DOCTOR OF PHILOSOPHY

The Role of Aquaporin 3 (AQP3) in Breast Cancer

Muhammad Arif

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Aston University

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Thesis summary

The increasing prevalence of breast cancer (BC) in different parts of the world, particularly in the UK, highlights the importance of research into the aetiology and pathology of the disease. BC is the most common malignancy affecting women worldwide. Aquaporins (AQPs) are membrane protein channels that regulate cellular water flow. Recently, studies have demonstrated that expression of AQP3 is up-regulated in cancerous breast tissue. The present study examines the role of AQP3 in BC cell biology. Examination of clinical cases of BC showed higher AQP3 gene and protein expression in cancer tissues compared to healthy border tissues. In distinct clinicopathological groups however there were no differences observed with regards to AQP3 expression, suggesting that AQP3 expression may not be a predictor of lymph node infiltration or tumour grade. shRNA technology was used to knockdown gene expression of AQP3 in the invasive MDA-MB-231 BC cellular model. Cellular proliferation, migration, invasion, adhesion and response to the 5-fluorouracil (5-FU) based chemotherapy treatment were investigated in parental and knockdown cell line. AQP3 knockdown cells showed reduction in cellular proliferation, migration, invasion and increase in cell sensitivity to 5-FU compared with wild type (WT) or scrambled control (SC) cells. The effects of AQP3 knockdown on cellular glycolytic ability and ATP cellular content were quantified. Indirect glucose uptake was also measured by quantifying reconditioned media. AQP3 knockdown cells showed significantly lower levels of glucose uptake as compared to WT or SC. However there was no difference in the glycolytic ability and ATP content of the cells suggesting AQP3 has no role in cancer cell energetics. These data collectively suggest AQP3 expression is associated with the BC disease clinically and plays a role in multiple important aspects of BC pathophysiology, thus AQP3 represents a novel target for therapeutic intervention.

Keywords: Water channel, breast cancer, glycerol channel.

*Dedicated to my parents
&
family*

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Last I would like to thank my parents, my brother and his family, my sister and her family, and my in-laws for their love and kindness. Words certainly cannot express my emotions but their support means a lot to me. Last one person without whom I couldn't do this thesis is my wife. Her love, sacrifice and patience has helped me to fully concentrate on the project during this study.

The sources of images published in this thesis are provided underneath the images. All other images (where the source is not mentioned) were purchased.

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Abbreviations

2-DG	2-deoxy-D-glucose
5-FU	5-Fluorouracil
A°	Angstroms
AC	Adenocarcinoma
ADH	Atypical ductal hyperplasia
AH	Atypical hyperplasia
Akt	Protein kinase B
ALH	Atypical lobular hyperplasia
ANOVA	Analysis of variance
AQP	Aquaporin
ar/R	Aromatic/Arginine
ATCC®	American type culture collection
ATP	Adenosine triphosphate
Au	Gold or Aurum
BC	Breast cancer
BME	Basement membrane extract
BMI	Body mass index
BRCA1	Breast cancer associated gene 1
BRCA2	Breast cancer associated gene 2
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CC	Craniocaudal
cDNA	Complementary DNA
cGMP	Cyclic guanosine monophosphate
CHIP	Channel forming integral protein

CK	Cytokeratin
CMF	Cyclophosphamide, methotrexate, fluorouracil
CO_2	Carbon dioxide
CTCs	Circulating tumour cells
$CuSO_4$	Copper sulphate
DAPI	4',6-diamidino-2-phenylindole
DCIS	Ductal carcinoma *in situ*
DHAP	Dihydroxyacetonephosphate
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECAR	Extracellular acidification rate
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
ER	Oestrogen receptor
ErbB	Erythroblastic leukaemia viral oncogene homolog
FAC	Fluorouracil, adriamycin, cyclophosphamide
FBS	Foetal bovine serum
FEC	Fluorouracil, epirubicin, cyclophosphamide
FFPE	Formalin fixed paraffin embedded
FGF-2	Fibroblast growth factor-2
FITC	Fluorescein isothiocyanate
Foxa2	Forkhead box a2
G3P	Glycerol-3-phosphate

G3PDH	Glycerol-3-phosphate dehydrogenase
GCNT1	Glucosaminyl (N-acetyl) transferase 1
GK	Glycerol kinase
HBSS	Hank's balanced salt solution
HCl	Hydrochloric acid
HER-2	Human epidermal growth factor receptor-2
HIF-1	Hypoxia-inducible factor-1
HRP	Horse radish peroxidase
HRT	Hormone replacement therapy
IDC	Invasive ductal carcinoma
ILC	Invasive lobular carcinoma
KO	Knockout
LB	Luria-Bertani
LCIS	Lobular carcinoma *in situ*
LFS	Li-Fraumeni syndrome
LN	Lymph node
MIP	Major intrinsic proteins
MLO	Mediolateral oblique
mRNA	Messenger ribonucleic acid
MSCs	Mesenchymal stem cells
mTOR	Mammalian target of rapamycin
NHS	National health service
NPA	Asparagine - proline – alanine
NSCLC	Non-small cell lung cancer
Ob-R	Leptin receptor
OXPHOS	Oxidative phosphorylation
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate-buffered saline
PI3K	Phosphatidylinositol-3-kinase
PKA	Protein kinase A
PLD2	Phospholipase D2
PR	Progesterone Receptor
qPCR	Real-time polymerase chain reaction
RCF	Relative centrifugal force
RFP	Red fluorescence protein
RNA	Ribonucleic acid
RNAi	RNA interference
ROI	Region of interest
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SC	Scrambled control
SCC	Squamous cell carcinoma
SCID	Severe combined immunodeficiency
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error mean
shRNA	Short hairpin RNA
TBS	Tris-buffered saline
TDLU	Terminal duct lobular unit
TNBC	Triple-negative breast cancer
TP53	Tumour suppressor 53 gene
UK	United Kingdom
USA	United States of America
WT	Wild type

Chapter 1

Introduction

Our understanding of life threatening diseases has increased dramatically in recent decades. Through medical and scientific advances, societies worldwide are better able to prevent and treat many diseases and have even eradicated some from existence. Despite these advances however, our understanding of many lethal diseases continues to be relatively poor and therefore the search for an improved understanding of many diseases continues.

The human body is an incredibly complex organism and the story of how each human develops is both complicated and beautiful. Human life starts from the fusion of two cells into one. The process by which this single cell then divides, develops and differentiates into a large multi-cellular, multi-organed organism is tightly regulated by a network of genes and proteins. There are approximately 20,000–25,000 genes in the human genome (International Human Genome Sequencing, 2004) and recently it was suggested that about ~572 of these genes are involved in somatic cell division or mitosis (Neumann *et al.*, 2010).

Cell division occurs via an elegant process consisting of an ordered sequence of controlled events, often termed as the cell cycle (see Figure 1.1), that produces two identical daughter cells containing an equal number of chromosomes (Nicklas, 1997). The fate of cells is often decided by the genetic material that exists inside the nucleus of each cell, consisting of deoxyribonucleic acid (DNA) and other associated macromolecules. Essentially, there are four phases in the cell cycle of an animal cell; gap phase (G1), the synthesis phase (S phase), a second gap phase (G2) and Mitosis (M phase) (Hartwell and Weinert, 1989; Norbury and Nurse, 1992). DNA replication takes place in S phase (Laskey *et al.*, 1989) and chromosomes separate during M phase (McIntosh and Koonce, 1989). Cells which have exited the cell cycle enter into a quiescent state referred to as G0 phase and can re-enter the cell cycle in response to stimulus (Cheung and Rando, 2013). Each cell must successfully duplicate its chromosomes before division so that it can produce its two daughter cells as a result of M phase followed by cytokinesis. The whole process of cell division is tightly regulated by molecular checkpoints in the cell cycle and the cell must pass through these checkpoints in order to divide (Hartwell and Weinert, 1989). This tight regulation can however fail

in certain situations. Exposure to ionising radiation, viral infection, and chemical insult may all

cause DNA damage or mutations. Structural or chemical alterations to the DNA of a cell can result

in a variety of cellular fates, ranging from programmed cell death (often termed as apoptosis) or

uncontrolled cellular proliferation (Hedgecock *et al.*, 1983; Ellis and Horvitz, 1986). If the cell

cycle becomes dysregulated, cellular division may in turn become uncontrolled. This can lead to

abnormal cells dividing in an uncontrolled fashion, increasing in number without the normal checks

and balances that regulate the proliferation of healthy cells. This uncontrolled growth may be able

to potentially give rise to a malignant tumour, commonly referred to as cancer.

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Figure 1.1   Stages of the cell cycle.

After cell division an active cell enters into the cell cycle after going through checkpoints whilst senescent, inactive and arrested cells exit cycle and enter into G0 phase. In the first stage (G1) the cell grows and duplicates its organelles and after passing through checkpoints enters into the S phase where DNA replication takes place. The next phase, G2 phase, is where replicated material grows and the cell prepares itself for division. After passing through another checkpoint, the cell enters in M phase where chromosomes segregation takes place followed by cytokinesis. The newly formed cells are ready to enter in G1phase once again.
(Image was purchased from NATOMIMAGES)

Cancer is a term that describes a collection of disease states involving dysregulation of cellular proliferation and failure to respond to the normal factors that control cell division. Tumours are generally classified as being benign or malignant (Peck and White, 1922). Benign tumours are generally not thought to be dangerous as proliferating cells in benign tumours are confined and do not invade other parts of the body. However, malignant tumours consist of cells that are able to invade their surrounding tissue and spread to distant parts of the body. This process, termed metastasis, requires a specific environment to which the cells are exposed. In malignant tumours, the metastatic process may consist of different phases.

Cancer is an ancient disease. The Greek physician Hippocrates, commonly referred to as the father of medicine, believed that human emotions and temperaments were caused by body fluids called humours and these humours consisted of blood, phlegm, yellow bile and black bile. Hippocrates believed that a healthy body would have all four humours stably maintained and that their imbalance would potentially cause diseases. Hippocrates believed that an excess of black bile in the body caused tumours (Sudhakar, 2009). Hippocrates named the disease as "karkinos", the Greek word for crab which was later modified to carcinoma and translated into the Latin term "Cancer" (Sung *et al.*, 2011). The name Karkinos was given because of the appearance of blood vessels in the tumour resembling a crab's claw reaching out and intruding the nearby tissues. After the discovery of the lymphatic system, the theory of black bile causing cancer was finally rejected in 17th century (Mankin, 2010).

There are known to be many different causes of cancer, as there are many different types of cancer. The causes are diverse, including the DNA damaging factors mentioned earlier. Additionally, life style factors and distinct morbidities can place an individual at an increased risk of developing cancer. Examples include obesity and diabetes, which are both strongly associated with cancer of many different organs (Abe *et al.*, 1976; Edney and Smith, 1988; Farrow *et al.*, 1989; Fairfield *et al.*, 2002; Calle and Thun, 2004; Gallagher *et al.*, 2010; Bansal *et al.*, 2013; Dubois *et al.*, 2013; El-Jurdi and Saif, 2013; Fang *et al.*, 2013; Farrell, 2013; Mazzarella *et al.*, 2013; Rundle *et al.*, 2013;

Vermorken *et al.*, 2013; Yang *et al.*, 2013; Hursting, 2014; Vanni and Bugianesi, 2014). As the worldwide incidence of these diseases continues to rise, despite increased awareness and improved medical care, the incidences of the cancers they are associated with are also rising (Jemal *et al.*, 2010). Previous data displayed that cancer was one of the most significant causes of morbidity and mortality in the 20th century (De Flora *et al.*, 2005). The World Health Organisation estimates that the global burden of cancer mortality will increase to 12 million deaths per year by 2030 (Thun *et al.*, 2010). Because of the vast numbers of cases and deaths reported each year, cancer has become an area of heavy focus in biomedical research over recent decades, and is considered as one of the primary threats to human health in the 21st century.

Breast cancer (BC) occurs worldwide in both developing and developed countries and is considered as a heterogeneous disease which can be classified according to its clinical features, expression of tumour markers, and histology (Li *et al.*, 2005). Like other cancers, the incidence of BC is increasing (Cutler *et al.*, 1971; Miller *et al.*, 1991; Olsen, 1997) as well and many factors contribute to this rise in incidence. Modern lifestyles, particularly in developed nations, including unbalanced dietary intake of saturated fats and simple carbohydrates may also contribute to the upsurge in cancer cases (Nkondjock *et al.*, 2006; Afolabi, 2007; Saxena *et al.*, 2012). This change in lifestyle may also contribute to earlier onset of menstrual periods and late menopause (Flint, 1978; van Noord and Kaaks, 1991; Khaw, 1992; van Noord *et al.*, 1997; Kato *et al.*, 1998) which in turn can increase the risk of BC.

1.1   The anatomy and physiology of the breast

Human breasts or mammary organs are located on the anterior chest wall and generally cover a significant proportion of its space. Breasts in females become developed through puberty and are responsible for the production of milk for the nourishment of children. Breasts differ in size and shape depending mostly on the amount of adipose tissue that is present surrounding the glandular

aspects (see Figure 1.2). The breasts are connected to several muscle groups including the pectoral and the intercostal muscles (Jinde, 2009).

Numerous milk ducts within the adult female breast open into a main collecting duct termed the lactiferous duct through which milk is transported. Usually there are approximately fifteen to twenty lactiferous ducts (Bannister *et al.*, 1995), however this figure is controversial with other reported figures being as low as nine (Ramsay *et al.*, 2005a). Each breast is made up of fifteen to twenty lobes arranged in a circular fashion and each lobe is made up of many bulb-shaped milk glands called lobules (Glasier and McNeilly, 1990; Bissell and Labarge, 2005). This whole area of ducts, glands and lobules is covered by stroma which includes fibrous tissue and adipose fat (Cowie, 1974). This complex ductal system terminates in small lobular units known as terminal duct lobular units (TDLUs) which are composed of lobules, and these TDLUs develop through a highly conserved process called branching morphogenesis (Gudjonsson *et al.*, 2005). The lobules are arranged around ducts and each lobule is composed of sac-like clusters called alveoli which consist of gland cells called lactocytes where milk is formed and stored (Bannister *et al.*, 1995). Each individual cell in an alveolus is a complete milk secretory unit that synthesizes fat, protein and carbohydrate (Cowie, 1974). The ducts terminate at the nipple connecting alveoli with cavities called ampullae or lactiferous sinuses. Each sinus is a dilated portion of the lactiferous duct where milk accumulates temporarily before secretion by cells in the nipple (Bannister *et al.*, 1995) although this too is controversial, as one study has demonstrated the complete absence of sinuses in lactating breast of mothers (Ramsay *et al.*, 2005b). The majority of milk is stored in the alveolar region until required by the suckling baby (Geddes, 2009). The stimulation of the nipple results in the production of the hormone oxytocin from the posterior pituitary into the bloodstream which then binds to the receptors of contractile cells near lactocytes and due to the contraction of these cells milk is transferred into the milk duct (Cowie, 1974; Geddes, 2009).

Figure 1.2   Schematic representation of the human breast.

Human breasts are located on the anterior chest wall and in adult women are responsible
for the production of milk for the nourishment of children. Each breast has a rich supply of
blood vessels, nerves and lymphatic vessels and nodes. The breasts are connected to
different types of muscles which include the pectoral major and minor muscles. Several
milk ducts open into a main collecting lactiferous duct through which milk is transported.
Each breast is made up of fifteen to twenty lobes which are made up of many lobules.
Sac-like clusters called alveoli are present in each lobule and composed of lactocytes
where milk is formed and stored. The ducts terminate at the nipple connecting alveoli with
cavities called lactiferous sinuses.
(Image was purchased from Dreamstime.com)

Breast tissue has a rich supply of blood vessels, nerves and lymphatic vessels and nodes (Montagna

and Macpherson, 1974). A breast is composed of two layers of epithelium, an inner luminal

epithelium and the outer basal layer of myoepithelium (Bissell and Labarge, 2005). These layers

are lined by inner polarised luminal epithelial cells and outer contractile myoepithelial cells (Sarrio

et al., 2012) respectively segregated from stroma by a structural barrier of basement membrane

which is mostly made up of laminin (Gudjonsson *et al.*, 2002). These two layers build the cellular architecture of the breast components (Anbazhagan *et al.*, 1998). The differentiated luminal cells in the alveoli act as lactocytes and the contractile cells are myoepithelial cells (Sleeman *et al.*, 2007). In addition to these cell lineages mammary stem cells are also present in the breast. Luminal and myoepithelial cells are thought to arise from common ancestral cells which act like stem cells. Indeed reports have indicated that a single stem cell is proficient enough of rebuilding the entire mammary tree (Kordon and Smith, 1998; Shackleton *et al.*, 2006; Stingl *et al.*, 2006).

1.2   Breast cancer

BC is one of the leading causes of cancer mortality in women worldwide. The prevalence of BC in different parts of the world, particularly in the UK, highlights the importance of on-going research into the mechanisms involved in its initiation, growth and spread. There is currently an estimated life-time risk of 1 in 8 women to develop BC in the UK (CancerResearch, 2013). BC is the second commonest cause of cancer death (16% of all women) in women after lung cancer (20.3%) in the UK (Ferlay *et al.*, 2010). Illustrating this risk, around, 11,600 women and 75 men died from BC in the UK in 2010 (CancerResearch, 2013) and in 2011, around 41,523 new cases of malignant BC were registered in women and 303 in men for England alone (Statistics, 2013). The prevalence of the disease is higher in less developed regions (around 324, 000 die each year) than more developed regions (around 198, 000 die each year) (Ferlay *et al.*, 2013). Most deaths occur due to metastatic BC as there are currently no permanent cures available once primary tumours have spread (Miller *et al.*, 2005). Importantly, metastatic relapse is observed within 5 years in almost 50% of women in the USA who undergo surgical treatment for their initial cancer (Lorincz and Sukumar, 2006).

The prevalence of BC coupled with its impact on women's lives and failure to find a permanent cure makes it a prime candidate for research. The main aim for the BC researcher is not only to find

a cure but also to prevent the disease. The burden of BC mortality could be reduced by evidence based strategies and more adequate treatment of patients (Anderson *et al.*, 2006).

BC is an ancient disease (Robinson, 1986) and the existence of BC was known even in ancient times when other diseases and even other types of cancers were less known. The Edwin Smith Papyrus, written around 1500 B.C.E., is based on an ancient Egyptian document dating possibly from 3000 B.C.E. and it is believed to be the first written document that describes breast tumours or ulcers (Breasted, 1930; Stiefel *et al.*, 2006). The document described one case (Case 45) of bulging tumours in male's breast. It was mentioned that there was no treatment available for bulging and cool-to-touch breast tumours (Donegan, 2006). The fact that advanced breast tumours are visible in the breast in the form of a lump, and that this mass increases with time may be the reason that BC was detected in such a primitive era.

1.3   Histopathological types of breast cancer

The transformation of normal breast cells into tumour cells is a complex process involving changes in the activity of a number of key genes and proteins. The development of a tumour in the breast is not in itself life threatening, however should this tumour moves to other tissues then the situation becomes grave. The route by which cancerous cells invade and migrate plays a critical role in the development of tumours from a non-invasive to an invasive and metastatic phenotype (Price *et al.*, 1997). The progression of BC is a multi-sequential phenomenon. It can start from stem cells in TDLUs of normal breast epithelia to give rise to atypical hyperplasia (AH) which then proceed to *in situ* carcinoma and then finally to invasive cancer (Allred *et al.*, 2001).

AH is a pre-cancerous stage, considered an early precursor lesion which consists of some but not all abnormal cells with indications of few somatic chromosomal alterations (Sinn *et al.*, 2010). If these cells are present in a duct then a diagnosis of atypical ductal hyperplasia (ADH) can be made

and in case of a lobular origin atypical lobular hyperplasia (ALH). These pre-cancerous lesions may proliferate, resulting in marked enlargement of ducts and TDLUs compared to normal tissue and should potentially be considered as tumours in spite of their small size (Allred *et al.*, 2001). Whilst BC risk is believed to be positively associated with both types of AH, it is more evident in cases affected with ALH (Page *et al.*, 1985; Marshall *et al.*, 1997). In addition, women with ALH are at an increased risk (about 3-fold increase) of developing BC in the same breast (Page *et al.*, 2003).

BC can found in different areas of the breast but most frequently it is initiated in ducts or lobules (Wellings, 1980a; Wellings, 1980b). Depending upon the origin of the cancer, cases are often clinically classified as ductal carcinoma or lobular carcinoma, the two most common histologic forms of the disease which together account for around 90% of all BC cases (Bertos and Park, 2011). There are several other types of BC but far less is known about these rarer histological BC types, including mucinous, tubular, inflammatory, medullary, and papillary carcinomas, which together account for the remaining 10% of cases (Li *et al.*, 2005).

There are two subtypes of ductal and lobular carcinomas. Ductal carcinoma in situ (DCIS) which is a non-invasive type and invasive ductal carcinoma (IDC) which as the name suggests is an infiltrating type of BC. Similarly, lobular carcinoma is also divided into two subtypes, lobular carcinoma in situ (LCIS) and invasive lobular carcinoma (ILC). DCIS occurs inside the lumen of lactiferous ducts and remains confined to the duct that it is initiated in. As the basement membrane is generally undamaged, the tumour cells do not move out of the duct into any of the healthy surrounding breast tissue, and therefore cancer is contained and does not spread. DCIS is the most commonly recorded type of non-invasive BC (Jara-Lazaro *et al.*, 2009). Generally, DCIS is detected by mammography, as it is not usually clinically palpable and often there are no visible changes to the patient's breast (Peters *et al.*, 2012). DCIS is generally not associated with a high mortality, although it can potentially increase the risk of developing an invasive cancer. DCIS

comprises between 15–33% of all breast tumours diagnosed within mammographic screening programs for women over 50 (Ernster *et al.*, 1996).

IDC is a cancer that is initiated in the cells of the lactiferous ducts of the breasts which has subsequently spread into the surrounding breast tissue or infiltrated into the nearby lymph nodes due to the migrating potential of these cells (see Figure 1.3A). In LCIS, irregular growth and cell morphology changes occur inside the breast lobes. Again the cells remain confined to their original position and do not infiltrate into surrounding tissue. LCIS does not present a significant risk to life if diagnosed early but it can increase the risk of getting ILC in the future. In ILC, cancer growth initiated in lobule cells subsequently invades into the surrounding breast tissue (see Figure 1.3B).

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Figure 1.3   Types of invasive breast cancer.

The invasive cancer cells spread and enter into the surrounding breast tissues. The two main types of invasive cancers are IDC and ILC. (A) In IDC, cancer cells break the basement membrane of the duct, invade into the surrounding tissues and then can metastasise to other parts of the body through lymphatic system. (B) ILC commonly starts in lobules and then metastasises to other tissues through the lymphatic system after breaking the basement membrane of the lobe.
(Image was purchased from NATOMIMAGES)

1.4   Epidemiology of BC

The incidence of BC is increasing worldwide, even though it is very rare in men, affecting more than 1 million women in 2012 (Ferlay *et al.*, 2013). According to a recent report, nearly 136 new cases of BC are diagnosed each day in the UK (CancerResearch, 2014). There are many risk factors that have been associated with the progression of BC, including gender, age, race, heredity, obesity, smoking, early menarche, late menopause, later age of first full-term pregnancy, hormonal replacement therapy, nulliparous women after age 40 years, diet, alcoholism and socioeconomic conditions. Gender is the most common risk factor as women are more affected than men. Some of these established risk factors are discussed in detail subsequently.

1.4.1   Age

As ageing is a universal process every human being is continuously ageing, consequently increasing their risk of age-associated diseases. Life expectancy has increased with the advancement in medical science but such increase come with a price to pay. Many human cancers are considered late-onset and occur when the body's natural defence and repair systems are unable to effectively cope with them. Cancer susceptibility in older adults depicts the accumulated effects of many underlying mechanisms which may include mutation, epigenetic changes, telomeric instability and altered stromal milieu (DePinho, 2000). The hypothesis that BC biology is significantly affected by increasing patient age is supported by some data (Eppenberger-Castori *et al.*, 2002). However women age 35-40 and younger BC patients often have a worse prognosis than their older counterparts, which is again an indication of dissimilarity in age-associated tumour biology (Nixon *et al.*, 1994; Chung *et al.*, 1996). Additionally, this worse prognosis was observed in younger patients in spite of a higher than expected positive hormone receptor status (El Saghir *et al.*, 2006).

The incidence of BC cancer rises with age culminating in a lifetime risk of developing BC of 1 in 12 for women before reaching the age of 75 years (Coleman, 1999). But this risk is lower in younger women and increases as they get older. BC risk is strongly correlated with age as 80% of cases occur in women aged 50 years and over (CancerResearch, 2013) and the incidence has been suggested to increase with age at doubling rates approximately every 10 years till menopause, after which the rate slows significantly (McPherson *et al.*, 2000). BC is the single commonest cause of death among women aged 40-50 (McPherson *et al.*, 2000). In the UK, around 1,200 women aged below 50 died from BC in 2010 (CancerResearch, 2013).

Ageing is thus a universal problem and predisposes an individual to many diseases associated with the biological decline that occurs as we age. There is no universally accepted theory of ageing. One specific theory of ageing involves telomere dysfunction and telomere length is also considered as a useful biomarker of biological ageing. Telomeres shorten with each cell division and this process is thought to be key in cellular ageing (Ershler and Longo, 1997). Telomeres are distinctively situated at the ends of chromosomes and consist of hexa-nucleotide tandem repeats of the sequence TTAGGG (Blasco, 2005). Somatic cells carrying chromosomes with shortened telomeres reach replicative senescence (known as the Hayflick limit after the scientist who first identified the phenomenon) which induces arrested cell growth after a finite number of cell divisions (Hayflick, 1965). Cellular proliferation beyond the Hayflick limit culminates in telomere attrition leading to a cellular crisis that can culminate in mutation or apoptosis (Chin *et al.*, 2004). However some cells are able to escape cellular crisis by evading cellular checkpoints due to inactivation of tumour suppressor 53 gene (*TP53*) and reactivating a telomere maintenance mechanism (Maser and DePinho, 2002) resulting in uncontrolled cell division. This mainly occurs at the G1 phase, and mutations involving the G1 checkpoint are extremely common in human cancers (Ershler and Longo, 1997).

Thus telomere dysfunction weakens the chromosomal stability and is associated with an increased risk of various cancers including BC (Wu *et al.*, 2003). Indeed, evidence has shown that short

telomere length is associated with increased BC risk and is more evident in premenopausal women (Shen *et al.*, 2007). Telomere shortening is also reported in DCIS (Meeker *et al.*, 2004). It is also suggested that telomere dysfunction and associated events occurred in BC during transition from ADH to DCIS (Chin *et al.*, 2004). Thus BC and ageing are correlated with each other and elderly women may suffer from BC due to mutation load, telomere dysfunction, epigenetic mechanisms and changes in stromal environment (DePinho, 2000).

In addition to these biological factors, the increased mortality of breast cancer in older women may also be due at least in part to disparities of diagnosis among elder women. A study has shown that elderly women with BC have late diagnosis, incomplete diagnostic evaluation, and often lack a uniform therapeutic tactic (Bouchardy *et al.*, 2003). Thus apart from genetic and other factors BC screening is also important.

1.4.2 Sex hormones

As mentioned earlier, BC in aged women is a major health problem and it is highly correlated with early menarche, late menopause, and first pregnancy at more advanced age (Russo *et al.*, 2005). A woman's risk of developing BC is inversely proportional to the number of children she has. Women whose first child was delivered at a very young age have a reduced lifetime risk of developing BC and this risk further decreases with more pregnancies (Lambe *et al.*, 1996). It has been reported that the risk of BC decreases for a woman, who gives birth for the first time aged under 18 years (Macmahon *et al.*, 1970). This observation is backed up with data from laboratory animals, where a recent study demonstrated in mice that a history of early pregnancy changes genetic profiles and reduces BC risk (Meier-Abt *et al.*, 2013). However child birth at an early age does not confer any protecting effect for carriers of mutations in breast cancer associated gene 1 (*BRCA1*) or breast cancer associated gene 2 (*BRCA2*) (Jernstrom *et al.*, 1999). Also consumption of

oral contraceptives for longer time period before the birth of first child may also lead to increased risk of cancer (Pike *et al.*, 1981).

Endocrine cells synthesise and secrete both steroid and peptide hormones including oestrogen, progesterone, androgens and glucocorticoids which are transported via the blood to their target cells where they bind to their specific receptors (Tsai and O'Malley, 1994) and elicit a response. The two most important sex hormones are oestrogen and progesterone. Oestrogen and progesterone are primarily produced in ovaries and testis in response to follicle stimulating hormone and luteinising hormone. These are steroid hormones that have significant roles in the development and growth of mammary glands and mammary cells in addition to many other functions (Fendrick *et al.*, 1998). The common functions of oestrogen include the stimulation of follicle production, proliferation of the uterine and vaginal epithelium and the development of external genitalia and secondary sexual characteristics (Bocchinfuso and Korach, 1997). To perform its function, oestrogen binds to its receptor (oestrogen receptor or ER) which has two major variants; ERα and ERβ (Ban *et al.*, 2008). Several different endogenous oestrogens are produced in a woman's body. Two of the main types of endogenous oestrogens that are found in the circulation are oestrone and oestradiol. Both oestrogen and progesterone are associated with the growth of normal breast cells and also with cancerous breast cells (Pike *et al.*, 1993; Travis and Key, 2003; Duss *et al.*, 2007). Similarly ER and progesterone receptor (PR) are known to be involved in BC pathology. Although circulating oestrogen in premenopausal women is formed mainly in the ovaries, endogenous oestrogen synthesis in adipose tissue plays a significant role in postmenopausal women (Ghosh *et al.*, 2007). Exposure to increase levels of oestrogen and progesterone substantially promote the lifetime risk of developing BC (Anderson, 2002). In 1896, George Beatson, a British surgeon, removed both ovaries of a 40-year-old premenopausal woman, who was suffering from a large tumour on her right breast, consequently reduced her pain and disease (Beatson, 1896). The case clearly demonstrated the role of sex hormones in BC pathogenesis. For a long time, it was believed that sex hormones were risk factors for developing BC and that therefore postmenopausal status would be important to reduce this risk. However, from the 20th century, postmenopausal status was

linked with increased BC risk (Gapstur *et al.*, 1992; Thacker, 2003; Kessenich, 2004; von Schoultz, 2009).

Studies using laboratory animals have shown that exposure to oestrogens promotes development of mammary tumours (Rose *et al.*, 2002). The variants of ER are expressed differentially, with around 75% of diagnosed BC cases having an ERα positive status and subsequently responding efficiently to hormone based therapy (Di Leva *et al.*, 2010). This suggests the importance of targeted therapies when patients express different isoforms of hormone receptors. Association of high levels of sex steroid hormones were found to be powerful predictors of BC (Berrino *et al.*, 1996). Higher concentrations of endogenous oestrogens are linked with around two fold increased risk of BC in postmenopausal women (Key *et al.*, 2002). In postmenopausal women serum oestrogen levels are usually at around one third of the lowest premenopausal level, and serum progesterone levels are almost zero (Pike *et al.*, 1993). Those with elevated oestrogen concentration are also at an increased risk of developing endometrial cancer (Campbell and McTiernan, 2007) as well.

There are a variety of influences that can control the circulating concentrations of sex hormones. These factors which include age at first pregnancy, number of children, and age at menarche can also influence the risk of BC (Colditz, 1998). It has been estimated that each child that a woman gives birth to reduces the risk of ER positive and PR positive BC subtype by 11%, whereas first pregnancy at old age increased the risk to this BC subtype by 27% (Ma *et al.*, 2006).

The use of hormone replacement therapy (HRT) for symptomatic relief during the menopause is relatively high in developed nations. It has been hypothesised that oestrogen replacement therapy increases BC risk and that addition of exogenous progesterone will further increase this risk (Pike *et al.*, 1993). Previously published work in a cohort of postmenopausal women showed that women taking oestrogen alone or both oestrogen and progesterone were at elevated risk of developing invasive BC, especially women over the age of 55 (Colditz *et al.*, 1995). Findings from another

study suggest that combined oestrogen and progesterone treatment was associated with an increased risk of BC compared to oestrogen alone (Schairer *et al.*, 2000). Recently it was demonstrated in a large population based study that those BC patients who showed menopausal symptoms including hot flushes, night sweats and vaginal dryness have better disease prognosis and reduced risk of relapse compared to patients without menopausal symptoms (Chen *et al.*, 2013). It was suggested that menopausal symptoms were associated with decreased recurrence because of oestrogen reduction (Chen *et al.*, 2013).

The underlying molecular mechanisms behind the role of sex hormones in the BC development and progression are not yet clear. Nonetheless it has been shown by various studies that sex hormones and their receptors increase the risk for developing BC. One possibility is that ovarian hormones stimulate mitosis in breast cells during menstruation (Key and Pike, 1988). Another possibility is that after menopause, when ovarian production of oestrogens has ceased, the circulating oestrogens are produced in the adipose tissue by fat cells (Rose *et al.*, 2004). Overall these studies support the hypothesis that there is an important role of endogenous hormones in the aetiology of BC.

1.4.3 Heredity

There are a selection of individuals that possess genotypes that can significantly predispose them to BC. Mostly germ-line mutations are the reasons for these selective variants predisposed to cancer but some *de novo* mutations may also cause BC. The two commonly known germ-line mutations *BRCA1* (Miki *et al.*, 1994) and *BRCA2* (Wooster *et al.*, 1995) are identified and linked with an increased risk of developing BC. The normal function of *BRCA1* and *BRCA2* proteins is to assist DNA repair and homologous recombination, thus maintaining chromosomal stability (Welcsh *et al.*, 2000). Mutation in these genes can therefore induce a decreased ability to repair damaged DNA and can significantly increase the risk of developing BC (Lidegaard *et al.*, 2005). Mutation in an autosomal dominant allele may lead to a risk of 50% of developing cancer in the offspring as in the

case of *BRCA* mutations. Approximately 5% to 10% of all BC cases specifically account for germ-line mutations (Mazhar and Waxman, 2006; de Bruin *et al.*, 2013) and most of these are due to *BRCA1* and *BRCA2* mutated genes (Ford and Easton, 1995). Research has suggested that the majority of *BRCA1*-associated tumours and sporadic basal-like tumours descend from luminal intermediate cells rather than from basal stem cells (Molyneux *et al.*, 2010).

Scientists confirmed the involvement of *BRCA1* in hereditary breast cancers by characterisation of germ-line mutations in 63 BC patients across 10 families with cancer linked to chromosome 17q21 (Friedman *et al.*, 1994). Interestingly, analysis of human tumour samples revealed that mutations in both *BRCA1* and *BRCA2* have different effects upon gene expression (Hedenfalk *et al.*, 2001). In some cases where only one allele is mutated, a more lethal effect is observed than cases of complete mutation. Such mutant *BRCA1* (mut/+) gene demonstrates lower *BRCA1* levels. This leads to a failure of producing luminal cells progeny indicating that *BRCA1* is a critical regulator of breast epithelial progenitor lineage (Liu *et al.*, 2008). Transcriptional repressors are present in the tissues to ensure the cell lineages remain intact. However, increased expression and stability of transcriptional repressors such as Slug are partly responsible for increased expression of basal-like phenotypes. Increased Slug expression is associated with *BRCA1* (mut/+), causing an increased tendency for the development of basal-like phenotype thus dictating progenitor cell fate (Proia *et al.*, 2011). Consequently, *BRCA1* mutations, whether partly or completely apparent, may result in the accumulation of genetically unstable breast stem cells, providing prime targets for further carcinogenic events (Liu *et al.*, 2008).

Thus *BRCA1* and *BRCA2* mutations can be important in families where the penetrance can be as high as 80% (Cuzick, 2003) and even in close relatives of *BRCA1/2* negative cases, there is a high risk of developing BC (Shahedi *et al.*, 2006). This highlights the importance of other genes associated with BC risk as mentioned in medical literature. One such gene associated with cancer is *TP53*. The *TP53* mutation is identified in most families diagnosed with Li-Fraumeni syndrome (LFS), which is a syndrome of autosomal dominant inherited cancers including BC (Malkin *et al.*,

1990). On the other hand genetic analysis in patients suffering from LFS revealed that the *TP53* gene was inactivated (Frebourg *et al.*, 1992). It was also observed that *TP53* inherited BC was linked with over expression of human epidermal growth factor receptor-2 (HER-2) (Wilson *et al.*, 2010). Overall about 20% of BC tumours show overexpression of HER-2 protein (Mukohara, 2011). In addition to being HER-2 positive, most BC cases of either DCIS or IDS associated with *TP53* mutations are hormone receptor positive (Masciari *et al.*, 2012). Such HER-2 positive cases have a family history of early-onset BC (Melhem-Bertrandt *et al.*, 2012). Recently a study reported a case of a young 35-year old woman with BC in both breasts (although negative for *BRCA1* and *BRCA2*) who was diagnosed with LFS due to mutations in *TP53*. This study emphasises the importance of gene testing for *BRCA1*, *BRCA2* and *TP53* mutations in individuals with a family history (de Bruin *et al.*, 2013).

BC is the most common tumour among women with germ-line *BRCA1*, *BRCA2* and *TP53* mutations; therefore it may at times be important to use genetic screening for diagnostic purposes. However genetic screening is very expensive and cumbersome procedure. As BC associated with germ-line *BRCA1* or *BRCA2* mutations have identifiable histologic phenotypes, therefore it is important to do histologic evaluation before genetic testing for susceptibility genes (Armes *et al.*, 1998). In cases where there is a family history of BC, presence of obesity significantly increases the risk of developing BC compared with slimmer women with a positive family history (Carpenter *et al.*, 2003).

1.4.4 Obesity

According to global estimates, in 2008 over 200 million men and around 297 million women were obese (Finucane *et al.*, 2011). A significant increase in obesity has been observed in England since 1993 as 13% of men and 16% women were regarded as obese in 1993 as compared with 24% and 26% respectively in 2011 .With the help of a simulation model, it is projected that the increase in

obese adults by year 2030 would be 65 million in the USA and 11 million in the UK (Wang *et al.*, 2011). Body mass index (BMI) is commonly used to classify underweight, overweight and obesity in adults and is defined as the weight in kilograms divided by the square of the height in metres (kg/m^2) (Plankey *et al.*, 1997). According to the health and social care information centre UK, adults having a BMI of 25 kg/m^2 to 29.9 kg/m^2 are considered to be overweight and with a BMI of 30 kg/m^2 or above to be obese (Health and Social Care Information Centre, 2013). A potential association between BC and obesity has been recognised for many years (De Waard *et al.*, 1964). Obese BC patients show evidence of a higher risk for lymph node metastasis and death when compared with non-obese BC patients (Calle *et al.*, 2003; Berclaz *et al.*, 2004). Whilst evidence suggested that women diagnosed with invasive BC have higher risk of developing BC with increasing obesity (Schapira *et al.*, 1990), the contrary has been suggested in another analysis, whereby an inverse association between elevated BMI and BC in premenopausal women was observed (Ursin *et al.*, 1995). The link therefore between obesity and BC is an intriguing and controversial one. Generally, evidence suggests that increased BMI among premenopausal women is not linked with BC risk, possibly via decreased endogenous oestrogen levels (Potischman *et al.*) but significantly increases risk for BC in postmenopausal women (Calle *et al.*, 2003). Recent data supports the overall notion that there is a differential link between menopause status and BC and that obese postmenopausal women are at higher risk level to develop BC. One recent meta-analysis showed that elevated BMI was linked inversely in premenopausal women and positively in postmenopausal women with ER^+PR^+ tumour subtype (Suzuki *et al.*, 2009). This analysis demonstrated that for each 5-unit increase in BMI, a 33% increase in BC risk was observed in postmenopausal women and 10% decreased risk in premenopausal women (Suzuki *et al.*, 2009). Furthermore, recently a large pooled analysis of invasive BC patients showed that BMI was strongly associated with hormone receptor positive tumours (Yang *et al.*, 2011b). Conversely, a different study recently reported that elevated BMI was significantly associated with increased BC risk in premenopausal women older than 35 years, but not postmenopausal women (Cecchini *et al.*, 2012).

The pathophysiologic mechanism by which obesity and menarche status interplay to enhance BC risk is poorly understood. The adipocyte represents one of the most abundant cell types in the stroma that surrounds breast epithelial cells and it has been demonstrated in the breast microenvironment that adipocytes hold the ability to influence their neighbouring cancer cells (Iyengar *et al.*, 2003). Adipocytes not only store surplus energy in the form of lipids but are also capable of secreting a number of proteins and non-protein hormones often referred to as adipokines (Rajala and Scherer, 2003). As both size and number of adipocytes increase in obese individuals, it is thought that a dysregulated secretion of adipokines might have an additional influence on BC (Schaffler *et al.*, 2007). Researchers have in fact demonstrated that both preadipocytes and fully differentiated adipocytes *in vitro* have the potential to enhance the growth and development of BC cells (Johnston *et al.*, 1992). One of the most studied adipokines produce in the body, mainly by subcutaneous white adipose tissue, is leptin. Most of the effects of leptin are mediated by its full length receptor (Ob-R) (Rajala and Scherer, 2003). It has been shown that mammary epithelial cells can also express and secrete leptin, with secretion being mainly associated with fat globules present in the milk (Smith-Kirwin *et al.*, 1998). Studies on cancer and non-cancer breast biopsies indicate that both leptin and Ob-R are present in the human breast tissue, suggesting that mammary cells can be influenced by leptin, potentially through paracrine, endocrine and even autocrine pathways (O'Brien S *et al.*, 1999; Garofalo *et al.*, 2006; Ishikawa *et al.*, 2006). Also, leptin has been shown to influence the growth of cultured BC cells *in vitro* (Rose *et al.*, 2004). Exposure to leptin also increases expression and enzymatic activity of aromatase (an important enzyme responsible for the synthesis of oestrogen) in cultured BC cells (Catalano *et al.*, 2003). However it has also been shown that oestrogen can regulate leptin levels in rats and human (Shimizu *et al.*, 1997), suggesting a potentially complicated situation in the breast. It was demonstrated *in vitro* that leptin secretion was significantly increased in adipose tissue samples from women only and not in men when stimulated by oestradiol and was unaffected by stimulations by oestrone and progesterone in both genders (Casabiell *et al.*, 1998).

Taken together these studies provide a possible mechanistic link between obesity and BC prognosis, however more concrete studies are required to investigate the link between these two. Complicating the situation is the observation that circulating leptin levels in premenopausal women diagnosed with DCIS were not significantly different from healthy volunteers (Mantzoros *et al.*, 1999). This suggests that although leptin has significant effects *in vitro*, the effects may not be as significant *in vivo*. It has however been shown that leptin is expressed in DCIS, ADH and normal border tissue around DCIS but not by healthy breast tissue specimens (Caldefie-Chezet *et al.*, 2005). This demonstrates the complex nature of both obesity and BC thus making it difficult to understand their relationship. Moreover, disparities between studies also make this linkup controversial.

1.5   Phenotypes of breast cancer

Although BC can present with the histological types previously mentioned, there are also a series of phenotypic classifications that can be applied to the disease. Recent research suggested that BC should be classified into 10 novel subgroups with distinct clinical outcomes on the basis of 10 integrative clusters (designated as IntClust 1–10) which are characterized by well-defined copy number anomalies (Curtis *et al.*, 2012). Although novel and interesting, this system is still to be clinically validated before it can be used in hospitals. Conventional classification is based upon gene expression profiling, whereby BC cases can be separated into five different major subtypes: luminal A, luminal B, HER-2 overexpressing, basal-like, and normal-like (Perou *et al.*, 2000; Sorlie *et al.*, 2001; Sorlie *et al.*, 2003; Cheang *et al.*, 2008). The majority of BC cases emerge from differentiated luminal cell populations. These subtypes differ in genomic complexity, key genetic alterations and clinical prognosis (Sorlie *et al.*, 2001). Among the five, the HER-2 enriched expression and basal-like BC have worse clinical outcomes, and luminal A has a better prognosis than luminal B (Cheang *et al.*, 2008). These subtypes are broadly conserved between Asian and Caucasian patients and are already present at the pre-invasive stage (Yu *et al.*, 2004). Indeed

studies demonstrated the presence of these subtypes in DCIS and suggested a potential precursor to invasive type (Bryan *et al.*, 2006; Livasy *et al.*, 2007).

The term triple-negative breast cancer (TNBC) is used to describe tumours that are PR and ER negative and do not overexpress HER-2 and accounts for approximately 15%–20% of all BC patients. The basal like group is composed of mostly triple negative cancers, characterized by the lack of any ER and PR immunoreactivity and of HER-2 over-expression (Viale *et al.*, 2009). Positive markers of this group of tumours are basal-cell cytokeratins including cytokeratins (CK) 5/6, 14 and 17 (Yehiely *et al.*, 2006). It has been previously demonstrated that expression of basal cytokeratins (CK 5/6 and/or CK 14/17) predicts poor outcome (Nielsen *et al.*, 2004; Dent *et al.*, 2007) in BC patients, and particularly in the node-negative breast carcinoma, expression of CK5/17 was a prognostic factor independent of size and grade of tumour (van de Rijn *et al.*, 2002).

A less common potential human subtype identified based on genomic studies is named as claudin-low subtype (Herschkowitz *et al.*, 2007). These tumours have worse prognosis than luminal A and are mostly triple negative (about 61%-71%) but closely bear a resemblance to mesenchymal and mammary epithelial stem cells and show enhanced epithelial-to-mesenchymal transition (EMT) features (Prat *et al.*, 2010). Previous studies have suggested that cases of normal-like subtype are actually significantly contaminated by normal tissues and therefore do not depict the true tumour (Prat *et al.*, 2010).

