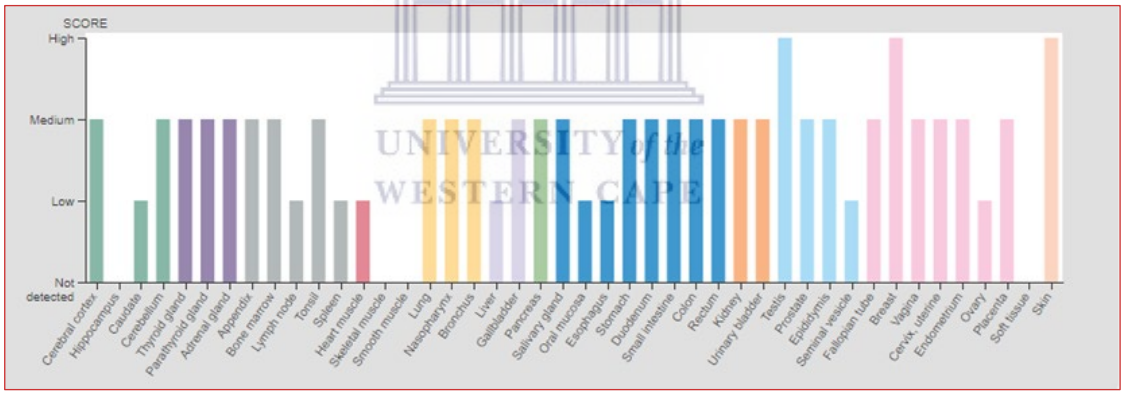

Figure 4.10: Protein expression overview of HDAC6 in various tissues according to HPA database score

On the other hand, MUC16 had no expression in the SKOV-3 cell similar to the HPA database (Figure 4.11) and neither did MUC16 expressed in OAW42 cells tested against the HPA database Figure 4.11). Correspondingly, four of the five genes (LPAR1, LPAR2, HDAC5 and HDAC6) also expressed in the cytoplasm of normal epithelial ovarian cells (HOSEpiC). Since comparing our results to Gene Cards data, we found LPAR1 expressed mainly in the endosome, plasma membrane and nucleus (Figure 4.12).

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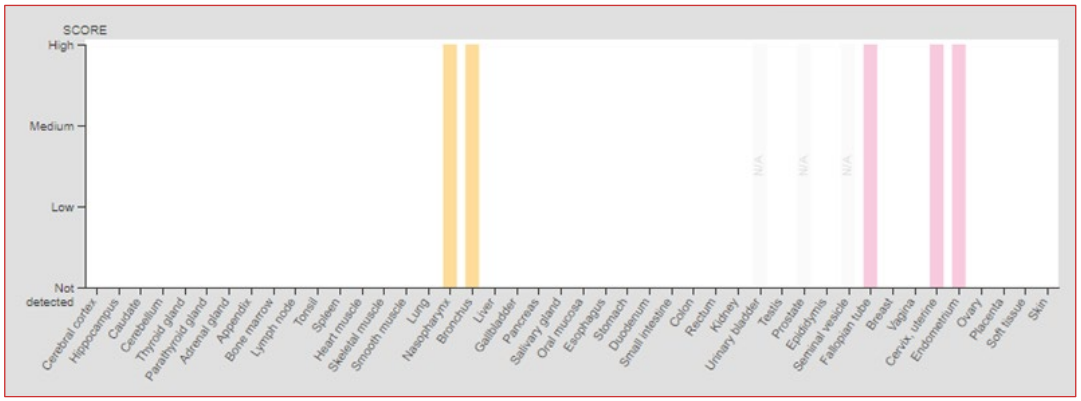
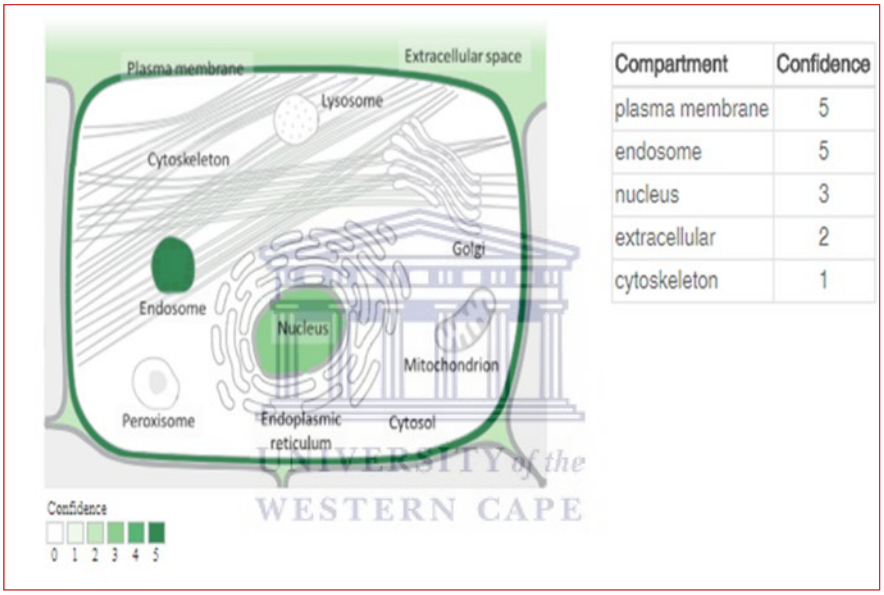