Based on this, the two most common subtypes of human BC are the luminal and the basal-like which show more aggressive behaviour, including invasion and supposed cancer stem cell activity (Sorlie *et al.*, 2001). Recently, Kim *et al*. (Kim *et al.*, 2012) showed that luminal-like cells were fully capable of generating much larger and more invasive tumours than basal-like cells in mice and suggested there are multiple stem-like cells with tumorigenic potential within a single tumour. The ability to form tumours and invasive potential of the luminal-like cancer cells relies strongly on the expression of the gene GCNT1, which encodes a key enzyme named as glycosyl transferase

controlling O-glycan branching (Kim *et al.*, 2012). Studies have also suggested that both subtypes (basal and luminal) may originate from the luminal epithelial lineage as shown by an *in vitro* model in which *BRCA1* mutation-associated basal-like tumours developed in luminal progenitors indicating that luminal progenitors are probably the cell type most commonly associated with the initiation of BC (Molyneux *et al.*, 2010).

1.6   Breast screening

Breast screening programs aim to both identify the presence of breast cancer at an early, more treatable stage and to target women who do not yet have cancer but may develop it due to the risk associated with BC. Whilst effective treatment of the disease is essential, preventing the disease is equally important and it is believed that improved screening and detection strategies, including the use of biomarkers is achievable and could potentially reduce the BC mortality rate (Wang and Srivastava, 2010). In order to be valuable any screening test must be relatively inexpensive, accurate and should be minimally invasive. About 1.6 million women per year are screened for BC in the UK (Ariyarathenam *et al.*, 2013). The national health service (NHS) Breast Cancer Screening Programme in the UK has invited women aged between 50-70 to breast screening every three years since 1988 (Independent, 2012). However it was recommended to change this age range and to invite all women ages 47-49 for breast screening without randomisation (Ariyarathenam *et al.*, 2013). BC screening is normally carried out by mammography (see Figure 1.4) in most parts of the world. Mammographic screening for BC is performed with the viewpoint to diagnose the disease at a preclinical and localised phase (Zorbas, 2003). Mammography has proved to be sensitive for women aged 50 years and older who have less denser breast as compared to women younger than 50 years (Kerlikowske *et al.*, 1996). Breast screening is still a debatable practice in the UK, with both merits and demerits of the system being apparent. According to a panel review, around 1,300 breast cancer deaths every year are saved due to screening, which is equal to 22,000 years of life, although about 11% of the cancers over-diagnosed as well (Independent, 2012).

Unsurprisingly a recent study showed that the participants of screening programs often are unaware of over-diagnosis (Waller *et al.*, 2013). Over-diagnosis cannot be avoided as screening increased the number of mastectomies by 20%, which in some cases should be avoided thus creating harm to affected women and even identifying slow-growing cancers and cell changes that are biologically benign is not beneficial (Gøtzsche and Nielsen, 2009). Screening reduces BC death by 15% and over-diagnosis is around 30% which means for every 2000 screened throughout 10 years, one will avoid death due to BC whereas 10 will be treated unnecessarily for non-invasive cancers (Gøtzsche and Jorgensen, 2013). About 25% of women aged 40-49 years with invasive cancer would be ill-advised that their mammograms are clear whereas 10% of women aged 50-69 years would be falsely diagnosed even after using a high-tech screening mammography (Zorbas, 2003).

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Figure 1.4   Representation of a typical mammogram.

Mammogram showing extensive areas of dense tissue. Routine mammogram comprises of Craniocaudal (CC) and mediolateral oblique (MLO) views. A) CC view of right breast (RCC) and left breast (LCC). (B) MLO view of right breast (RMO) and left breast (LMO). Arrows show abnormal tiny micro-calcifications which were clinically proven to be ductal carcinoma.
(Image was purchased from Dreamstime.com)

Therefore an effective screening program is needed which can increase early detection of cancer and it should also confer some advantage over current screening programs (Bleyer and Welch, 2012). Consequently, screening has benefits and yet it may also be harmful. Some of the studies which have considered that screening is beneficial are not accurate as they do not include deaths due to other causes (such as heart attack) which may be due to the effects of radiotherapy. If we consider that breast screening reduces mortality, then the number of women over-diagnosed and over-treated is also incredibly large. Therefore effective and accurate screening is necessary and should also consider other biomarkers of the disease before surgery and adjuvant therapies. This will certainly lower the psychological distress and pain of the affected women and their families.

1.7 Metastasis

The phenomenon by which cancer cells spread from the organ of origin to secondary organs of the body is termed metastasis. BC cells can be transported through blood and lymph vessels to distant tissues. Generally organs such as bone, brain, liver, and lung are affected from metastatic BC cells. However, for BC metastasis, bone and lung are two of the most frequently observed sites (Minn *et al.*, 2005) and inflict different fates in terms of overall survival (Solomayer *et al.*, 2000). Metastasis was explained as being comparable to seed dispersion in plants by a famous theory of Stephen Paget in 1889. He suggested that metastasis was like viable seeds being dispersed and carried to all directions, but only those can survive and grow which fall on a nurturing soil (Paget, 1889). Likewise all cancer cells are similar to individual seeds and only those cells grow which find an appropriate environment (Paget, 1889). The hypothesis of seed and soil was questioned over a period and was accepted only after many studies indicated that tissue microenvironment does play an important role in the development of secondary tumours.

The mechanism of metastasis in a malignant tumour was accurately described by Isaac Levin in 1913. According to Levin, the process of metastasis starts when cancer cells are discharged from

the initial tumour and transported into distant parts of the organism where these cells divide and form secondary tumours (Levin, 1913). The presence of the right microenvironment to permit the seeding of tumour cells and therefore growth of tumour at the secondary site away from the primary tumour is essentially the major cause of death in the majority of cancer patients. Metastasis can more accurately be described as is the property of malignant cells to migrate, disseminate, intravasate, extravasate, and proliferate at a location far away from the primary source (Welch *et al.*, 2000). Consequently metastasis is a multiphase disease consists of interlinked steps each of these can be rate limiting and can stop the entire phenomenon (Fidler, 2003b). As the normal development of breast tissue during puberty exhibits inherent phenomena of invasion, cell proliferation, resistance to apoptosis, and angiogenesis which are all hallmarks of cancer, there is no great surprise why it is a likely site of tumour progression (Wiseman and Werb, 2002). Metastatic tumour cells have to escape the challenging regulation of homeostatic and immune mechanisms that are present in the surrounding tissues of the primary site, as well as the secondary site. Even though there are significant hurdles preventing a cancerous cell from invading new sites around the body they manage to overcome this in many cases (Gupta and Massague, 2006). Enabling this is the observation that these migrating cells, often referred to as circulating tumour cells (CTCs), undergo multiple changes during the progression of the disease to acquire a metastatic phenotype. Published data suggests that a tumour can undergo a series of changes during the course of the disease and tumour cells can attain genetic variation which leads to tumour progression (Fidler, 2003a). These variations ensure the emergence of genetically diverse cancer cell populations, driving a 'selection' process between CTCs. Only those cells which are strong enough to survive the metastatic process, and are therefore better adapted to the host tissue environment, will survive and proliferate at a secondary site. These multicellular populations are often already present in parent tumours and have heterogeneous metastatic potential (Fidler and Kripke, 1977; Fidler, 2003a). As such, it has also been shown that those tumour cells which demonstrated a higher metastatic potential are highly mutable as compared to other tumorigenic cells (Cifone and Fidler, 1981). Furthermore the secondary tumour is essentially formed from a

unicellular ancestral dividing cell even when different ancestral cells reached the final destination presenting a single clonal selection model (Talmadge *et al.*, 1982; Fidler and Talmadge, 1986).

As mentioned earlier, *in situ* cancers are confined within the site of origin by a basement membrane and for metastasis breast tumour cells need to break through this membrane barrier. Thus, invasive cancers must achieve this task before they can invade to other tissues. This destruction of the basement membrane is achieved with the help of cellular and non-cellular components of stroma which are effectively conscripted by the tumour. Stroma is the supportive framework which is generally present in the surrounding environment of bilayered epithelial cells and not only enhances the growth of the primary cancer but also facilitates its metastatic distribution to secondary sites (Joyce and Pollard, 2009). The stroma consists of multiple components which include endothelial cells, adipocytes, fibroblasts, blood vessels, inflammatory cells, and the extracellular matrix (ECM) (Wiseman and Werb, 2002; Joyce and Pollard, 2009). Stromal cells communicate with epithelial cells through the ECM and the ECM is frequently being remodelled by this interaction (Davies, 2001). This three-dimensional interplay between the tumour, stromal cells and the ECM is important in deciding the fate of metastasis. During tumour progression some or all of these components are modified as well. These may include, activated endothelial and lymphatic cells, transformed fibroblasts, infiltrating lymphocytes, modified adipocytes, pericytes, bone marrow derived cells such as stimulated mesenchymal stem cells (MSCs), myeloid derived suppressor cells, macrophages, neutrophils and mast cells (Erler and Weaver, 2009; Joyce and Pollard, 2009). In particular, recruitment of MSCs by tumours (through the release of various homeostatic signals) is similar to general tissue remodelling during injury and it speeds up the process of metastasis and invasion (Karnoub *et al.*, 2007). This modification or alteration in stroma during tumour transformation and progression consequently degrades the ECM (Shekhar *et al.*, 2003). Thus, disseminating cancer cells are released from the primary site which subsequently invading the nearby tissues, travel through the vasculature or lymphatic systems, reached at a distant site, extravasate and then colonised and proliferate from a single clonal cell in order to achieve a successful metastasis (Talmadge, 2007).

1.8 Treatments for BC

The range of treatments available today to BC patients continues to improve and with advancements in medical, surgical and pharmaceutical fields new effective therapies are being developed. The main aim of treatment should be to remove any traces of the disease post-operation and to reduce the risk of relapse. Different types of treatment are given to patients depending on the type of BC that is present, and these treatments over time subsequently lead to delayed mortality. As BC is not a single disease, primary tumours may respond differently to similar lines of treatment. Consequently, it is important that therapies should in theory at least be specifically targeted against phenotypically different types of BC which are based on gene expression profiling. Intensive energy has been employed to evolve therapies which are appropriate and more effective to cure different types of BC. A breast tumour can be removed surgically by lumpectomy, however in some cases where there is risk of cancer spreading larger portions of tissue or even the whole effected breast may be surgically removed (see Figure 1.5) to prevent recurrence. The managing of BC treatment often involves either mastectomy or lumpectomy plus an adjuvant therapy (Buxton *et al.*, 2010). These adjuvant therapies include endocrine therapy, immunotherapy and chemotherapy which are used alone or in combination depending upon the type and stage of cancer.

Early-detected BC patients with confined tumours or even with local lymph node infiltration can be surgically treated by removal of tissue and undetected remaining deposits of neoplastic disease can be treated with a course of radiotherapy preventing these deposits from eventually developing into a clinically detectable recurrence (Early Breast Cancer Trialists' Collaborative Group, 2000). It is believed among most of the medical fraternity that radiotherapy is a powerful tool in preventing local relapse in BC patients. Radiotherapy is mainly used as a local adjuvant treatment to destroy the remaining BC cells in the chest wall or regional lymph nodes after mastectomy (National Institutes of Health Consensus Development, 2001). Breast conserving surgery followed by adjuvant radiotherapy is the standard treatment used for BC patients with an early detection of disease. Adjuvant whole breast radiotherapy following breast conserving surgery improves local

control rates over breast conserving surgery alone (National Institutes of Health Consensus Development, 2001). Research trials in the past have shown that the relative risk of local recurrence is reduced by 65%-70% by radiotherapy, but that this reduction in local recurrence did not reduce the overall mortality rate (Early Breast Cancer Trialists' Collaborative Group, 2000; Cuzick, 2005). Similarly for patients treated with total mastectomy and axillary dissection, addition of radiotherapy to adjuvant chemotherapy reduces local recurrences and improves survival (Overgaard *et al.*, 1997). However there are some conflicting reports published which show potential dangers of using radiotherapy. It has been reported that radiotherapy can produce a small increase in the mortality rate from causes other than BC (Early Breast Cancer Trialists' Collaborative Group, 1995). One meta-analysis acknowledged the value of radiotherapy but also found a 62% increase in cardiac deaths in women who received adjuvant radiation (Cuzick *et al.*, 1994).

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Figure 1.5   Mastectomy of cancerous breast.

Surgical removal of a whole breast in patients with lymph node infiltration to avoid relapse and metastasis of the disease.
(Image was purchased from Dreamstime.com)

Nevertheless recent reports have proposed that careful radiotherapy has lowered the risk related to the therapy. Indeed, the mortality rate of developing heart disease linked with radiation in BC patients has noticeably reduced with the passage of time according to a population based analysis (Giordano *et al.*, 2005).

Chemotherapy treatment involves exposing BC patients to drugs that target rapidly proliferating cells. These drugs commonly include combinations of cyclophosphamide, methotrexate, fluorouracil (CMF) and anthracycline-based combinations such as fluorouracil, adriamycin also called doxorubicin, cyclophosphamide (FAC) or fluorouracil, epirubicin, cyclophosphamide (FEC). FAC or FEC treatment procedures are generally considered more effective than CMF (Early Breast Cancer Trialists' Collaborative, 2005). Treatment for 6 months with FAC or FEC reduces the annual BC mortality rate by approximately 38% for women younger than 50 years of age when diagnosed and by approximately 20% for those women aged 50–69 years when diagnosed (Early Breast Cancer Trialists' Collaborative, 2005). In patients (younger than 50 or between ages 50-69) with little or negative ER expression, multi-chemotherapy significantly reduces the recurrence and breast cancer mortality rates (Early Breast Cancer Trialists' Collaborative *et al.*, 2008). Not all patients respond well to chemotherapy as this is often age-related (Fisher *et al.*, 2004).

As most BC cases are ER-positive, targeting ERs is often an effective treatment strategy which is usually achieved by endocrine therapy. The standard anti-oestrogenic drug used as endocrine therapy for treating ER-positive BC patients is tamoxifen. Tamoxifen is used as a gold standard first-line treatment of ER-positive metastatic breast cancer and as adjuvant therapy for early BC patients with ER-positive tumours (Buzdar, 2003). The binding of oestrogen to the ER is blocked by tamoxifen, thus preventing oestrogen-mediated growth and proliferation of BC cells (Early Breast Cancer Trialists' Collaborative Group, 1998; Choueiri *et al.*, 2004). The application of adjuvant treatment with tamoxifen for 1, 2, and 5 years reduces the risk of recurrence of BC by 13%, 26% and 47% respectively (Early Breast Cancer Trialists' Collaborative Group, 1998). Tamoxifen administration also significantly reduces the rate of treatment failure at contralateral

breast, primary and secondary sites and the rate of tumour recurrence after lumpectomy and breast irradiation (Fisher *et al.*, 1989). The 5-year adjuvant tamoxifen therapy reduces the annual mortality rate by 31% irrespective of age, chemotherapy and PR status in ER-positive cases (Early Breast Cancer Trialists' Collaborative, 2005). However previous trials have also shown that exposure of tamoxifen therapy for longer than five years did not improve rate of recurrence (Fisher *et al.*, 1996), suggesting that 5 years is as long as tamoxifen therapy needs to be. In fact, long-term tamoxifen exposure induces toxicity after 5, 10, and 15 years of follow up, including increases in the incidence of endometrial cancer and thromboembolism (Bruzzi, 1998). Therefore, the use of other available agonist post-tamoxifen therapy is important. Tamoxifen and aromatase inhibitors such as letrozole are currently of the mainstay of endocrine treatment in BC patients (Haynes *et al.*, 2003).

Immunotherapy is another type of treatment based on antibodies successfully used for patients. Trastuzumab (also called herceptin), a monoclonal antibody targeted the HER-2 protein, was approved in 1998 as a first-line treatment for HER-2-positive BC. HER-2 expression is amplified in 25% of invasive breast cancers, meaning that these cancer types are targetable by trastuzumab (Nahta *et al.*, 2006). Trastuzumab increases the clinical benefit of first-line therapy in amplified HER-2 metastatic BC patients (Slamon *et al.*, 2001). Data has shown that trastuzumab therapy improves prognosis, with a 44% reduction in death risk as compared with women with HER-2 negative disease (Dawood *et al.*, 2010). Additionally the combination of pertuzumab (another antibody) and trastuzumab is dynamic and better tolerated in metastatic HER-2-positive BC patients who had encountered progression during prior trastuzumab therapy and demonstrated a clinical benefit rate of 50% (Baselga *et al.*, 2010). However, in some HER-2 overexpressed and ER positive BC cases, resistance has been encountered during disease relapse, so a combination of endocrine and immune therapies may prove to be a more sensible treatment option in these cases.

Another strategy to combat BC is to use combinations of these various treatments. Correspondingly a trial study demonstrated that addition of letrozole and lapatinib (another aromatase inhibitor)

significantly increased the benefit rates in metastatic BC patients with positive hormonal status and HER-2 (Johnston *et al.*, 2009). This treatment when combined with chemotherapy drugs (paclitaxel after doxorubicin and cyclophosphamide) produced improved outcomes in surgically removed HER-2 positive BC patients (Romond *et al.*, 2005). Similarly, adjuvant radiotherapy when combined with tamoxifen in premenopausal BC patients has been associated with a lower risk of relapse and a longer disease-free survival (Overgaard *et al.*, 1999). Radiotherapy can be used for *in situ* cases and even for invasive tumours with good prognosis (Cuzick, 2005).

Thus, these various treatments either alone or in combination are employed successfully to some extent against BC. However, care must be taken when treating patients because one treatment approach beneficial for one patient may not bear fruits for other patients. Treatments should be planned according to individual needs and depending on the stage of the disease.

1.9   Cellular models of BC

Research into the biology of BC is often done using primary tissue or animal models. These tissues are not always available however there are several cellular models of BC available for use. These cell models vary from normal, non-cancerous breast epithelial cells, through non-invasive BC cells to invasive BC cell models, with both hormone-sensitive and insensitive models in common use. As mentioned above, TNBC is usually associated with poor outcome and patients show worse prognosis than patients affected with luminal breast cancers (Korsching *et al.*, 2008). There are many established TNBC cell lines available which represent TNBC model. Some of these TNBC cell lines include BT20, BT-549, HCC 3153, MDA-MB-157, MDA-MB-435, MDA-MB-468 and MDA-MB-231 (Chavez *et al.*, 2010). MDA-MB-231 cells are characterized as triple-negative basal-B mammary carcinoma (Tate *et al.*, 2012). Employing a BC cell line in mouse xenograft studies is an excellent strategy to evaluate the effectiveness of therapeutic treatments against breast tumours particularly TNBC. The MDA-MB-231 cell line represents an exceptional model for *in*

vivo xenograft studies using severe combined immunodeficiency (SCID) mice (Tate *et al.*, 2012). The MDA-MB-231 cell line is also frequently used as a model for breast adenocarcinoma (AC).

This study therefore hypothesised that reducing AQP3 expression in the TNBC cellular model (MDA-MB-231) potentiates cancer cells to respond better to 5-FU based treatment. TNBC lacks a conventional therapeutic target therefore conventional chemotherapy is the only available treatment (Schneider *et al.*, 2008). The MDA-MB-231 BC cell line was originally derived in 1973 at M. D. Anderson Cancer Centre from a pleural effusion of a 51-year old Caucasian BC patient who also had an intraductal carcinoma (Cailleau *et al.*, 1974). These cells were spindle shaped cells and represent epithelial-like morphology. The cell line grew very swiftly and cells had to be transferred every 5-8 days at dilutions of 1:10 or higher (Cailleau *et al.*, 1974). The MDA-MB-231cell line imitates most of the important genomic abnormalities found in primary breast tumours indicating that MDA-MB-231cells have retained most of the genomic abnormalities of the original tumours (Neve *et al.*, 2006). Moreover, it was observed that MDA-MB-231 cell line possess a small fraction of self-renewing stem cells thus representing an excellent model for the development and testing of novel therapeutics against cancer stem cells (Fillmore and Kuperwasser, 2008).

MDA-MB-231 cells are highly aggressive, invasive and poorly-differentiated and are characterized by the expression of epidermal growth factor receptor (EGFR) (Wang *et al.*, 2009). EGFR, which is also known as HER-1, is a 170-kD glycoprotein that consists of an extracellular receptor domain, a transmembrane region, and an intracellular domain with tyrosine kinase function (Herbst, 2004). EGFR belongs to the erythroblastic leukaemia viral oncogene homolog (ErbB) tyrosine kinase family, which consists of EGFR (c-erbB-1), HER-2 (c-erbB-2), HER3 (c-erbB-3), and HER4 (c-erbB-4) (Yarden *et al.*, 2001; Nie *et al.*, 2012). BC subtypes which retain high EGFR expression showed reduced ER expression. Additionally, the absence of ER expression in human BC cell lines is associated with higher levels of functional EGFR protein and messenger ribonucleic acid (mRNA) (Davidson *et al.*, 1987). Although MDA-MB-231 cells express EGFR, they do not show a

proliferative response to epidermal growth factor (EGF) (Davidson *et al.*, 1987) due to an active

K-ras (Price *et al.*, 1999).

1.10 Aquaporins

Aquaporins (AQPs) are integral transmembrane proteins that belong to the major intrinsic protein

(MIP) family found across species (Benga, 2009). The major function of AQPs is to permit the

movement of water molecules across membranes through a central pore. The presence of a water

channel protein was first identified in the erythrocyte membrane by Benga and colleagues in 1986

(Benga *et al.*, 1986a; Benga *et al.*, 1986b). Later Agre and colleagues accidently purified the

channel protein and thus named it CHIP28 (channel forming integral protein of 28 kDa) (Agre *et

al.*, 1987) which was later named as AQP1 (Agre *et al.*, 1993). AQP1 (CHIP28) complementary

DNA (cDNA) was subsequently isolated from human bone marrow (Preston and Agre, 1991) and

revealed to be closely related to 26kD MIP (Gorin *et al.*, 1984; Smith and Agre, 1991), the major

intrinsic protein of ocular lens (Broekhuyse *et al.*, 1976). Identification of rare genetically AQP1-

deficient humans (Colton-null individuals who lack AQP1 antigen) have shown no obvious clinical

phenotype (Saison *et al.*, 2012). The break through study, which demonstrated the function of

CHIP as a water-selective channel in *Xenopus* oocytes was carried out by Agre and his team

(Preston *et al.*, 1992). Peter Agre was subsequently awarded the Noble Prize for Chemistry in 2003

for his discovery of this family of water channels.

To date, thirteen different AQPs, named AQP0-12, have been identified. All AQPs in non-

glycosylated form have molecular weights of approximately 26–30 kDa, with glycosylated forms

having molecular weights in the range of 35–60 kDa (Benga, 2009). Amino acid similarity between

AQP family members ranges from 20% to 50% (Kobayashi *et al.*, 2004). Distribution and

expression of AQPs in cells is controlled by distinct regulatory mechanisms (Kobayashi *et al.*,

2004). Different AQPs facilitate the passage of water across membranes in diverse types of cells

and tissues possibly due to the difference in the requirement of water in these cells/tissues (King *et al.*, 2004). AQPs are associated with selective transport of water across biological membranes, which is essential to maintain a variety of cellular functions including the cellular electrochemical potential. The opening of aquaporin pores allows rapid diffusion of water in and/or out of the cell, which highlights the importance of their role in water homeostasis. It is estimated that approximately $3x10^9$ water molecules/sec/protein molecule move through AQP1 (Sansom and Law, 2001), demonstrating the rapid nature of water transport via AQPs.

A subclass of the AQP family exists, whose members are commonly referred to as aquaglyceroporins (AQP3, 7, 9 and 10). These AQPs transport glycerol and some other small molecules in addition to water across cell membranes. Glycerol, a three-carbon backbone tri-alcohol, is a key component of the majority of phospholipids and an important metabolite in glucose metabolism (Hagopian *et al.*, 2008). Glycerol plays a fundamental role in various physiological processes, including gluconeogenesis, lipid synthesis, glucose homeostasis and osmoregulation (Stein and Stein, 1967; Klein and Mindich, 1976; Herrmann and Gercken, 1982; Gleeson *et al.*, 1986; Brisson *et al.*, 2001; Yazdi *et al.*, 2008; Bassarak *et al.*, 2011).

Most AQPs form functional homo-tetramers that span the cell membrane, in which one or two monomers may be glycosylated (Smith and Agre, 1991; Verbavatz *et al.*, 1993), with each monomer acting as an individual water channel (van Hoek *et al.*, 1991; Preston *et al.*, 1993). Structurally, each AQP protein consists of six tilted transmembrane α-helices (see Figure 1.6) connected with five inter-helical loops (A–E) (Gorin *et al.*, 1984; Smith and Agre, 1991) having both the amino and the carboxyl terminals located in the cytoplasm (Smith and Agre, 1991; Preston *et al.*, 1994). The three loops A, C and E are extracellular whereas the B and D loops are cytoplasmic (Jung *et al.*, 1994). Loop B and E consist of highly conserved asparagine-proline-alanine (NPA) amino acid motifs (Reizer *et al.*, 1993), which are involved in the formation of the selective aspect of the water pore (Jung *et al.*, 1994). The constricted aromatic/Arginine (ar/R) region is formed about 8 A° above the NPA region which consists of a tetrad formed by two

residues from helices 2 & 5 and two residues from loop E (Wallace and Roberts, 2004). Loop B and loop E are connected within the membrane forming a single, narrow channel that provides each monomer of AQP with an hourglass-like conformational shape (Jung *et al.*, 1994). Water can only pass through the pore in a single file as the narrow pore cannot hold more than two water molecules therefore the energetic barrier for such dehydration is high (Fu *et al.*, 2000). With the help of computer simulations, oxygen atoms of water molecules have been shown facing downwards through this narrow channel, whereas in the midway a fine-tuned dipole inversion results in the oxygen atoms facing upwards due to the effect of the local electrical field exhibited by the amino acid residues of the channel (de Groot and Grubmuller, 2001). This work has also demonstrated that all AQPs have two selectivity filters; one at ar/R and the second at the NPA motifs. The first filter prevents the passage of protons and the second prevents larger molecules from passing through the pore (de Groot and Grubmuller, 2001).

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Figure 1.6   Structure of a typical aquaporin.

Diagrammatic representation of the structure of aquaporin in the plasma membrane. Each monomer of AQP has six transmembrane α-helices (shown as cylinders) which are interlinked with helical loops to form conserved NPA (asparagine, proline and alanine) motifs. These NPA motifs form a pore through which water molecules can transport in/out of the cell.
Source: Clinical update on renal aquaporins. *Biology of the Cell.*(Chen *et al.*, 2005)
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The presence of AQPs across most species including humans, plants, animals and bacteria is well established, highlighting the importance of control of water transport in biological processes. This accurate control of water transport in cells is therefore an essential necessity for our existence. The occurrence of AQPs in different organs of the body indicates their involvement in the physiology of various tissues and equally potentially in their pathology. In addition to their role in water transport, AQPs also play a role in the formation of cell-to-cell contacts and establishment of epithelial cell polarity (Nejsum and Nelson, 2007). A single gene mutation in AQP structure can cause disease as in the case of mutations in AQP0 or AQP2 causing congenital cataracts and nephrogenic diabetes insipidus respectively (Kozono et al., 2002). AQP2 deficiency is the primary cause of nephrogenic diabetes insipidus in which large volumes of dilute urine are excreted due to a total absence of water reabsorption in the collecting ducts of the kidney (King et al., 2000). Therefore, malfunctions of AQPs are known to lead to imbalance in water homeostasis both in epithelial and non-epithelial cells; for example in skin cells, primary astrocytes and fibroblasts (Mola *et al.*, 2009).

1.11 Trafficking of AQP

AQP mediated transport of solutes across biological membranes is considered to be bidirectional and controlled by either the osmotic or solute gradients that are present across a membrane (Zelenina, 2010). Although concentration gradients do play an important role in AQP-mediated transport of water and other materials across membranes, it is thought that the number of AQPs present in membrane and their single channel permeability (Zelenina, 2010) is also of key importance. Cells exposed to hyperosmotic conditions (leading to an increase in the ionic gradient across the plasma membrane) can induce expression of AQPs in an attempt to alleviate the stress (Bell *et al.*, 2009). To deal with changes in the extracellular environment, cells recruit conventional intracellular vesicles to facilitate incorporation of AQPs into their membranes. Thus, translocation of AQPs is a key mechanistic process by which living cells respond to the swiftly changing

extracellular environment. More than one type of AQP may be present in the membrane of a single cell, which may or may not work synergistically to perform their functions. For example, AQP3 co-expresses with AQP4 in the basolateral plasma membrane of kidney collecting duct principal cells and when needed moves towards the plasma membrane using separate vesicles based on cytoplasmic domain sorting signals (Arnspang *et al.*, 2013). This shows that although more than one AQP is present in the membrane of the cell, their delivery is specific and independent of each other. Thus, movement of AQPs can control overall cellular homeostasis and may influence many physiological and pathophysiological processes.

AQP expression appears to be controlled in both the short- and long-term depending on cellular requirement. Short-term regulation is facilitated by conformational change or channel gating, which can regulate the abundance of AQP within seconds or minutes, whereas long-term regulation depends on AQP mRNA/protein synthesis or degradation rates, which covers the time period of hours to days (Zelenina, 2010). Phosphorylation of specific residues in AQPs may prompt translocation of protein or channel gating as has been observed in plants (Engel and Stahlberg, 2002). Indeed, phosphorylation of residues in AQPs is established as a mechanism of AQP trafficking. Translocation of AQP2 is known to be regulated in cells of the renal collecting duct cells by the hormone vasopressin, which induces AQP2 translocation from intracellular vesicles to the plasma membrane (Nielsen *et al.*, 1995; Sabolic *et al.*, 1995). The translocation of AQP2 is facilitated by cyclic adenosine monophosphate (cAMP) through activation of protein kinase A (PKA) (Nielsen *et al.*, 1995) which phosphorylates AQP2 at serine residues (Klussmann *et al.*, 1999; Tamma *et al.*, 2003). The cellular vesicles carrying AQP2 are translocated toward the plasma membrane via microtubules, actin and myosin and finally insert into the membrane by exocytosis (Brown *et al.*, 1998). Whilst involvement of PKA is important in the trafficking of AQP2 towards the plasma membrane, other kinases are thought to be involved in AQP trafficking independent of PKA and cAMP pathway. For example, elevation of cyclic guanosine monophosphate (cGMP) levels under both hormonal or chemical influence induces AQP2 translocation in cultured epithelial cells by activating protein kinase G (Bouley *et al.*, 2000). Similarly, it has been shown that

hypotonicity mediated rapid translocation of AQP1 to the plasma membrane requires elevated calcium levels and is dependent on both calmodulin and protein kinase C (Conner *et al.*, 2012). Whilst phosphorylation of AQPs residues by different kinase enzymes is important in the trafficking of some AQPs towards the plasma membrane, the precise mechanisms and pathways for the trafficking of the majority of AQPs is not yet clear.

1.12 Role of AQPs in migration

Migration is an extremely energetic and vital asset of living cells performed by all cells at some stage during their life span. Some living cells like neurons only migrate until they differentiate and become mature, whilst some cell types like epithelial cells move along a basement membrane and others like white blood cells move constantly throughout their life (Friedl and Wolf, 2010). Any stimulus, physical or chemical, in the adjacent environment of a mobile cell can induce it to move. Cellular migration has an important role in various physiological and pathological processes including embryogenesis, immune response, wound-healing, formation of new blood vessels, osteoporosis, rheumatoid arthritis, multiple sclerosis, mental retardation and cancer metastasis (Nobes and Hall, 1999; Ridley *et al.*, 2003). In an ordinary case, migration of cells takes place during skin injury. As it is important for an organism to heal and rebuild the skin in order to stop blood loss, therefore cells migrate to the site of injury and repair the damage. This heterogeneous multistep process of cellular migration involves upsurge of a cell membrane protrusion, formation of substratum for stable attachments near the leading edge of the protrusion, forward translocation of the cell body, and the detachment of substratum (Farooqui and Fenteany, 2005; Harishkumar *et al.*, 2013). To move, a cell becomes polarised and protrudes its cytoplasm in one direction via forming a thin structure called lamellipodium which attaches to a substrate and this polarisation of actin filaments occurs due to the presence of active barbed ends and passive pointed ends which helps in membrane protrusion (Ridley *et al.*, 2003). The polarised actin filaments derives protrusion of leading edge at the front of the cell and retraction at the rear by attaching and detaching the

protrusion to the ECM respectively (Lauffenburger and Horwitz, 1996). The contractile force generated by areas of the leading edge or the entire cell body together with forces generated by acto-myosin network leads to pulling the cell forward (Friedl and Wolf, 2003; Ananthakrishnan and Ehrlicher, 2007). The cell constantly observes the signal direction during the migration and moves accordingly (Ananthakrishnan and Ehrlicher, 2007).

AQP have recently emerged as potential regulators of cancer and metastasis. Migration of tumour cells requires cell volume regulation and changes in cellular shape (Papadopoulos *et al.*, 2008) that may be facilitated by entry of water and therefore AQPs can play a key role in cellular migration. AQP facilitated cell migration has been detected in tumour cells, suggesting that it may have an important role in tumour angiogenesis and spread. The AQP mediated cell migration has an important role in angiogenesis, metastasis, wound healing and glial scarring (Papadopoulos *et al.*, 2008). Deletion of AQPs significantly reduces tumour growth and migration while up-regulation of AQPs increases the migration and metastatic potential in many cancer types. The simplest explanation for AQP mediated cell migration is the formation of cell membrane protrusions as a result of increased water flow through AQP channels. For example, cellular migration was greatly reduced in AQP1-deficient cells, with abnormal micro vessel formation *in vitro* whereas AQP1 expressing cells showed AQP1 polarisation in lamellipodia with rapid water flow at the leading edge (Saadoun *et al.*, 2005a). Migration was similarly impaired in wild-type astroglia after ∼90% reduction in AQP4 protein expression by RNAi (Saadoun *et al.*, 2005b).

As AQP function can be tied to both physiological and pathophysiological processes, identifying agents capable of blocking AQPs is of potential clinical importance. Equally, inhibitors of AQP expression, trafficking or function are also an interesting approach for treatment of diseases involving disruption in water homeostasis such as brain oedema, stroke, heart-failure etc. (Mola *et al.*, 2009) . Research has shown that the transporting capabilities of various AQPs are inhibited by $CuSO_4$ and mercurial compounds (Preston *et al.*, 1993), however these substances are very non-specific and extremely toxic to living cells. Ammonium compounds and sulfonamide-based

carbonic anhydrase inhibitors have both been suggested to be selective modulators of AQP1 and AQP4, but subsequent measurements in multiple water transport systems have not confirmed this (Mola *et al.*, 2009). Other metal compounds have also been suggested as potential AQP blockers, including gold (Au) complexes which may work as novel metal-based scaffolds for AQP drug development (Martins *et al.*, 2012). Phloretin (a plant derived polyphenol) was also used in some studies to inhibit functional activity of several AQPs including AQP3 (Yang *et al.*, 2001; Huber *et al.*, 2007). The discovery of these inhibitors demonstrates the potential for targeting AQPs in disease related pathologies.

1.13 Aquaporin 3

Aquaporin 3 (AQP3), which belongs to the aquaglyceroporin subgroup of AQPs, acts as a plasma membrane channel that facilitates transport of water and other small solutes (including glycerol and urea) across the cell membrane (Bell *et al.*, 2009). AQP3 was first cloned in 1994 by Ishibashi & colleagues (Ishibashi *et al.*, 1994), and its expression has been reported in various mammalian tissues including breast, skin epidermis, urinary tract, lungs, kidney, and in human red blood cells (Ecelbarger *et al.*, 1995; Roudier *et al.*, 1998; Matsuzaki *et al.*, 1999; Sougrat *et al.*, 2002; Mobasheri *et al.*, 2005b; Liu *et al.*, 2007). In skin, AQP3 has been linked with a diverse range of biological roles including regulation of hydration, wound healing and keratinocyte proliferation and differentiation. AQP3 has also been shown to regulate the migration of dendritic cell populations (Song *et al.*, 2011). The expression of the AQP3 gene is known to be regulated by a number of hormonal factors, including insulin (Higuchi *et al.*, 2007).

Since the discovery of AQPs and work detailing their involvement in various pathologies, many studies have investigated expression of AQPs in normal and pathological tissues. In order to study the function of a gene, it is often either overexpressed or silenced/knocked-down. To study the function of AQP3, scientists have produced AQP3 null mice. AQP3 deficient mice displayed

delayed wound healing, selectively reduced epidermal glycerol content and water holding capacity after stratum corneum removal (Hara *et al.*, 2002; Ma *et al.*, 2002) which were improved by administration of glycerol (Hara and Verkman, 2003). This experiment elegantly demonstrated that knocking-down AQP3 would result only in minor phenotypic differences, namely slowing down of cell proliferation and wound healing rates and dry skin. However, it is entirely likely that due to the size and variety of the AQP family that more than one AQP is present on a cell membrane, which may potentially compensate for the loss of AQP3. Co-expression of AQ3 with other AQPs is reported by many studies and is related to important cellular functions in normal as well as cancerous tissues. This was demonstrated in the early stage human and mouse embryos, and suggested that AQP3 and AQP7 might play a role in pre-implantation mouse embryo development (Xiong *et al.*, 2013). However coordinated co-expression of AQPs in cancer cells can also be harmful. Recently, relative co-overexpression of AQP5 and AQP3 was observed in tumour cells of squamous cell carcinoma of the tongue (Ishimoto *et al.*, 2012). Similarly another study has demonstrated that AQP3 is co-expressed with AQP5 in oesophageal squamous cell carcinoma patients and high expression of both AQP3 and AQP5 together (not alone) is an independent poor prognostic factor in these patients (Liu *et al.*, 2013b). The high co-expression of AQP3 and AQP5 was both correlated with invasion depth, lymph node (LN) status and distant metastasis in these patients (Liu *et al.*, 2013b). This suggests that in some tissue these AQPs may perform well coordinated or additive functions and produce a cumulative pathological effect.

Solo expression of AQPs has also been observed in high-grade tumour cells originated from a variety of tissues (Hu and Verkman, 2006). Likewise, some recent studies have reported the expression of AQP3 in several tumour types. AQP3 expression is positively correlated with microvascular density of tissue samples from patients diagnosed with non-small cell lung cancer (NSCLC) and AQP3 expression is significantly higher in these cells than in than normal tissue samples, with higher expression in male patients than female patients (Li *et al.*, 2012). Up-regulation of AQP3 expression has also been linked with the differentiation, lymph node and distant metastasis in patients and thus could be a potential therapeutic target in colon cancer (Li *et*

al., 2013b). It has however been revealed that the faecal water content in the colon is mediated by AQP3 and knockdown of AQP3 expression results in severe diarrhoea (Ikarashi *et al.*, 2012b). Thus, overexpression leads to tumour pathogenesis and down-regulation prompted diarrhoea. This suggests that balance and homeostasis is very important in AQP3 expression and any change in its expression will lead to dysregulation. These findings will assist in future to understand the role of AQP3 in different diseases.

Increased expression of AQP3 was also observed in MCF-7 (breast carcinoma) and HT29 (colon adenocarcinoma) cell lines when cells were treated with deoxy-5-fluorouridine and gemcitabine and is related to increase in cell volume and cell cycle arrest (Trigueros-Motos *et al.*, 2012a). In some studies down-regulation of AQP3 was associated with tumour pathogenesis thus making the picture penetratingly complex. The enzyme phospholipase D2 (PLD2) and AQP3 coordinate in normal keratinocytes to slow down the cell proliferation and abnormalities or deregulation in the AQP3/PLD2 signalling cascade resulted in enhanced cell proliferation in skin cancers (Voss *et al.*, 2011), although contrary to this report it was also mentioned that AQP3 is overexpressed in keratinocytes and helps in cancer cell proliferation (Hara-Chikuma and Verkman, 2008a; Hara-Chikuma and Verkman, 2008b). Likewise it has been shown that there is an association between AQP3 expression and tumour stage and grade and loss of AQP3 may play a role in the progression of bladder cancer (Rubenwolf *et al.*, 2012b).

It is believed that AQP localisation from the cytoplasm to the plasma membrane via intracellular vesicles is a key mechanism by which the cell can control water and glycerol homeostasis. AQP3, like other aquaglyceroporins, is a relatively weak transporter of water (compared to non-aquaglyceroporins) but an efficient transporter of glycerol (Yang and Verkman, 1997). Garcia and colleagues (Garcia *et al.*, 2011) proposed that AQP3 localises near the plasma membrane in keratinocytes shortly after an osmotic stress. It has been demonstrated that transport of glycerol and water via AQP3 is reversible and furthermore influenced by changes in pH. AQP3 can act both as a glycerol and water channel at a physiological pH, but will selectively act as a glycerol channel at

lower pH values (around pH 6.1) with water being transported at neutral pH (Zeuthen and Klaerke, 1999). AQP3 can only act as a glycerol channel by becoming hydrophobic when the site of its pore is protonated thus any interaction between glycerol and H^+ results in removal of the H^+ by the glycerol molecule and subsequent glycerol transport (Zeuthen and Klaerke, 1999). This suggests that both water and glycerol are transported through AQP3 by the formation of hydrogen bonds between the pore and the permeating molecule. Interestingly, it has been suggested that expression of AQP3 may depend on cellular glycerol content (Zeuthen and Klaerke, 1999).

1.14 Aquaporins and cancer

An increasing volume of research has identified many members of the AQP family as being involved in a wide variety of different cancer states. Several studies have collectively suggested that AQPs have a regulatory role in tumour progression, invasion, and metastasis, presenting the family as potential targets for novel anticancer treatments (Verkman, 2009a). Researchers recently reviewed the available literature surrounding the involvement of AQPs in tumour growth, angiogenesis and metastatic processes and suggested that a possible curative technique may exist via altering or inhibiting the function or expression of AQPs (Nico and Ribatti, 2010).

Most research published at the time of writing has focussed on the role of AQP1 in cancer as it was the first AQP to be described and therefore has subsequently been the most studied. AQP1 is widely expressed in epithelial and endothelial cells in various tissues and it has been associated with angiogenesis, cell migration and metastasis in human malignancies (Verkman, 2009a). Previous research has reported that AQP1 expression is associated with brain, prostate and colon cancer (Saadoun *et al.*, 2002b; Moon *et al.*, 2003a; Mobasheri *et al.*, 2005a). Expression of AQP1 has also been observed in the two major histological subtypes of NSCLC; adenocarcinoma (AC) and squamous cell carcinoma (SCC) (Mobasheri *et al.*, 2005a; Hoque *et al.*, 2006). Many different AQP family members have been implicated in cancer of various forms (see Table 1.1).

Table 1.1   Expression of common aquaporins in different cancer types.

Research has indicated the differential expression of AQPs in various cancer types. The table indicates that most AQPs are up-regulated in common cancer whereas only few AQPs were reported to be down-regulated in different cancers.

Level of Expression	AQPs	Cancer type	References
Up	AQP1	Brain tumour	(Saadoun *et al.*, 2002b; Chen *et al.*, 2006; Wang and Owler, 2011)
		Breast cancer	(Mobasheri *et al.*, 2005a; Otterbach *et al.*, 2010b; Shi *et al.*, 2012)
		Colorectal carcinoma	(Moon *et al.*, 2003b; Mobasheri *et al.*, 2005a)
		Glioblastomas	(Oshio *et al.*, 2003; Oshio *et al.*, 2005; Hayashi *et al.*, 2007)
		Lung cancer	(Mobasheri *et al.*, 2005a; Hoque *et al.*, 2006; Kola and Yeldandi, 2010; López-Campos *et al.*, 2011; Machida *et al.*, 2011; Xie *et al.*, 2012)
		Ovarian cancer	(Mobasheri *et al.*, 2005a; Yang *et al.*, 2006a)
		Pancreatic cancer	(Burghardt *et al.*, 2003)
		Prostate cancer	(Mobasheri *et al.*, 2005a)
		Renal cancer	(Morrissey *et al.*, 2010)
	AQP3	Breast cancer	(Shi *et al.*, 2012; Cao *et al.*, 2013)
		Colorectal carcinoma	(Moon *et al.*, 2003b)
		Gastric carcinoma	(Huang *et al.*, 2010; Shen *et al.*, 2010)
		Lung cancer	(Liu *et al.*, 2007; Machida *et al.*, 2011)
		Oesophageal cancer	(Kusayama *et al.*, 2011)
		Renal cancer	(Kafé *et al.*, 2004)
		Skin cancer	(Hara-Chikuma and Verkman, 2008b; Hara-Chikuma and Verkman, 2008a)
	AQP4	Brain tumour	(Saadoun *et al.*, 2002a; Hu *et al.*, 2005; Wang and Owler, 2011)
		Glioblastomas	(Warth *et al.*, 2004; Hu *et al.*, 2005; Warth *et al.*, 2007b; Xie *et al.*, 2012)
		Lung cancer	(Xie *et al.*, 2012)
	AQP5	Breast cancer	(Jung *et al.*, 2011; Shi *et al.*, 2012)
		Cervical cancer	(Zhang *et al.*, 2011)
		Chronic myelogenous Leukaemia	(Chae *et al.*, 2008a)
		Colorectal carcinoma	(Moon *et al.*, 2003b; Kang *et al.*, 2008)
		Gastric carcinoma	(Watanabe *et al.*, 2009; Shen *et al.*, 2010)
		Lung cancer	(Chae *et al.*, 2008b; Zhang *et al.*, 2010; Machida *et al.*, 2011)
		Ovarian cancer	(Yang *et al.*, 2006b)
		Pancreatic cancer	(Burghardt *et al.*, 2003)
	AQP7	Ovarian cancer	(Yang *et al.*, 2011a)
	AQP9	Brain tumour	(Warth *et al.*, 2007a; Fossdal *et al.*, 2012)
		Ovarian cancer	(Yang *et al.*, 2011a)
Down	AQP3	Bladder cancer	(Rubenwolf *et al.*, 2012a)
		Skin cancer	(Voss *et al.*, 2011)
	AQP4	Brain tumour	(Xu *et al.*, 2009; Warth *et al.*, 2011; Fossdal *et al.*, 2012)
		Breast cancer	(Shi *et al.*, 2012)
		Gastric cancer	(Shen *et al.*, 2010)
	AQP8	Colorectal carcinoma,	(Fischer *et al.*, 2001)
		Hepatocellular carcinoma	(Jablonski *et al.*, 2007)
	AQP9	Hepatocellular carcinoma	(Jablonski *et al.*, 2007)

1.15 Aquaporins and breast cancer

Demonstration of AQPs in other cancers has stimulated interest in the potential role of AQPs in carcinoma of breast as well. Despite this, not enough research has been done to firmly elucidate the role of AQPs in BC progression and metastasis. What is known so far is that moderate AQP1 expression has been observed in the basolateral membranes of mammary ducts, glands and endothelial barriers of normal human breast samples using tissue microarrays (Mobasheri and Marples, 2004). Subsequently it was found that AQP3 was also expressed in the human breast, where it was found to be highly expressed in basolateral membranes of mammary ducts and glands of healthy breast tissue samples (Mobasheri *et al.*, 2005b). Another study using tissue microarray analysis revealed that AQP1 expression was higher in advanced mammary tumour samples as compared to control samples (Mobasheri *et al.*, 2005a).