Figure 4.11: Protein expression overview of MUC16 in various tissues according to HPA database score



Compartment	Confidence
plasma membrane	5
endosome	5
nucleus	3
extracellular	2
cytoskeleton	1

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Figure 4.12: LPAR1 localization in the normal cell

LPAR2 is highly expressed in the plasma membrane (Figure 4.13). Figures 4.14 and 4.15 show that HDAC5 and HDAC6 are abundantly expressed in the nucleus, cytosol, Golgi apparatus, whereas HDAC6 shows high expression profiles in the plasma membrane and endosome as well (Figure 4.15). MUC16 had protein expression in the cytoplasm of HOSEpiC, which agree with the Gene Cards database that showed the expression of MUC16 in the normal cell, but it is localized in the Golgi apparatus, plasma membrane and extracellular compartment (Figure 4.16). In conclusion, LPAR1, LPAR2, HDAC5, HDAC6 and MUC16 were confirmed as biomarkers to

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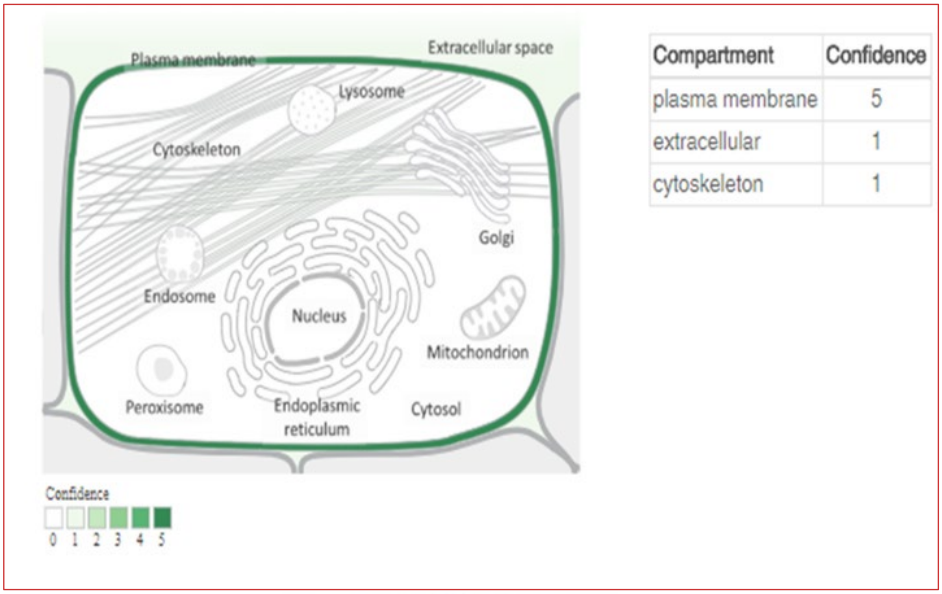
diagnose the epithelial ovarian cancer since these genes are also expressed in the normal ovarian cell line.



Compartment	Confidence
plasma membrane	5
extracellular	1
cytoskeleton	1

Figure 4.13: LPAR2 localization in the normal cell

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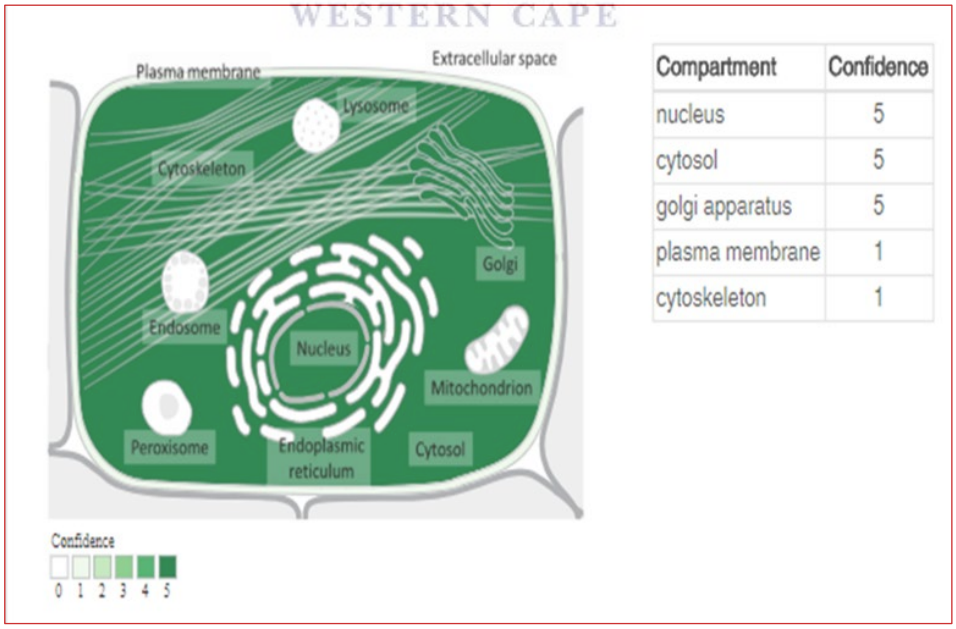