The major published study that elucidated the potential link between AQP1 and breast carcinoma by using immunohistochemistry was performed on 203 invasive breast carcinomas. According to the authors, AQP1 expression was detected in 11 cases (5.4%) and showed a significant correlation with high tumour grade and TNBC (basal-like phenotype) with poor prognosis (Otterbach *et al.*, 2010a). In this study AQP1 expression was proposed to be the strongest marker of poor prognosis among all other basal-like parameters studied even though researchers only detected AQP1 expression in only 11 (5.4%) out of 203 cases. Brushing aside the critique on the data, this study however highlighted the link between AQPs and BC.

At the start of the current project only the above mentioned studies were published. During the progression of the current study several more studies have been published investigating the role of AQPs in BC. The first of these studies was published in 2012 by Shi *et al* (Shi *et al.*, 2012) and they suggested that several AQPs have an important role in human breast carcinogenesis. The study showed mRNA and protein expression of AQP1, AQP3, AQP4, AQP5, AQP10, AQP11 and AQP12 in normal and cancer human breast tissues. It was demonstrated that AQP1, AQP3, AQP4

and AQP5 exhibited differential expression between the normal and cancer tissues with AQP4 being down-regulated and the remaining three up-regulated in cancer tissues (Shi *et al.*, 2012). This was the first study to clearly show expression profiles of different AQPs in BC. Though their mRNA and protein data of AQP3 also showed differential expression they only selected AQP5 to compare it with established clinicopathological factors and elucidated that AQP5 was associated with cellular differentiation, lymph node invasion, and tumour grade.

A second recently published study revealed that AQP5 was overexpressed in IDC tumour samples which also exhibit lymph node metastasis as compared to IDC samples without lymph node infiltration (Jung *et al.*, 2011). The authors suggested that AQP5 is likely to play a role in proliferation and migration of BC cells. The final study to date was published in 2013 (February), it demonstrated the requirement of AQP3 for fibroblast growth factor-2 (FGF-2) facilitated cell migration in MDA-MB-231 cultured cells (Cao *et al.*, 2013). The authors speculated that FGF-2 up-regulates AQP3 expression via its signal transduction pathways and suggested AQP3's role in migration and metastasis of human BC cells.

All these reported studies indicate the importance of AQPs in the physiology and pathophysiology of the cancers. Hence regulating AQP function and/or expression might play an important part in delaying the migration and proliferation of BC cells.

1.16 Cellular energy homeostasis

All cells require energy to perform their vital tasks. The metabolic rate in living cells depends upon both the metabolic demand and stock of substrate in the body. Glycerol and glucose can both act as a substrate for oxidative and synthetic reactions and needed phosphorylation to become active substrates (Bortz *et al.*, 1972). Mitochondria are the cellular organelle most heavily involved in the production and regulation of cellular energy. Mitochondria utilise macronutrients derived from

dietary sources including carbohydrates, fats and proteins to produce the energy rich molecule adenosine triphosphate (ATP) (Vander Heiden *et al.*, 2009). Glucose metabolism is initiated in most mammalian cells by a process called glycolysis (Kaelin and Thompson, 2010). This phenomenon occurs in the cytosol and its by-products are subsequently metabolised within mitochondria. Glucose catabolism in the cytoplasm, in addition to ATP, also forms pyruvate molecules which subsequently enter the mitochondria where they are further metabolised by the well characterised series of reactions known as the Krebs cycle (also known as the citric acid cycle) and electron transport chain (Vander Heiden *et al.*, 2009). Glucose is commonly utilised as a rapid energy source when required by cells, however during the fasting state, the liver maintains the glucose levels by synthesising glucose in a process known as gluconeogenesis which utilises molecules such as lactate and glycerol (Montminy and Koo, 2004).

Glycerol is produced in the body as an intermediate metabolite after the metabolism of proteins, triglycerides and dietary fats and act as the link between several metabolic pathways (Hagopian *et al.*, 2008). Glycerol is therefore an important metabolite that enters into the glycolytic pathway. Indeed, it has been reported that in both lean and obese individuals that plasma glycerol is almost completely converted to glucose under fasting conditions (Bortz *et al.*, 1972). Two enzymes which are important in the regulation of glycerol metabolism are glycerol kinase (GK) and glycerol-3-phosphate dehydrogenase (G3PDH). Glycerol is initially converted by phosphorylation to glycerol-3-phosphate (G3P) by GK and then to dihydroxyacetonephosphate (DHAP) by G3PDH (Hagopian *et al.*, 2008).

The regulation of hepatic lipid and carbohydrate metabolism is controlled primarily by insulin and glucagon through activation of metabolic transcription factors (Montminy and Koo, 2004). One such transcription factors is forkhead box a2 (Foxa2). Insulin is possibly the most important anabolic hormone involved in key metabolic processes such as glycolysis, gluconeogenesis, glycogen synthesis, protein synthesis, lipogenesis, and lipolysis (Saltiel and Pessin, 2002). It is known that GK expression levels are modulated in tissues by insulin (Lin, 1977)**.** In adipocytes,

during fasting triglycerides are liberated from lipid droplets and hydrolysed to release glycerol and free fatty acids which then released into the bloodstream and are taken up by organs rich in GK such as the liver where these are utilised via gluconeogenesis to maintain plasma glucose levels (Kishida *et al.*, 2000). The expression and function of aquaglyceroporins is therefore relevant as they are the transporting channels of glycerol and can continuously supply glycerol during starvation.

Several AQPs are known to be regulated in some part by insulin. For example it has been demonstrated that insulin activation of the PI3K/Akt/mTOR signalling pathway regulates the expression of AQP3 and AQP7 in adipose tissues, facilitating glycerol efflux from adipose tissue while also modulating the glycerol influx into hepatocytes via AQP9 (Rodriguez *et al.*, 2011). Insulin also represses AQP3 gene expression through Foxa2 and it has been suggested that AQP3 is involved in fatty acid oxidation and glycometabolism given its permeability to glycerol (Higuchi *et al.*, 2007). Fasting or glucagon exposure activates Foxa2 which subsequently activates lipid metabolism and is inhibited via insulin-PI3K-Akt signalling-mediated phosphorylation (von Meyenn *et al.*, 2013). It has been shown in normal mice that Foxa2 was inhibited by plasma insulin through nuclear exclusion and in the fasted (low insulin) state, Foxa2 activated the lipid metabolism; however hyperinsulinaemic mice showed inactive Foxa2 permanently located in the cytoplasm of hepatocytes which prevented the stimulation of triglyceride degradation and fatty acid oxidation consequently promoting lipid accumulation and insulin resistance (Wolfrum *et al.*, 2004). This accumulation of lipids represents potential warning of tumour formation as a number of studies have indicated that lipogenesis is closely linked to tumour formation in breast cancer. These studies have demonstrated different types of lipids in human cancerous tissues (Chajes *et al.*, 1995; Hilvo *et al.*, 2011), serum (Feldman and Carter, 1971; Alexopoulos *et al.*, 1987), human hair samples (Mistry *et al.*, 2012), cultured cells (Katz-Brull *et al.*, 2002; Moreau *et al.*, 2006; Eliyahu *et al.*, 2007; Hilvo *et al.*, 2011) and in murine mammary tumours (Monteggia *et al.*, 2000), which may involve in malignant transformation and tumour progression.

Similarly in most mammalian cells, glycolysis is inhibited under aerobic conditions (known as the Pasteur Effect), a situation that allows mitochondria to oxidize pyruvate to carbon dioxide and water. However cancer cells display increased glycolysis and lactic acid production even in aerobic conditions, a phenomena referred to as the Warburg effect or aerobic glycolysis (Gatenby and Gillies, 2004; Alirol and Martinou, 2006). Aerobic glycolysis is selectively beneficial to tumour cells for the production of energy and it is accepted that cancer cells generally prefer glycolysis to oxidative phosphorylation (OXPHOS) as a source of cellular energy (Reitzer *et al.*, 1979). The energy state of the cell greatly influences the shape, number and function of mitochondria. In the case of enhanced respiration and activated OXPHOS, the mitochondrial phenotype changes to enlarged cristae and interconnected mitochondrial network whereas in enhanced glycolytic state and low OXPHOS, the mitochondrial phenotype appears small and fragmented with expanded matrix, and displaying reduce intracristae space (Alirol and Martinou, 2006). Difference between mitochondria of normal and cancer cells was reported in literature. It was shown in an aggressive human osteosarcoma cell line that mitochondria increase in size and number and had elevated mitochondrial DNA levels (Shapovalov *et al.*, 2011). It has long been suggested that faults in mitochondrial function are associated with the progression of cancer but it not clear yet, how changes in mitochondrial shape are involved in cancer.

Oxygen is essential for all living organisms to respire and carry out important physiological functions in the body. If enough oxygen is not available then this results in hypoxia. Generally oxygen consumption is low in tumours tissues as compared to normal tissues and hypoxic cells are more inclined to show more aggressive metastatic phenotype (Vaupel *et al.*, 1998). It has been demonstrated that hypoxia is not necessary to influence the glucose metabolic pathway as some hypoxic tumours cells displayed modest glucose metabolism, on the other hand some immensely metabolic tumour cells are not hypoxic (Rajendran *et al.*, 2004). Also hypoxia can stimulate hypoxia-inducible factor 1 (HIF-1), which enhances the expression of glucose transporters and glycolytic enzymes so that the hypoxic cancer cells can adapt to anaerobic glycolysis (Dang *et al.*, 2008).

1.17 Aims and objectives

The broad aim of this study was to investigate the role of AQP3 in BC using clinical specimens and an *in vitro* model. Considering the present scenario of BC related problems, the objective of the current study was to evaluate the role of AQPs in BC biology and pathology and to relate it with clinical laboratory parameters, which may be useful for early detection, disease monitoring and slowing down cancer progression.

The following were the specific objectives of this project:

- To assess the clinical relevance of AQP3 expression in BC specimens collected from BC patients.
- To develop and validate an *in vitro* model of AQP3 knockdown cell line in MDA-MB-231 using short hairpin RNA (shRNA) silencing technology.
- To identify the effect of AQP3 gene knockdown on BC cell biology *in vitro*.
- To use the model to investigate the role of AQP3 on glycerol transport and energy metabolism.

Chapter 2

Materials
and
Methods

2.1 RNA extraction and gene manipulation from snap frozen tissue samples

Fresh cancer tissue and normal border tissue samples were collected under NHS Research Ethics Committee approval (study number: 11/WM/0424) from Russells Hall hospital, Dudley, and immediately stored at -80˚C. RNA was isolated from all samples using a proprietary RNA isolation kit (AllPrep® DNA/RNA/Protein Mini Kit, QIAGEN, UK). Approximately 30 mg of tissue sample was homogenised using a homogeniser in lysis buffer came with the kit. RNA was subsequently purified using RNeasy Mini Kit (QIAGEN, UK) in accordance with the manufacturer's instructions, and subsequently quantified using the NanoDrop1000 spectrophotometer (Thermoscientific, UK) by measuring absorbance of light at 260/280nm. Sample concentrations were recorded, and any samples having 260/280 ratios of below 1.7 were discarded. 500 ng of the total RNA from each sample was subsequently reverse transcribed using a proprietary cDNA synthesis kit with oligo dT primer (Primerdesign, UK) for 20 minutes at 55°C. The reaction was heat inactivated by incubation at 75°C for 15 minutes. Resulting cDNAs were diluted 1 in 10 with RNase free water and stored at -20°C until needed for amplification by polymerase chain reaction. The real-time polymerase chain reaction (qPCR) was carried on cDNA as described below.

2.2 Polymerase chain reaction

SYBR® Green qPCR was performed on diluted cDNA (5 μL of diluted cDNA in a final volume of 20 μL) using sequence specific prevalidated primers for AQPs purchased from Primerdesign, UK. Normalising housekeeping genes primers (Invitrogen, UK) used to normalise data, were chosen from a pool of candidate normalising genes with the two most stable being employed (beta actin and YWHAZ). The primer sequences of forward and reverse primers of YWHAZ and beta actin used in qPCR reactions were:

Beta actin forward primer (CTGGAACGGTGAAGGTGACA)

Beta actin reverse primer (AAGGGACTTCCTGTAACAATGCA)

YWHAZ forward primer (ACTTTTGGTACATTGTGGCTTCAA)

YWHAZ reverse primer (CCGCCAGGACAAACCAGTAT)

Stability of gene expression was established in previous work within the group. Samples were analysed on a Stratagene MX3000P™ thermal cycler (Stratagene, UK). Cycling conditions were as follows: 10 minutes at 95°C, 15 seconds at 95°C and 1 minute at 60˚C for 40 cycles, 30 seconds at 95°C, 30 seconds at 55°C and 30 seconds at 95°C. Comparisons were made for each sample between the average crossing point (C_t) obtained from the genes of interest and the geometric mean of the housekeeping genes beta-actin and YWHAZ (ΔCt). ΔC_t values were calculated for each sample by subtracting the C_t value of housekeeping gene from the C_t value of the gene of interest. The level of each transcript relative to housekeeping gene levels could then be calculated from the equation $2^{-\Delta\Delta Ct}$ where $\Delta\Delta C_t$ is the ΔC_t value of the reference transcript subtracted from the ΔC_t value of the gene of interest transcript. Any sample that showed a C_t value of 35 and above was considered to be not significant expression.

2.3   SDS-PAGE and Western blotting

Samples were prepared in 2X Laemmli sample buffer (deionised H_2O, 25% glycerol, 62.5 mM Tris-HCl pH 6.8, 2% SDS, 0.01% bromophenol blue and addition of freshly prepared 5% β-mercaptoethanol each time) and loaded into separate wells in the gel. 5 μL of PageRuler™ Plus prestained protein Ladder (Fermentas, UK) was used as a molecular weight marker. Samples were subsequently electrophoresed to separate proteins by size and charge using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Samples were electrophoresed in 1X running buffer (containing 25 mM Tris, 192 mM Glycine and 0.1% SDS, Geneflow, UK) using the Mini Protean® 3 Cell (Biorad, UK). 4% stacking gel and 12% resolving gel were prepared according to a standard recipe. Samples were separated by running electrophoresis at 40mA for approximately 1.5 h until the bromophenol blue reached the bottom of the gel.

Glass plates were then carefully separated and the gel was placed in 1X transfer buffer (containing 25 mM Tris, 192 mM Glycine, Geneflow, UK to which final concentration of 20% (v/v) Methanol was added fresh) for a brief wash to remove accumulated salts. Following SDS-PAGE, sandwich cassettes were prepared and gel was placed under a pure 0.45 micron pore size nitrocellulose blotting membrane (VWR International, U.K.) and transfer electrophoresis was performed with 1X transfer buffer at 100V for 1 h to transfer the proteins from the gel to the nitrocellulose membrane. After that the membrane was stained with Ponceau S solution (Sigma, UK) for few minutes to confirm protein transfer before being destained with 1X Tris-buffered saline (TBS) (8g NaCl, 0.2g KCl, 3g Tris-base in 1 L H_2O). Subsequently, membranes were blocked by using filtered 5% (w/v) non-fat milk (Marvel) in 1X TBS buffer containing 0.1% (v/v) Tween 20 (TBS-T) for one hour on a "rocker" at room temperature. After one hour blocking, the membrane was washed three times in 1X TBS-T (15 minutes for each wash) and finally once in TBS buffer without tween (15 minutes) before being incubated overnight at 4°C with the anti AQP3 rabbit primary antibody. A commercially available AQP3 polyclonal antibody raised in rabbit (sc-20811, Santa Cruz Biotechnology, USA) directed against AQP3 protein was used at 1/200 dilution for Western blotting. Post primary incubation, the membrane was again washed three times in TBS-T and once in TBS buffer (as mentioned above) and incubated at room temperature for one hour with the anti-rabbit secondary antibody. The secondary antibody used was anti-rabbit horse radish peroxidase (HRP) conjugated raised in goat (ab97051, Abcam Ltd, Cambridge, UK) at 1/2 000 dilution.

Following secondary antibody incubation, the membrane was washed three times in TBS-T and once in TBS buffer (as above) and subsequently incubated (in a dark environment) with EZ-ECL (enhanced chemiluminescence) western blotting detection system (Geneflow, UK) for 5 minutes. Bands were then visualised using G-BOX (Syngene) and GeneSys software (Syngene) and quantified using free image analysis software (ImageJ, NIH). Blots were stripped using a membrane stripping buffer (formulated by Abcam, UK) before being analysed for beta-tubulin expression as a loading control using the same protocol as described above for AQP3 expression. A tubulin antibody raised in mouse (ab28439, Abcam Ltd, Cambridge, UK) directed against α-tubulin

protein was used at 1/200 dilution as a primary antibody and donkey anti-mouse HRP conjugated (sc-2314, Santa Cruz Biotechnology, USA) diluted at 1/2 000 concentration was used as secondary antibody.

2.4 Protein isolation from tissue samples and Western blotting

Total cellular protein was isolated using a proprietary protein isolation kit (All Prep®️ DNA/RNA/Protein Mini Kit, QIAGEN, UK). As the tissue lysates contained dithiothreitol (DTT), it was not possible to accurately quantify protein in samples so equal volumes of lysates were used in Western blotting. Lysates were loaded on 12% PAGE gel for AQP3 and tubulin expression according to the protocol described in section 2.3. A commercially available AQP3 polyclonal antibody raised in rabbit (sc-20811, Santa Cruz Biotechnology, USA) directed against AQP3 protein was used at 1/200 dilution for Western blotting. A tubulin antibody raised in mouse (ab28439, Abcam Ltd, Cambridge, UK) directed against α-tubulin protein was used at 1/200 dilution to normalise the AQP3 expression. The secondary antibodies goat anti-rabbit horseradish peroxidase conjugated (ab97051, Abcam Ltd, Cambridge, UK) and donkey anti-mouse horseradish peroxidase conjugated (sc-2314, Santa Cruz Biotechnology, USA) were diluted at 1/2000 concentrations. Bands were then visualised using G-BOX (Syngene) and GeneSys software (Syngene) and then quantified using free image analysis software (Image J, NIH). Blots were stripped using a proprietary membrane stripping reagent before being analysed for beta-tubulin expression as a loading control using the same protocol as described previously. All AQP3 bands were normalised with respective tubulin band intensity.

2.5 Immunohistochemistry

All archived tissue samples used in this study were fixed in formalin and embedded in paraffin wax in Russells Hall Hospital, Dudley. Each block was labelled with its unique project identifier

number and ten 3 μm thick sections from each block were cut and placed onto charged slides. These slides of Formalin fixed paraffin-embedded (FFPE) tissues were then collected from the hospital for further investigations. The slides were placed in a rack and to remove paraffin off the slides following treatments were performed. First slides were treated twice with Xylene for 10 minutes each. Then the slides were washed with Xylene and 100% Ethanol in 1:1 combination for 3 minutes. Then slides were washed with 100% Ethanol twice for 3 minutes. After that a series of 95% (v/v), 70% (v/v) and 50% (v/v) Ethanol washes were performed each for 3 minutes. The slides were left in cold water for few minutes. To retrieve antigen, heat induce method was applied. After deparaffinisation and rehydration of the tissues, slides were incubated with Sodium citrate buffer (10 mM Sodium citrate, 0.05% (v/v) Tween 20, pH 6.0) in a vegetable steamer. The slides were carefully placed in the steamer with the pre-heated antigen retrieval buffer. The rack of slides initially brought the temperature of the solution down but it was return to 95-100°C within several minutes. The slides container was kept in the steamer for 20-30 minutes from this point. After that the rack was removed from the steamer and was placed in the cold water for 10 minutes. The slides were blocked with 10% (w/v) bovine serum albumin (BSA) in TBST for 2 h at room temperature and then washed three times in TBS for 5 minutes each time. All slides excluding the negative controls were subsequently incubated for overnight at 4°C with the mixture of desired primary antibodies for AQP3 raised in (sc-20811, Santa Cruz Biotechnology, USA) and Cytokeratin 5/8 raised in mouse (sc-32328, Santa Cruz Biotechnology, USA) at 1:50 and 1:250 concentrations respectively diluted in 1% (w/v) BSA-TBST. Post primary incubation, the slides were washed three times each for 5 minutes duration with TBS. After washing, all slides were incubated with a mixture of goat anti-mouse FITC (fluorescein isothiocyanate) conjugated (ab6785, Abcam Ltd, Cambridge, UK) and sheep anti-rabbit Texas Red conjugated (ab6793, Abcam Ltd, Cambridge, UK) secondary antibodies diluted at 1:1000 and 1:2000 concentrations respectively for one hour in a dark environment at room temperature. At the end of the secondary antibody incubation, slides were washed three times each for 5 minutes duration in dark with TBS. Following the final wash, excess wash fluid was removed by gently tapping the edge with the tissue paper. A drop of Vectashield (Vector Laboratories, UK) containing 4',6-diamidino-2-phenylindole (DAPI) (to stain

cell nuclei) was mounted onto each slide and then each slide was covered with a clean coverslip. The slides were then immediately viewed on a Leica TCS SP5 (II) confocal laser scanning microscope using FITC, Texas red and DAPI filters. Alternatively, if fluorescence microscopy was not performed on the same day, the slides were stored (in tin foil) in the dark at 4°C until viewing.

2.6   Protein isolation from FFPE and Western blotting

FFPE tissue samples were scratched off the slides and then transferred into 1.5 mL eppendorfs. The dewaxing was carried out by treatment of Xylene followed by three different dilutions of Ethanol. Briefly all samples after treatment with 200 µL Xylene were vortexed for 15 seconds and then incubated at 25°C for 10 minutes. The samples were then centrifuged at 16000 relative centrifugal force (RCF) for 2 minutes and the supernatant was discarded. The same treatment was repeated again with fresh Xylene. After two Xylene treatments, all samples were washed twice with 200 µL of 100%, 95% (v/v) and 70% (v/v) Ethanol treatments exactly in similar way to Xylene. After discarding the supernatant the pellets were dissolved in the extraction buffer supplied with the kit. Protein was isolated from these samples using proprietary Qproteome FFPE Tissue kit (QIAGEN, UK) according to the manufacturer's protocol. Proteins were quantified using a modified Lowry method (DC Protein Assay, Bio-Rad, UK) and photometrically measured at 750 nm using a Thermo multiscan EX 96-well plate reader (Thermofisher, UK). Protein quantification protocol was followed as per manufacturer's instructions. All samples were compared to a standard curve using BSA of a known concentration. Samples were run in triplicate and the average taken. Briefly 5 µL of each diluted lysate (sample was diluted 1:4 in 1X RIPA buffer) was used for protein analysis. Similarly 5 µL diluted concentration of BSA standard (standard was diluted 1:4 in 1X RIPA buffer; ranging from 250-1500 µg/mL) and 1X RIPA buffer which acted as a BSA free control were added to a 96-well microplate. The microplate was then incubated at room temperature for 20 minutes and then absorbance was detected as mentioned above.

Western blotting was performed to quantify the AQP3 protein expression in these tissue samples. Lysates were loaded on 12% PAGE gel for AQP3 and tubulin expression according to the protocol described in section 2.3. A commercially available AQP3 polyclonal antibody raised in rabbit (sc-20811, Santa Cruz Biotechnology, USA) directed against AQP3 protein was used at 1/200 dilution for Western blotting. A tubulin antibody raised in mouse (ab28439, Abcam Ltd, Cambridge, UK) directed against α-tubulin protein was used at 1/200 dilution to normalise the AQP3 expression. The secondary antibodies goat anti-rabbit horseradish peroxidase conjugated (ab97051, Abcam Ltd, Cambridge, UK) and donkey anti-mouse horseradish peroxidase conjugated (sc-2314, Santa Cruz Biotechnology, USA) were diluted at 1/2 000 concentrations. Bands were then visualised using G-BOX (Syngene) and GeneSys software (Syngene) and then quantified using free image analysis software (ImageJ, NIH). Blots were stripped using a proprietary membrane stripping reagent before being analysed for beta-tubulin expression as a loading control using the same protocol as described previously. All AQP3 bands were normalised with respective tubulin band intensity.

2.7   RNA extraction and gene manipulation of FFPE samples

FFPE tissue samples were scratched off the slides and then transferred into 2 ml eppendorfs. For RNA isolation from these samples the PureLink® FFPE RNA isolation kit (Invitrogen, UK) was used. The paraffin was removed according to the manufacturer's protocol and RNA was eluted. Quantification of total RNA was performed using NanoDrop1000 spectrophotometer (Thermoscientific, UK) by measuring absorbance of light at 260/280nm. The kit manufacturer mentioned that the RNA isolated using this kit has an OD260/280 of 1.5-2.0, therefore during measurement of RNA concentrations of specimens any samples having 260/280 ratios of below 1.5 were disregarded. 1 µg of total RNA from each sample was subsequently reverse transcribed using a proprietary cDNA synthesis kit with oligo dT primer (Primerdesign, UK) for 20 minutes at 55°C. The reaction was heat inactivated by incubation at 75°C for 15 minutes. Resulting cDNAs were

diluted 1 in 10 with RNase free water and stored at -20°C until needed for amplification by polymerase chain reaction. The qPCR was carried on cDNA as described in section 2.2.

2.8   Cell culture (general)

All materials used were purchased from Fisher Scientific (UK) unless stated otherwise. The human BC cell line MDA-MB-231 used as a model of invasive BC in this study was kindly donated by Dr Stephane Gross, Aston University. Cells were stored in a supplemented media containing 5% (v/v) dimethyl sulphoxide (DMSO) as per American type culture collection (ATCC®) protocol in liquid nitrogen storage until needed. From frozen state, cells were defrosted quickly by placing a vial in warm water, and were subsequently cultured in Roswell Park Memorial Institute (RPMI) 1640 (PAA, UK) media supplemented with 10% (v/v) foetal bovine serum i.e. FBS, (PAA, UK), 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were microscopically examined regularly to assess freedom from infection, confluence levels and morphological appearance. Cells were cultured as monolayers in 25 and 75cm^2 flasks at 37°C in a humidified atmosphere containing 5% carbon dioxide (CO_2).

The medium in each flask was replenished every 2-3 days and cells were sub-cultured when confluent, usually after 5-6 days. Subculture of MDA-MB-231 was carried out following media aspiration and washing with 10 mL of Hank's balanced salt solution (HBSS, PAA, UK). Briefly, after washing, cells were detached following incubation with 3 mL of accutase (PAA, UK) for 10-15 minutes at 37°C, resulting in a single cell suspension. Subsequently 7 mL complete media was added to neutralise the activity of accutase. Cell suspensions were decanted into a 15 mL tube and cells were spun down in centrifuge at 500 RCF for 5 minutes to collect the cell pellets. Pellets were resuspended in culture medium and cells were then cultured again in a 75 cm^2 flask at a dilution of 1 in 15. All cell treatments used for this study were preceded by an overnight serum-starvation in

serum free medium (supplemented with 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin) in order to synchronise cells within populations unless stated.

2.9   Cell culture of transfected stable cell lines

Cells that were successfully transfected with AQP3 siRNA plasmids, including positive and negative controls, were cultured in similar RPMI 1640 (PAA, UK) media with the addition of 2 µg/mL puromycin (Invitrogen, UK). Subculturing was performed as stated in section 2.8 except that AQP3-knockdown stable cells were subcultured at a ratio of 1:10 instead of 1:15 due to slower growth.

Importantly for all the experiments, the same media was used for both wild type (WT) MDA-MB-231 and transfected cells as puromycin was removed prior to treatment. All cell treatments were preceded by an overnight serum-starvation in serum free medium (supplemented with 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin) in order to synchronise cells within populations unless stated.

2.10   Cryopreservation of cells

Both transfected and parental cell lines were cryopreserved in the gaseous phase of liquid nitrogen. Cells in T75 cm^2 flask were washed with 10 mL HBSS after media aspiration. After that cells were detached following incubation with 3 mL of accutase for 10-15 minutes at 37°C in an incubator. Accutase was neutralized by adding in 7 mL of the complete media. Cells were decanted into a 15 mL tube and cells were spun down in centrifuge at 500 RCF for 5 minutes to collect the pellets of the cells. The supernatant was discarded and cell pellets were resuspended in 3 mL freezing solution which is made by adding 5% (v/v) DMSO as a cryoprotectant to the complete growth

medium. The cell solution was aliquoted into cryovials and stored in a cell freezing box at -80°C for 24 h. The cryovials were then transferred to liquid nitrogen container.

2.11 RNA isolation and gene manipulation of cultured cell samples

For all cell culture based gene expression experiments in this study, total RNA was extracted from cultured cells using the following protocol. All reagents used for this study were of molecular biology grade. Cells were grown in and collected using accutase as stated earlier in section 2.10. Cell samples were transferred into 15 cm^2 tubes and centrifuged at 500 RCF for 5 minutes. Cell supernatant was discarded and the pellets were collected and frozen down at -80°C if not immediately lysed. RNA was isolated from all samples using a proprietary RNA isolation kit (E.Z.N.A. ® Total RNA Kit I, Omega Bio-tek, UK). Quantification of total RNA was performed using nanodrop technology by measuring absorbance of light at 260/280nm. Sample concentrations were recorded, and any samples having 260/280 ratios of below 1.7 were discarded.

1 μg of total RNA from each sample collected was subsequently reverse transcribed using a proprietary cDNA synthesis kit (Primerdesign, UK) that utilised oligo (dT) primers for 20 minutes at 55°C in thermocycler. The reaction was heat inactivated by incubation at 75°C for 15 minutes. Resulting cDNAs were diluted 1 in 10 with RNase free water and stored at -80°C until needed for amplification by polymerase chain reaction. The qPCR was carried on cDNA as described in section 2.2.

2.12 Protein isolation from cell lysates and Western blotting

All cell types used in this study had protein isolated by the following method. Cells were collected as described in section 2.10. Cell samples were transferred into 15 cm^2 tubes and centrifuged at 500 RCF for 5 minutes. Cell supernatant was discarded and the pellet was placed on ice (4˚C) and

resuspended in 500 µL of 1X RIPA buffer (Millipore, UK). The solution was transferred to a 1.5 mL eppendorf (still on ice) and vortexed. Samples were subsequently agitated for 1 h at 4°C. Following this 1 h extraction period, samples were centrifuged at 13,000 RCF for 15 minutes at 4°C to remove cellular debris. The supernatant containing total cellular protein was collected and the pellet discarded. Samples were stored at -20°C if SDS-PAGE was not performed on the day of extraction. Cell lysate protein content was quantified using a modified Lowry method (DC Protein Assay, Bio-Rad, UK) as described in section 2.6. The microplate was then incubated at room temperature for 20 minutes and then absorbance was detected as mentioned previously.

For Western blot analysis of AQP3 protein expression, samples were prepared in 2X Laemmli sample buffer as described previously. 60 µg of protein per sample was denatured by heating at 70°C for 10 minutes in heat block. Western blotting was performed on these samples as described in section 2.3.

2.13 Immunocytochemical analysis of AQP3 expression in MDA-MB-231 cells

For immunocytochemical analysis of AQP3 expression in cultured cells, all washes and incubations were performed at room temperature unless otherwise stated. Cells were cultured as per standard method and grown on sterile glass coverslips placed in 6-well plates. Cells were grown to approximately 60% confluence before fixation. The cells were fixed and permeabilised by bathing in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 10 minutes followed by a series of washes in ice cold PBS, before 30 minutes incubation in 0.5% (v/v) Tween-20 in PBS. Once fixed, the cells were blocked with 1% (w/v) BSA in PBS-T for 30 minutes. Following the blocking phase the coverslips were washed three times in PBS for 5 minutes each time. All coverslips excluding the negative controls were subsequently incubated for 1 h at room temperature with the AQP3 primary antibody (described previously) at 1:50 concentration diluted in 1% (w/v) BSA-PBS. Post-primary incubation, the coverslips were washed three times in PBS for 5 minutes

each time and then incubated with goat anti-rabbit DyLight488® conjugated secondary antibody (ab96899, Abcam Ltd, Cambridge, UK) diluted at 1:500 concentration for one hour in a dark environment. At the end of the secondary antibody incubation, coverslips were washed three times with PBS in the dark to prevent light exposure. Following the final wash, excess wash fluid was removed by gently tapping the edge of the coverslip onto tissue paper. Each coverslip was then mounted onto a clean slide with one drop (approximately 25 μL) of Vectashield (Vector Laboratories, UK) containing DAPI (to stain cell nuclei), and sealed with clear nail varnish. The slides were then immediately viewed on a Leica DMI4000 B inversion microscope with GFP (for DyLight488) and A4 (for DAPI) filters. Alternatively, if fluorescence microscopy was not performed on the same day, the slides were stored (in tin foil) in the dark at 4°C until viewing.

2.14 Puromycin sensitivity

Since AQP3 shRNA vectors include integrated puromycin resistance elements, MDA-MB-231 kill curve analysis of puromycin exposure was performed in order to investigate the minimum concentration of puromycin that caused minimal cell death. Cell viability was evaluated by PrestoBlue® reagent (Invitrogen, UK) that functions as a cell viability indicator. After adding to the cells, the blue resazurin cell viability solution turns into red resorufin by the reducing environment of the viable cells and becomes highly fluorescent. MDA-MB-231 cells were plated in 96-well plates at a density of 2×10^4 per well for 24 h. Media was aspirated and the cells were exposed to a concentration range of puromycin in triplicate for 24, 48 and 72 h. On the day of cell viability detection, treatment media was replaced with 90 μL complete culture media and 10 μL PrestoBlue® reagent was added to all wells containing cells and three empty wells which were used as blanks. The plate was incubated at 37°C for 2 h and then the solution from the all wells was transferred to a 96-well fluorescence plate. Fluorescence measurements using SpectraMAX GeminiXS fluorescence microplate reader (Molecular Devices, UK) and SoftMaxPro software were taken at Ex/Em 544/590 nm.

2.15 Transformation and amplification of shRNA vectors

HuSH hairpin shRNA expression vectors targeting AQP3 were purchased from Origene, USA. These vectors consist of 29-mer sequences designed against AQP3. Four shRNA constructs were purchased to enable the best level of gene silencing. The 4 shRNA constructs were designated as 841, 842, 843 and 844 according to their product codes and the non-silencing scrambled sequence control (SC) designated as 015. These constructs have integrated turbo red fluorescence protein (RFP) element.

Transformations of shRNA vectors were carried out in DH5α cells. shRNA vectors contained a chloramphenicol resistance sequence which was used as a selection marker in DH5α competent cells. Agar plates were prepared by using following protocol: 20g Luria-Bertani (LB) broth (Invitrogen, UK) was dissolved in 1 L distilled water. Then 15 g bacto-agar (Fisher Scientific, UK) was added and the solution was autoclaved at 121°C. Liquid LB broth was similarly prepared but excluded the bacto-agar. Solutions were subsequently allowed to cool to approximately 50-55°C. Sterile chloramphenicol solution at a final concentration of 34 μg/mL was added to the LB agar media and LB broth. The agar media was then poured into sterile plates and left to set overnight at room temperature.

On the following day, approximately 5 ng (around 5 μL) of each shRNA plasmid was added into a separate labelled sterile 1.5 ml eppendorf and kept on ice. DH5α competent cells were defrosted on ice for 15 min and 100 μL of competent cells were aliquoted into each eppendorf containing plasmid. The eppendorfs were flicked to mix both plasmid/cells and immediately placed on ice for 30 min. After 30 min, eppendorfs were placed on a heat-block at 37°C for 1 min and then immediately placed back on ice for 5 min. Then 500μl of liquid LB (without chloramphenicol) media was added to each eppendorf and all the eppendorfs were shaken at 200 rpm on a shaker set at 37°C for 1 h. During this time, chloramphenicol agar plates were dried upside down in an incubator at 37°C to remove excess moisture. After 1 h, a 200μl spot of each plasmid/cell mixture

was placed into the middle of labelled chloramphenicol plates and was spread with a sterile spreader. All plates were placed upside down in a 37°C incubator overnight until colonies grew.

The following day, single bacterial colonies from streaked plates were picked using sterile wooden sticks and placed in flasks containing 30 mL LB media (with chloramphenicol). The bacterial cells were allowed to grow on a shaker at 200 rpm set at 37°C for 6 h. These mini-cultures were then inoculated into larger flasks containing 200 mL LB media (with chloramphenicol) overnight.

On the following day, cells were lysed and DNA plasmids isolated and purified using Machery-Nagel endotoxin-free plasmid DNA purification kit. The plasmid DNA was resuspended in 500 uL sterile double distilled water and concentration of each plasmid was determined. The shRNA plasmids were stored at -20°C.

2.16 Transfection of AQP3 shRNA vectors

In order to transfect, 3×10^5 cells per well were seeded into a 6-well plate and cultured for 18 to 24 h in RPMI 1640 complete culture media prior to transfection so that the cell density reached an optimal 50-70% confluency by the time of transfection. Dulbecco's Modified Eagle Medium (DMEM) (PAA, UK) enriched with high glucose (4.5 g/L) was used for transfecting AQP3 shRNAs according to the protocol supplied with GenJet™ reagent. DMEM high glucose medium with 10% (v/v) FBS without antibiotics was freshly added to each well 30~60 minutes before transfection. For each well, 2 μg of AQP3 shRNA plasmid was diluted into 100 μL of serum-free DMEM high glucose medium. Similarly for each well, 6 μL of GenJet™ solution was diluted into 100 μL of serum-free DMEM high glucose medium. The two solution tubes were vortexed and briefly centrifuged. 100 μL GenJet™ solution was then added to the 100 μL plasmid solution and the mixture was vortexed immediately and briefly centrifuged. The mixture was incubated for 10-15 minutes at room temperature. The 200 μL GenJet™/ DNA mixture was then added drop-wise

onto the medium in each well and the mixture was homogenized by gently swirling the plate. The plate was subsequently incubated at 37°C and 5% CO_2 in a humidified atmosphere. The medium on the cells was replaced with complete DMEM media without antibiotics 4~5 h post transfection according to the above mentioned protocol. After 24 h, media was replaced by complete RPMI 1640 media. Transfection efficiency was observed 24 h post transfection using fluorescence microscopy to detect RFP expression. After 48 h, transfected cells were reseeded into a new 6 well plate. The cells were then exposed to 2 µg/mL puromycin for selection purpose. Medium containing this concentration of puromycin was changed every 2-3 days to allow the formation of single cell colonies.

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Figure 2.1   The shRNA plasmid vector for AQP3 RNAi.
The vector has an integrated turboRFP element and it includes puromycin resistance element suitable for selection purpose
(source: http://www.origene.com/shRNA/vector_information.aspx).

2.17 Isolation of single cell colonies

Once colonies of puromycin resistant cells appeared in the 6-well plate, they were identified for RFP expression under fluorescence microscope (Leica DMI4000 B inversion microscope). RFP expressing colonies were marked by a circle with a permanent marker on the lower outside portion of the well plate. Colonies were then carefully isolated by using cloning cylinders (Sigma, UK) with silicone grease on one end. Briefly, medium was removed from the plate and each well was washed once with 2 mL HBSS. The greased cylinders were placed over the colonies marked using sterile forceps. The greased cylinders were pressed gently to create a seal between cylinder and the plate. 100 µL of accutase was added into each cylinder and colonies were incubated for 5-10 minutes at 37°C. Culture medium was added, as soon as cells had detached, to resuspend the cells and then cells were plated in 24 well plates containing 2 µg/mL puromycin and incubated at 37°C in an incubator with CO_2.

2.18 Generation of stable cell lines

Mammalian culture cells are widely used for studying the function of genes as it is very easy to transfect cells with shRNA against the gene of interest. The ability to produce knockdown cells helps in biochemical analysis and understanding of phenotypes over long period (Paddison *et al.*, 2002). Cells generated from individual colonies of all shRNA constructs were then further purified by serial dilution method. Cell suspension was prepared at $2\text{x}10^4$ and 1:2 serial dilution was performed in a 96 well plate for this method. Briefly, 100 µL culture medium was added to all the wells of a 96 well plate except first well (A1). 200 µL of the cell suspension (about 4000 cells) was added to the A1 well. Then 100 µL of the suspension was transferred to the B1 well and cells were mixed with gentle pipetting. Then 100 µL cells were transferred from B1 to C1 well. Cells were transferred to all the wells in the first column with the same strategy. 100 µL of 200 µL suspension from final H1 well was discarded so that all wells in column 1 had 100 µL of cell suspension. Then

100 μL of fresh medium was added in column 1 (A1 to H1 wells) to make the final volume of 200 μL in these wells. Then 100 μL cell suspension was transferred from each well in column 1 to the corresponding well in column 2 and then to all columns (from A1 to A2, from A2 to A3 and onwards). Final 100 μL of cell suspension was discarded from all the wells in column 12. Plates for all cell types were prepared in the same way and incubated at 37°C in 5% CO_2 incubator until single cell colonies appeared in the wells. Three different clones for each AQP3 shRNA construct were selected based on observation of homogenous RFP intensity under the fluorescence microscope, resulting in a total of 15 clonal cell lines being generated (three clones for each of shRNA 841, 842, 843, 844 and 015 constructs). All clones were then plated in 12 well plates and subsequently 6 well plates to continue growing in puromycin containing media. Once cells in 6 well plates were confluent they were transferred into 25 cm^2 flasks. Each flask was subsequently subcultured into 75 cm^2 flasks. Every 2-3 days, medium was aspirated from all the flasks and replaced with fresh medium containing 2μg/mL puromycin. They were further subcultured for an additional one month before sufficient cells were available for cryopreservation in liquid nitrogen.

2.19 Cellular proliferation assay

All cell counts were performed using a Countess® Automated Cell Counter (Life technologies, UK). To measure cellular proliferation, WT, SC and 844 cells were grown to confluence and serum-starved overnight in serum free medium to synchronise the cell cycle. 2 x 10^5 cells were seeded into 25cm^2 cell culture vessels and allowed to grow for 24, 48 and 72 h at 37°C in a CO_2 incubator under standard conditions. Cells were subsequently detached using accutase (PAA, UK) before being re-counted. For each count, both chambers of a Countess slide were used and an average figure recorded.

2.20 Cellular migration assays

Two separate methodologies were employed to assess cell migration. A conventional wound scratch assay and automated Cell-IQ® system were used.

2.20.1 Scratch wound assay

The phenomenon of cell migration can be observed *in vitro* easily by utilising wound-healing scratch assays (Poujade *et al.*, 2007). Scratch assays have been used to study cell polarization, matrix remodelling and cellular migration (Yarrow *et al.*, 2004). This process can be started by breaking the confluent monolayer of cells through scratching with the help of pipette tip. This disruption induces the process of wound closure by the migration of the cells into the wound area (Farooqui and Fenteany, 2005). This new artificial gap on a monolayer allows the cells near the edge of the scratch to migrate towards the wound in order to heal it by establishing new cell–cell contacts (Liang *et al.*, 2007). These new contacts are launched again due to an increased concentration of growth factors at the wound edge which help the healing the wound through amalgamation of proliferation and migration (Yarrow *et al.*, 2004).

Cellular migration of WT, SC, and 844 cells was assessed using a standard wound scratch assay method as previously described elsewhere (Koblinski *et al.*, 2005). Cells were seeded in 6-well plates and cultured until confluent and subsequently serum depleted overnight. To perform a wound healing assay, a cross-shaped scratch was introduced into each cell monolayer using a pipette tip. A sterile 20 µL pipette tip was used to produce a cross shaped scratch in the middle of each well. Cells were then washed with HBSS to remove any detached cells and fresh complete RPMI 1640 media was added. Wound healing was observed over a time course of 0–72 h. Images were taken using a Leica DMI4000 B inversion microscope at time points 0, 24, 48 and 72 h. Wound scratch analysis software TScratch developed by the Koumoutsakos group (CSE Lab, ETH

Zurich) (Geback *et al.*, 2009), was used to calculate the percentage wound closure for all four cell types for all time points. The images were analysed by the software on time-series basis therefore 0 h for all respective cell types was used as a control and analysis was done comparing 0 vs 24, 0 vs 48 and 0 vs 72 h. The analysed data from the software was used in Graph pad prism to test the statistical significance and to draw a graph. Statistical significance was tested by one way analysis of variance (ANOVA) with Dunnett's post-hoc test.

2.20.2 Cell-IQ® migration assay

Cellular migration was also assessed by Cell-IQ® automated image capture system (CM Technologies, Finland). Cells were seeded into 6-well plates and allowed to become confluent prior to overnight serum starvation. Cells were then scratched in vertical axis with a 20 µL pipette tip, and washed twice with HBBS to remove floating cells. Cells were treated with 5 µg/ml mitomycin C to inhibit cellular proliferation. The plates were placed on the Cell-IQ® system and two regions of interest (ROI) were selected for each cell type. The images were obtained for every 15 minutes continuously over a 24 h period. Cell migration was expressed as the percentage of the wound closure relative to the initial wound scratched area. Digitised images were then analysed by Cell-IQ® Analyser™ software to calculate the percentage of wound closure. Data represent the mean of three separate experiments. The averaged analysed data was used to obtain graph and p value was calculated by Student's t test using graph pad software.