Compartment	Confidence
nucleus	5
cytosol	5
golgi apparatus	5
plasma membrane	1
cytoskeleton	1

Figure 4.14: HDAC5 localization in the normal cell

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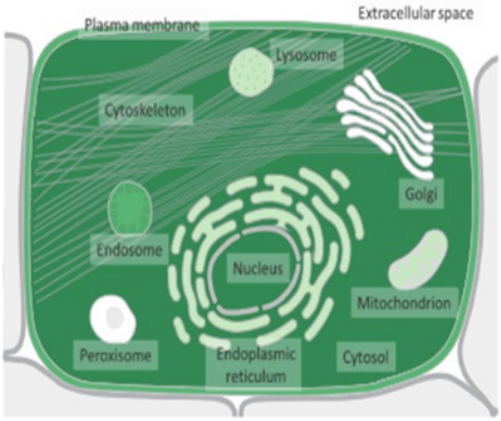
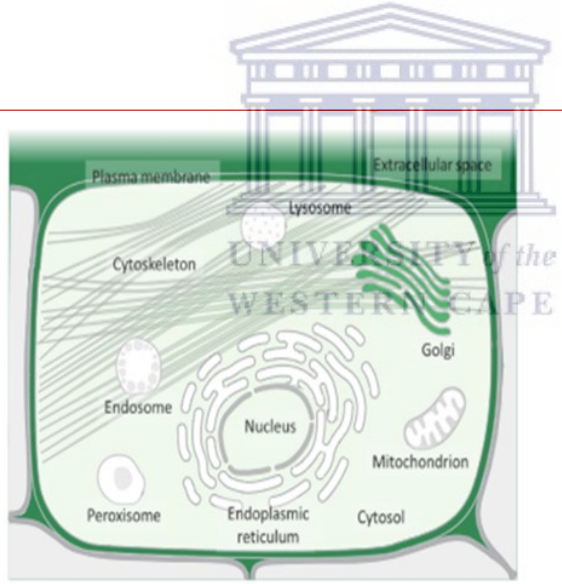


Compartment	Confidence
cytoskeleton	5
nucleus	5
cytosol	5
plasma membrane	4
endosome	4
mitochondrion	2
endoplasmic reticulum	2
lysosome	2

Figure 4.15: HDAC6 localization in the normal cell



Compartment	Confidence
plasma membrane	5
extracellular	5
golgi apparatus	4
cytoskeleton	2
nucleus	1
cytosol	1

Figure 4.16: MUC16 localization in the normal cell

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CHAPTER 5

General Discussion and Conclusion

5.1 Ovarian Cancer Epidemiology

Cancer in women has become an important health issue due to the morbidity and mortality outcome annually. The estimation for 2030 is that about 22.2 million new cancer cases and 12.7 million-cancer-related deaths will occur in women globally [377, 382]. Indeed, the availability and access to early, accurate diagnosis and quality care will assist to identify more than half of new cases and two-thirds of cancer deaths that will occur in low- and middle-income countries (LMIC) where this is urgently required [30], especially in black women in South Africa where cancer is detected at a relatively advanced stage.

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Universally, ovarian cancer is the fifth leading cause of cancer-related deaths [383], where it accounted for 2.5% of all female cancer cases [3], and about 240 000 cases are investigated every year, of which 140 200 cases were recorded in 2016 [23, 43, 61, 63]. It is estimated that 22 240 new ovarian cancer cases will be diagnosed and 14 070 ovarian cancer deaths will occur in the US alone [3]. Ovarian cancer has not precursor lesions to recognize, has more complex histological features and early stage cancer samples are more difficult to obtain when comparing to colorectal cancer [384]. Thus, there is 19% of women morbidity from breast cancer compared to 63% of ovarian cancer [385].

5.2 Ovarian Cancer Molecular Diagnostics, Therapeutics and Prognosis

Ovarian cancer is categorized with unique biology and behaviour at the clinical, cellular and molecular levels [66]. Regarding the most common type in ovarian cancer called Epithelial Ovarian cancer (EOS), 90% from all ovarian cancer types originate from the single layer of epithelial cells

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that covering the ovary surfaces, called Human Ovarian surface Epithelium (HOSE) [26]. Most women present with epithelial ovarian cancer symptoms at an advanced stage and thereafter cancer spread beyond the ovary to include the peritoneal cavity, which results in the high mortality rate, associated with EOC. At first, 80% of EOC cases responded to treatment, but most cases relapse. There is no real progress in the rate of cure although some data suggest that survival after the initial diagnosis of EOC has improved over time [27]. In this respect, from 12% in the 1970s to only 14% by the year 2000, the EOC diagnosis in the United States has improved which is a minor improvement [386].

Therefore, these emergency statistics shows the importance of research in ovarian cancer and the need for novel treatment approaches, therapies, and better prognostic and predictive biomarkers. Since the absence of ovarian cancer symptoms in the early stages leads to the poor prognosis, detection of definite molecular biomarkers/panels is emerging as an essential requirement for early detection of ovarian cancer. In this regard, an abundant of biomarkers / panels have been investigated and showed promise for ovarian cancer diagnosis based on two dimensional gel electrophoresis, mass spectrometry (MS), and protein microarrays and in combination with advanced bioinformatics, this have become powerful tools to detect proteins associated with the development of cancer [132].

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To this end, gene expression arrays have become powerful tools for ovarian cancer biomarker discovery[64, 80, 387-389] and have presented valuable evidence with respect to the biology of ovarian cancer, molecular changes in altered isotypes and signatures that predict prognosis and response to ovarian cancer treatment [80, 390-392]. Moreover, gene expression array has been an important tool to find the changes in mRNA expression that reflect the level of protein in tumour and these proteins are shed into body fluids where they can be isolated [80].

5.3 Contributions of This Study to the Ovarian Cancer Landscape

This study identified genes to screen and detect pre-symptomatic ovarian cancers that could

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facilitate earlier diagnosis and treatment. In addition, to identify potential tumour markers for the early detection of epithelial ovarian cancer, we chose samples that would exemplify the histological heterogeneity of the ovarian cancer by using gene expression array (RT-QPCR) in combination with protein microarrays (immunocytochemistry) which contributed to identify putative genes that expressed differentially in ovarian cancers when compared with normal ovarian surface epithelium.

In this study, we chose to investigate a panel of 11 target genes, including their expressed transcripts in ovarian cancer cells via normalization to the normal ovarian surface epithelium. Furthermore, validation was carried out using HPRT as a stable control gene. Q-PCR was used to quantity the RNA transcription level of various candidate target genes. Gene expression levels were calculated by Q-RT-PCR, expression variability was evaluated via the ΔΔCt method, and sample variation was determined by calculating the standard deviation.

In this study, we estimated the expression profiles of HDAC class I (HDAC1, HDAC2, HDAC3, HDAC8) [393, 394] HDAC class II (HDAC5, HDAC7) [394], LPAR1, LPAR2 [395, 396], and FOSL1[224, 225] are new markers for ovarian cancer and MUC16 as a standard biomarker of ovarian cancer [140], and investigated their expression profile using RT-QPCR. This was applied in various ovarian cell lines, including OAW42 (cystadenocarcinoma), SKOV-3 (adenocarcinoma), HOSEpiC (human ovarian surface epithelial cell), and using the relative expression tool REST® to analyse the expression profiles of the targets genes in each cell.

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The expression profiles of some targets were not the same in the mentioned cells. According to what was observed in Chapter 3, FOSL1, MUC16, LPAR2 and HDAC6 were expressed in significantly high levels in SKOV-3, and LPAR1 was upregulated in OAW42, i.e., these would be as good biomarkers for these cells respectively complemented with advanced studies. On the other hand, in OAW42 cells, HDAC1, HDAC7, HDAC8, LPAR2 gene profile expressions were 0.2, 0.4, 0.2, and 0.6, respectively, i.e., very low expression, which imply they could used synergically with another good profile ovarian cancer biomarker. Similarly, given the low expression profiles of

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HDAC2 and HDAC8 in SKOV-3 (0.9 and 0.7, respectively), they too could be used in combination with other biomarkers. Although the other genes (HDAC3 and HDAC5) are also moderately expressed in ovarian cancer cells (OAW42 and SKOV-3), the expression levels of these genes are not as high as the expression levels of FOSL1, MUC16, LPAR2 and HDAC6 in SKOV-3, and the expression level of LPAR1 in OAW42. As mentioned in Chapter 2, all QPCR based on using HPRT fulfilled most criteria as a suitable housekeeping gene (HKG) in that it was steadily expressed, and it did display the least fluctuation.

The QPCR expression profiles of the five genes were confirmed by ICC staining. The use of marker panels, including RT-QPCR and ICC tests, increased the sensitivity to 60% while keeping up 100% specificity. Hence, although further studies are clearly required, our data recommend that a limited number of markers in combination might detect most ovarian cancers. In addition, our results suggest two valuable methods for epithelial ovarian cancer detection in the future. First, the methods show that a panel of individual markers may be more accurate than a single marker. Second, the evaluation of global gene expression patterns in cancers with RT-QPCR and ICC tests can reveal unexpected copies that provide new opportunities for biomarker identification. The integration of these two approaches is attractive in that additional markers can simply be added to the panel as they are discovered, retaining specificity through high cut-offs while expanding sensitivity.