2.21 Cellular invasion assay

Cell invasion assays were carried out using 96-well Transwell® plates with 8-µm pores in the Cultrex® BME and Cultrex® Collagen IV Cell Invasion Assay systems (R&D, UK). Cells were starved in serum free medium for 18 h prior to the beginning of assays. On the day of experiment, the top chamber plates were coated with BME (Basement Membrane Extract) and collagen and

incubated for 4 h at 37°C in a CO_2 incubator. For all invasion experiments, 50 μL of 0.5X BME and 50 μL 0.5X collagen was used to coat the top well chamber. When the Transwell® plates were ready for the experiment, serum deprived cells were harvested by using accutase and then counted by Cell countess machine as mentioned before.

Cells were centrifuged at 250 RCF for 10 minutes and then washed with 1X wash buffer (came with the kit). Cell pellets were resuspended at 1×10^6 cells/mL in serum free medium. BME and Collagen solution was aspirated from the top chambers of cell invasion device and 50,000 cells for each cell type were seeded by adding 50 μL of cell suspension per well to top chamber. 150 μL of culture medium was added in wells of the bottom chamber. The cells in the chamber device were incubated at 37°C in CO_2 incubator for 48 h. After 48 h top chambers and bottom chambers of invasion devices were carefully aspirated and washed with 100 μL and 200 μL of 1X Wash Buffer. 100 μL of cell dissociation solution mixed with Calcein-AM (1.67 μg/μL) was added to bottom chambers and incubate at 37°C in CO_2 incubator for one hour. The top chambers were removed and plates were read on a SPECTRAmax® GEMINI-XS Spectrofluorometer (Molecular Devices Corporation, USA) at 485 nm excitation, 538 nm emission.

2.22 Cellular adhesion assay

The cell adhesion assay was carried out on commercially available pre-coated cell culture plates. 5×10^4 cells of WT, SC and 844 were seeded into each well of 96-well plates coated with fibronectin (R&D, UK), where adherent cells were captured. Plates were incubated for 4 h at 37°C in CO_2 incubator. After 4 h cells were washed twice with HBSS, and the adherent cells are were exposed to Calcein-AM solution before fluorescence was taken at on a SPECTRAmax® GEMINI-XS Spectrofluorometer (Molecular Devices Corporation, USA) at 485 nm excitation and 538 nm emission.

2.23 Effect of 5-Fluorouracil on cell viability

Cell viability assay was performed to examine the effects of exposure to high concentrations of 5-Fluorouracil (5-FU) (TOCRIS bioscience, UK). Cytotoxicity of 5-FU against WT, SC and 844 cells was evaluated using PrestoBlue® (Invitrogen, UK) cell viability reagent. WT, SC and 844 cells were plated in 96-well plates at a density of 2 x 10^4 per well for 24 h. Medium was aspirated and the cells were exposed to a concentration range of 5-FU in triplicate for 24 and 48 h. On the day of cell viability detection, treatment media was replaced with 90 µL complete culture media which was also added to three empty wells. 10 µL PrestoBlue® reagent was added to all wells containing cells and three empty wells which were used as blanks. The plate was incubated at 37°C for 2 h and then the solution from the all wells was transferred to a 96-well fluorescence plate. Fluorescence measurements using SpectraMAX GeminiXS fluorescence microplate reader (Molecular Devices, UK) and SoftMaxPro software were taken at Ex/Em 544/590 nm

2.24 Glycolysis stress test

Stock solutions of 1 mM oligomycin (Sigma, UK) and both 2.5 M glucose (Sigma, UK) and 1 M 2-deoxy-D-glucose (2-DG) (Sigma, UK) were prepared in DMSO and water respectively and aliquots were frozen. DMEM (D5030, Sigma) powder (1bottle) was dissolved in 990 mL of tissue culture-graded water and then 1.85g of sodium chloride (NaCl) (Sigma, UK) was added for a final concentration of 143mM. The pH was adjusted to 7.35 +/- 0.05 and then water was added to make it to 1000 mL solution. This media was filter sterilised and stored at 4°C. On the day of experiment, 200 mL of this base medium was used to prepare glycolysis stress test assay medium by adding 2mL of (200 mM) L-glutamine solution for a final concentration of 2mM and pH was adjusted to 7.4. To measure glycolysis stress, the Seahorse XF24 analyser was used. Cells (2×10^4 cells/well in 10 wells for each cell type and 4 wells left blank) were seeded into 24-well XF24 microplates (Seahorse Bioscience, North Billerica, MA) containing 100 µL standard growth medium and plates

were subsequently incubated at 37 °C with 5% CO_2. After one hour of incubation, once cells were adhered, 150 μL of growth medium was added into each and plate was incubated for 23 h in the same incubator. XF24 extracellular sensor cartridges (Seahorse Bioscience, North Billerica, MA) were calibrated overnight with XF calibrant solution (Seahorse Bioscience, North Billerica, MA) at 37°C in an incubator without CO_2. Next day aliquots of stock solutions of glucose, oligomycin and 2-DG were thawed at room temperature. Then 0.5 mL glucose stock solution was diluted in 14.5 mL of freshly prepared glycolysis stress media and 27 μL oligomycin was diluted in 2273 μL. After 23 h of seeding the cells, the culture medium from XF24 culture plate was aspirated and wells were rinsed twice with 500 μL of XF stress test glycolysis assay medium. Then 560 μL of this test medium was added to each well and the culture plate was incubated at 37°C in an incubator without CO_2 for one hour prior to the assay. Meanwhile, 80 μL of glucose (the final concentration of glucose in the assay was 10 mM), 80 μL of oligomycin (final concentration 1 μM) and 80 μL of 2-DG (final concentration 100 mM) solutions were injected into A, B and C ports of the cartridge in each well respectively except 4 blanks wells in which only stress test media was injected. The sensor cartridge plate was placed into the XF analyser for the automatic calibration of optical sensors of the analyser. The cell culture plate was inserted into the instrument once it was incubated for one hour. The pre-set glycolysis stress test program of the Seahorse XF24 analyser was used according to the manufacturer's recommendation and the data are expressed in mpH/min/10^4 cells for extracellular acidification rate (ECAR).

2.25 Estimation of glucose content in cultured medium

Glucose levels in the conditioned media of cultured cells were measured by using a Glucose Oxidase Assay Kit (GAGO, Sigma, UK). The assay is based on enzymatic reaction where glucose undergoes oxidation by glucose oxidase and forms gluconic acid and hydrogen peroxide (H_2O_2). Then H_2O_2 oxidises the reduced dianisidine colourless compound to an oxidised brown coloured dianisidine which then reacts with sulphuric acid (H_2SO_4) to form a more stable pink coloured

oxidised dianisidine. 2 x 10^5 cells were seeded into 25cm^2 flasks vessels and allowed to grow in normal medium for 24 and 48 h after which reconditioned media was collected in 15 mL tubes. For assay all media samples were diluted in 1:50 with deionized water. Standards were prepared using a glucose solution (1mg/mL) supplied with the kit in the concentration range between 10 μg/mL and 60 μg/mL. The assay was modified for a 96-well microplate. 50 μL of standards, blank and samples were added per well in triplicate. Then 100 μL glucose (GO) assay reagent was added into all wells containing samples, blank control and standards. The microplate was covered with aluminium foil to protect it from light and was incubated at room temperature for 30 minutes. Post incubation, 100 μL of 12N H_2SO_4 solution was added to stop the reaction. Absorbance was measured at 540 nm using a Thermo multiscan EX 96-well plate reader (Thermofisher, UK).

2.26 Analysis of glycerol induced cell death

Cell viability assay was performed to examine the effects of exposure to high levels of glycerol. Cell viability was evaluated using a proprietary modified MTS assay, PrestoBlue® (Invitrogen, UK) cell viability reagent. Cells were seeded in 96-well plates at a density of 2 x 10^4 for 24 h. The cells were then exposed to a concentration range of glycerol in triplicate for 24, 48 and 72 h. On the day of cell viability detection, treatment media was replaced with 90 μL complete culture media and 10 μL PrestoBlue® reagent was added to all wells containing cells and three empty wells which were used as blanks. The plate was incubated at 37°C for 2 h and then the solution from the all wells was transferred to a 96-well fluorescence plate. Fluorescence measurements using Spectra MAX Gemini XS fluorescence microplate reader (Molecular Devices, UK) and Soft-MaxPro software were taken at Ex/Em 544/590 nm.

2.27 Determination of cellular ATP levels

Cell pellets were collected as described in section 2.10. The somatic cell ATP releasing reagent (Sigma, UK) was used to lyse the cells. The level of ATP was measured based on bioluminescence assay for quantitative determination of ATP. The ATP Determination Kit (A22066, Molecular Probes, Invitrogen UK) was used to measure the intracellular ATP levels in cell lysates. ATP levels were measured according to the manufacturer's instructions. Briefly, 10 µL of samples and standards were added into a 96-well plate, and 90 µL of the reaction mixture was added (reaction mixture was prepared according to the protocol provided by the manufacturer). The standards (ranging from 10-1000 nM) were prepared according to the kit's protocol. The plate was incubated for 10 minutes at room temperature protected from light. The luminescence was recorded using Spectra MAX Gemini XS microplate luminometer (Molecular Devices, UK) and Soft-MaxPro software.

Chapter 3

AQP3 Expression in Clinical Cases of Breast Cancer

3.1 Introduction

Recent research on AQPs and their involvement in different cancer tissues has demonstrated expression of AQPs in BC tissue. BC tissues are graded clinically depending on various features. The histological grading of tumour into grades 1, 2 and 3 score was based on three histological features; tubule formation, nuclear variation (Bloom and Richardson, 1957) and mitotic rate as explained by Elston *et al*. (Elston *et al.*, 1982). According to this system tumours with visible lumen and well-formed tubules, regular nuclei with very less variation and lesser mitotic activity were considered as grade 1 tumours (Elston *et al.*, 1982). Tumours with moderate tubules, moderate nuclear variation and moderate mitotic activity were considered as grade 2 tumours (Elston *et al.*, 1982). Tumours in which cells growing in sheets with little or no tubule formation, showing marked variation in nuclear shape and size and showing higher mitotic activity were considered as grade 3 tumours (Elston *et al.*, 1982). Based on this classification, grade 1 tumours are characterised as well defined tumours and grade 3 tumours poorly differentiated tumours, whereas grade 2 tumours belong to an intermediate category of moderately defined tumours. It is generally considered that grade 1 tumours can progress to become grade 3 tumours, however some studies suggested that the majority of grade 1 tumours did not progress to grade 3 tumours indicating the diversity of breast tumour grades (Roylance *et al.*, 1999). The complexity of BC disease therefore demands more efficient research and new strategies to combat the disease in order to save precious lives.

The main aim of this chapter is to assess the expression levels and clinical relevance of AQP3 and other AQPs in both frozen tissues and paraffin embedded BC specimens. This was done by measuring expression of all AQPs in selected tumour samples from both grade 1 and grade 3 cases.

3.2　Materials and Methods

The qPCR reactions, Western blot analysis and immuohistochemical analysis were performed as described in Chapter 2 in detail. Statistical analysis of data was performed using GraphPad Prism 6 software. One-way ANOVA with Dunnett's post-hoc test for comparisons of > 3 groups or Student's unpaired t-test for comparisons of <3 groups were performed for the statistical analysis between experimental conditions; p values <0.05 were considered significant. Band density analysis for blots was carried out using Image J (NIH) and data were analysed in GraphPad prism.

3.3　Results

3.3.1　AQPs gene expression in snap frozen tissue samples

To examine the mRNA expression of all 13 mammalian AQPs in snap frozen cancerous and healthy border tissue samples from 8 BC patients, qPCR was performed. As stated before, the housekeeping genes beta-actin and YWHAZ were used to normalise gene expression and relative mRNA expression of all AQPs was measured using $2^{-\Delta\Delta CT}$ method by comparing the mRNA expression between normal and cancer tissues. Statistical significance was examined by unpaired t test.

The mRNA expression levels of all AQPs varied between normal and cancer tissue samples (Figures 3.1 to 3.26) with few AQPs showing differences; however the only statistically significant difference was observed for AQP3 (Figure 3.7 & Figure 3.8) which was found to be expressed at a significantly higher level (p = 0.0258) in cancer tissues as compared to healthy border tissues.
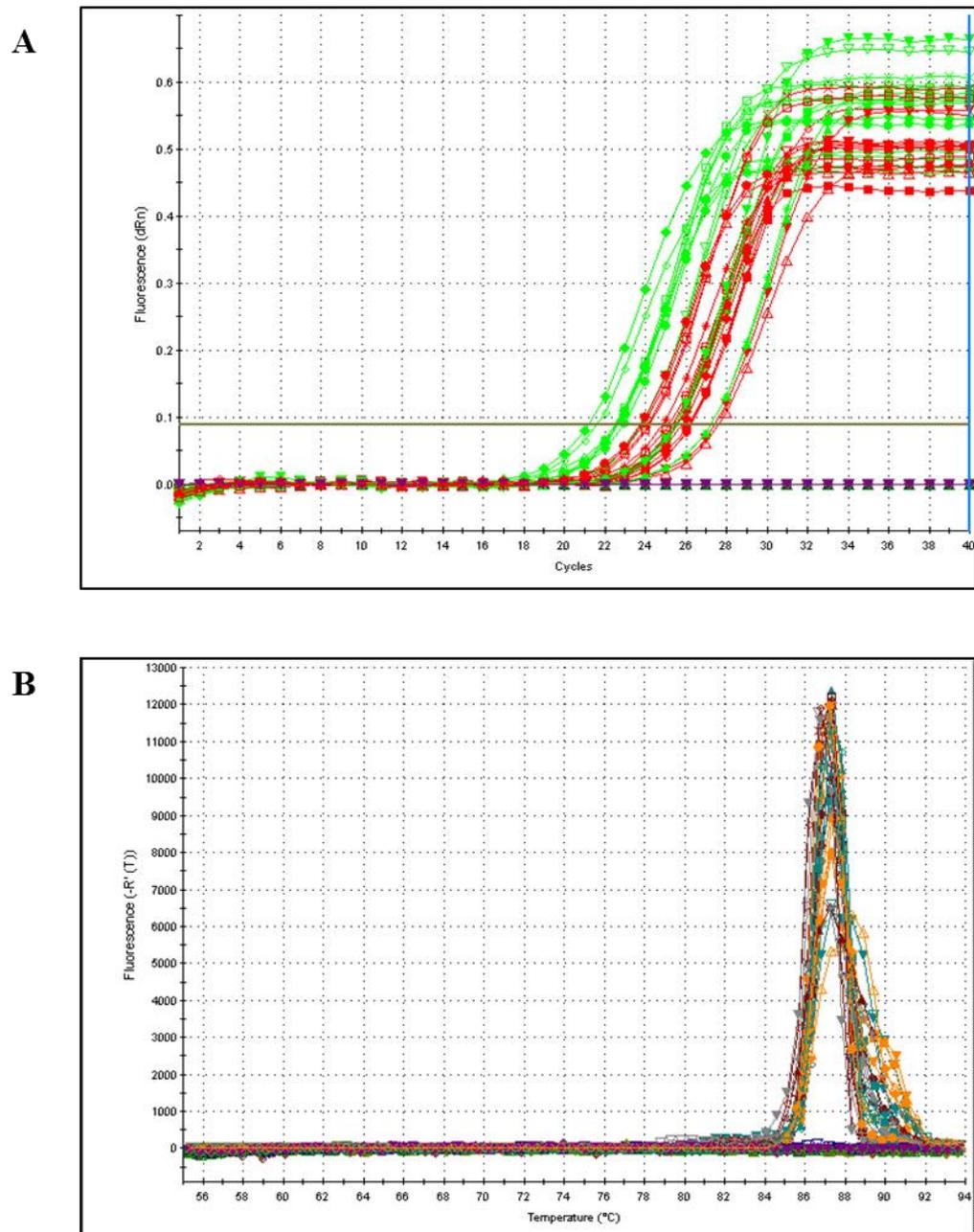
Figure 3.1 SYBR® Green real-time PCR analysis of AQP0 gene expression.

Gene expression of AQP0 was assessed in diluted cDNA samples of cancer and corresponding adjacent normal tissue samples using pre-validated primers. (A) Amplification curves generated for AQP0. Red and green curves represent cancer and normal tissue samples respectively. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 87°C showing adequate primer specificity; n=8.
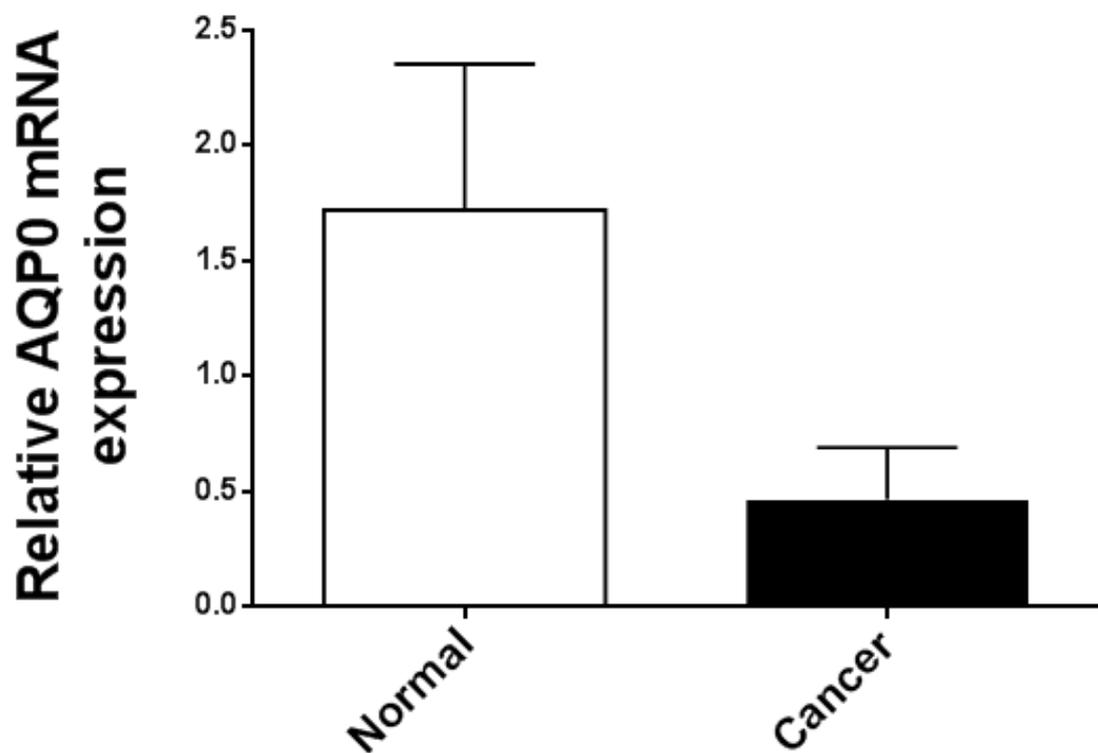
Figure 3.2   Expression of AQP0 mRNA in snap frozen tissue samples.

qPCR analysis of AQP0 mRNA expression in cancer and corresponding adjacent normal tissue samples showed that there was no significant difference between them. Bar graph displaying the corresponding relative fold difference of normal and cancerous samples internally normalised to housekeeping genes. Relative fold expression was calculated by considering normal tissue samples as control. Data represent mean ± standard error mean (SEM); p value = 0.084, n=8.
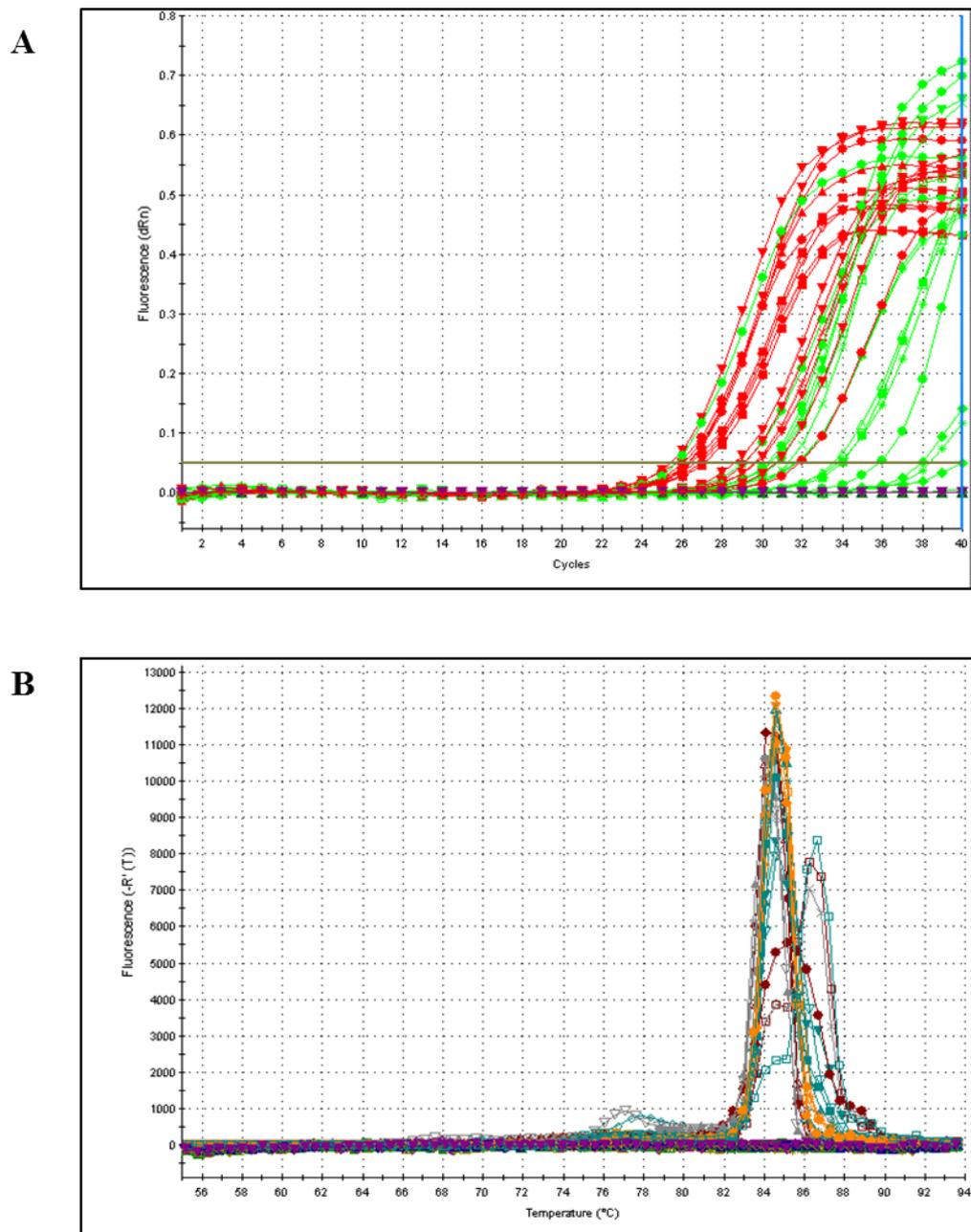
Figure 3.3   SYBR® Green real-time PCR analysis of AQP1 gene expression.

Gene expression of AQP1 was assessed in diluted cDNA samples of cancer and corresponding adjacent normal tissue samples using pre-validated primers. (A) Amplification curves generated for AQP1. Red and green curves represent cancer and normal tissue samples respectively. (B) Dissociation curve analysis of most samples showed peak dissociated at approximately 85°C suggesting adequate primer specificity; n=8.
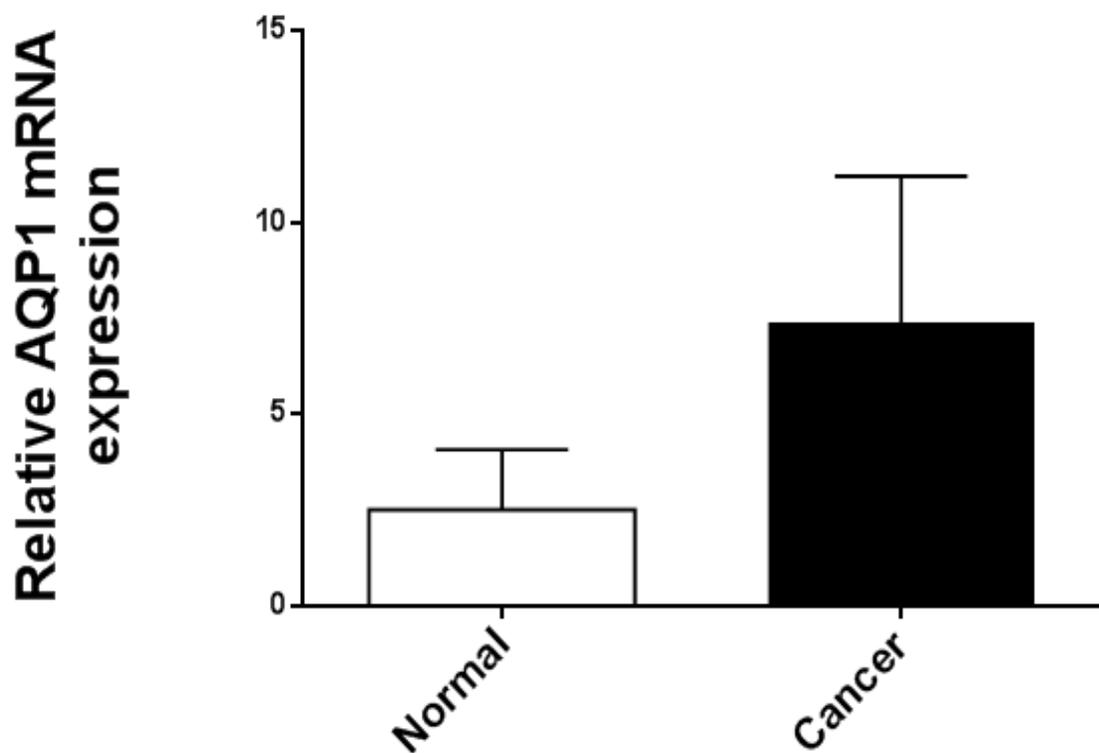
Figure 3.4   Expression of AQP1 mRNA in snap frozen tissue samples.

qPCR analysis of AQP1 mRNA expression in cancer and corresponding adjacent normal tissue samples showed that there was no significant difference between them. Bar graph displaying the corresponding relative fold difference of normal and cancerous samples internally normalised to housekeeping genes. Relative fold expression was calculated by considering normal tissue samples as control. Data represent mean ± SEM; p value = 0.256, n=8.
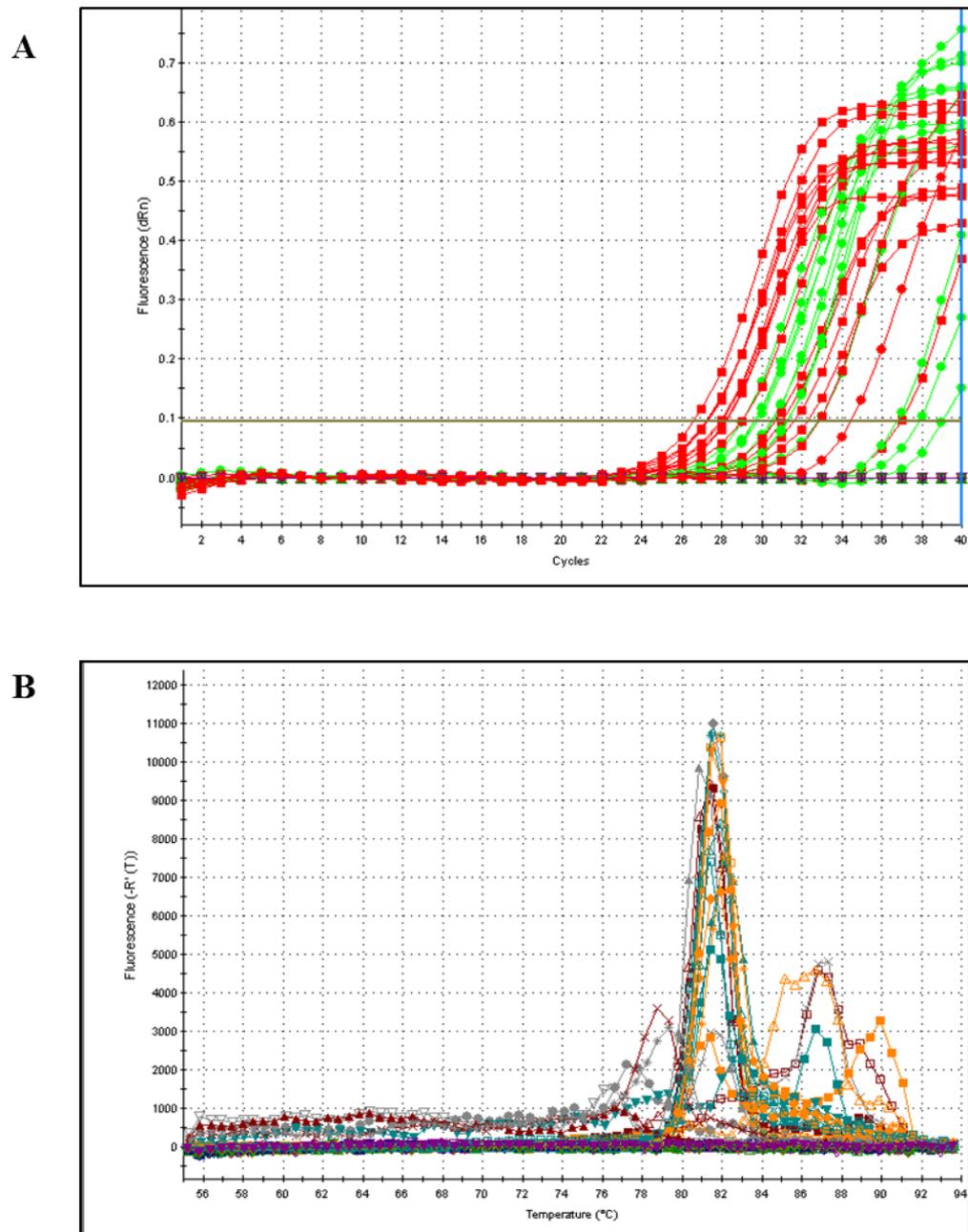
Figure 3.5   SYBR® Green real-time PCR analysis of AQP2 gene expression.

Gene expression of AQP2 was assessed in diluted cDNA samples of cancer and corresponding adjacent normal tissue samples using pre-validated primers. (A) Amplification curves generated for AQP2. Red and green curves represent cancer and normal tissue samples respectively. (B) Dissociation curve analysis of most samples showed dissociation peak that dissociated at approximately 82°C showing adequate primer specificity, however few samples showed nonspecific peaks; n=8.
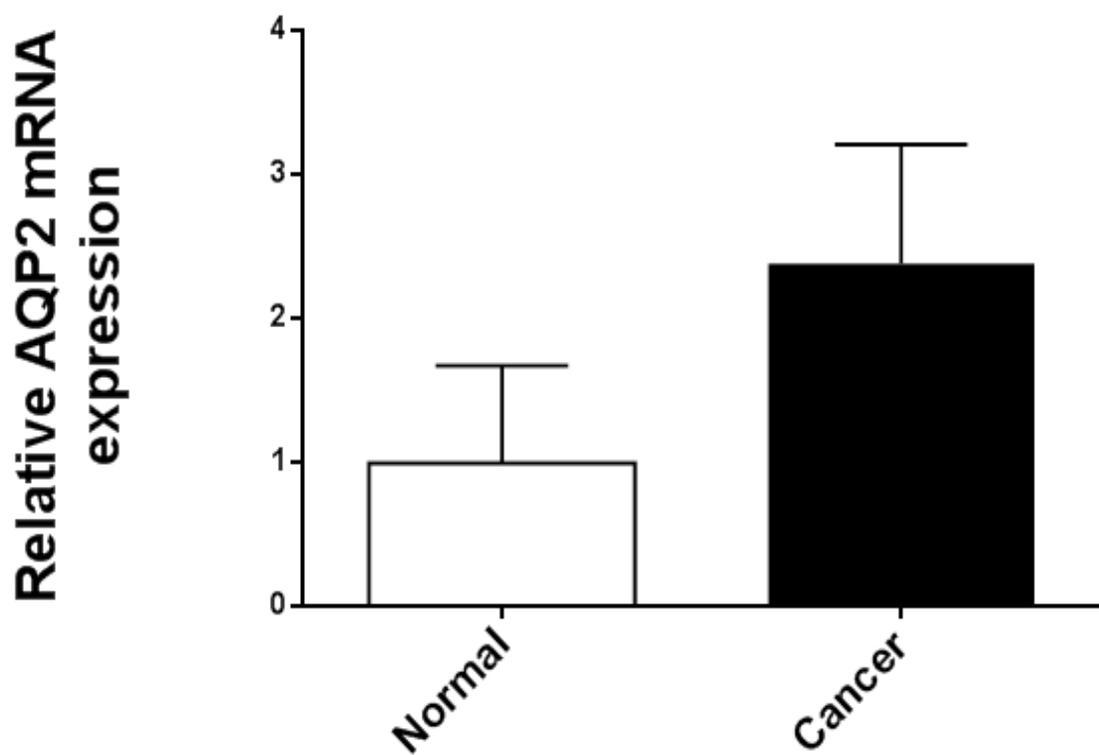
Figure 3.6 **Expression of AQP2 mRNA in snap frozen tissue samples.**

qPCR analysis of AQP2 mRNA expression in cancer and corresponding adjacent normal tissue samples showed that there was no significant difference between them. Bar graph displaying the corresponding relative fold difference of normal and cancerous samples internally normalised to housekeeping genes. Relative fold expression was calculated by considering normal tissue samples as control. Data represent mean ± SEM; p value = 0.216, n=8.
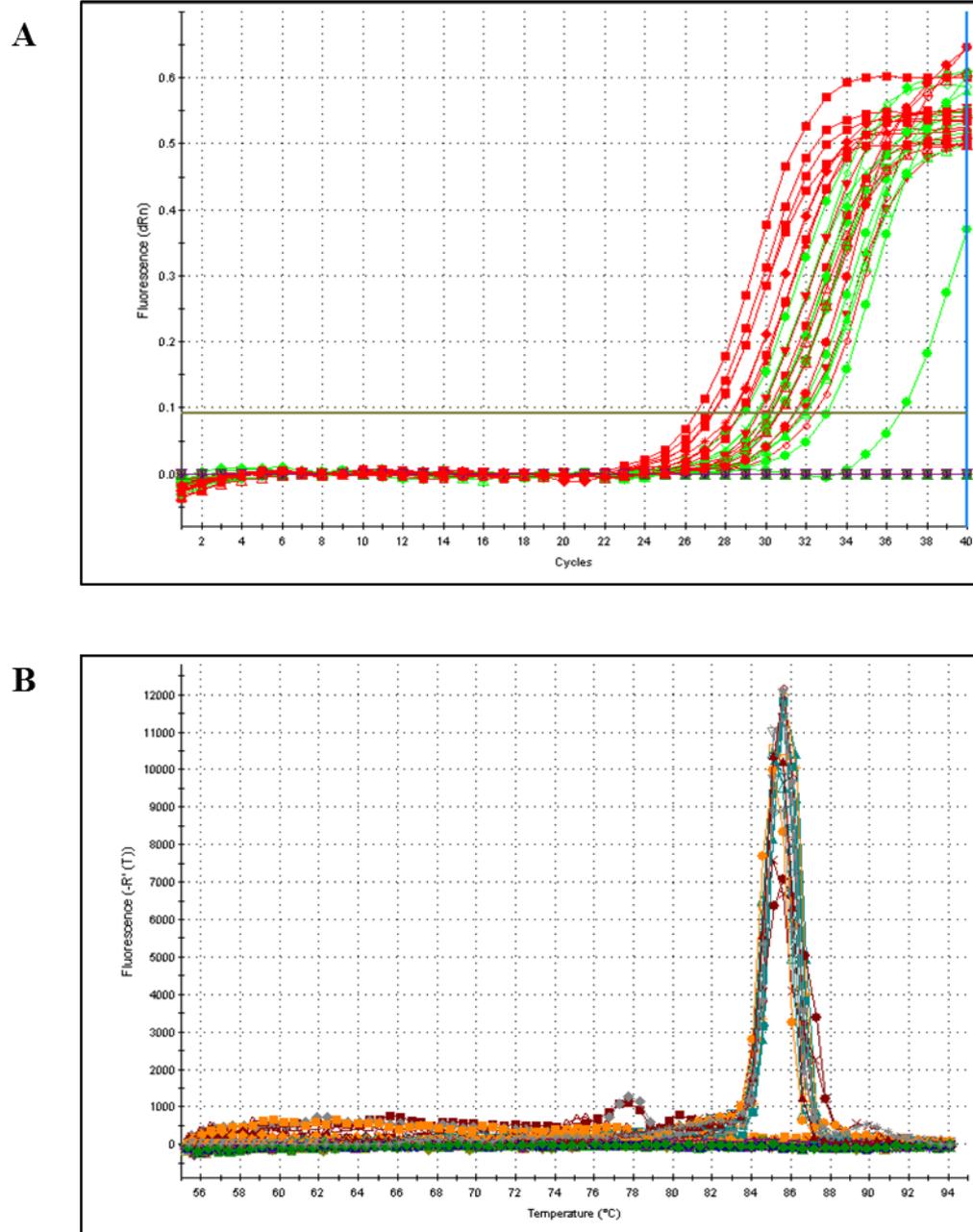
Figure 3.7   SYBR® Green real-time PCR analysis of AQP3 gene expression.

Gene expression of AQP3 was assessed in diluted cDNA samples of cancer and corresponding adjacent normal tissue samples using pre-validated primers. (A) Amplification curves generated for AQP3. Red and green curves represent cancer and normal tissue samples respectively. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 85°C suggesting excellent primer specificity; n=8.
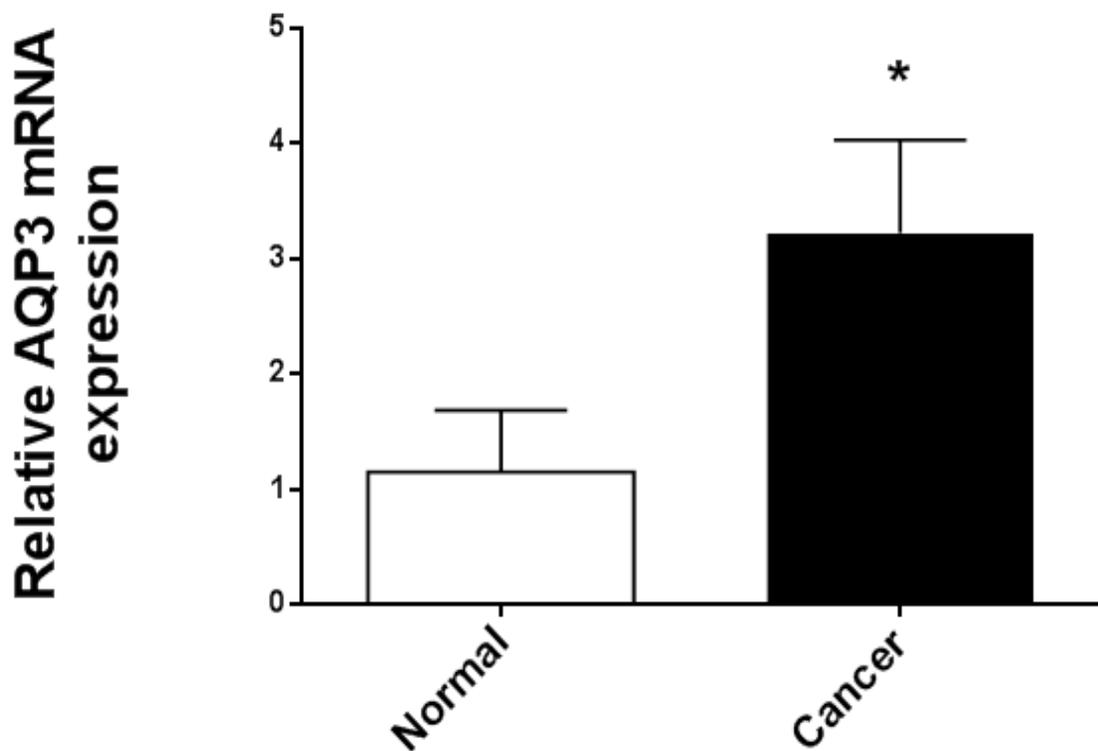
Figure 3.8   Expression of AQP3 mRNA in snap frozen tissue samples.

qPCR analysis of AQP3 mRNA expression in cancer and corresponding adjacent normal tissue samples showed that AQP3 expression level in cancer tissue was significantly higher than in corresponding normal tissue. Bar graph displaying the corresponding relative fold difference of normal and cancerous samples internally normalised to housekeeping genes. Relative fold expression was calculated by considering normal tissue samples as control. Data represent mean ± SEM; *p = 0.026, n=8.
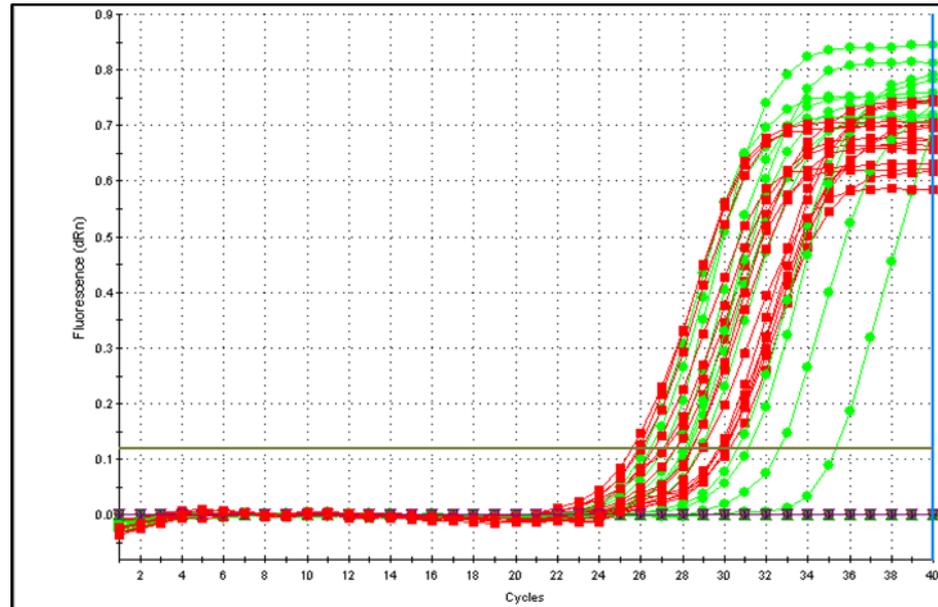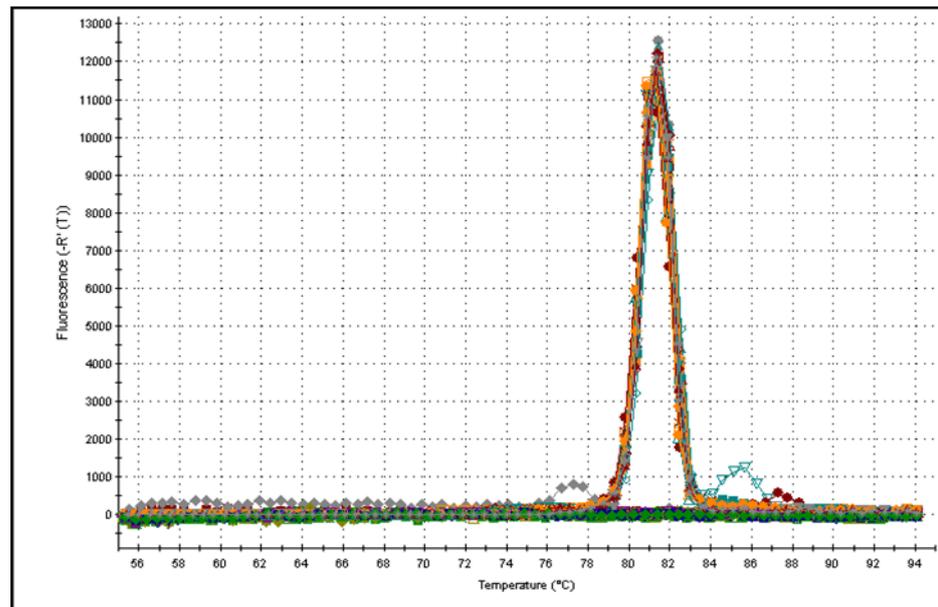
Figure 3.9   SYBR® Green real-time PCR analysis of AQP4 gene expression.