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Five biomarkers or candidate genes (HDAC5, HDAC6, LPAR1, LPAR2 and MUC16) that are expressed differentially in ovarian cancers compared to other tissues or cancer types have already been identified by RT-QPCR and confirmed by ICC analysis and efforts to generate monoclonal antibodies to the other six genes (HDAC1, HDAC2, HDAC3, HDAC7, HDAC8 and FOSL1) encoded proteins are underway. In conclude, here we provide strong evidence suggesting that HDAC5, HDAC6, LPAR1, LPAR2, except MUC16 are up regulated in ovarian cancer. These data were confirmed by examining Human Protein Atlas (HPA) databases, in addition to protein expression of HDAC5, HDAC6, LPAR1, LPAR2 and MUC16 in cells cytoplasm.

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5.4 Future Perspectives

Further experimentation is necessary to establish the usefulness of these markers for diagnosis, prognosis and treatment of ovarian cancer. Finding adequate biomarkers for early diagnosis of ovarian cancer remain limited and require additional investigation [395, 397]. In this study, we emphasized the diagnostic values of HDAC5, HDAC6, LPAR1, LPAR2 and MUC16 for early ovarian cancer. For future perspectives, the actual screening with serum tumour markers, protein expression will be strengthened by using other techniques that assess the variant expression that could explain the release of these gene candidates into the circulation. In addition, future work should focus on developing assays with the candidate genes and combinations identified in this study.

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APPENDIX

Chapter 3: Supplementary Information

App. 1. Melting Curves and Melting Peaks

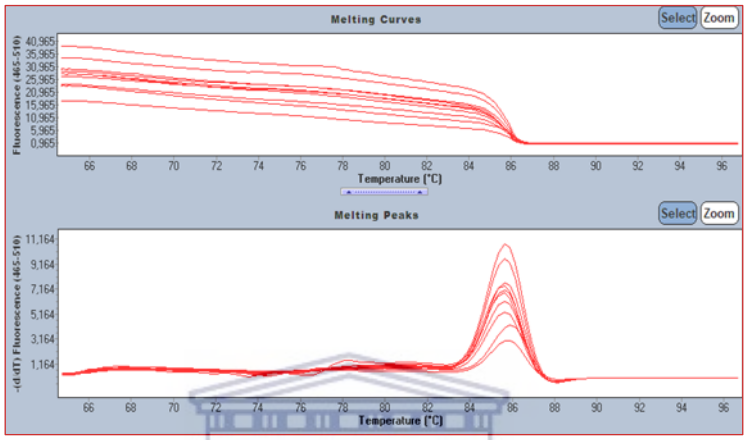

Figure App. 1: Melting curve and melting peak of GAPDH in HOSEpiC cells

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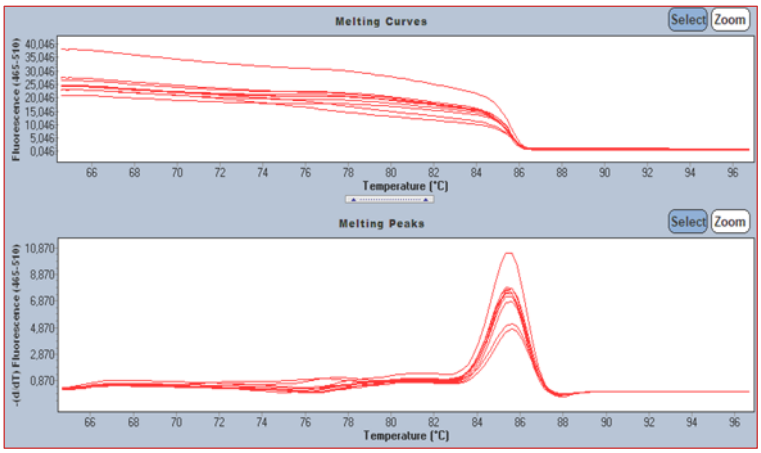

Figure App. 2: Melting curve and melting peak of GAPDH in OAW42 cells

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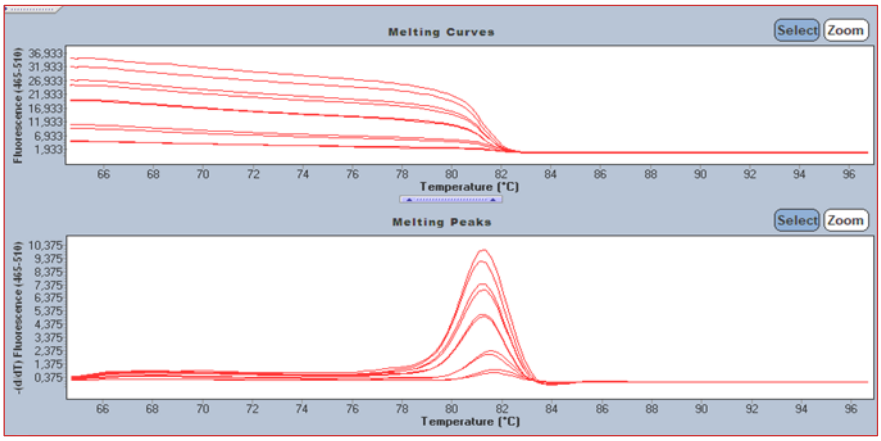

Figure App. 3: Melting curve and melting peak of HPRT in HOSEpiC cells

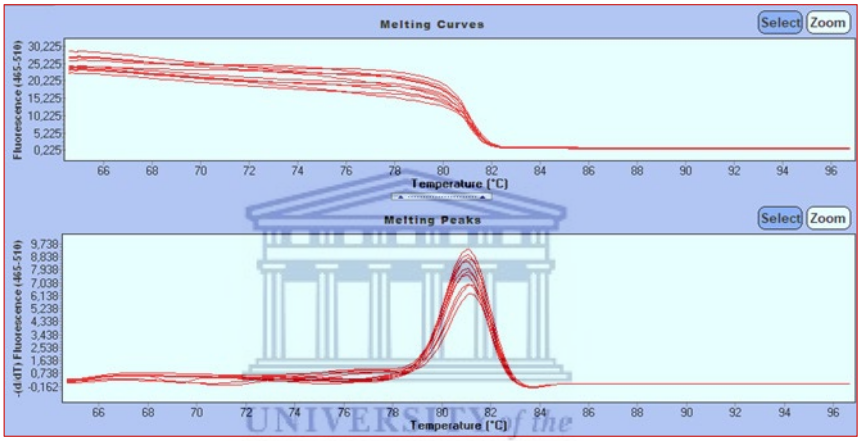

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Figure App. 4: Melting curve and melting peak of HPRT in OAW42 cells

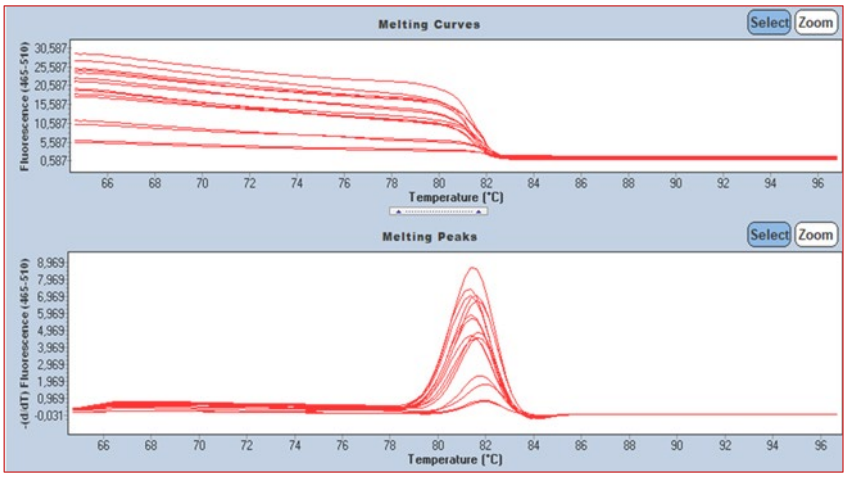

Figure App. 5: Melting curve and melting peak of HPRT in SKOV-3 cells

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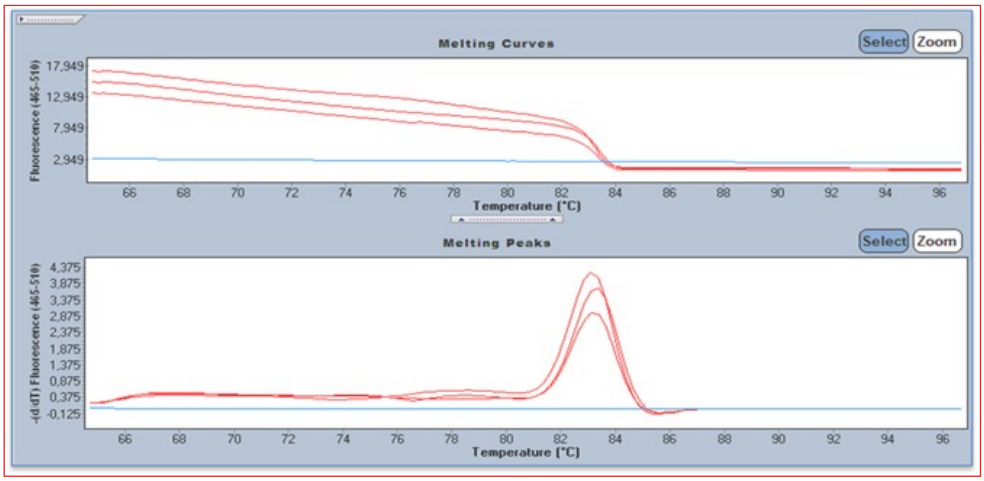