Gene expression of AQP4 was assessed in diluted cDNA samples of cancer and corresponding adjacent normal tissue samples using pre-validated primers. (A) Amplification curves generated for AQP4. Red and green curves represent cancer and normal tissue samples respectively. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 81°C suggesting excellent primer specificity; n=8.
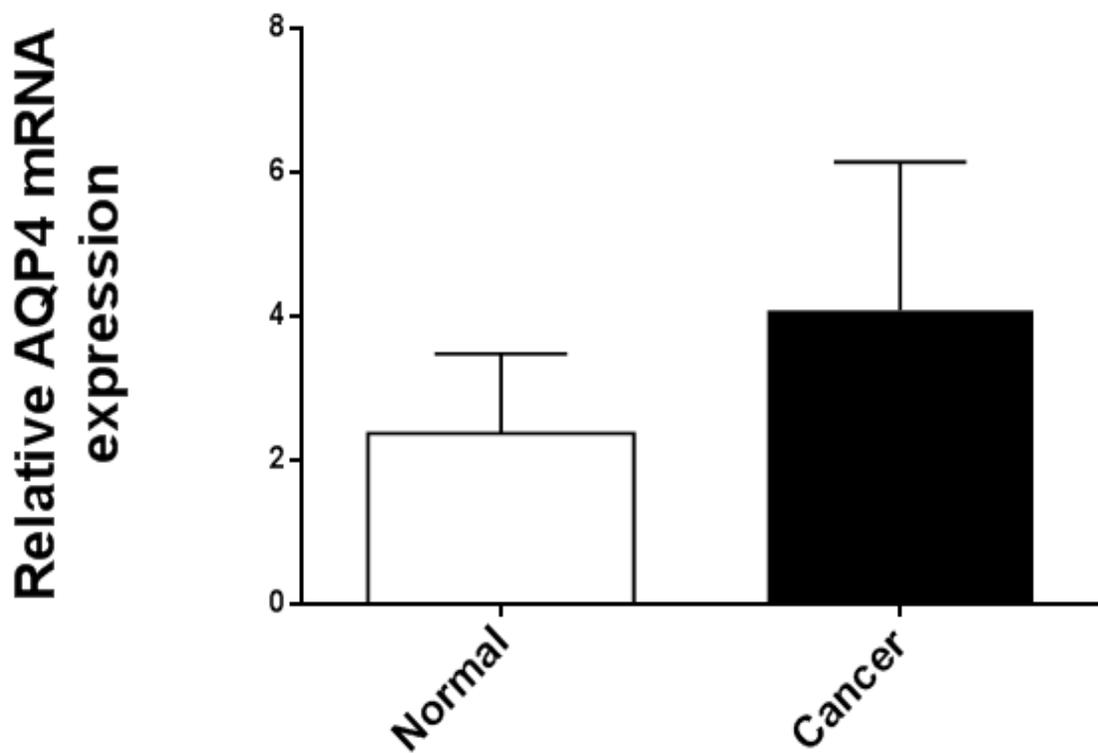
Figure 3.10   Expression of AQP4 mRNA in snap frozen tissue samples.

qPCR analysis of AQP4 mRNA expression in cancer and corresponding adjacent normal tissue samples showed that there was no significant difference between them. Bar graph displaying the corresponding relative fold difference of normal and cancerous samples internally normalised to housekeeping genes. Relative fold expression was calculated by considering normal tissue samples as control. Data represent mean ± SEM; p value = 0.476, n=8.
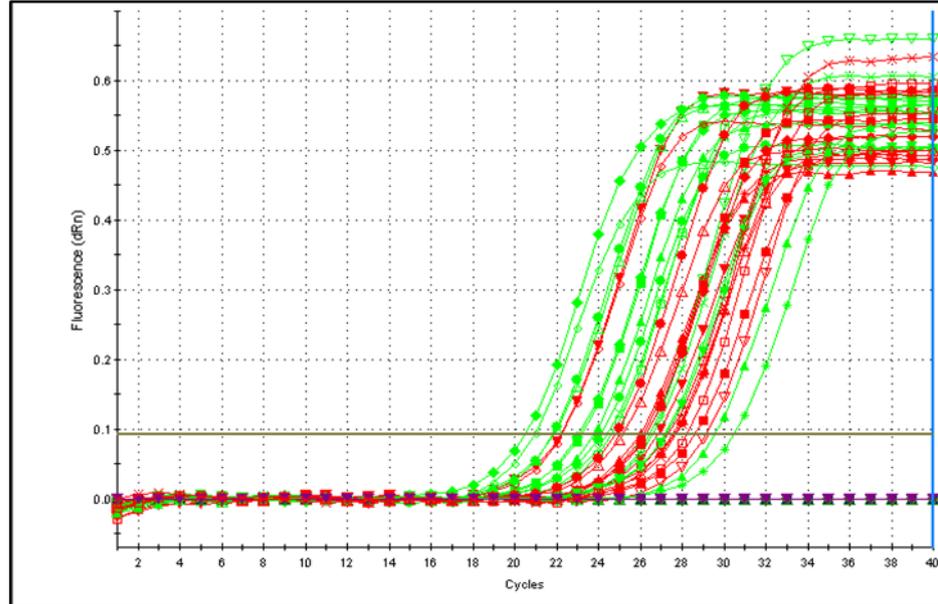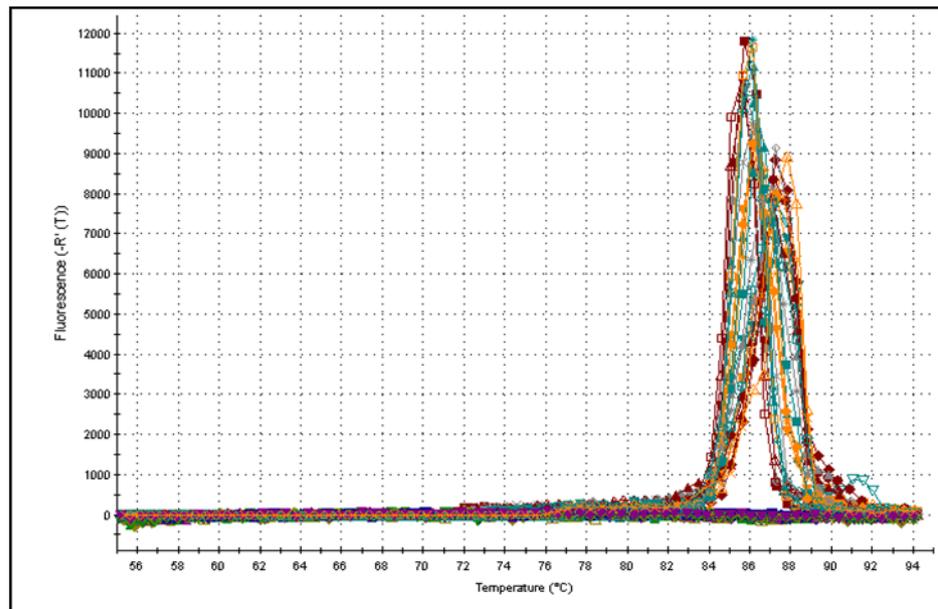
Figure 3.11   SYBR® Green real-time PCR analysis of AQP5 gene expression.

Gene expression of AQP5 was assessed in diluted cDNA samples of cancer and corresponding adjacent normal tissue samples using pre-validated primers. (A) Amplification curves generated for AQP5. Red and green curves represent cancer and normal tissue samples respectively. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 86°C showing adequate primer specificity; n=8.
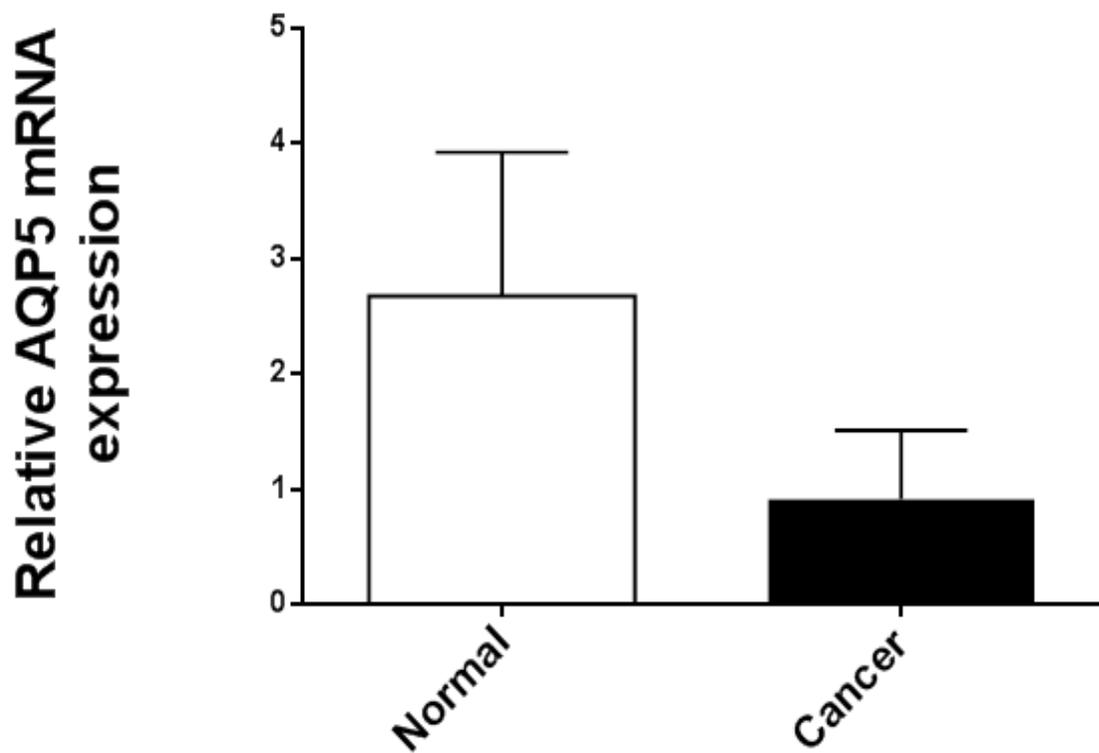
Figure 3.12   Expression of AQP5 mRNA in tissue samples.

qPCR analysis of AQP5 mRNA expression in cancer and corresponding adjacent normal tissue samples showed that there was no significant difference between them. Bar graph displaying the corresponding relative fold difference of normal and cancerous samples internally normalised to housekeeping genes. Relative fold expression was calculated by considering normal tissue samples as control. Data represent mean ± SEM; p value = 0.223, n=8.
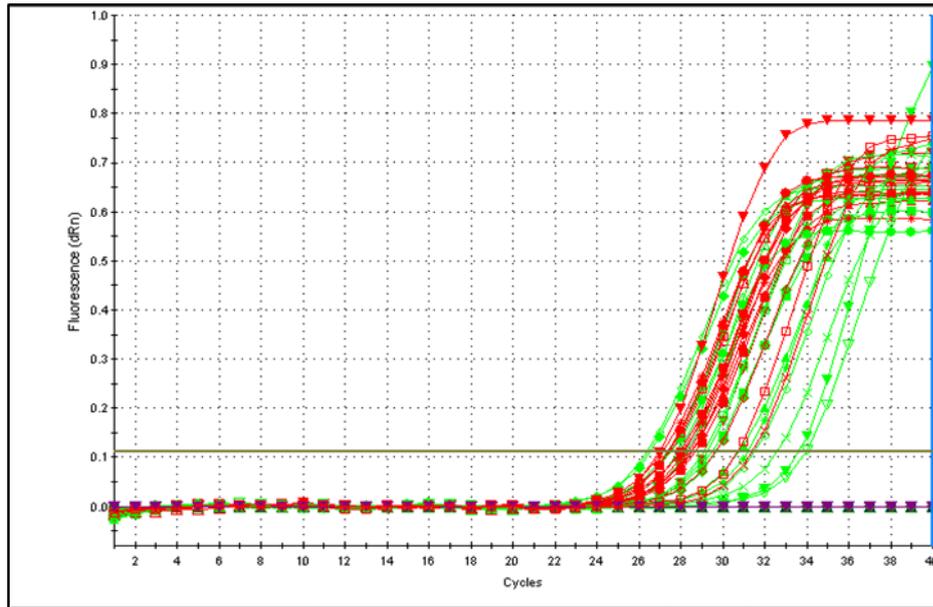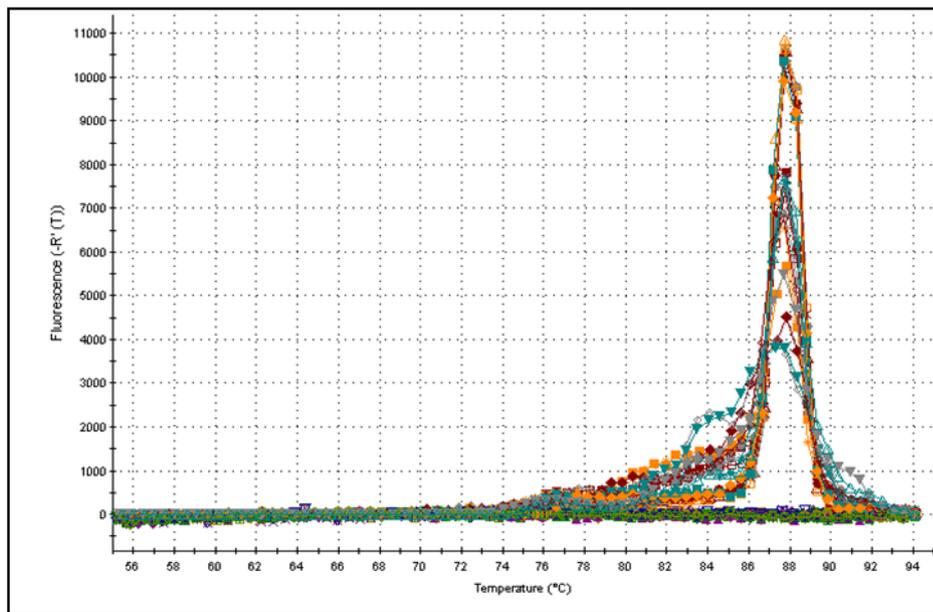
Figure 3.13   SYBR® Green real-time PCR analysis of AQP6 gene expression.

Gene expression of AQP6 was assessed in diluted cDNA samples of cancer and corresponding adjacent normal tissue samples using pre-validated primers. (A) Amplification curves generated for AQP6. Red and green curves represent cancer and normal tissue samples respectively. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 88°C showing adequate primer specificity; n=8.
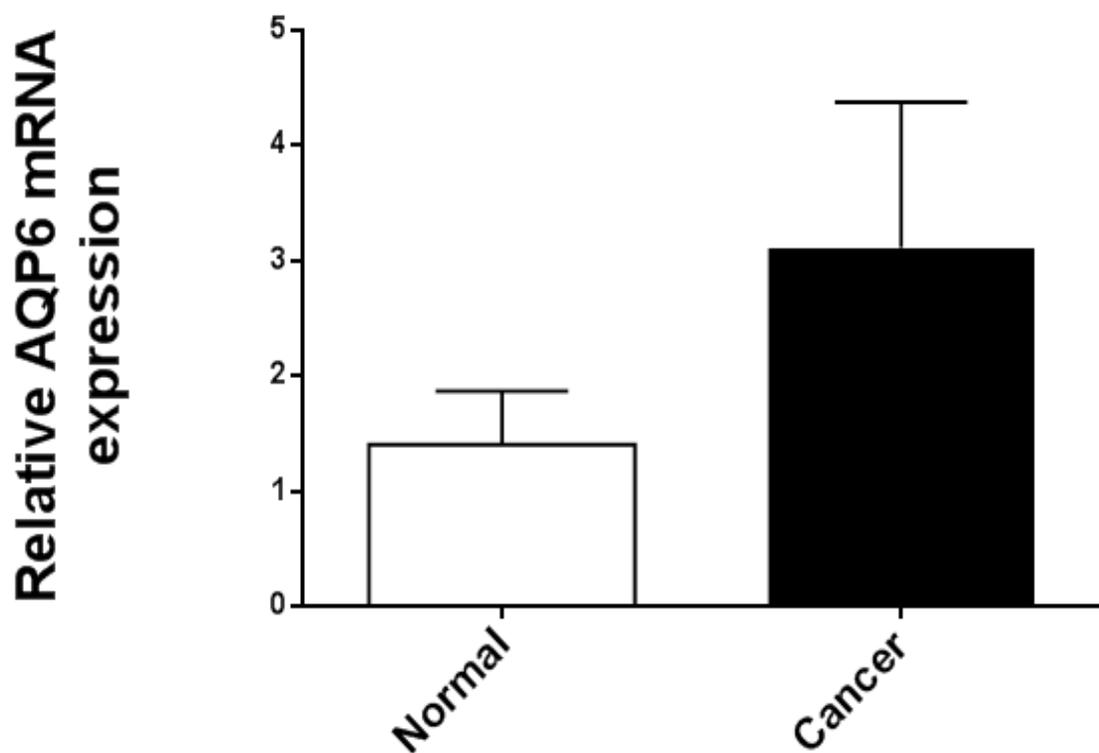
Figure 3.14   Expression of AQP6 mRNA in snap frozen tissue samples.

qPCR analysis of AQP6 mRNA expression in cancer and corresponding adjacent normal tissue samples showed that there was no significant difference between them. Bar graph displaying the corresponding relative fold difference of normal and cancerous samples internally normalised to housekeeping genes. Relative fold expression was calculated by considering normal tissue samples as control. Data represent mean ± SEM; p value = 0.227, n=8.
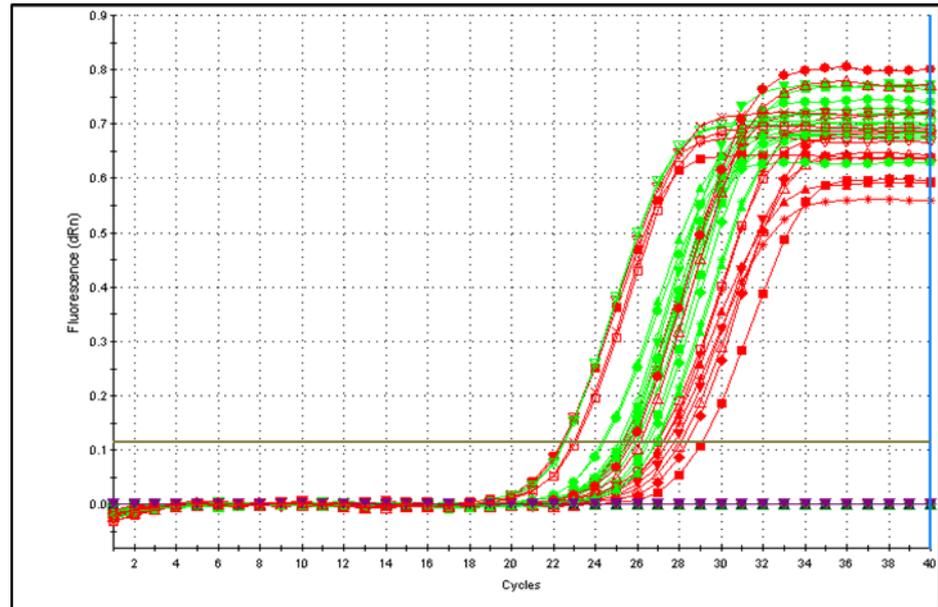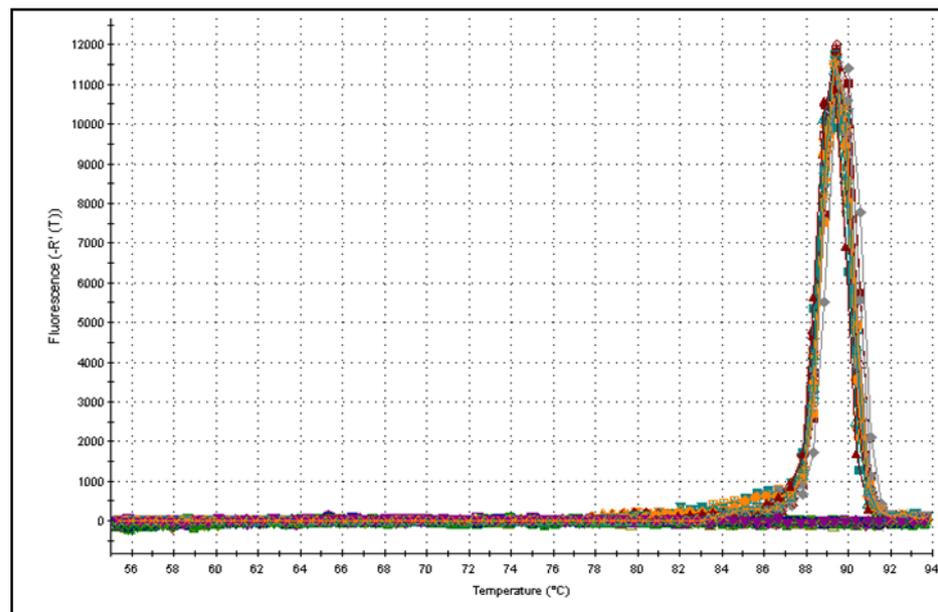
Figure 3.15   SYBR® Green real-time PCR analysis of AQP7 gene expression.

Gene expression of AQP7 was assessed in diluted cDNA samples of cancer and corresponding adjacent normal tissue samples using pre-validated primers. (A) Amplification curves generated for AQP7. Red and green curves represent cancer and normal tissue samples respectively. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 89°C showing excellent primer specificity; n=8.
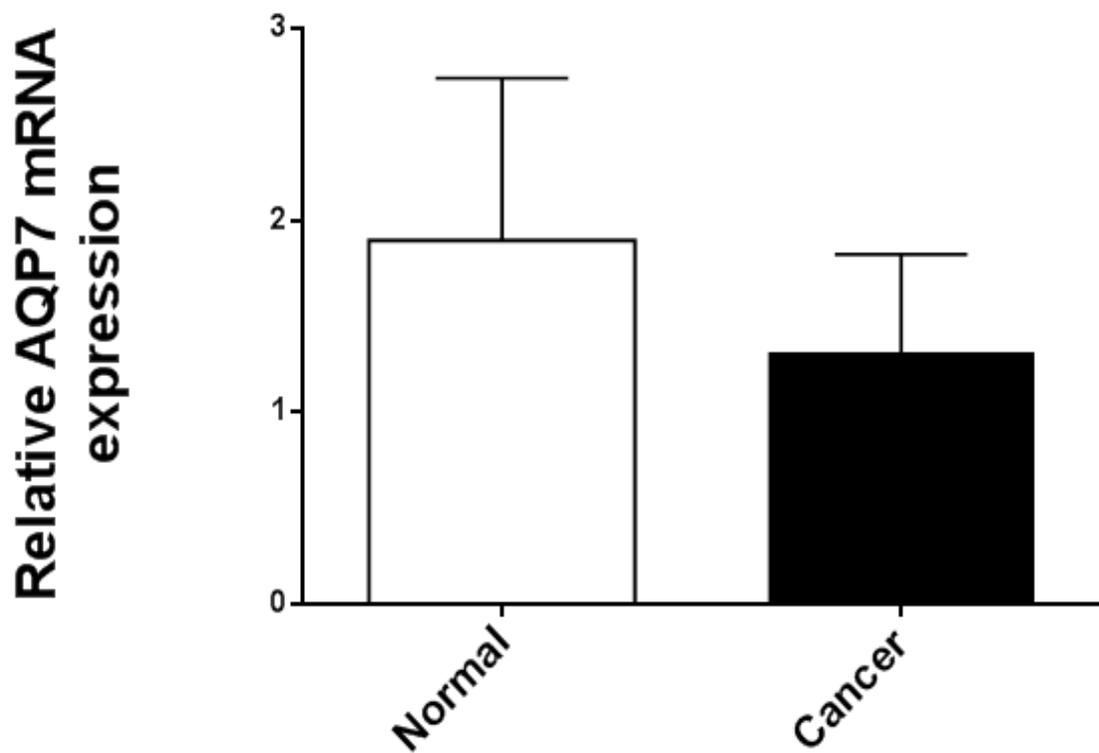
Figure 3.16   Expression of AQP7 mRNA in snap frozen tissue samples.

qPCR analysis of AQP7 mRNA expression in cancer and corresponding adjacent normal tissue samples showed that there was no significant difference between them. Bar graph displaying the corresponding relative fold difference of normal and cancerous samples internally normalised to housekeeping genes. Relative fold expression was calculated by considering normal tissue samples as control. Data represent mean ± SEM; p value = 0.564, n=8.
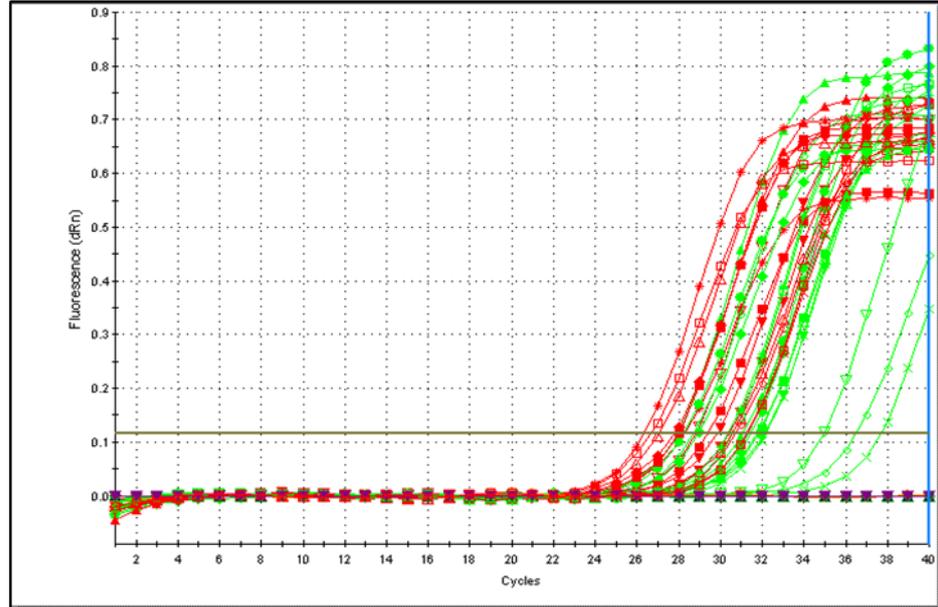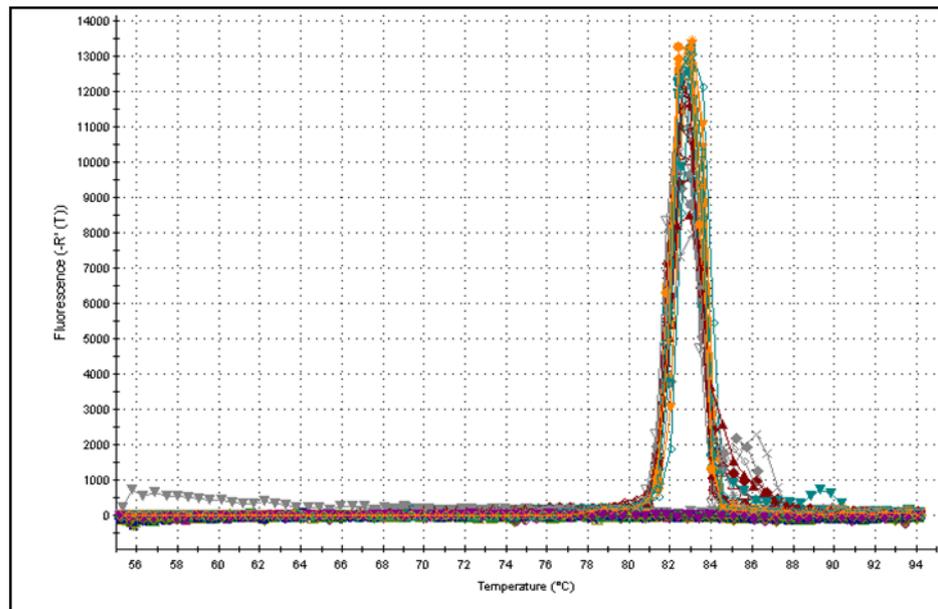
Figure 3.17   SYBR® Green real-time PCR analysis of AQP8 gene expression.

Gene expression of AQP8 was assessed in diluted cDNA samples of cancer and corresponding adjacent normal tissue samples using pre-validated primers. (A) Amplification curves generated for AQP8. Red and green curves represent cancer and normal tissue samples respectively. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 83°C suggesting excellent primer specificity; n=8.
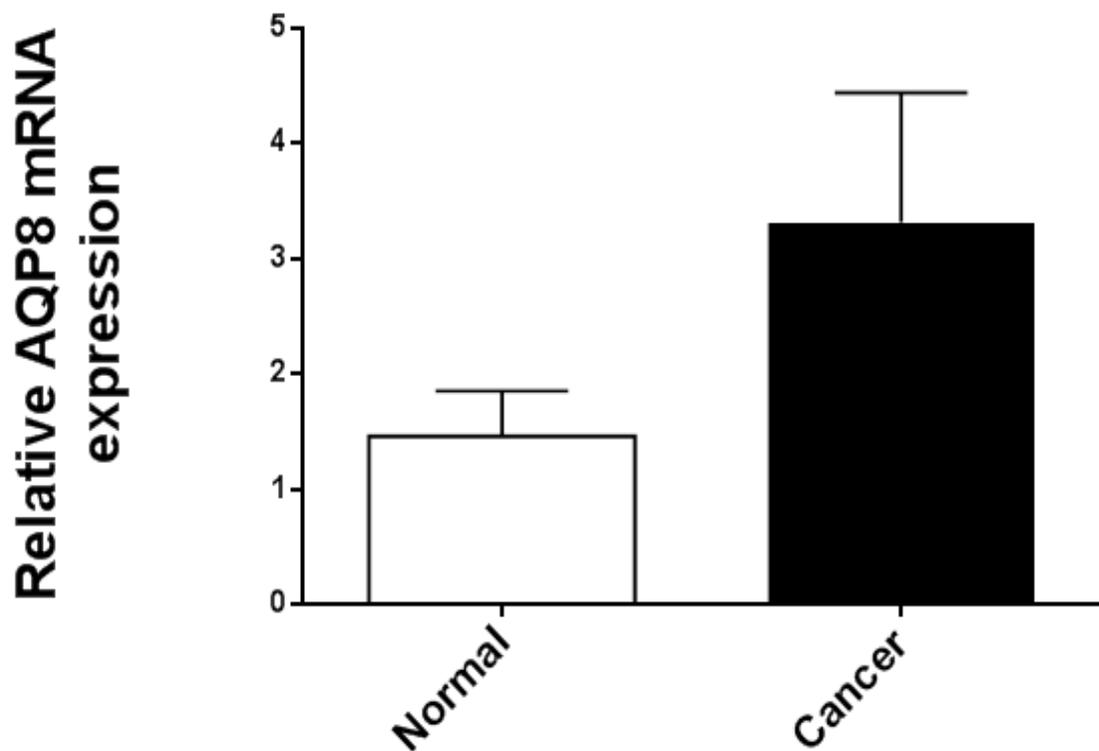
Figure 3.18 Expression of AQP8 mRNA in snap frozen tissue samples.

qPCR analysis of AQP8 mRNA expression in cancer and corresponding adjacent normal tissue samples showed that there was no significant difference between them. Bar graph displaying the corresponding relative fold difference of normal and cancerous samples internally normalised to housekeeping genes. Relative fold expression was calculated by considering normal tissue samples as control. Data represent mean ± SEM; p value = 0.106, n=8.
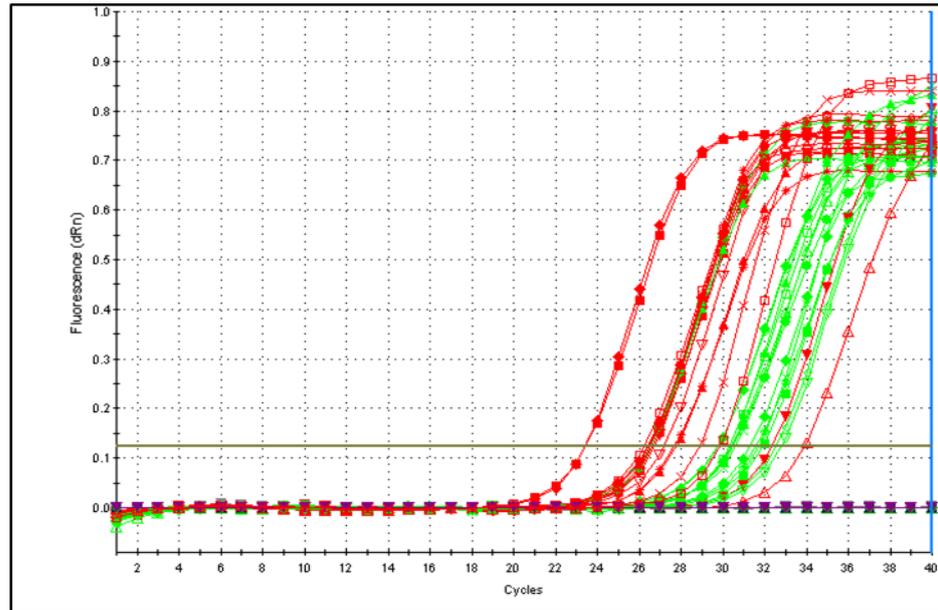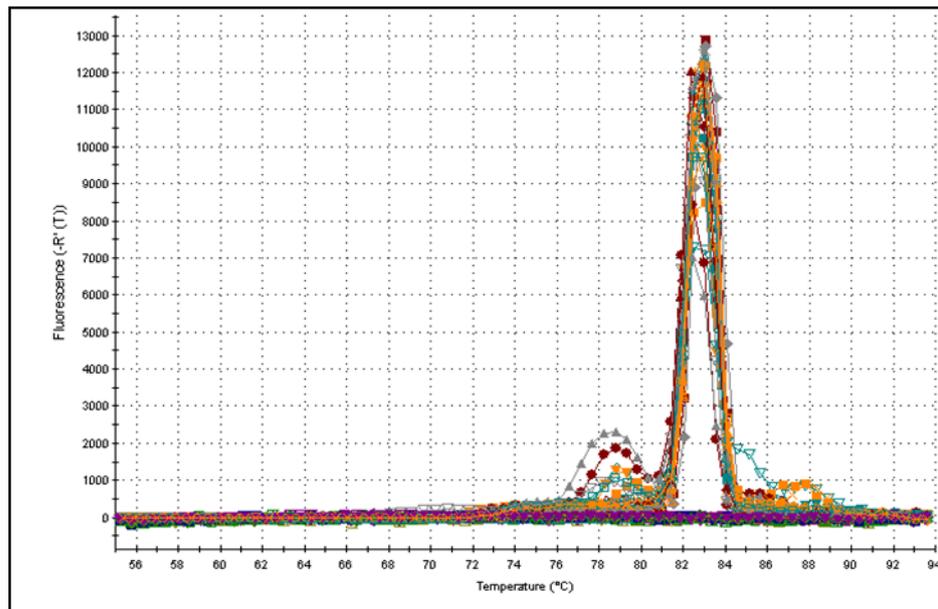
Figure 3.19   SYBR® Green real-time PCR analysis of AQP9 gene expression.

Gene expression of AQP9 was assessed in diluted cDNA samples of cancer and corresponding adjacent normal tissue samples using pre-validated primers. (A) Amplification curves generated for AQP9. Red and green curves represent cancer and normal tissue samples respectively. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 83°C suggesting excellent primer specificity; n=8.
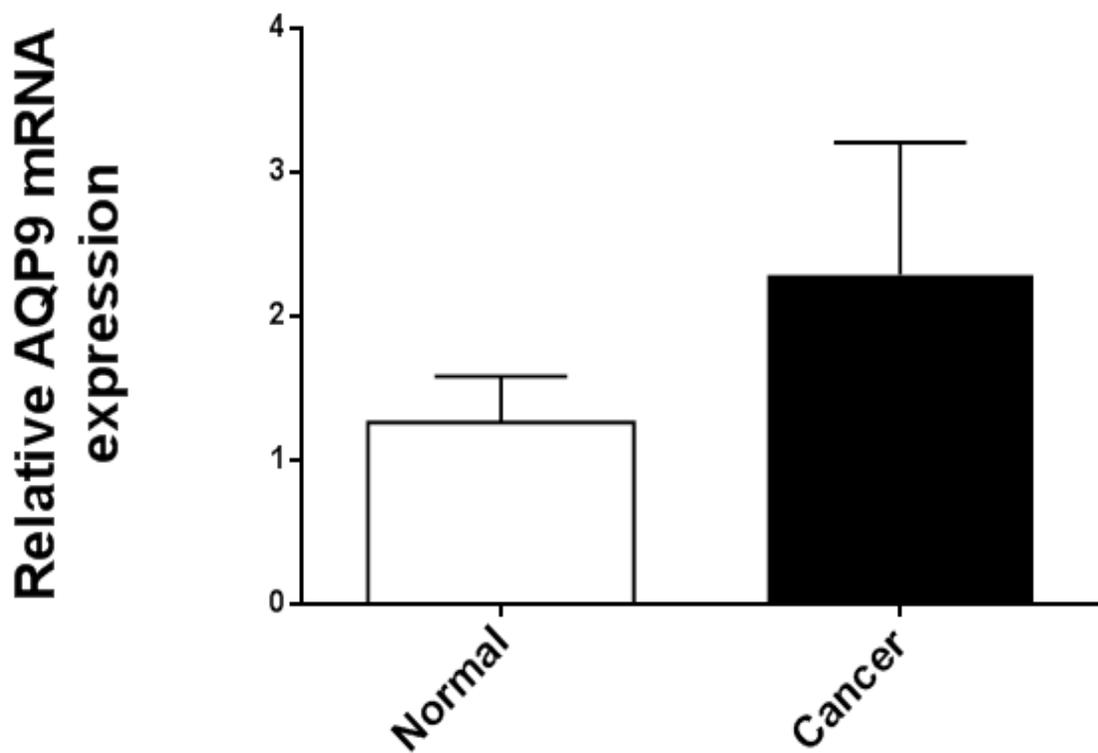
Figure 3.20   Expression of AQP9 mRNA in snap frozen tissue samples.

qPCR analysis of AQP9 mRNA expression in cancer and corresponding adjacent normal tissue samples showed that there was no significant difference between them. Bar graph displaying the corresponding relative fold difference of normal and cancerous samples internally normalised to housekeeping genes. Relative fold expression was calculated by considering normal tissue samples as control. Data represent mean ± SEM; p value = 0.212, n=8.
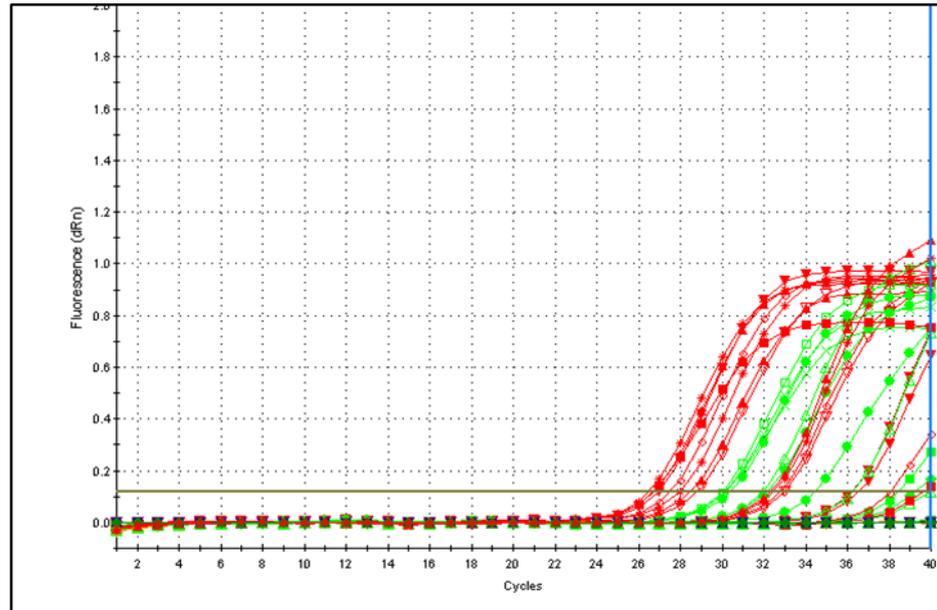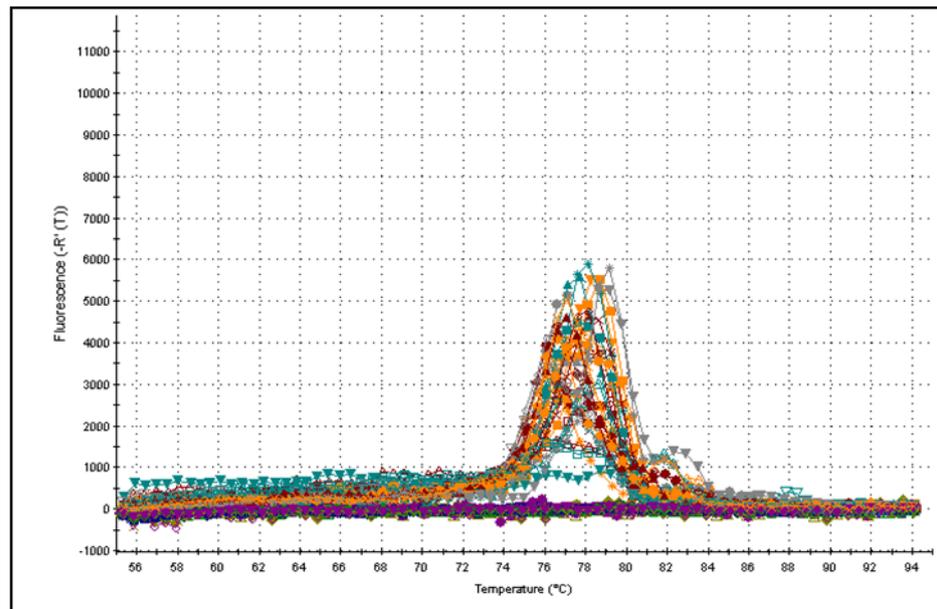
Figure 3.21   SYBR® Green real-time PCR analysis of AQP10 gene expression.

Gene expression of AQP10 was assessed in diluted cDNA samples of cancer and corresponding adjacent normal tissue samples using pre-validated primers. (A) Amplification curves generated for AQP10. Red and green curves represent cancer and normal tissue samples respectively. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 78°C showing adequate primer specificity; n=8.
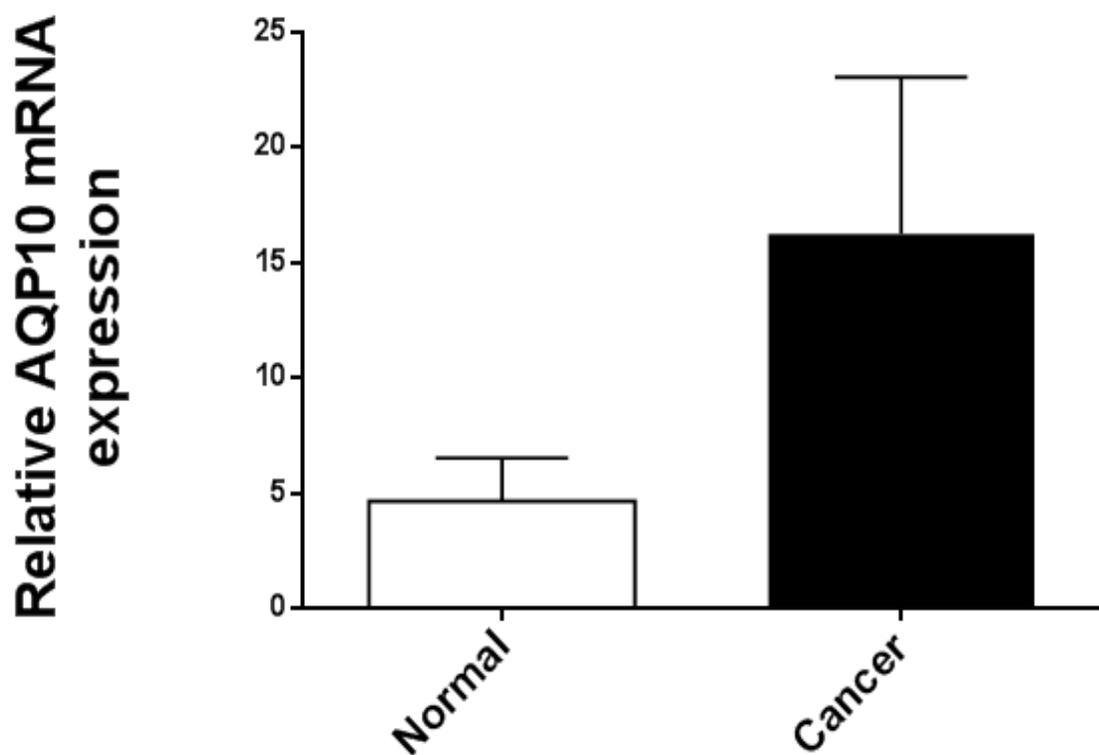
Figure 3.22   Expression of AQP10 mRNA in snap frozen tissue samples.

qPCR analysis of AQP10 mRNA expression in cancer and corresponding adjacent normal tissue samples showed that there was no significant difference between them. Bar graph displaying the corresponding relative fold difference of normal and cancerous samples internally normalised to housekeeping genes. Relative fold expression was calculated by considering normal tissue samples as control. Data represent mean ± SEM; p value = 0.123, n=8.
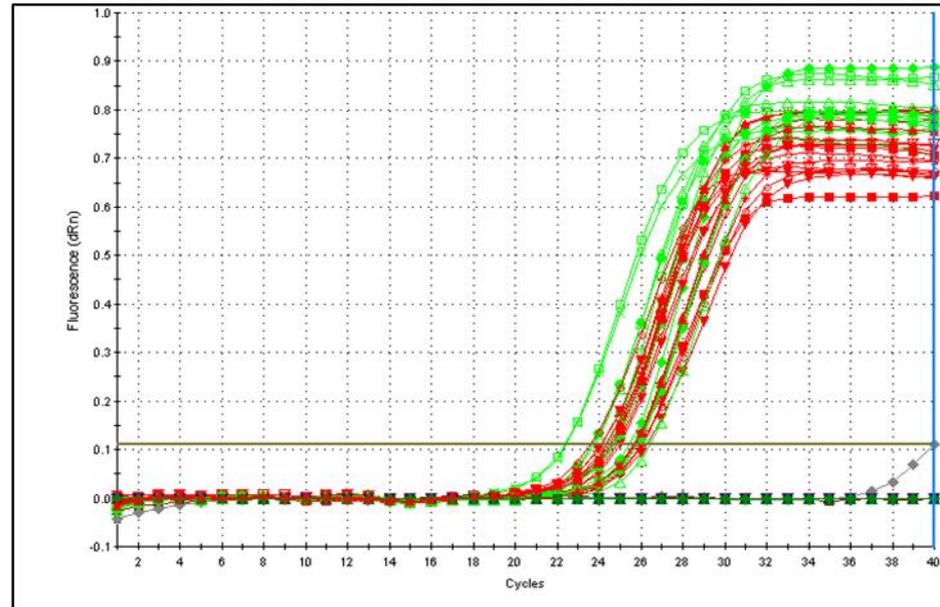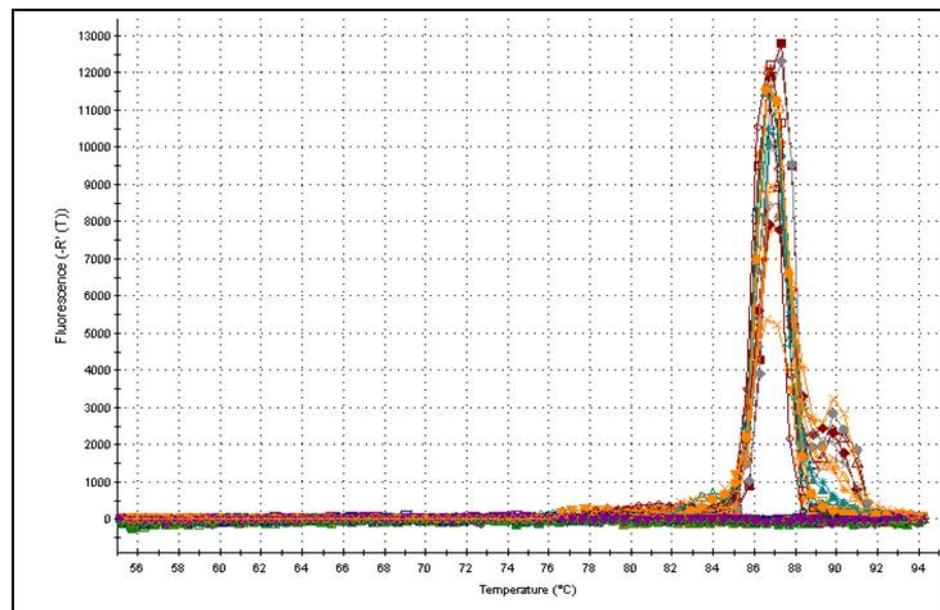
Figure 3.23   SYBR® Green real-time PCR analysis of AQP11 gene expression.

Gene expression of AQP11 was assessed in diluted cDNA samples of cancer and corresponding adjacent normal tissue samples using pre-validated primers. (A) Amplification curves generated for AQP11. Red and green curves represent cancer and normal tissue samples respectively. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 87°C showing excellent primer specificity; n=8.
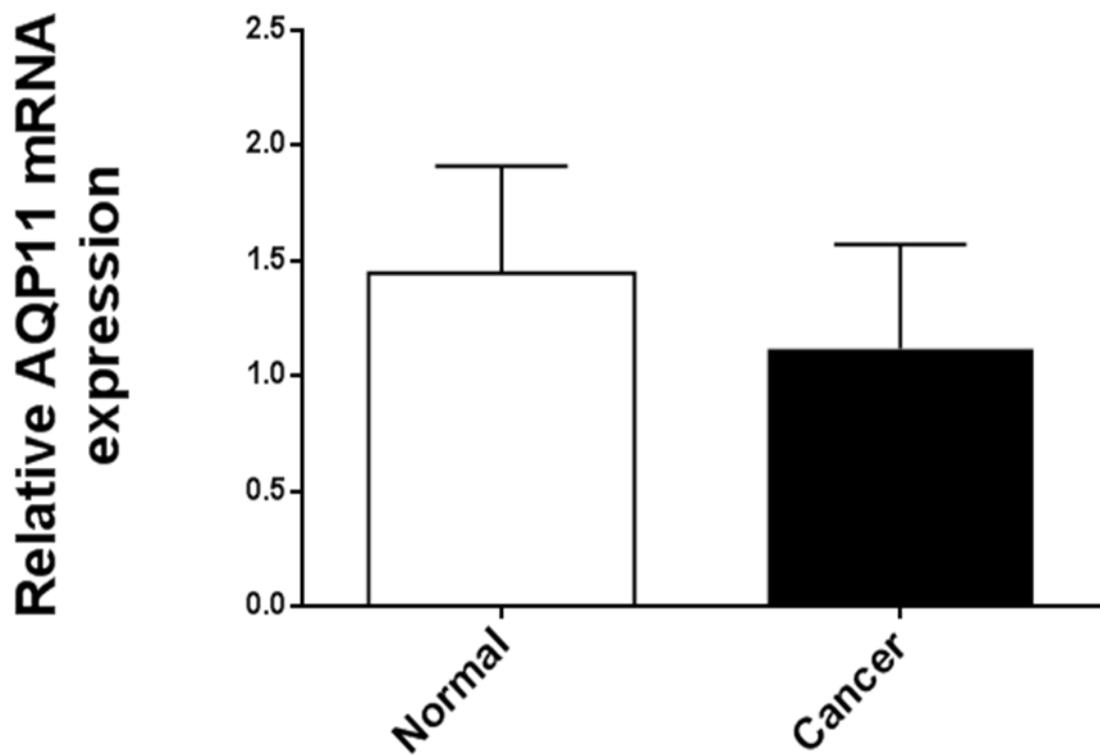
Figure 3.24   Expression of AQP11 mRNA in snap frozen tissue samples.

qPCR analysis of AQP11 mRNA expression in cancer and corresponding adjacent normal tissue samples showed that there was no significant difference between them. Bar graph displaying the corresponding relative fold difference of normal and cancerous samples internally normalised to housekeeping genes. Relative fold expression was calculated by considering normal tissue samples as control. Data represent mean ± SEM; p value = 0.620, n=8.
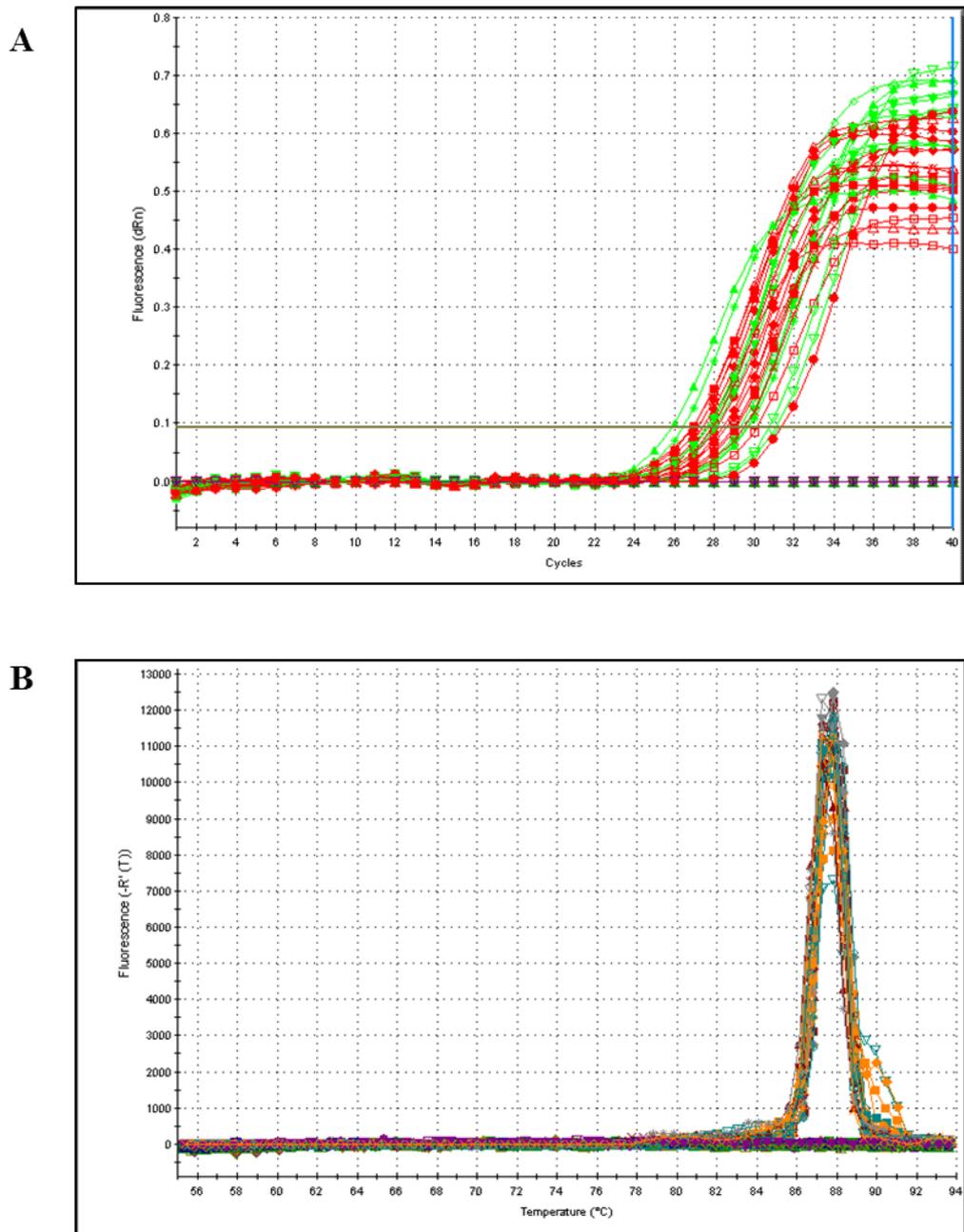
Figure 3.25   SYBR® Green real-time PCR analysis of AQP12 gene expression.

Gene expression of AQP12 was assessed in diluted cDNA samples of cancer and corresponding adjacent normal tissue samples using pre-validated primers. (A) Amplification curves generated for AQP12. Red and green curves represent cancer and normal tissue samples respectively. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 87°C suggesting excellent primer specificity; n=8.
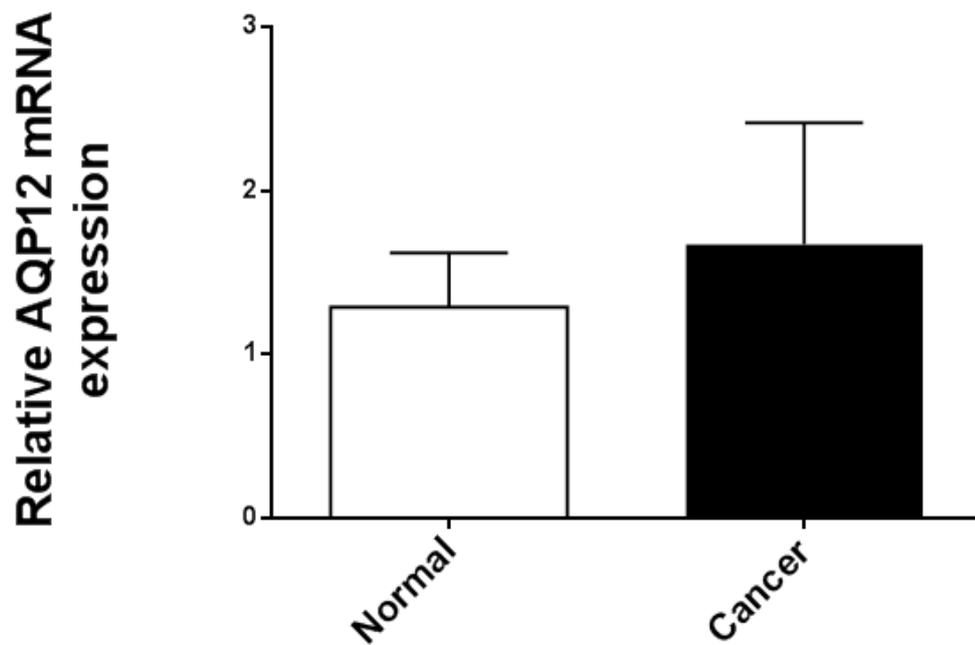
Figure 3.26   Expression of AQP12 mRNA in snap frozen tissue samples.

qPCR analysis of AQP12 mRNA expression in cancer and corresponding adjacent normal tissue samples showed that there was no significant difference between them. Bar graph displaying the corresponding relative fold difference of normal and cancerous samples internally normalised to housekeeping genes. Relative fold expression was calculated by considering normal tissue samples as control. Data represent mean ± SEM; p value = 0.643, n=8.

Table 3.1   Mean C_t values and mean fold expression of all AQPs in Snap frozen tissue samples

AQPs	Normal tissue samples		Cancer tissue samples		P value
	Mean C_t value of all 8 samples	Mean fold value of all 8 samples	Mean C_t value of all 8 samples	Mean fold value of all 8 samples	
AQP0	24.17	1.72	25.69	0.46	0.084
AQP1	31.37	2.52	29.62	7.40	0.256
AQP2	31.23	1.00	30.66	2.38	0.216
AQP3	31.93	1.15	30.12	3.22	**0.026***
AQP4	28.91	2.38	27.98	4.09	0.476
AQP5	24.90	2.68	26.55	0.91	0.223
AQP6	29.83	1.41	28.63	3.11	0.227
AQP7	25.54	1.90	25.79	1.31	0.564
AQP8	31.68	1.46	29.07	3.32	0.106
AQP9	30.53	1.26	29.26	2.29	0.212
AQP10	33.79	4.67	31.24	16.25	0.123
AQP11	24.76	1.45	25.11	1.12	0.620
AQP12	29.33	1.29	28.76	1.67	0.643

3.3.2 AQP3 protein expression in snap frozen tissue samples

To determine whether the differential expression of AQP3 mRNA in FFPE samples corresponds to an altered level of AQP3 protein expression, Western blot analysis was performed to assess AQP3 protein expression. Although AQP3 protein expression in has been described recently in MDA-MB-231 cells (Cao *et al.*, 2013), AQP3 protein expression in human breast normal and cancer tissues has not been characterised so far. Thus this is the first report showing differential expression of AQP3 in normal and cancer tissue samples. Western blot analysis revealed AQP3 bands in both cancer and normal tissue samples (see Figure 3.27A). Densitometry of the AQP3 (see Figure 3.27B) bands of normal and cancer tissue samples revealed a significantly higher level of AQP3 protein expression in cancerous tissue compared to healthy border tissue (n = 8, p = 0.0029).
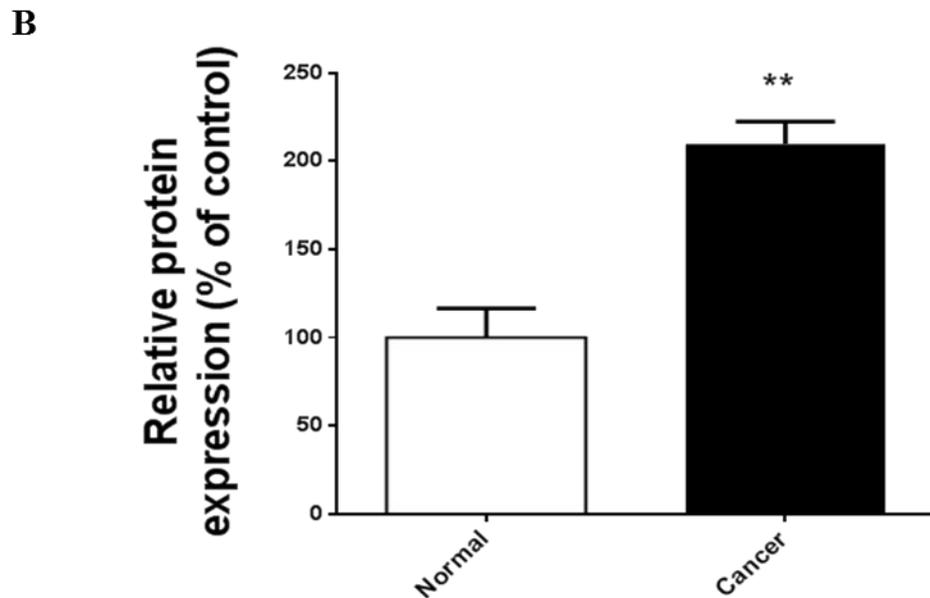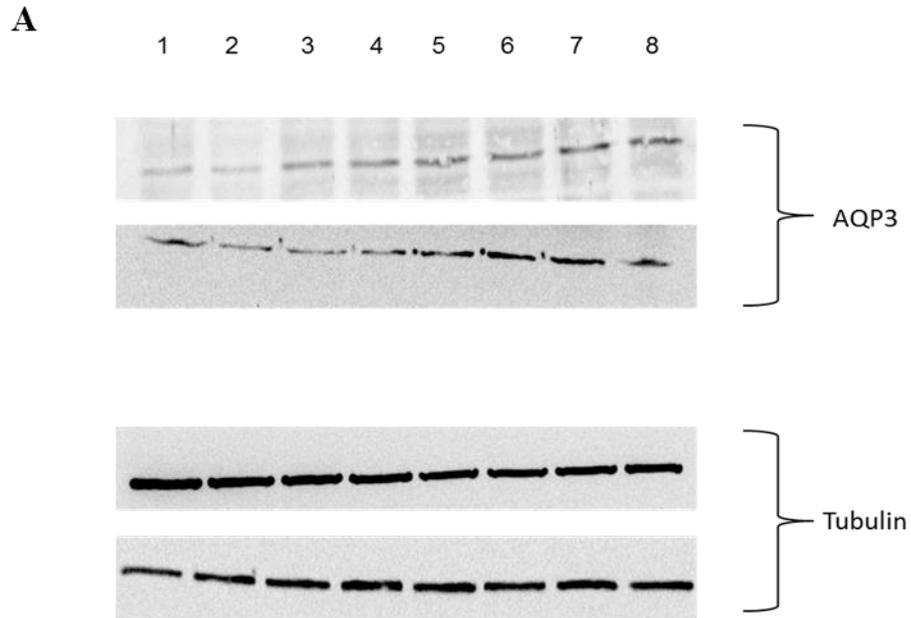
Figure 3.27   AQP3 Protein Expression in snap frozen tissue samples.

Western blot analysis of AQP3 protein expression in snap frozen tissue samples shows that cancerous tissues exhibit significantly higher AQP3 protein expression than corresponding normal tissues (A) Representative Western blots of bands generated for AQP3 and tubulin protein expression. Lane 1, 2, 3 and 4 represent normal tissue samples while lane 5, 6, 7 and 8 represent cancer tissue samples. (B) Graph displaying relative levels of AQP3 protein expression in normal and cancer tissue samples. Normal tissue was used as a control group to calculate the relative AQP3 protein expression (in % of control) for cancerous tissue. Data represent mean ± SEM; n=8, **p=0.0029.

3.3.3 AQP3 expression in FFPE tissues by immunohistochemistry

Immunohistochemistry analysis in breast tumour sections was performed by double immunofluorescence labelling for AQP3 and cytokeratin in FFPE. The tumour section was double stained with rabbit anti-AQP3 antibody and with mouse anti-cytokeratin 5/8 antibody. AQP3 immunoreactivity was broadly detected in the breast tumour sections with particularly strong immunoreactivity being detected in the blood vessels as red blood cells are known to express high levels of AQP3 (Roudier *et al.*, 1998). However positive staining was observed in most slides in negative control experiments in which all breast tissue slides were directly incubated with each secondary antibody (i.e., without prior incubation with primary antibodies) were performed. Therefore immunohistochemistry was not used to quantify the differential expression of AQP3 in different tumour types.
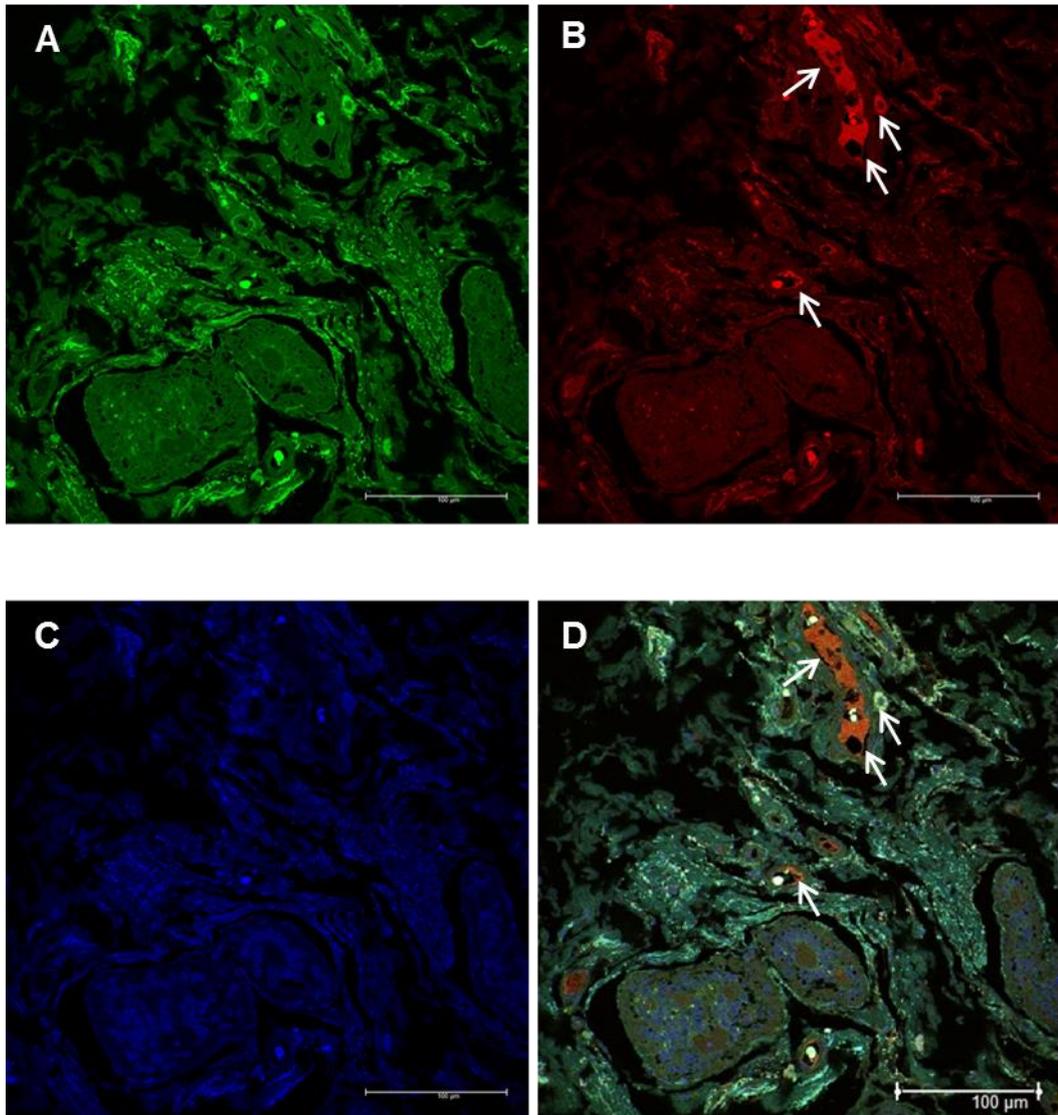
Figure 3.28   AQP3 expression in FFPE tumour section by immunohistochemistry

Immunohistochemical analysis of AQP3 protein in breast tumour sample stained with (A) FITC staining for cytokeratin 5/8 protein (B) Texas red staining for AQP3 protein (C) DAPI staining for nucleus (D) merged image of A, B and C stained for cytokeratin (green), AQP3 (red) and nucleus (blue). The white arrows represent possible AQP3 expression on panel B and D. Bar =100 μm.
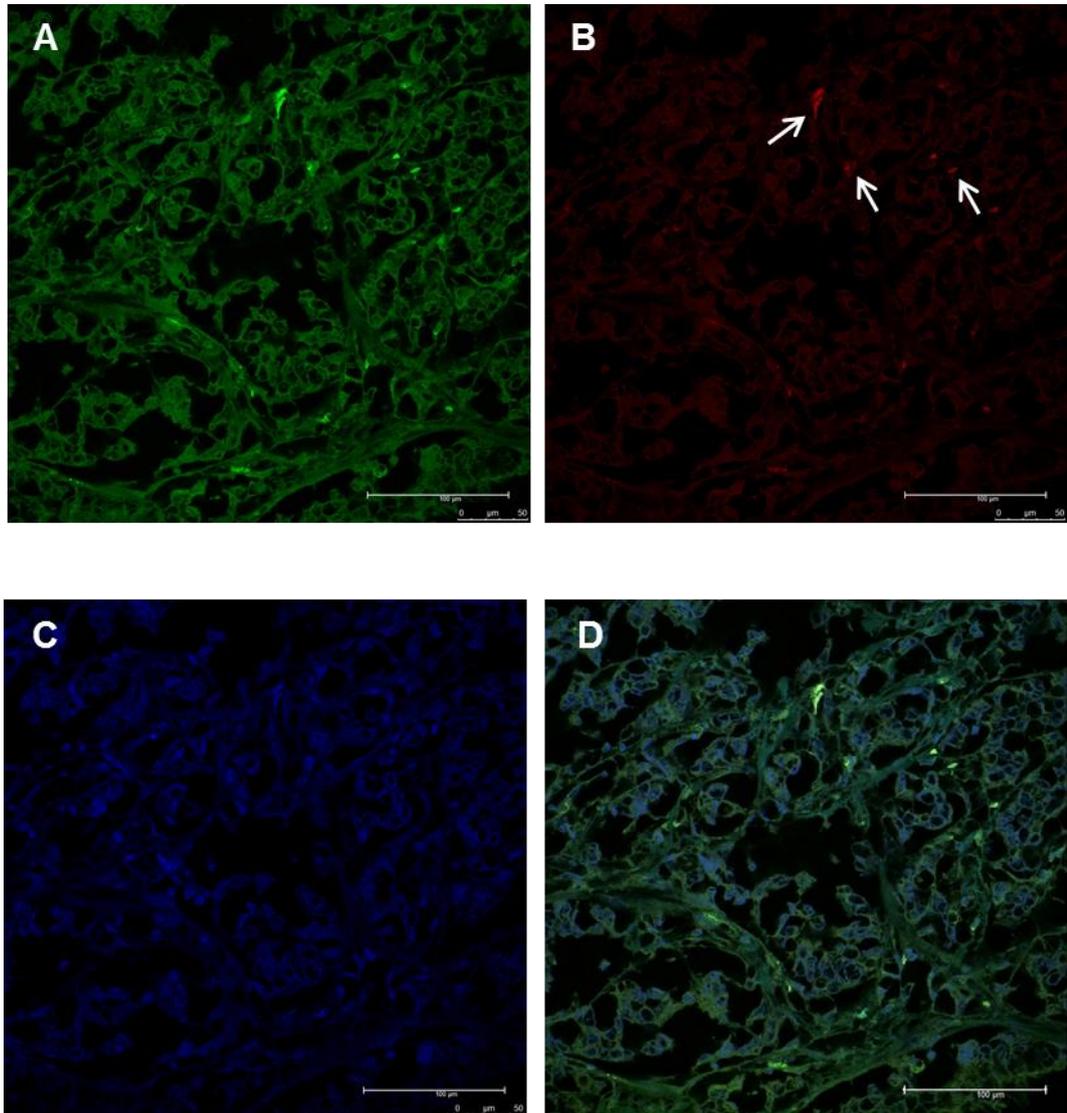
Figure 3.29   Representative false staining of secondary antibodies in FFPE tumour sample in a control experiment.

Representation of the negative control sample with each secondary antibody directly incubated with breast tissue section. Positive staining was observed and secondary antibodies showing unspecific binding (A) FITC conjugated staining (B) Texas red conjugated (C) DAPI staining for nucleus (D) merged image of A, B and C. The arrows on panel B show nonspecific staining with Texas Red conjugate. Bar =100 µm.

3.3.4 AQP3 protein expression in FFPE tissue samples

AQP3 protein expression in human breast tumour samples with LN infiltration and histological grade has not been characterized so far. Therefore, Western blot analysis was performed to assess the AQP3 protein expression in FFPE cancer tissue samples. AQP3 protein level was investigated in all four clinicopathological groups; LN negative with grade 1 tumour, LN negative with grade 3 tumour, LN positive with grade 1 tumour and LN positive with grade 3 tumour samples. Western blot analysis revealed AQP3 bands in all tumour samples see Figure 3.30A. Densitometry of the AQP3 bands (see Figure 3.30B) in all samples were normalised with respective tubulin bands. The statistical significance was checked by using one way ANOVA with Dunnett's multiple comparisons test. Western blot analysis showed that there was no significant difference in AQP3 protein expression between different clinical groups (n = 4, p = 0.683).
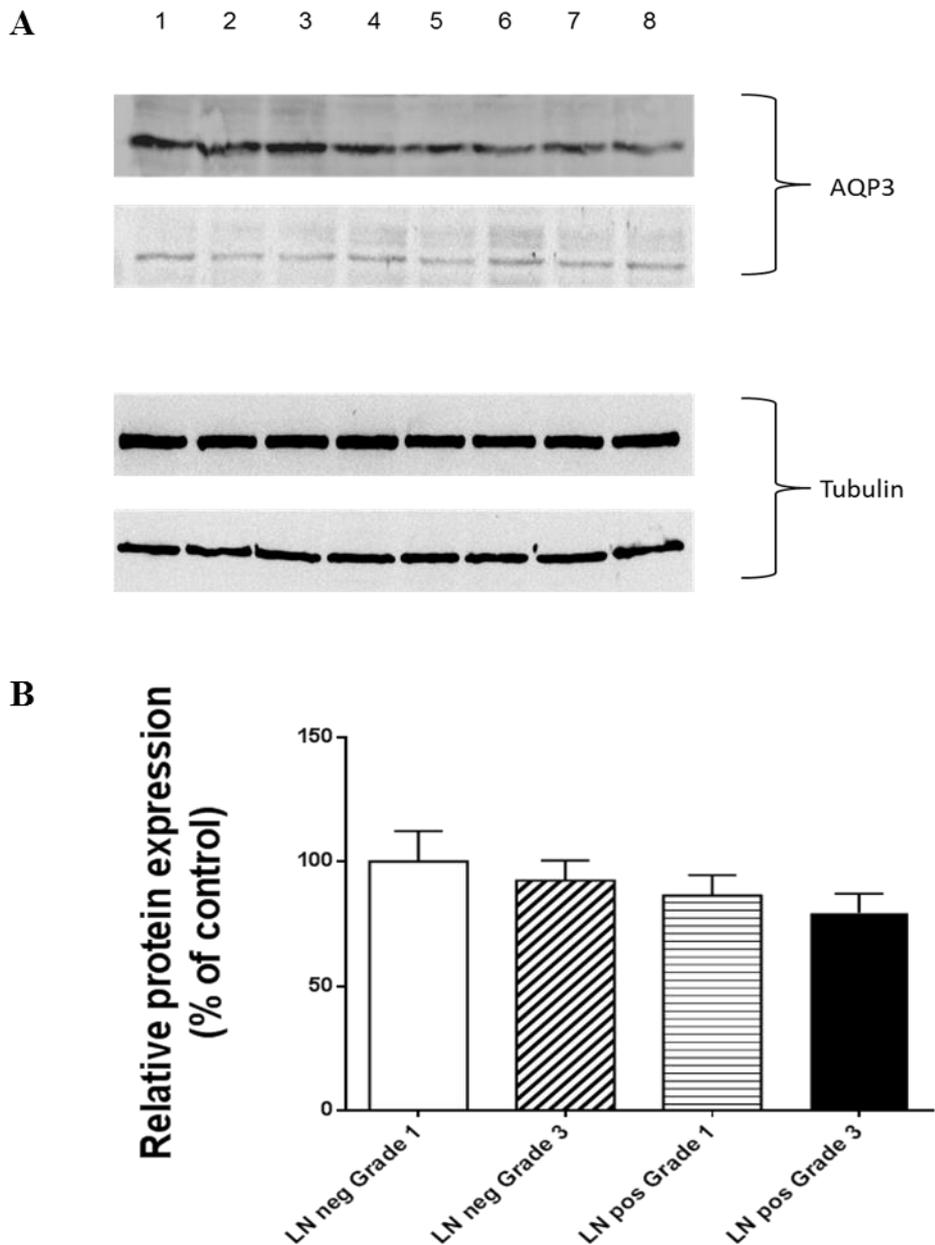
Figure 3.30   AQP3 protein expression in FFPE tissue samples.

Western blot analysis of AQP3 protein expression in FFPE tissue samples shows that there is no significant difference in expression of AQP3 protein in different clinicopathological groups of breast cancer. (A) Representative Western blots of bands generated for AQP3 and tubulin protein expression. Lane 1 and 2 represent LN negative status with grade 1 tumour samples, lane 3 and 4 represent LN negative status with grade 3 tumour samples, lane 5 and 6 represent LN positive status with grade 1 tumour samples and lane 7 and 8 represent LN positive status with grade 3 tumour samples. (B) Graph displaying relative levels of AQP3 protein expression in the FFPE tissue samples. LN negative status with grade 1 tumour group was used as a control group to calculate the relative AQP3 protein expression (in % of control) for all the other groups. Data represent mean ± SEM. All p values > 0.683, n=4 cases per group.

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3.3.5   AQPs gene expression in FFPE cancer tissues

Samples were identified from patients categorised into four groups on the basis of LN infiltration status and tumour grade. LN negative status with grade 1 tumour was used as a control group and compared with LN negative status with grade 3 tumour, LN positive status with grade 1 and grade 3 tumours. Gene expression was internally normalised with the previously named housekeeping genes and expression of LN negative status with grade 1 tumour was used to calculate the fold difference among other groups.

The mRNA expression of all the AQPs was investigated in these four groups. The statistical significance was checked by using one way ANOVA with Dunnett's multiple comparisons test. The expression of all AQPs in these four groups varied but none was shown to be statistically significantly different (see Figures 3.31 to Figures 3.56) in clinicopathological groups.
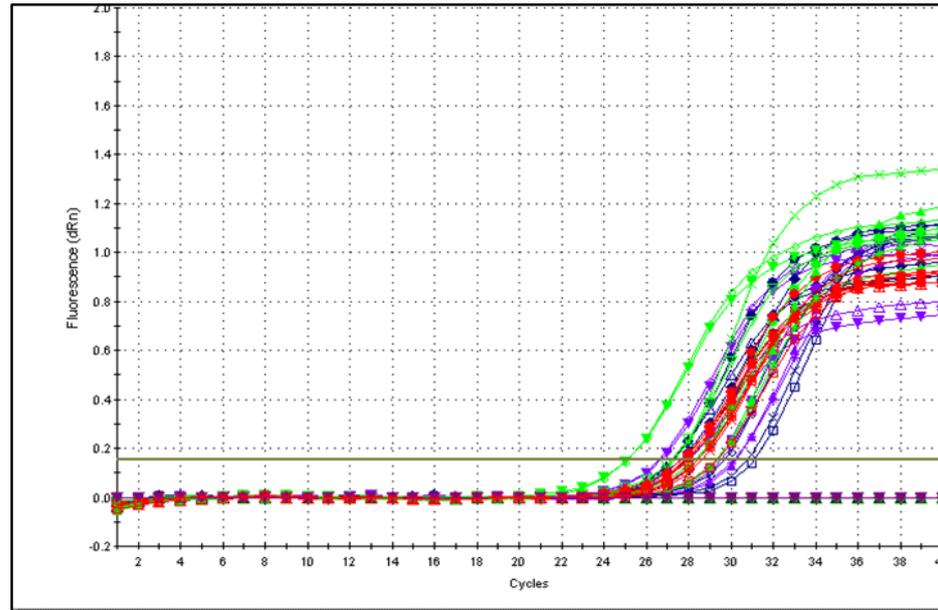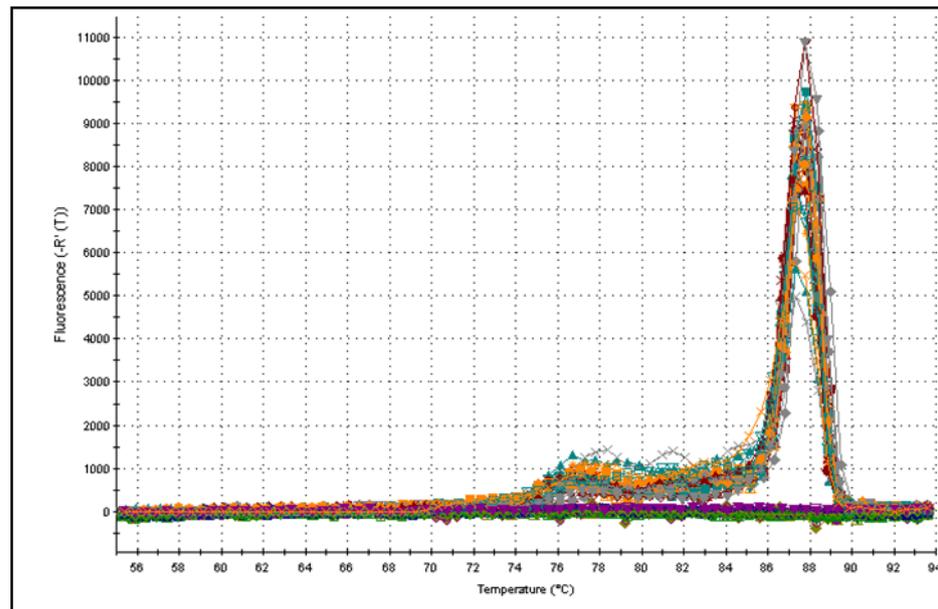
Figure 3.31   SYBR® Green real-time PCR analysis of AQP0 gene expression.

Gene expression of AQP0 was assessed in diluted cDNA of FFPE cancer tissue samples using pre-validated primers. (A) Amplification curves generated for AQP0. Blue and green curves represent tissue samples of negative LN status with grade 1 and grade 3 respectively. Purple and red curves represent tissue samples of positive LN status with grade 1 and grade 3 respectively. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 87°C showing excellent primer specificity; n=4.
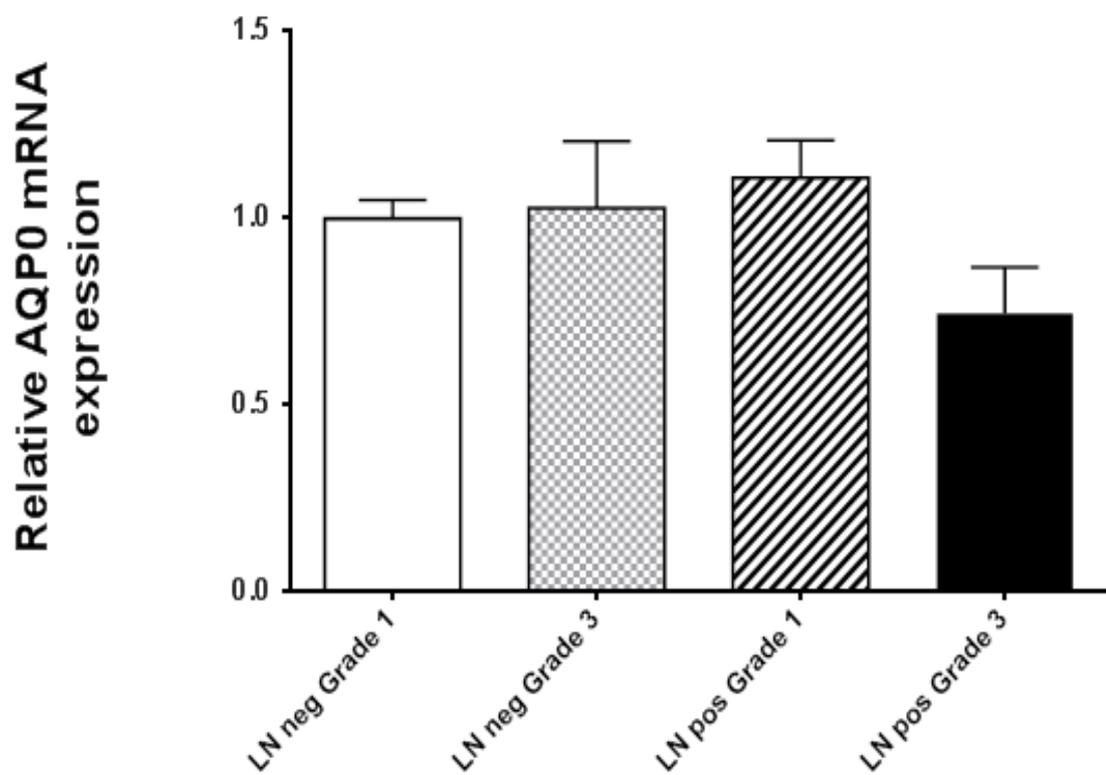
Figure 3.32   Expression of AQP0 mRNA in FFPE tissue samples.

qPCR analysis of AQP0 mRNA expression in FFPE cancer tissue samples showed that there were no significant difference in expression of AQP0 mRNA between the different clinicopathological groups of breast cancer. Bar graph displaying the corresponding relative fold differences normalised to housekeeping genes for each group.   Data represent mean ± SEM. All p values > 0.231 n=4 cases per group.
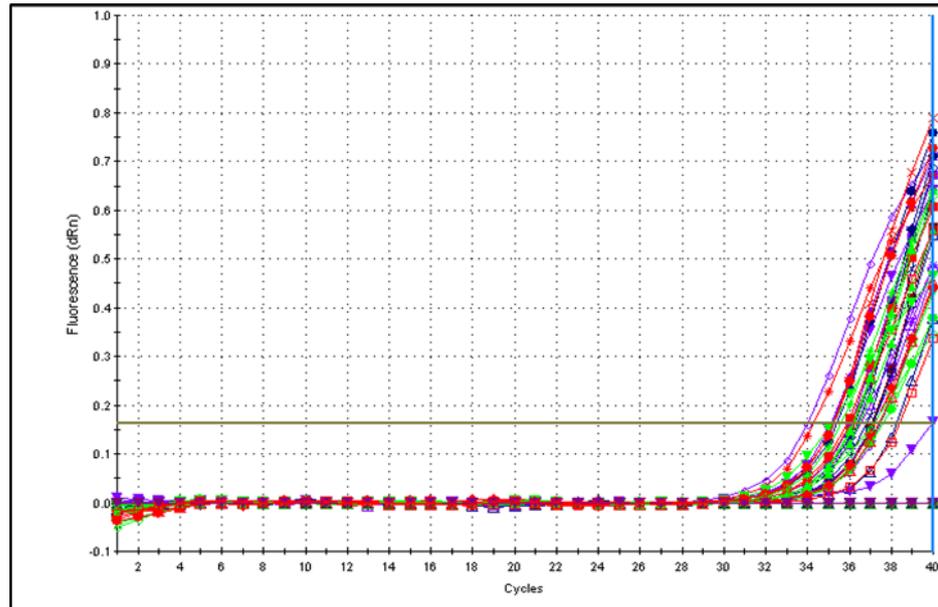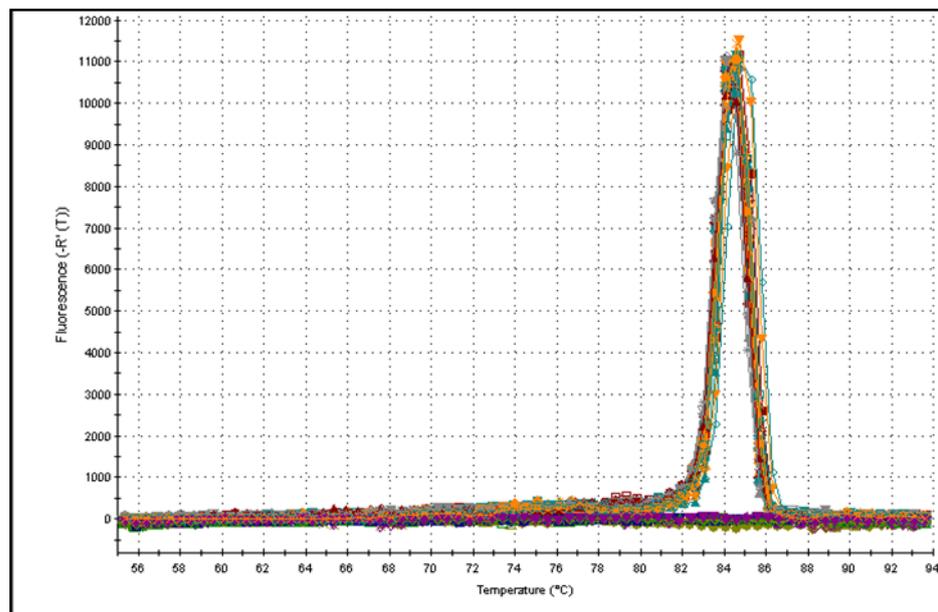
Figure 3.33   SYBR® Green real-time PCR analysis of AQP1 gene expression.