Figure App. 6: Melting curve and melting peak of HDAC1 in SKOV-3 cells

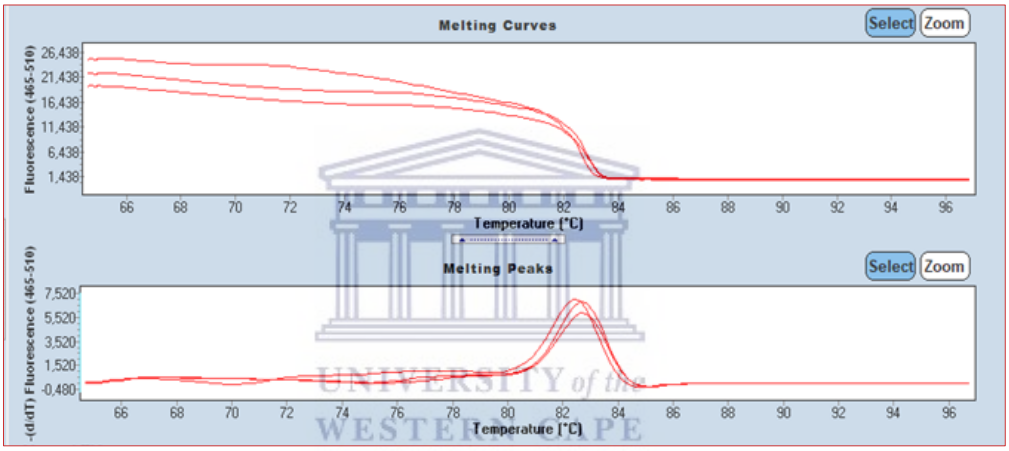

Figure App. 7: Melting curve and melting peak of HDAC1 in OAW42 cells

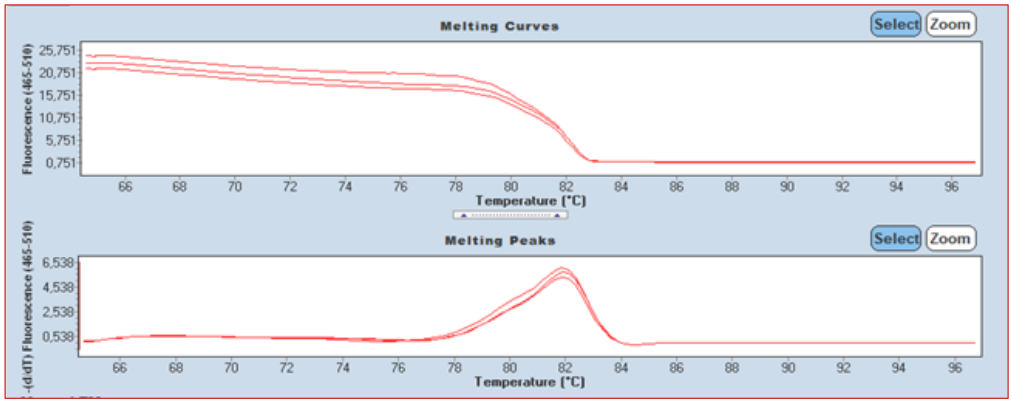

Figure App. 8: Melting curve and melting peak of HDAC2 in OAW42 cells

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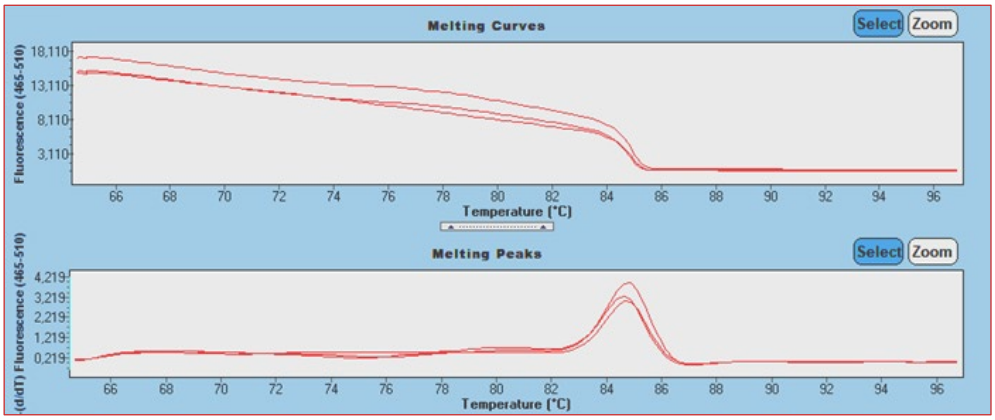

Figure App. 9: Melting curve and melting peak of HDAC3 in OAW42 cells

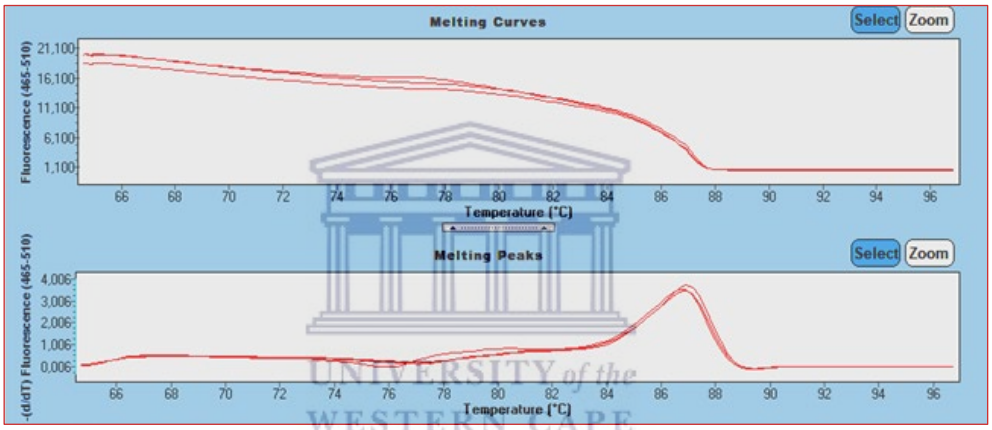

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Figure App. 10: Melting curve and melting peak of HDAC5 in OAW42 cells