Gene expression of AQP1 was assessed in diluted cDNA of FFPE cancer tissue samples using pre-validated primers. (A) Amplification curves generated for AQP1. Blue and green curves represent tissue samples of negative LN status with grade 1 and grade 3 respectively. Purple and red curves represent tissue samples of positive LN status with grade 1 and grade 3 respectively. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 85°C showing excellent primer specificity; n=4.
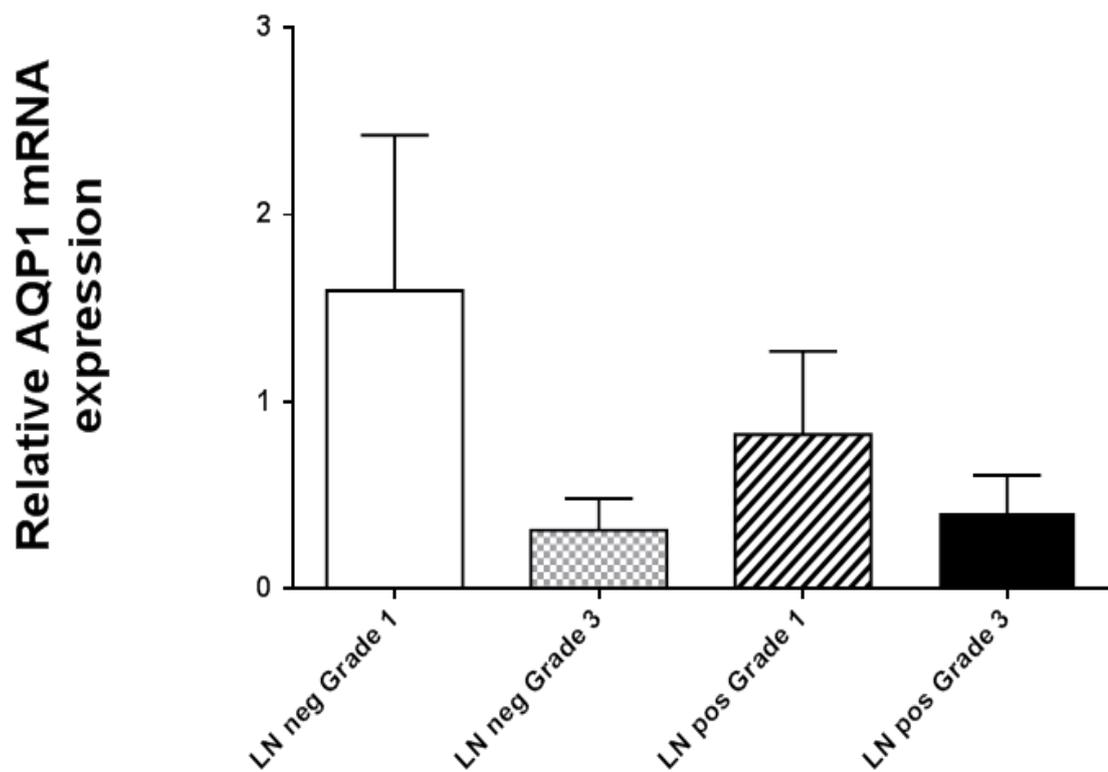
Figure 3.34   Expression of AQP1 mRNA in FFPE tissue samples.

qPCR analysis of AQP1 mRNA expression in FFPE cancer tissue samples showed that there were no significant difference in expression of AQP1 mRNA between the different clinicopathological groups of breast cancer. Bar graph displaying the corresponding relative fold differences normalised to housekeeping genes for each group. Data represent mean ± SEM. All p values > 0.285 n=4 cases per group.
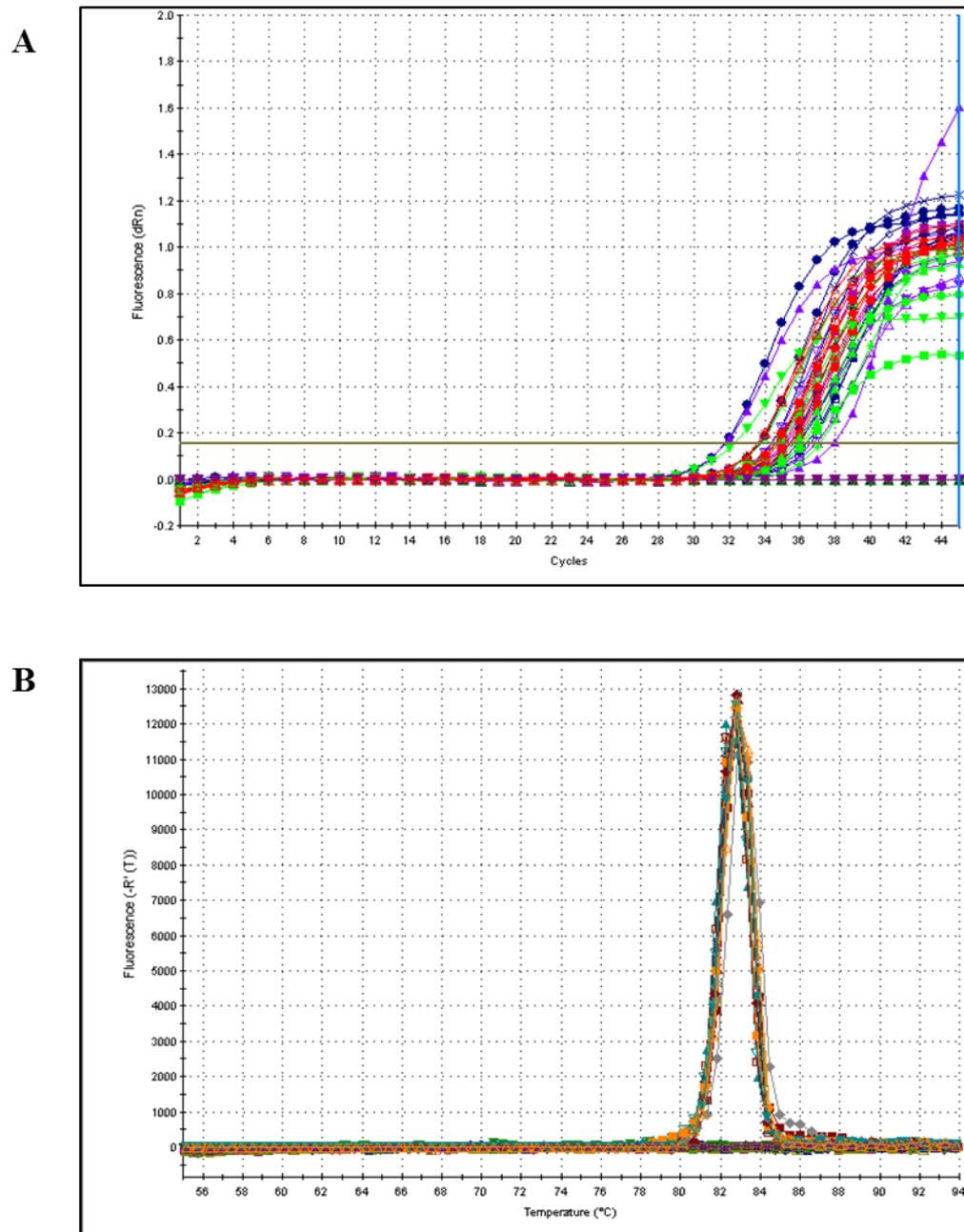
Figure 3.35   SYBR® Green real-time PCR analysis of AQP2 gene expression.

Gene expression of AQP2 was assessed in diluted cDNA of FFPE cancer tissue samples using pre-validated primers. (A) Amplification curves generated for AQP2. Blue and green curves represent tissue samples of negative LN status with grade 1 and grade 3 respectively. Purple and red curves represent tissue samples of positive LN status with grade 1 and grade 3 respectively. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 83°C.showing excellent primer specificity; n=4.
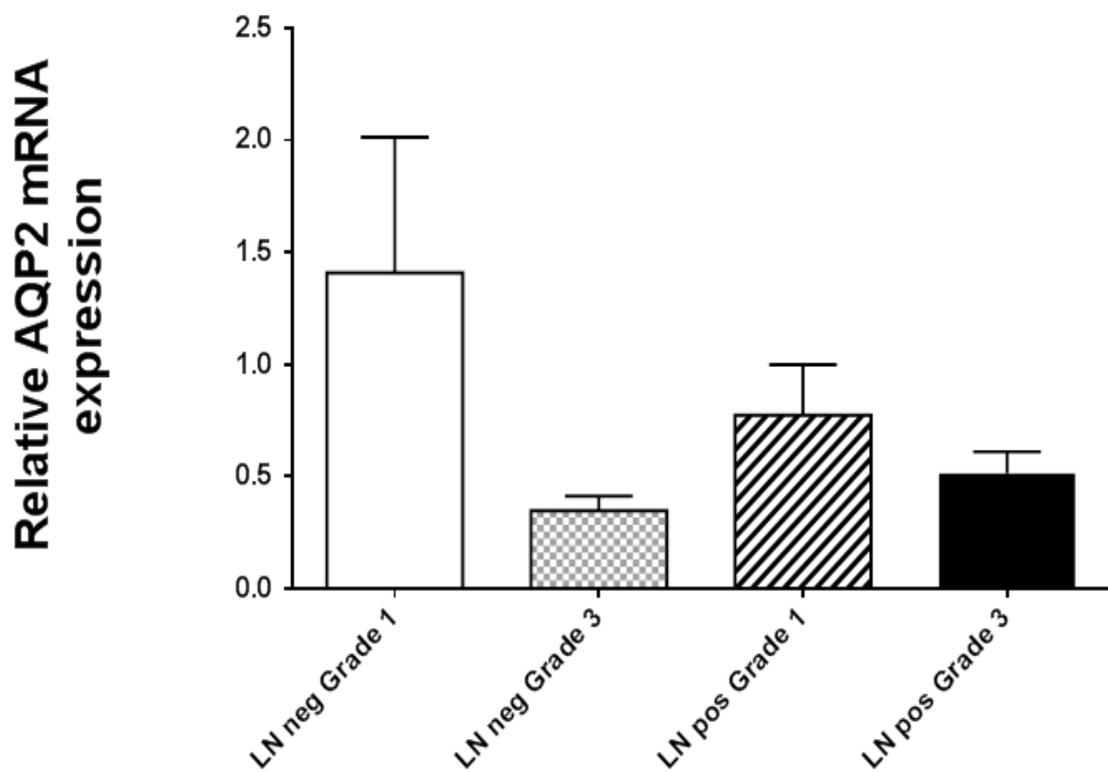
Figure 3.36   Expression of AQP2 mRNA in FFPE tissue samples.

qPCR analysis of AQP2 mRNA expression in FFPE cancer tissue samples showed that there were no significant difference in expression of AQP2 mRNA between the different clinicopathological groups of breast cancer. Bar graph displaying the corresponding relative fold differences normalised to housekeeping genes for each group. Data represent mean ± SEM. All p values > 0.164 n=4 cases per group.
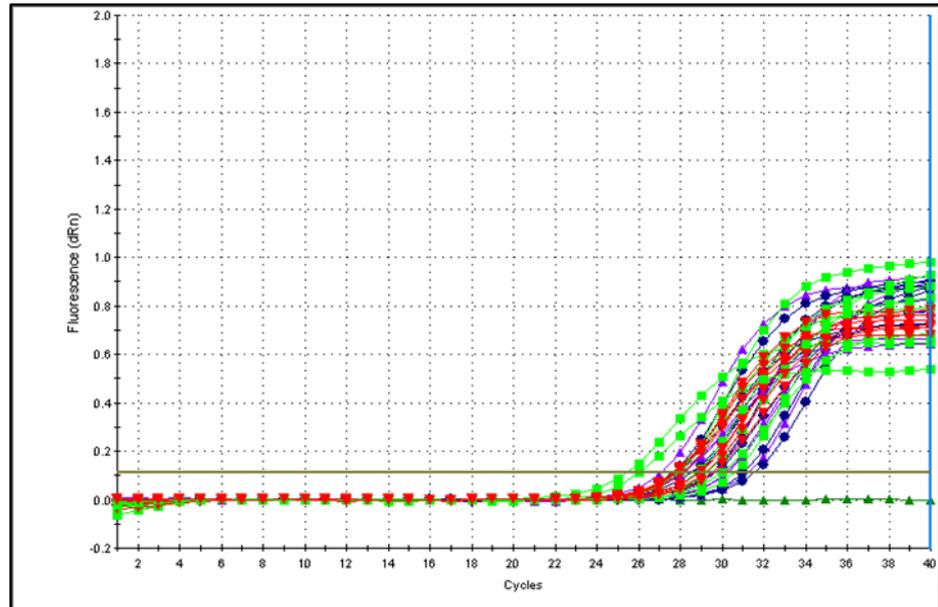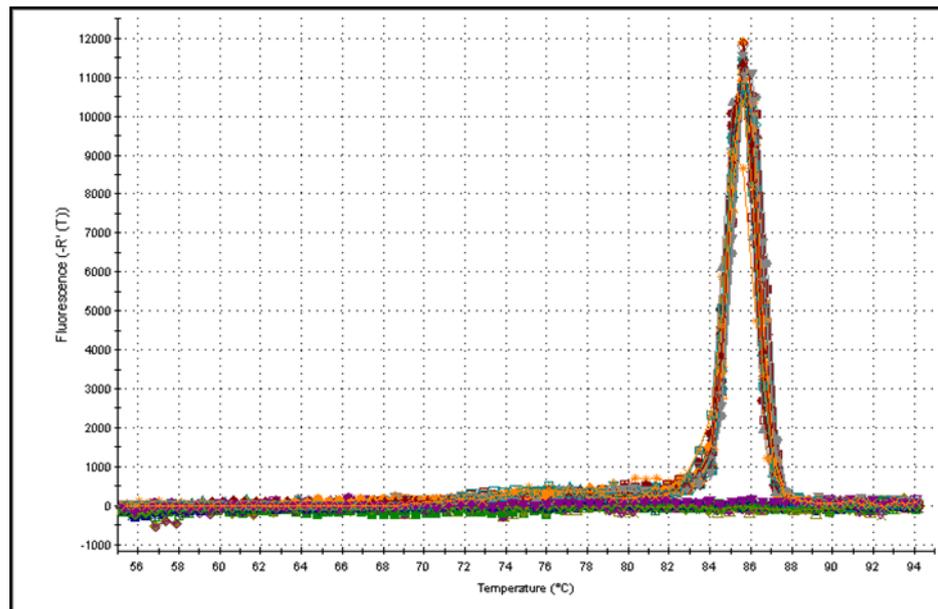
Figure 3.37   SYBR® Green real-time PCR analysis of AQP3 gene expression.

Gene expression of AQP3 was assessed in diluted cDNA of FFPE cancer tissue samples using pre-validated primers. (A) Amplification curves generated for AQP3. Blue and green curves represent tissue samples of negative LN status with grade 1 and grade 3 respectively. Purple and red curves represent tissue samples of positive LN status with grade 1 and grade 3 respectively. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 85°C showing excellent primer specificity; n=4.
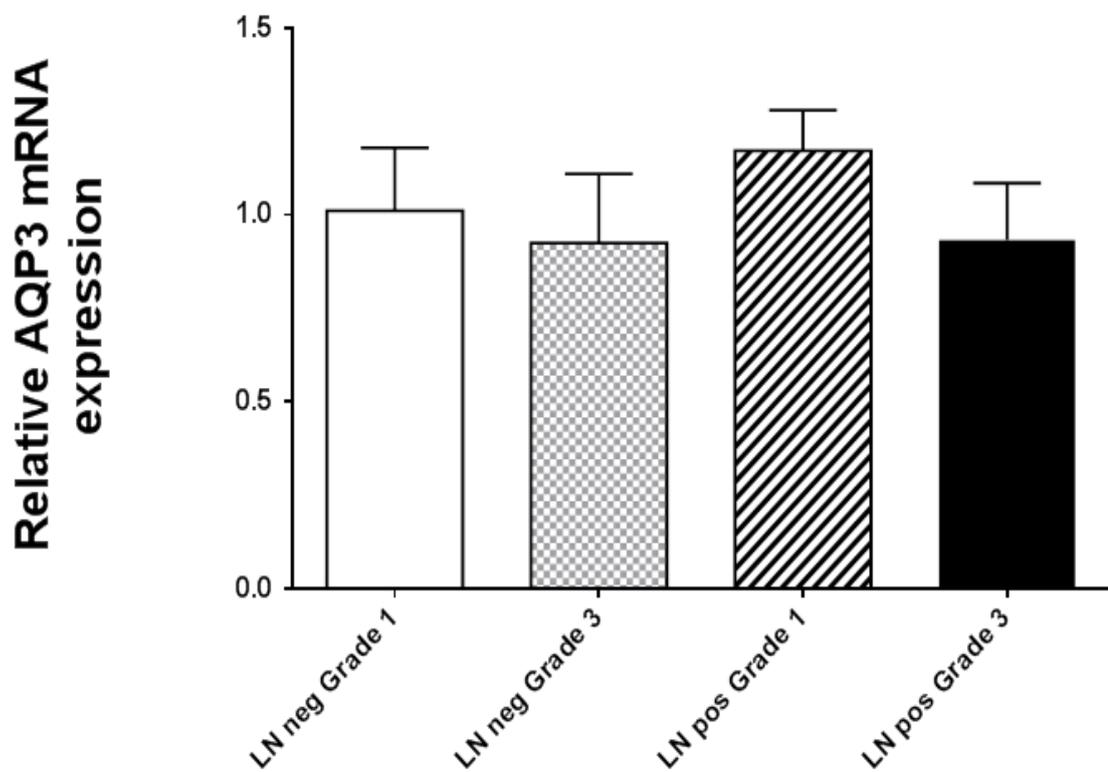
Figure 3.38   Expression of AQP3 mRNA in FFPE tissue samples.

qPCR analysis of AQP3 mRNA expression in FFPE cancer tissue samples showed that there were no significant difference in expression of AQP3 mRNA between the different clinicopathological groups of breast cancer. Bar graph displaying the corresponding relative fold differences normalised to housekeeping genes for each group. Data represent mean ± SEM. All p values > 0.664 n=4 cases per group.
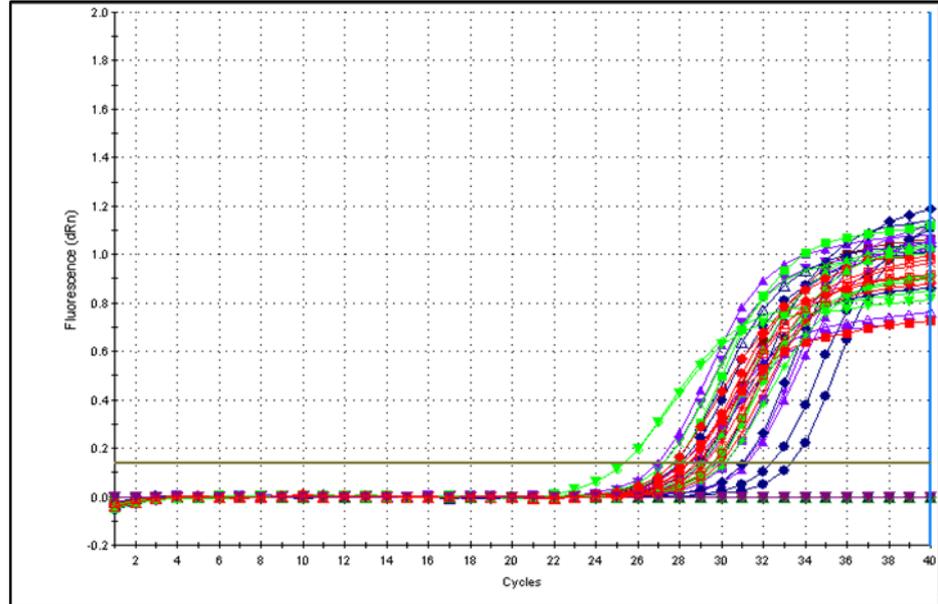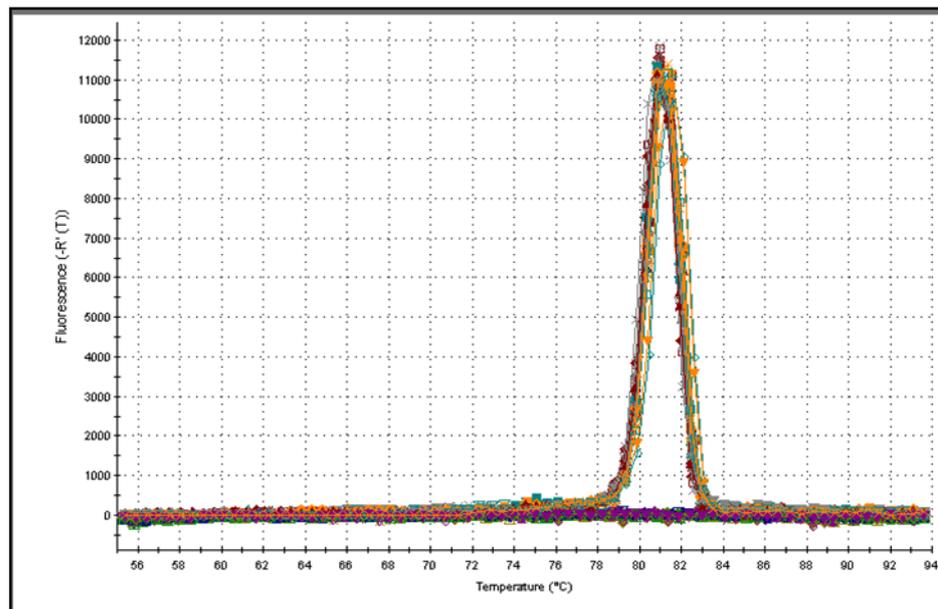
Figure 3.39   SYBR® Green real-time PCR analysis of AQP4 gene expression.

Gene expression of AQP4 was assessed in diluted cDNA of FFPE cancer tissue samples using pre-validated primers. (A) Amplification curves generated for AQP4. Blue and green curves represent tissue samples of negative LN status with grade 1 and grade 3 respectively. Purple and red curves represent tissue samples of positive LN status with grade 1 and grade 3 respectively. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 81°C showing excellent primer specificity; n=4.
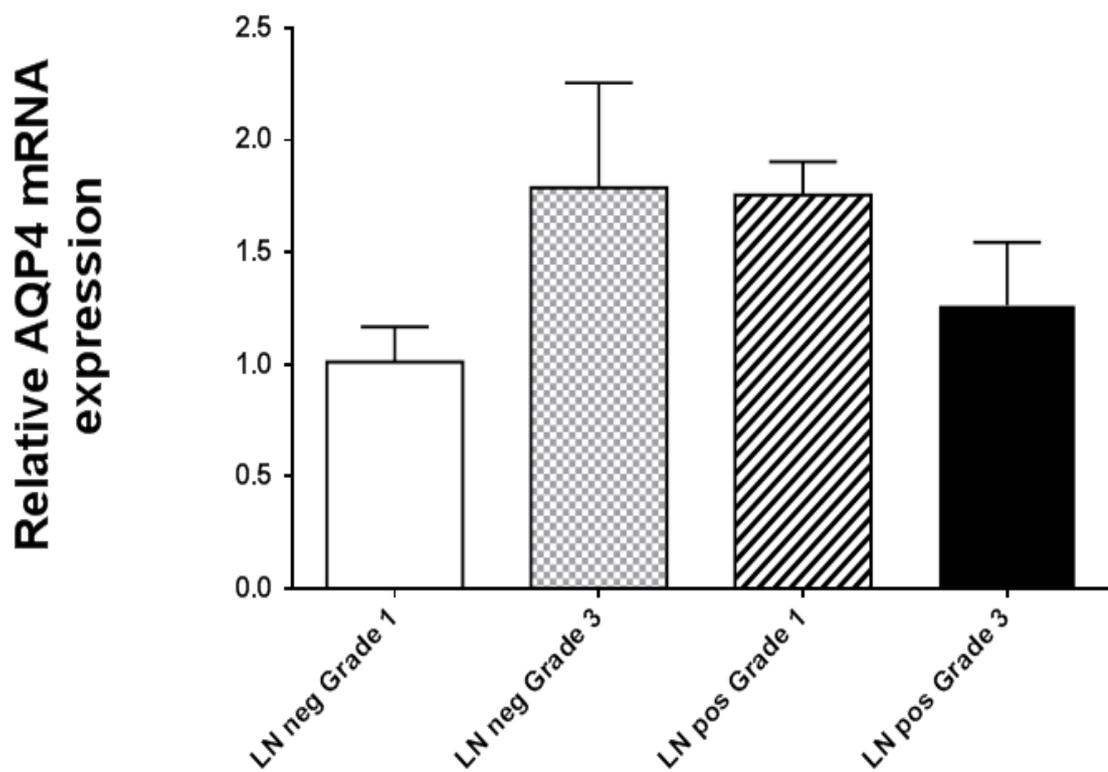
Figure 3.40   Expression of AQP4 mRNA in FFPE tissue samples.

qPCR analysis of AQP4 mRNA expression in FFPE cancer tissue samples showed that there were no significant difference in expression of AQP4 mRNA between the different clinicopathological groups of breast cancer. Bar graph displaying the corresponding relative fold differences normalised to housekeeping genes for each group. Data represent mean ± SEM. All p values > 0.225 n=4 cases per group.
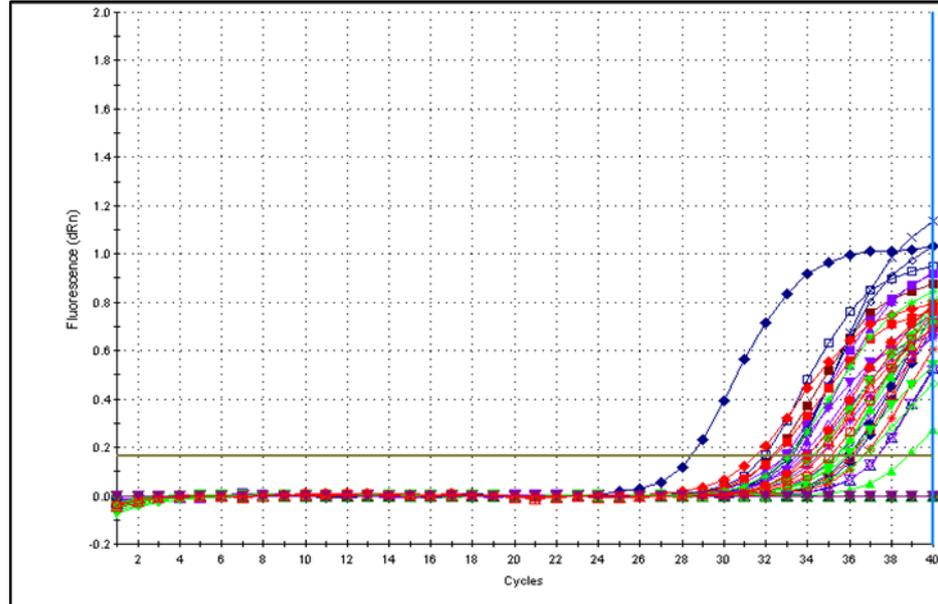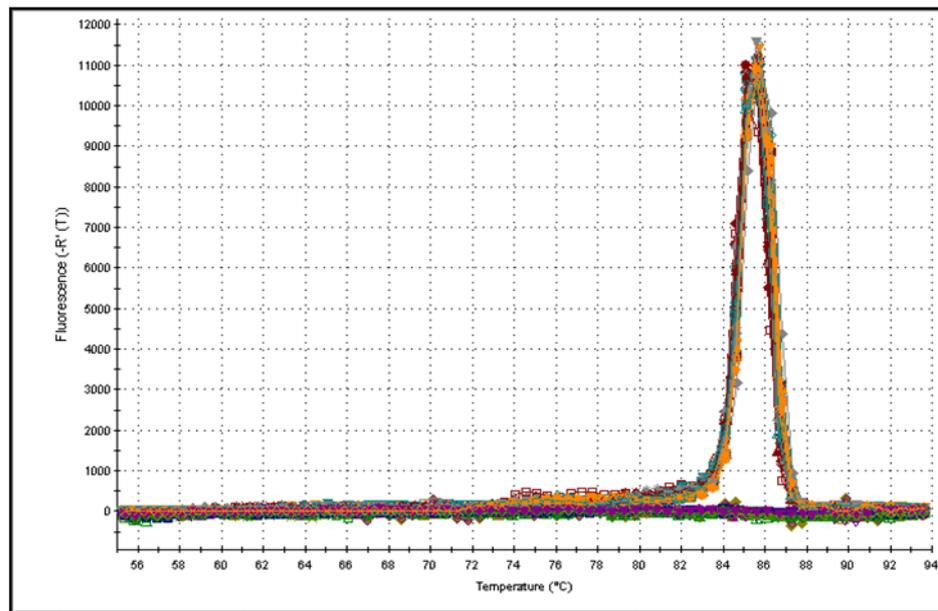
Figure 3.41   SYBR® Green real-time PCR analysis of AQP5 gene expression.

Gene expression of AQP5 was assessed in diluted cDNA of FFPE cancer tissue samples using pre-validated primers. (A) Amplification curves generated for AQP5. Blue and green curves represent tissue samples of negative LN status with grade 1 and grade 3 respectively. Purple and red curves represent tissue samples of positive LN status with grade 1 and grade 3 respectively. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 85°C showing excellent primer specificity; n=4.
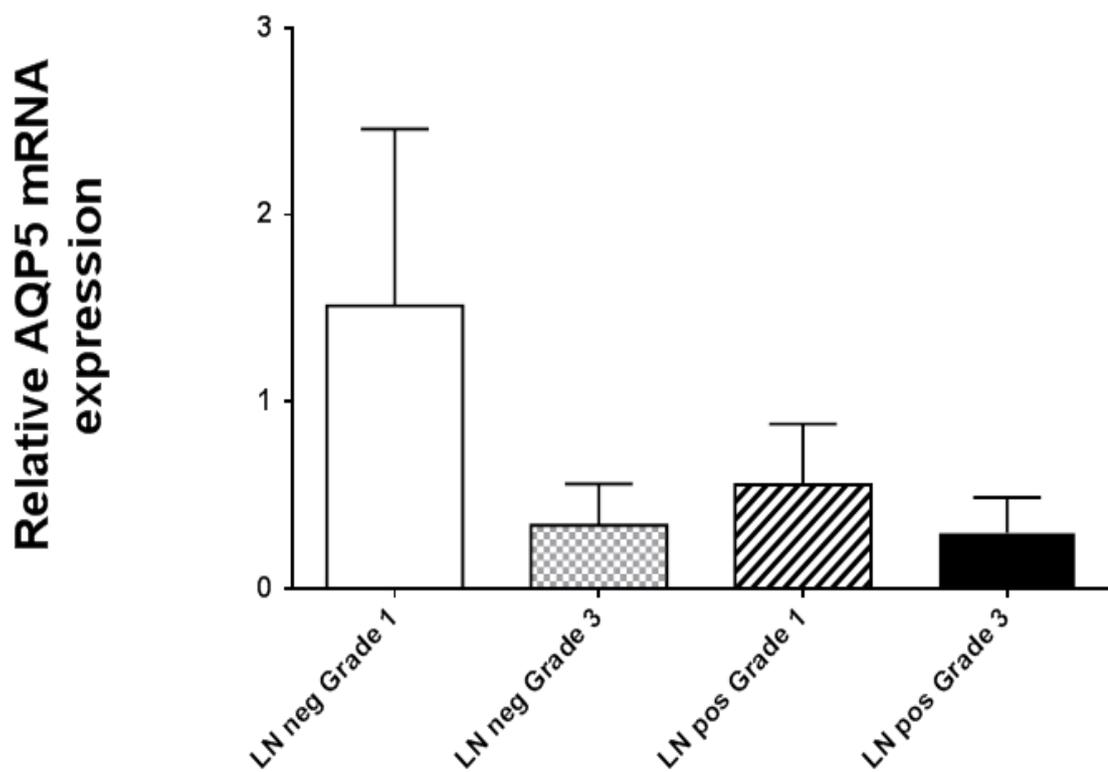
Figure 3.42   Expression of AQP5 mRNA in FFPE tissue samples.

qPCR analysis of AQP5 mRNA expression in FFPE cancer tissue samples showed that there were no significant difference in expression of AQP5 mRNA between the different clinicopathological groups of breast cancer. Bar graph displaying the corresponding relative fold differences normalised to housekeeping genes for each group. Data represent mean ± SEM. All p values > 0.478 n=4 cases per group.
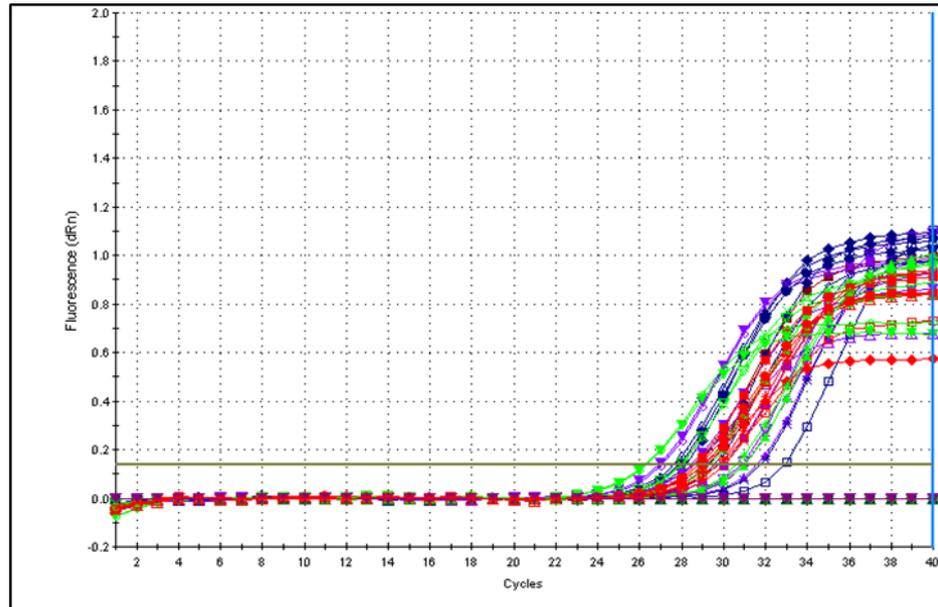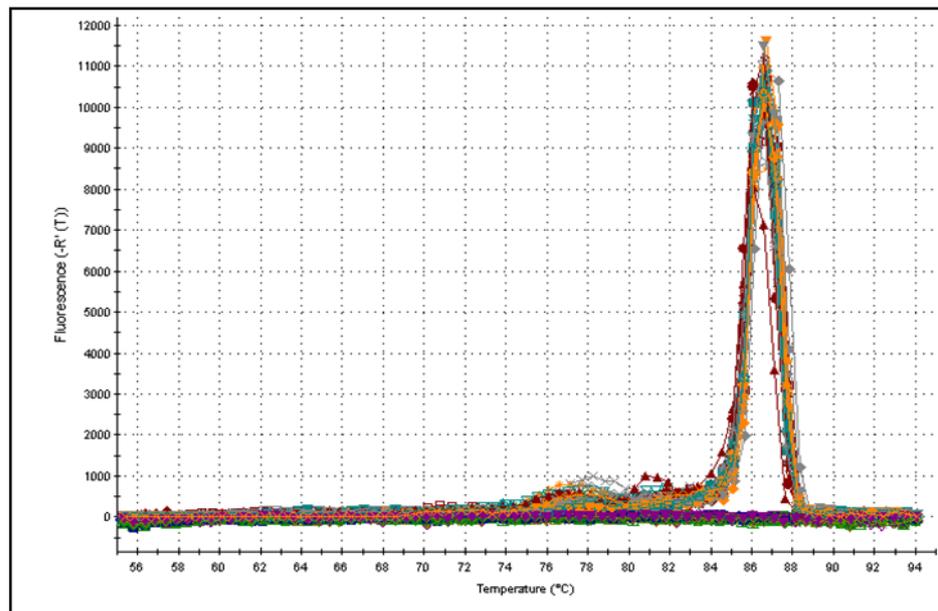
Figure 3.43   SYBR® Green real-time PCR analysis of AQP6 gene expression.

Gene expression of AQP6 was assessed in diluted cDNA of FFPE cancer tissue samples using pre-validated primers. (A) Amplification curves generated for AQP6. Blue and green curves represent tissue samples of negative LN status with grade 1 and grade 3 respectively. Purple and red curves represent tissue samples of positive LN status with grade 1 and grade 3 respectively. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 87°C showing excellent primer specificity; n=4.
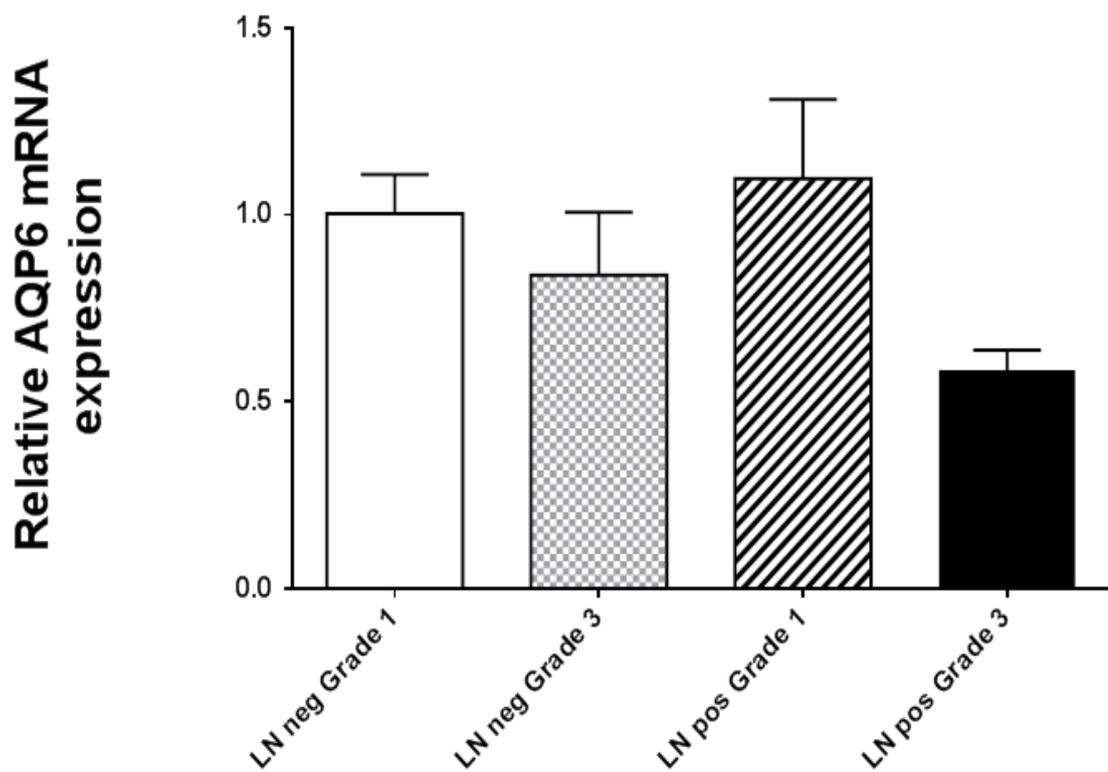
Figure 3.44   Expression of AQP6 mRNA in FFPE tissue samples.

qPCR analysis of AQP6 mRNA expression in FFPE cancer tissue samples showed that there were no significant difference in expression of AQP6 mRNA between the different clinicopathological groups of breast cancer. Bar graph displaying the corresponding relative fold differences normalised to housekeeping genes for each group. Data represent mean ± SEM. All p values > 0.128 n=4 cases per group.
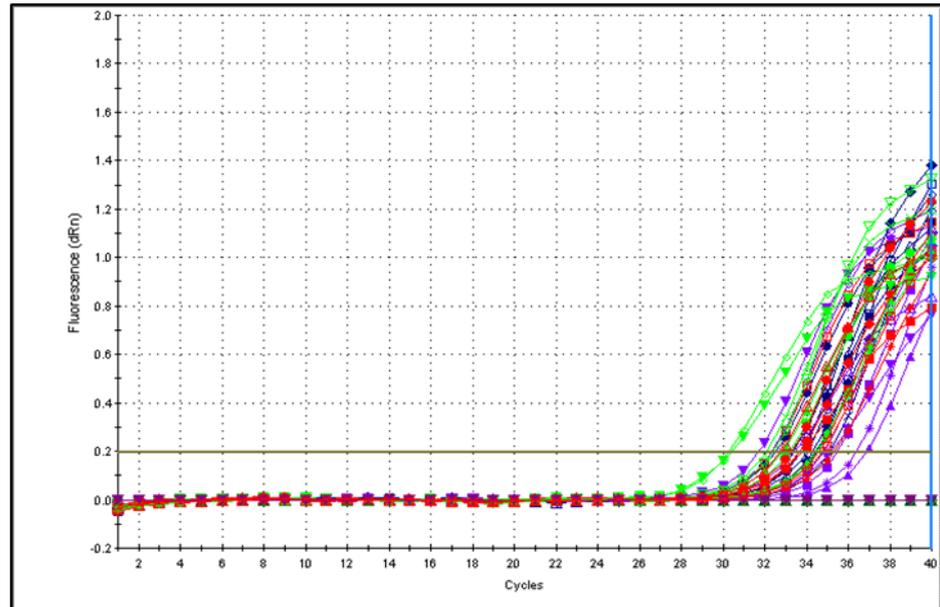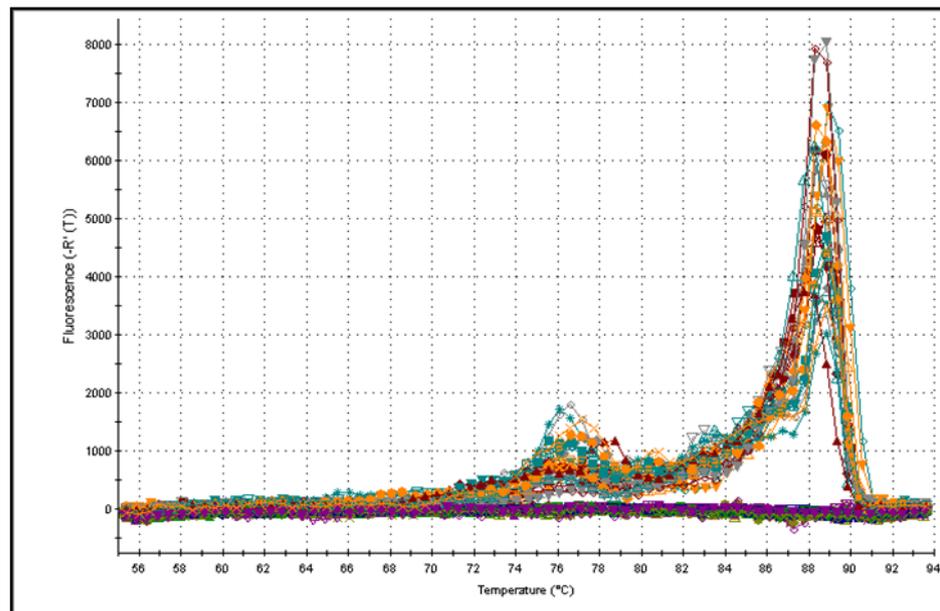
Figure 3.45   SYBR® Green real-time PCR analysis of AQP7 gene expression.

Gene expression of AQP7 was assessed in diluted cDNA of FFPE cancer tissue samples using pre-validated primers. (A) Amplification curves generated for AQP7. Blue and green curves represent tissue samples of negative LN status with grade 1 and grade 3 respectively. Purple and red curves represent tissue samples of positive LN status with grade 1 and grade 3 respectively. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 89°C showing adequate primer specificity; n=4.
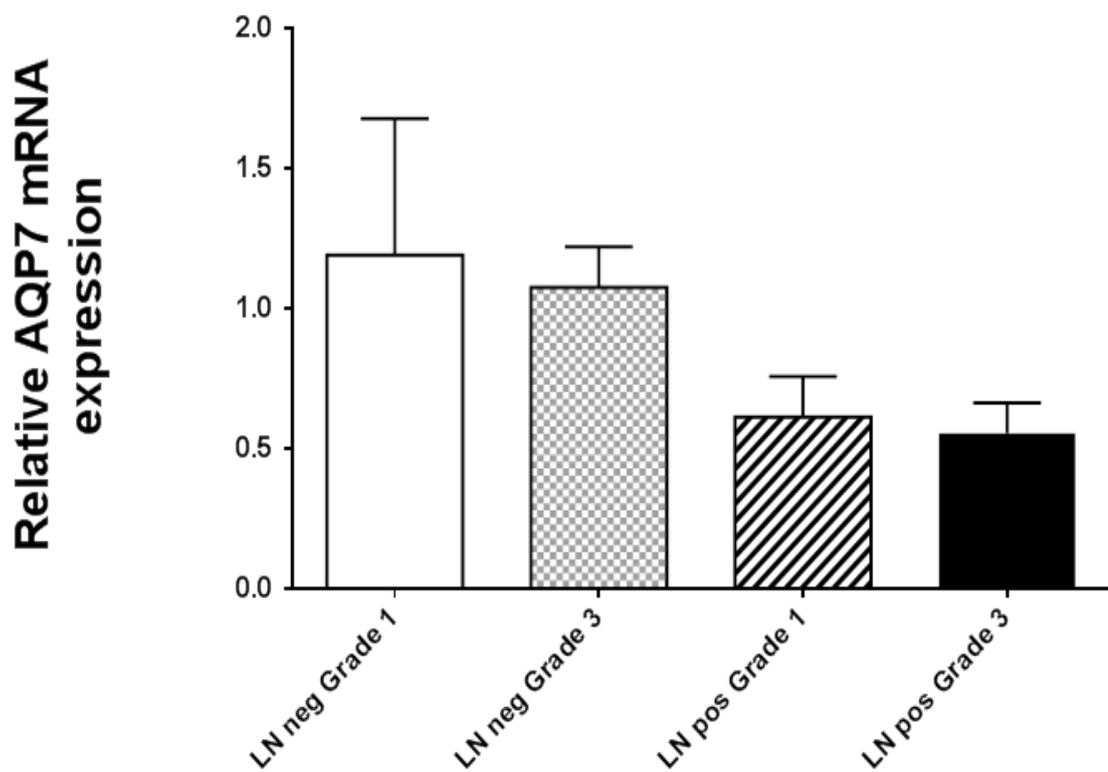
Figure 3.46   Expression of AQP7 mRNA in FFPE tissue samples.

qPCR analysis of AQP7 mRNA expression in FFPE cancer tissue samples showed that there were no significant difference in expression of AQP7 mRNA between the different clinicopathological groups of breast cancer. Bar graph displaying the corresponding relative fold differences normalised to housekeeping genes for each group. Data represent mean ± SEM. All p values > 0.283, n=4 cases per group.
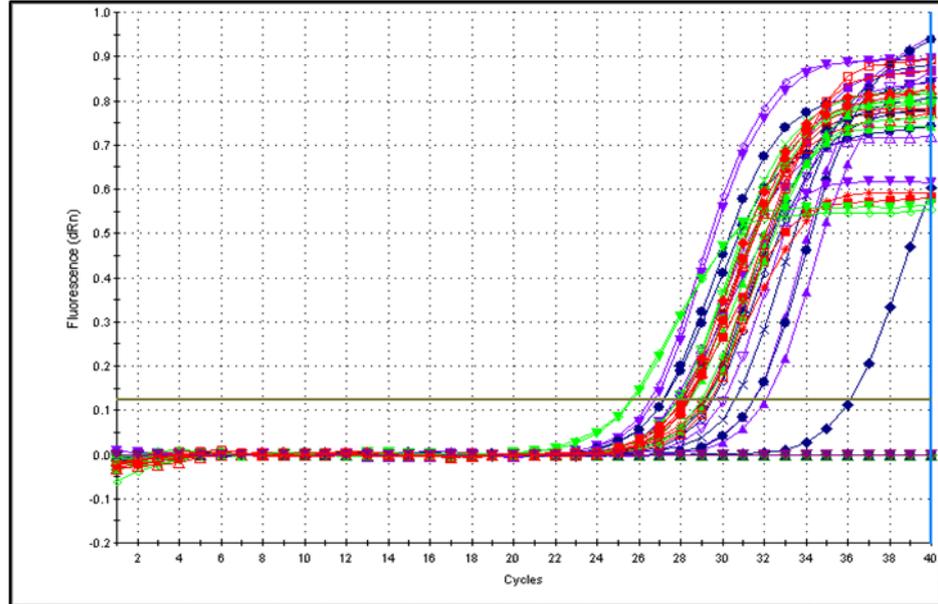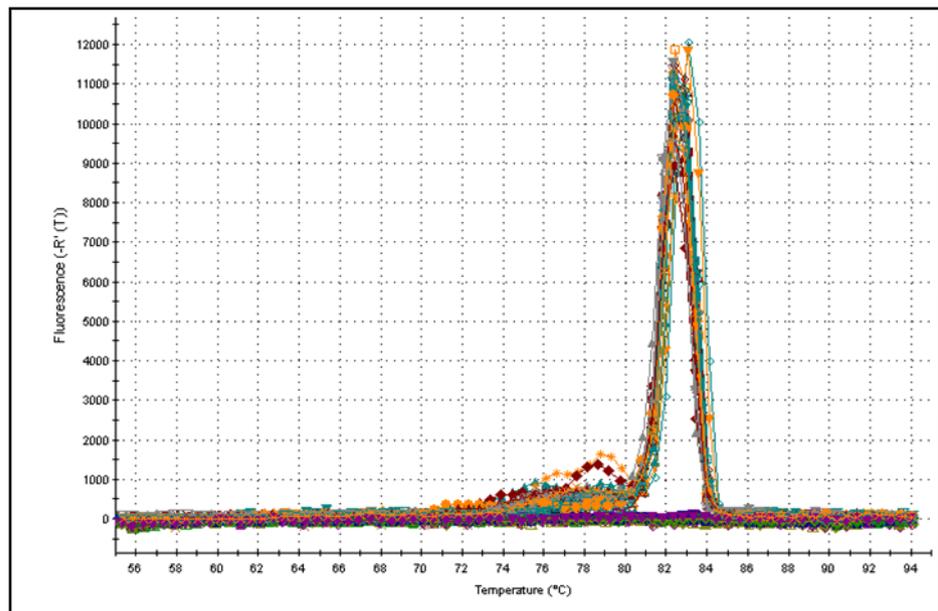
Figure 3.47   SYBR® Green real-time PCR analysis of AQP8 gene expression.