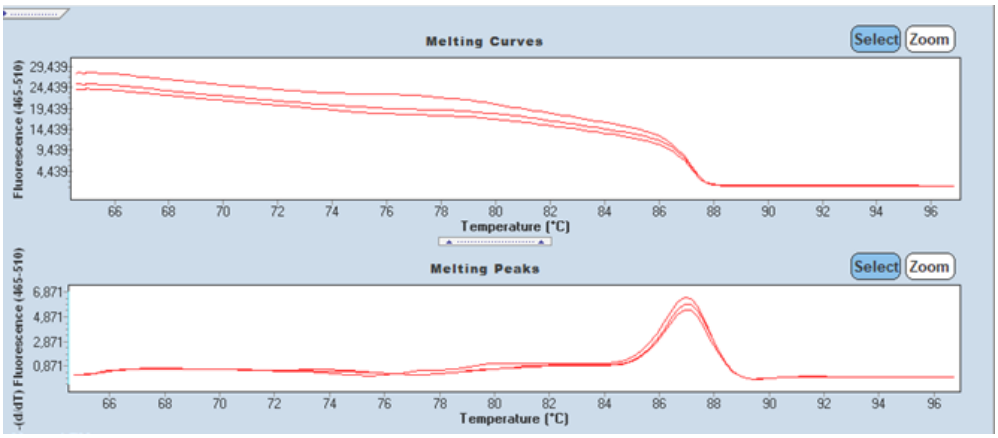

Figure App. 11: Melting curve and melting peak of HDAC7 in OAW42 cells

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App. 2 Best-Fit Values

Table App. 1: Best-fit values ± SE

Slope	**-3.4 ± 0.01587**	**-3.32 ± 0.006667**	**-3.367 ± 0.01491**	**-3.383 ± 0.01575**	**-3.363 ± 0.008389**	**-3.393 ± 0.005443**
Y-intercept	31 ± 0.02295	27.99 ± 0.009639	25.61 ± 0.02155	28.03 ± 0.02278	27.61 ± 0.01213	26.74 ± 0.00787
X-intercept	9,117	8,432	7,608	8,285	8,21	7,881
1/slope	-0,2941	-0,3012	-0,297	-0,2956	-0,2973	-0,2947
95% Confidence Intervals						
Slope	-3.451 to -3.349	-3.341 to -3.299	-3.414 to -3.319	-3.433 to -3.333	-3.39 to -3.337	-3.411 to -3.376
Y-intercept	30.92 to 31.07	27.96 to 28.02	25.54 to 25.68	27.96 to 28.1	27.57 to 27.65	26.72 to 26.77
X-intercept	8.986 to 9.251	8.379 to 8.485	7.505 to 7.714	8.167 to 8.407	8.147 to 8.274	7.841 to 7.92
Goodness of Fit						
R square	0,9999	1	0,9999	0,9999	1	1
Sy.x	0,05018	0,02108	0,04714	0,04981	0,02653	0,01721
Is slope significantly non-zero?						
F	45900	248004	51005	46129	160750	388622
DFn, DFd	1, 3	1, 3	1, 3	1, 3	1, 3	1, 3

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P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Deviation from zero?	Significant	Significant	Significant	Significant	Significant	Significant
Equation	Y = -3.4*X + 31	Y = -3.32*X + 27.99	Y = -3.367*X + 25.61	Y = -3.383*X + 28.03	Y = -3.363*X + 27.61	Y = -3.393*X + 26.74
Data						
Number of X values	5	5	5	5	5	5
Maximum number of Y replicates	1	1	1	1	1	1
Total number of values	5	5	5	5	5	5
Number of missing values	0	0	0	0	0	0

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App. 3 Quantitative Real-Time PCR Cycling Parameters

Programs

Program Name	Cycles	Analysis Mode
pre incubation	1	None
Amplification	40	Quantification
melting	1	Melting Curves
cooling	1	None

Temperature Targets

Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)
95	None	00:03:00	4.4		0	0	0

Amplification Temperature Targets

Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)
95	None	00:00:10	4.4		0	0	0
61	None	00:00:20	2.2		0	0	0
72	Single	00:00:01	4.4		0	0	0

melting Temperature Targets

Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)
95	None	00:00:05	4.4				
65	None	00:01:00	2.2				
97	Continuous		0.11	5			

cooling Temperature Targets

Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)
40	None	00:00:40	2.2		0	0	0

Figure App. 12: Quantitative real-time PCR cycling parameters

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