Gene expression of AQP8 was assessed in diluted cDNA of FFPE cancer tissue samples using pre-validated primers. (A) Amplification curves generated for AQP8. Blue and green curves represent tissue samples of negative LN status with grade 1 and grade 3 respectively. Purple and red curves represent tissue samples of positive LN status with grade 1 and grade 3 respectively. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 83°C showing excellent primer specificity; n=4.
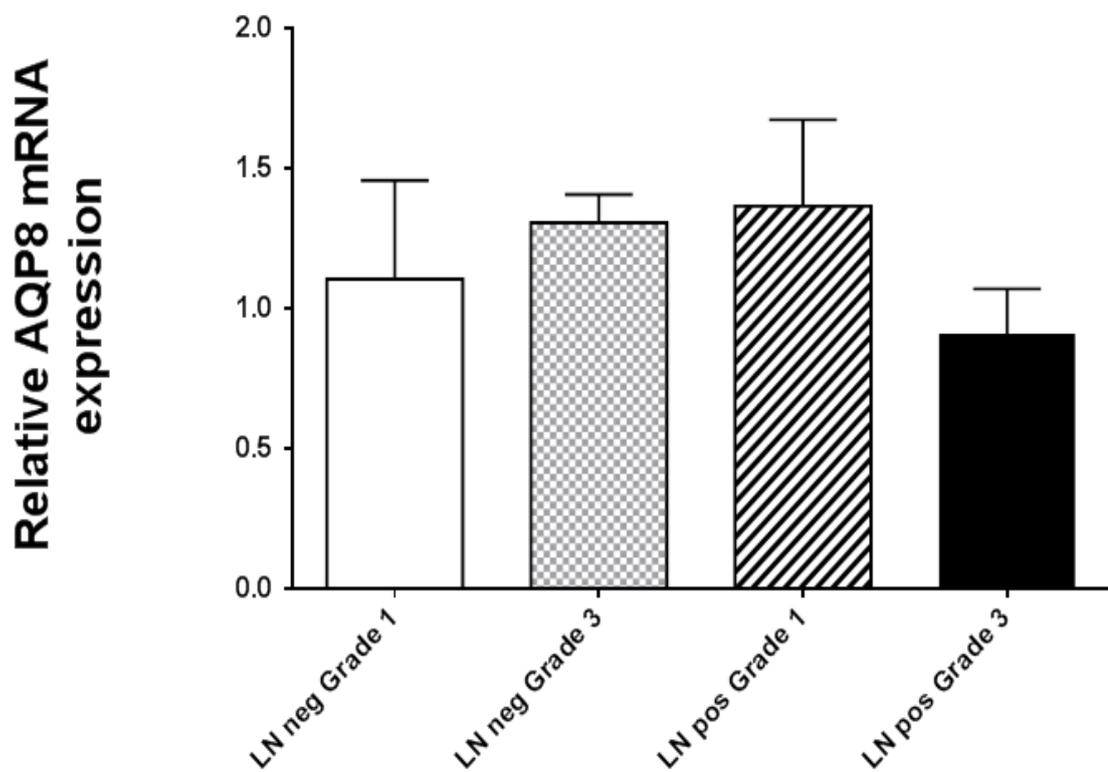
Figure 3.48   Expression of AQP8 mRNA in FFPE tissue samples.

qPCR analysis of AQP8 mRNA expression in FFPE cancer tissue samples showed that there were no significant difference in expression of AQP8 mRNA between the different clinicopathological groups of breast cancer. Bar graph displaying the corresponding relative fold differences normalised to housekeeping genes for each group. Data represent mean ± SEM. All p values > 0.585, n=4 cases per group.

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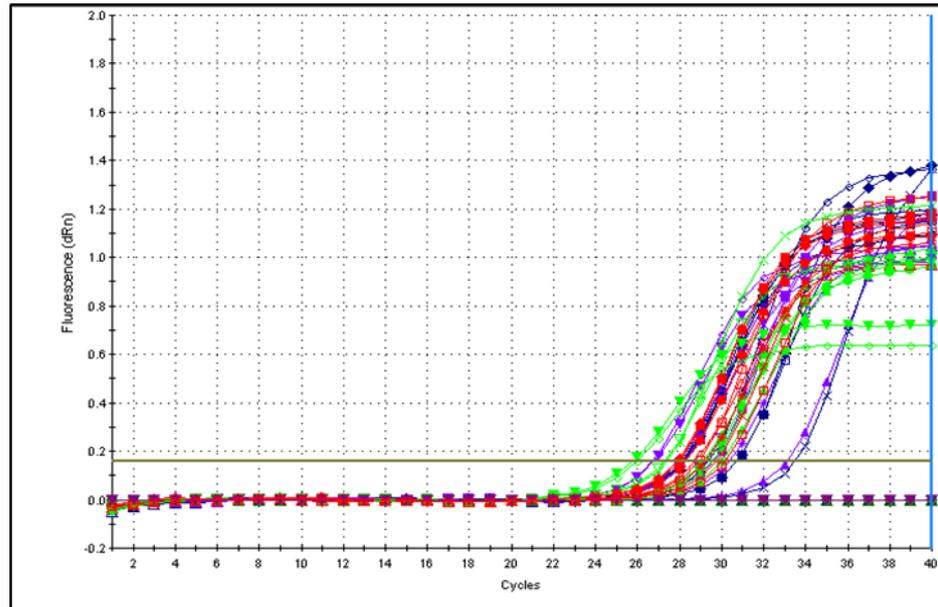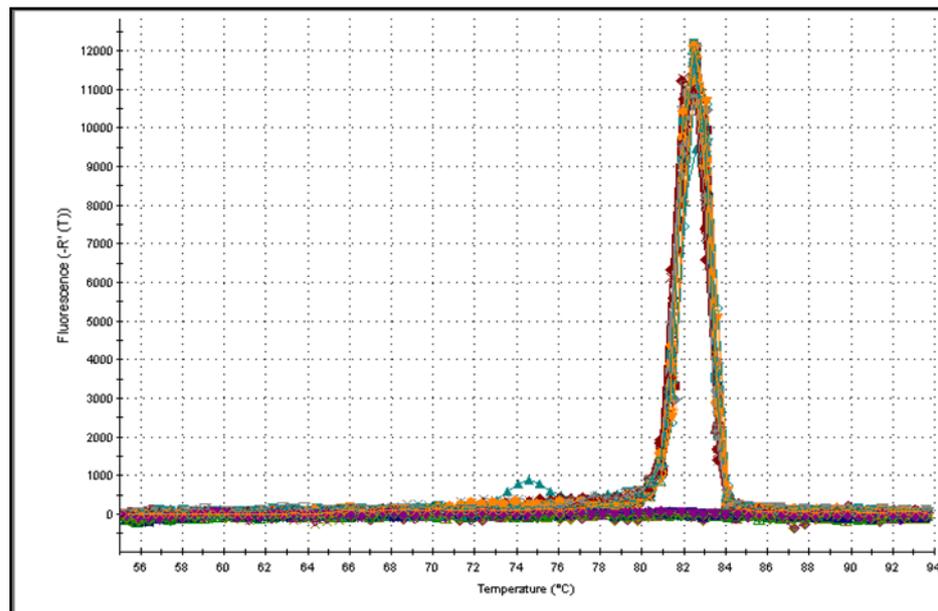
Figure 3.49 SYBR® Green real-time PCR analysis of AQP9 gene expression.

Gene expression of AQP9 was assessed in diluted cDNA of FFPE cancer tissue samples using pre-validated primers. (A) Amplification curves generated for AQP9. Blue and green curves represent tissue samples of negative LN status with grade 1 and grade 3 respectively. Purple and red curves represent tissue samples of positive LN status with grade 1 and grade 3 respectively. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 83°C showing excellent primer specificity; n=4.
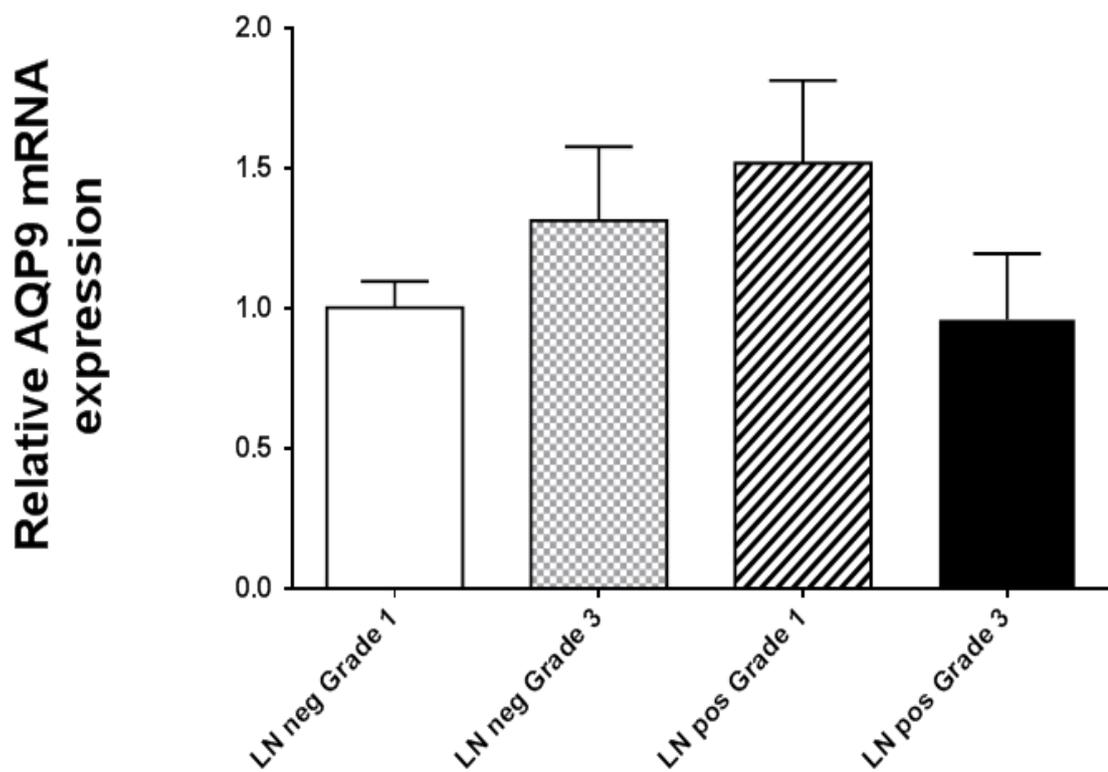
Figure 3.50   Expression of AQP9 mRNA in FFPE tissue samples.

qPCR analysis of AQP9 mRNA expression in FFPE cancer tissue samples showed that there were no significant difference in expression of AQP9 mRNA between the different clinicopathological groups of breast cancer. Bar graph displaying the corresponding relative fold differences normalised to housekeeping genes for each group. Data represent mean ± SEM. All p values > 0.326, n=4 cases per group.
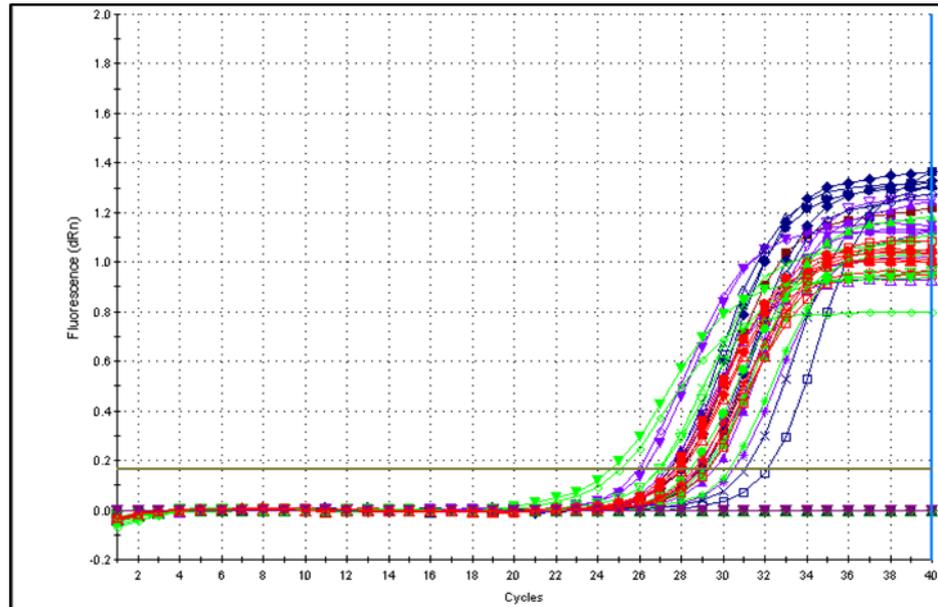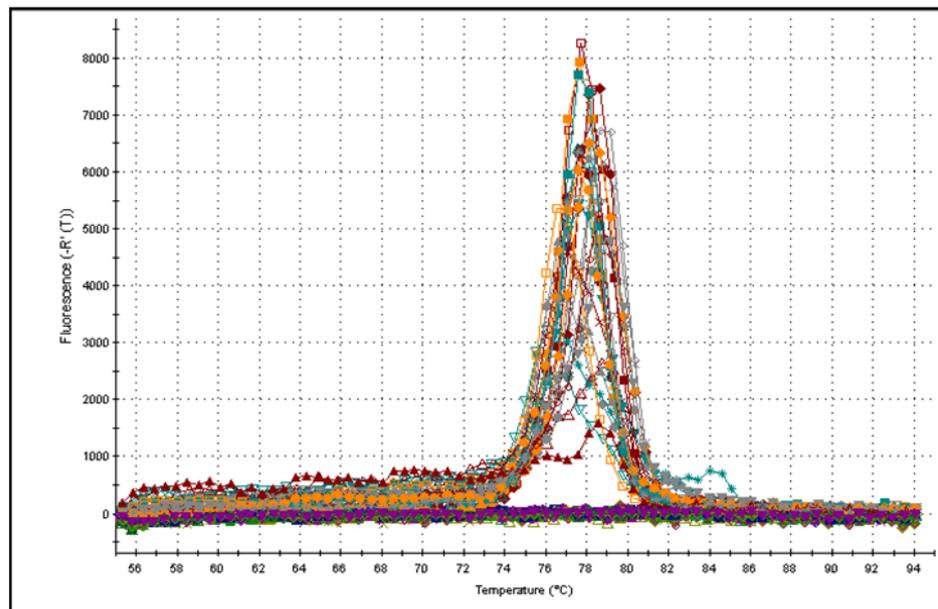
Figure 3.51   SYBR® Green real-time PCR analysis of AQP10 gene expression.

Gene expression of AQP10 was assessed in diluted cDNA of FFPE cancer tissue samples using pre-validated primers. (A) Amplification curves generated for AQP10. Blue and green curves represent tissue samples of negative LN status with grade 1 and grade 3 respectively. Purple and red curves represent tissue samples of positive LN status with grade 1 and grade 3 respectively. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 78°C showing adequate primer specificity; n=4.
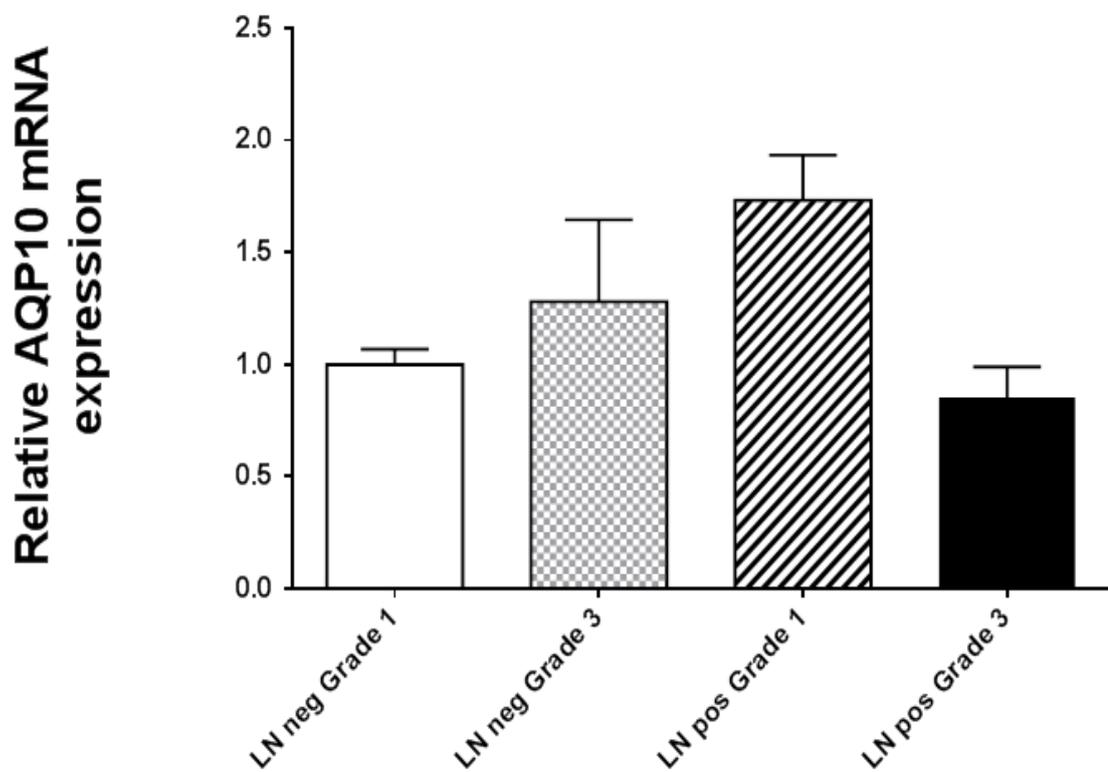
Figure 3.52   Expression of AQP10 mRNA in FFPE tissue samples.

qPCR analysis of AQP10 mRNA expression in FFPE cancer tissue samples showed that there were no significant difference in expression of AQP10 mRNA between the different clinicopathological groups of breast cancer. Bar graph displaying the corresponding relative fold differences normalised to housekeeping genes for each group. Data represent mean ± SEM. All p values > 0.071, n=4 cases per group.
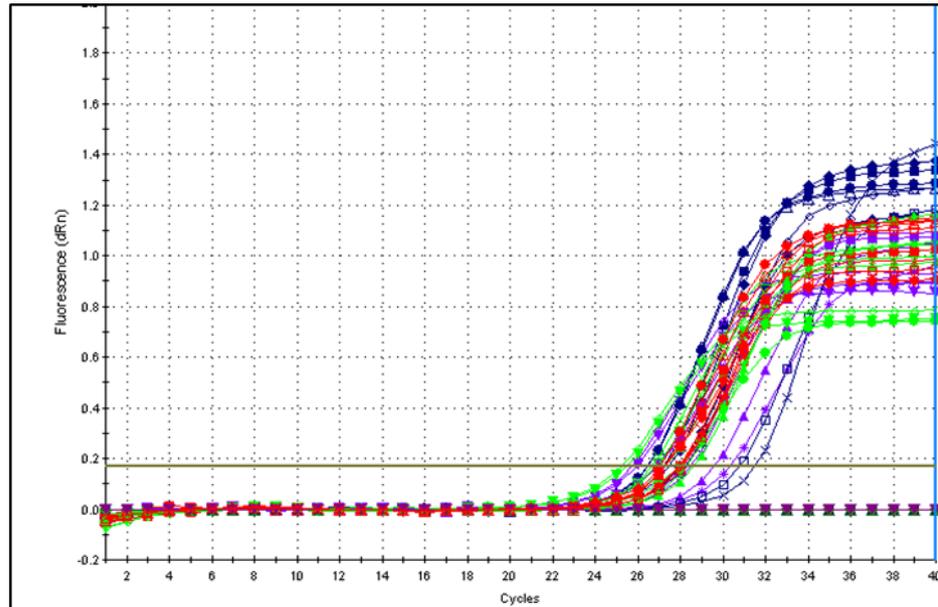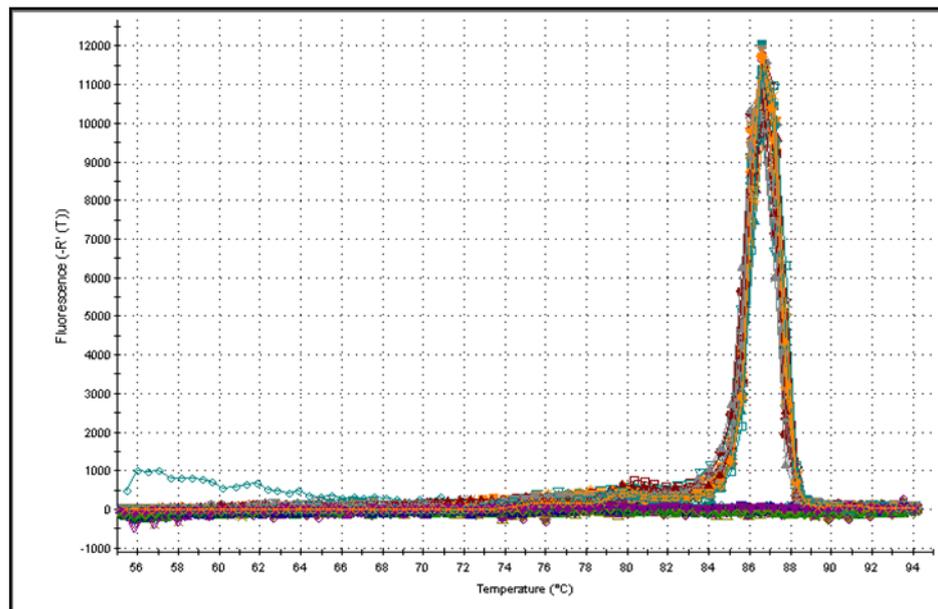
Figure 3.53   SYBR® Green real-time PCR analysis of AQP11 gene expression.

Gene expression of AQP11 was assessed in diluted cDNA of FFPE cancer tissue samples using pre-validated primers. (A) Amplification curves generated for AQP11. Blue and green curves represent tissue samples of negative LN status with grade 1 and grade 3 respectively. Purple and red curves represent tissue samples of positive LN status with grade 1 and grade 3 respectively. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 87°C showing excellent primer specificity; n=4.
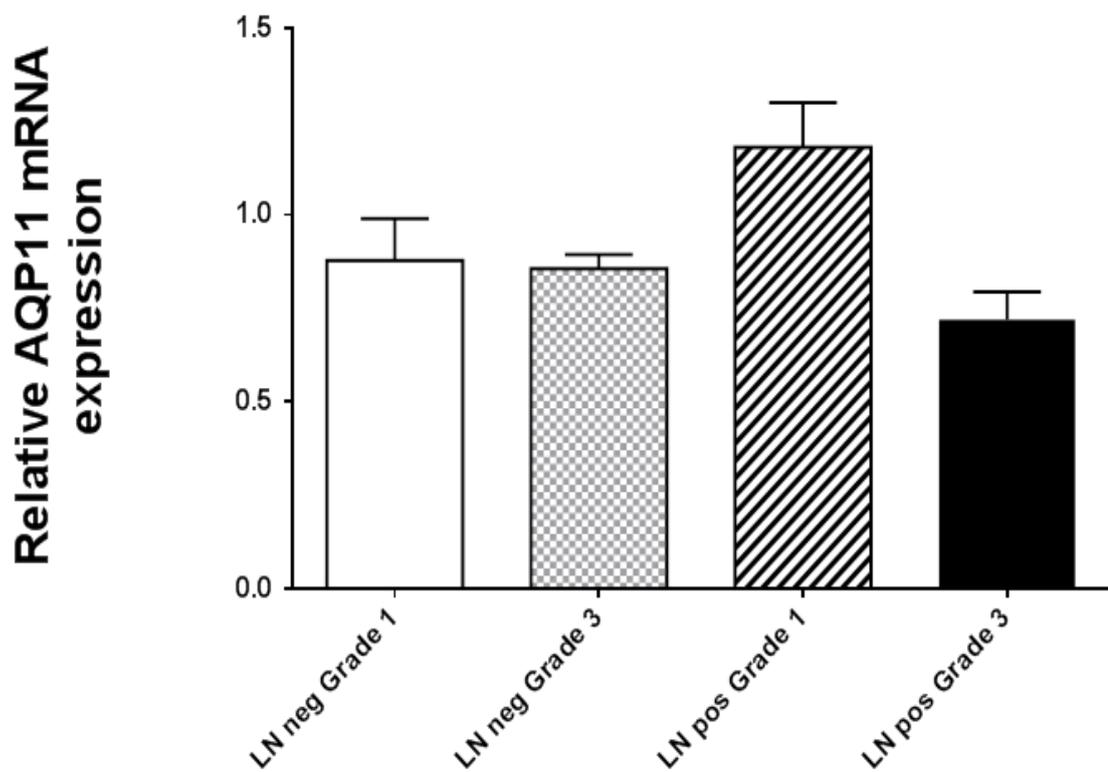
Figure 3.54   Expression of AQP11 mRNA in FFPE tissue samples.

qPCR analysis of AQP11 mRNA expression in FFPE cancer tissue samples showed that there were no significant difference in expression of AQP11 mRNA between the different clinicopathological groups of breast cancer. Bar graph displaying the corresponding relative fold differences normalised to housekeeping genes for each group. Data represent mean ± SEM. All p values > 0.240, n=4 cases per group.
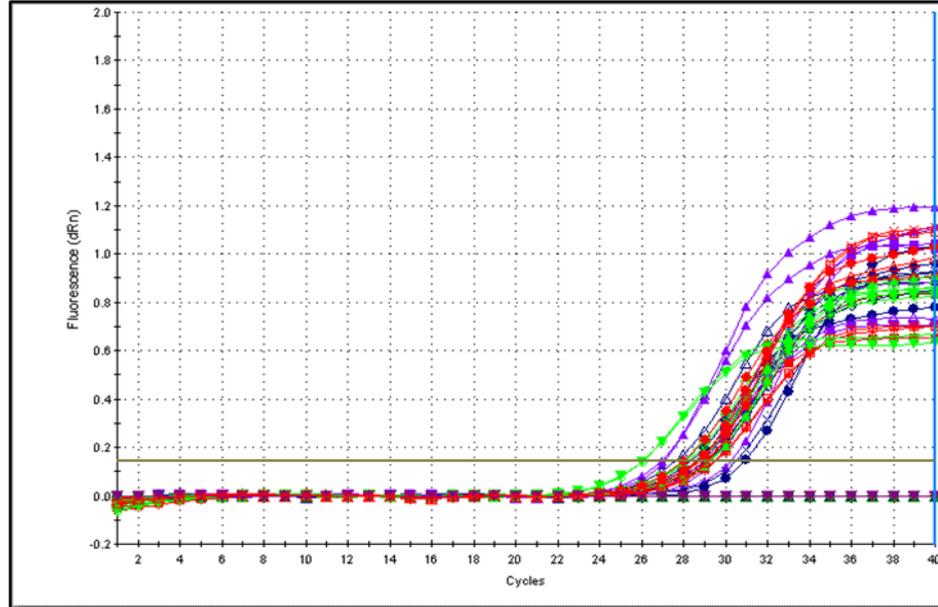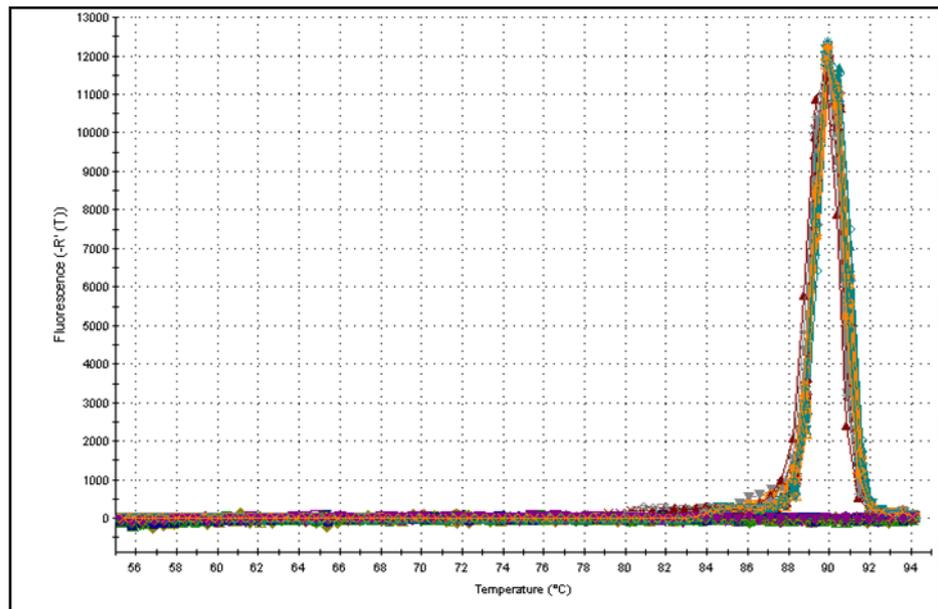
Figure 3.55   SYBR® Green real-time PCR analysis of AQP12 gene expression.

Gene expression of AQP12 was assessed in diluted cDNA of FFPE cancer tissue samples using pre-validated primers. (A) Amplification curves generated for AQP12. Blue and green curves represent tissue samples of negative LN status with grade 1 and grade 3 respectively. Purple and red curves represent tissue samples of positive LN status with grade 1 and grade 3 respectively. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 90°C showing excellent primer specificity; n=4.
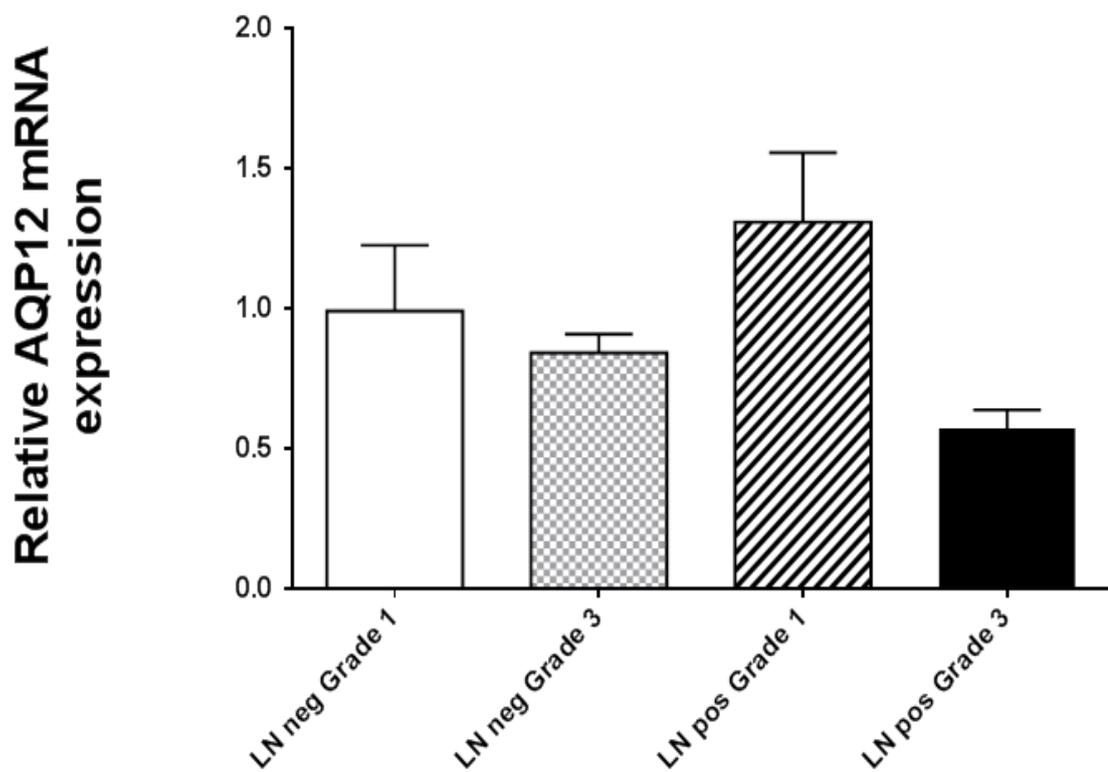
Figure 3.56   Expression of AQP12 mRNA in FFPE tissue samples.

qPCR analysis of AQP12 mRNA expression in FFPE cancer tissue samples showed that there were no significant difference in expression of AQP12 mRNA between the different clinicopathological groups of breast cancer. Bar graph displaying the corresponding relative fold differences normalised to housekeeping genes for each group. Data represent mean ± SEM. All p values > 0.072, n=4 cases per group.

Table 3.2   Mean C_t values and mean fold expression of all AQPs in FFPE tissue samples

AQPs	LN negative Grade 1		LN negative Grade 3		LN positive Grade 1		LN positive Grade 3		P value
	Mean C_t	Mean fold	Mean C_t	Mean fold	Mean C_t	Mean fold	Mean C_t	Mean fold	
AQP0	28.83	1.00	27.58	1.03	28.60	1.11	28.34	0.74	0.231
AQP1	35.38	1.60	36.12	0.31	36.10	0.82	35.90	0.41	0.285
AQP2	33.69	1.41	33.80	0.35	34.01	0.77	33.55	0.51	0.164
AQP3	29.67	1.01	28.55	0.93	29.31	1.17	28.82	0.93	0.664
AQP4	30.10	1.01	28.13	1.79	29.18	1.76	28.89	1.26	0.225
AQP5	33.39	1.51	35.40	0.34	34.07	0.56	34.16	0.30	0.478
AQP6	29.61	1.00	28.68	0.84	29.43	1.10	29.41	0.59	0.128
AQP7	33.75	1.19	32.34	1.08	34.37	0.61	33.64	0.55	0.283
AQP8	30.05	1.11	27.93	1.31	29.15	1.37	28.80	0.91	0.585
AQP9	29.79	1.00	28.22	1.31	29.18	1.52	29.03	0.96	0.326
AQP10	29.15	1.00	27.72	1.28	28.28	1.73	28.47	0.85	0.071
AQP11	28.20	0.88	26.94	0.86	27.66	1.18	27.50	0.72	0.240
AQP12	29.37	0.99	28.24	0.84	28.84	1.31	29.10	0.57	0.072

3.4 Discussion

This chapter deals with the investigation of AQP expression in snap frozen cancer tissue and paraffin-embedded tissue samples of breast cancer. Initially mRNA expression was examined using qPCR on newly collected frozen tissue samples for all 13 AQPs. These samples were also examined for AQP3 protein expression by Western blotting. Immunohistochemistry was performed on FFPE tissue slices for AQP3 expression and although there were qualitatively higher amounts of AQP3 expression in some cases immunohistochemistry was not used to quantify the AQP3 expression as some of the negative slides showed unspecific binding of secondary antibodies. Therefore it was decided to extract RNA and protein from these paraffin-embedded samples and qPCR and Western blotting was used to assess the expression of AQP3. Paraffin-embedded samples were divided into four groups, hypothesising that AQP3 expression may be associated with histological tumour grade and lymph node infiltration status. Lymph node negative with grade 1 tumours were selected as control because they represent primary tumours without metastasis. The findings of this study were that AQP3 is expressed at a higher level in breast cancer tissues than healthy border tissues but that expression levels of AQP3 are not significantly different between different clinical groups.

There are few recent studies that have reported the expression of AQPs in breast carcinoma (reviewed herein chapter 1). Only one study has reported the expression AQPs profiling of all 13 AQPs in breast carcinoma and corresponding normal tissues by RT-PCR technique. Of all the 13 AQPs examined, it was reported previously that AQP0, 2, 6, 7, 8 and 9 mRNA were absent in both cancer and corresponding normal tissues whereas it was also reported that expression of AQP10, 11 and 12 mRNA was detected in both cancer and adjacent normal tissues but no significant difference was found in their expression levels (Shi *et al.*, 2012). In the present study, qPCR analysis revealed the presence of expression of all AQPs in both cancer and normal tissues to some extent. There was however no significant difference in expression of all the AQPs in cancer and normal tissues except AQP3, which was exhibited a significantly higher level of expression in BC

tissue as compared to healthy border tissue. Shi *et al*. (Shi *et al.*, 2012) also reported increased expression of AQP3 in BC tissues compared to adjacent tissues by RT-PCR. In previous studies, expression of AQP1 and AQP5 was also reported to be higher in BC issues (Jung *et al.*, 2011; Shi *et al.*, 2012) however in this study no significant differences were observed.

Previously, research demonstrating immunohistochemical assessment of AQP5 expression established that AQP5 expression was significantly higher in the invasive ductal carcinoma with LN metastasis as compared to the invasive ductal carcinoma without LN metastasis with similar tumour grade (Jung *et al.*, 2011). However this was not observed in the present study and AQP5 mRNA expression was appeared to be markedly reduced in all three tumour groups as compared to control lymph node negative with grade 1 tumours but statistically it was not significant and also the C_t values were above 35 for some cases. Interestingly, AQP5 mRNA expression in the frozen cancer tissues was also reduced as compared to normal tissue samples. Remarkably, it was also demonstrated in the same study (Jung *et al.*, 2011) that AQP5 labelling intensity was gradually decreased during the cancer progression. The difference in expression in these two studies may be due to different techniques used to assess the expression. The qPCR used in present study is more sensitive and quantitative as compared to RT-PCR. However since sample size was small in the present study therefore this study is limited in this regard. More importantly, the expression of AQP3 was found significantly higher in cancer tissues as reported previously. The implications of higher AQP3 expression in cancer tissues might be important in disease pathogenesis. AQP3 has been involved in cellular migration and proliferation described by previous studies.

This study showed for the first time the differential AQP3 protein expression in breast tumour tissues and corresponding normal tissues by Western blotting. As mentioned above, a previous study also showed higher mRNA expression of AQP3 by less sensitive RT-PCR but did not try to investigate the protein expression. This novel observation therefore confirms that AQP3 is expressed at a higher level for both mRNA and protein in breast cancer tissue. It remains to be seen

if this change in expression is causative or correlative and future experiments might investigate this.

Immunohistochemistry was performed on limited paraffin-embedded cases. A double immunofluorescence technique was used to investigate the cytokeratin 5/8 and AQP3 expression. Red bloods cells consistently showed AQP3 expression whereas some epithelial cells showed AQP3 expression as well. However immunohistochemistry was not used to quantify the AQP3 expression in these tissue samples as some negative slides showed nonspecific secondary antibodies binding. Then it was decided to use the more sensitive qPCR and Western blotting techniques to investigate the association between AQP3 expression and tumour grade with lymph node metastasis. Western blot analysis showed that AQP3 was expressed in all the 4 tumour groups however no significant difference was observed between the groups. This indicates the complexity of breast cancer disease and involvement of more than one marker in the disease pathogenesis.

AQP3 was not associated with tumour grade and lymph node metastasis. Furthermore, the study showed that AQP3 protein expression was more prominent in cancer tissues as compared to normal tissues. Overexpression of AQP3 in tissue may suggest some important roles of AQP3 in breast cancer. Gaining of AQP3 expression might be important in fluid homeostasis and in metabolic pathways in cancer tissues specifically related to glycerol. The signal pathways that stimulate AQP3 overexpression in breast carcinoma is unknown, but might include growth factors and antiapoptotic factors.

Chapter 4

AQP Expression in MDA-MB-231 Cells and Production of Stable AQP3-Knockdown Cell Clones

4.1 Introduction

The wide and varied tissue distribution of AQP family members has been discussed previously, including the expression of AQPs in breast tissue. The genome of eukaryotic cells is a compelling target for scientific manipulation of gene expression. RNA silencing is one naturally conserved strategy developed in the genome to supress and degrade the foreign invaders soon after they have entered the body. This RNA silencing restricts the transcript level by either suppressing transcription or by posttranscriptional gene silencing process termed as RNA interference (RNAi) (Agrawal *et al.*, 2003). Gene manipulation by RNAi was first demonstrated in *Caenorhabditis elegans* (Fire *et al.*, 1998) and later on RNAi was independently observed by researchers in many different organisms including fungi, plants and animals (Baulcombe, 1999; Cogoni and Macino, 1999; Lohmann *et al.*, 1999; Wianny and Zernicka-Goetz, 2000).

RNAi is a regulatory phenomenon by which homologous double-stranded RNA (dsRNA) molecules block the expression of endogenous genes (Fjose *et al.*, 2001; Hammond *et al.*, 2001). One way of achieving RNAi is the use of shRNA technology to silence the expression of endogenous genes. Many recent studies have used shRNA technology to silence AQPs protein expression in order to investigate their functions in different cell lines.

The main aim of this part of the study was to knockdown AQP3 in MDA-MB-231cells and to generate stable knockdown cell lines by using shRNA. AQP3 was targeted, as it was the most highly expressed AQP family member identified in clinical samples.

4.2   Material and Methods

All methods for analysis of AQP gene expression, transfection of AQP3 shRNA into MDA-MB-231 cells, Western blotting and immunocytochemical analysis of cells were performed as described in Chapter 2.

To investigate the gene expression of all 13 AQPs in MDA-MB-231 cell line, SYBR$^{®}$ green real-time PCR reaction was performed. The expression of housekeeping genes was also analysed under the same conditions in order to use these as internal controls. The reaction conditions and amount of cDNA used for real time PCR were mentioned in detail in chapter 2. AQP3 expression was investigated by immunocytochemical assay and was performed on MDA-MB-231 cells as described in section 2.13.

Once enough cells were available, cell pellets were collected for each clone of the shRNA constructs for RNA and protein extraction. Total RNA from all clones of each shRNA construct was extracted and reverse transcribed as mentioned in chapter 2. The AQP3 gene expression in all clones was investigated by performing qPCR. The reaction conditions were same as described before in Chapter 2. Western Blotting (as described in Chapter 2) was also performed on selected clones to further verify the AQP3 protein expression.

Statistical analysis of data was performed using GraphPad Prism 6 software. One-way ANOVA with test post-hoc test (Dunnett's multiple comparisons) for comparisons of > 3 groups or Student's t-test for comparisons of <3 groups were performed for the statistical analysis between experimental conditions and p values < 0.05 were considered significant.

4.3 Results

4.3.1 mRNA expression profile of AQPs in MDA-MB-231 cell line

SYBR® Green real-time PCR (qPCR) was performed to investigate the gene expression of all AQPs and housekeeping genes (beta actin and YWHAZ) in the MDA-MB-231 cell line. MDA-MB-231 cells exhibited expression of several AQP family members but expressed AQP3 at a significantly higher level compared to all other AQPs. The expression of AQPs was normalised to beta actin and YWHAZ housekeeping genes. The amplification and dissociation curves for all 13 AQPs plus the normalising genes beta actin and YWHAZ are displayed in Figures 4.1 to 4.15. Relative expression of all AQPs was compared to AQP0 using the $2^{-\Delta\Delta Ct}$ method. The averaged Ct values generated in qPCR reaction were shown in Table 4.1. The mRNA expression of AQP3 (see Figure 4.16) was significantly (relative mean fold value 17.24, p <0.0001) higher than all other AQPs demonstrated by One way ANOVA with Dunnett's post-hoc test.
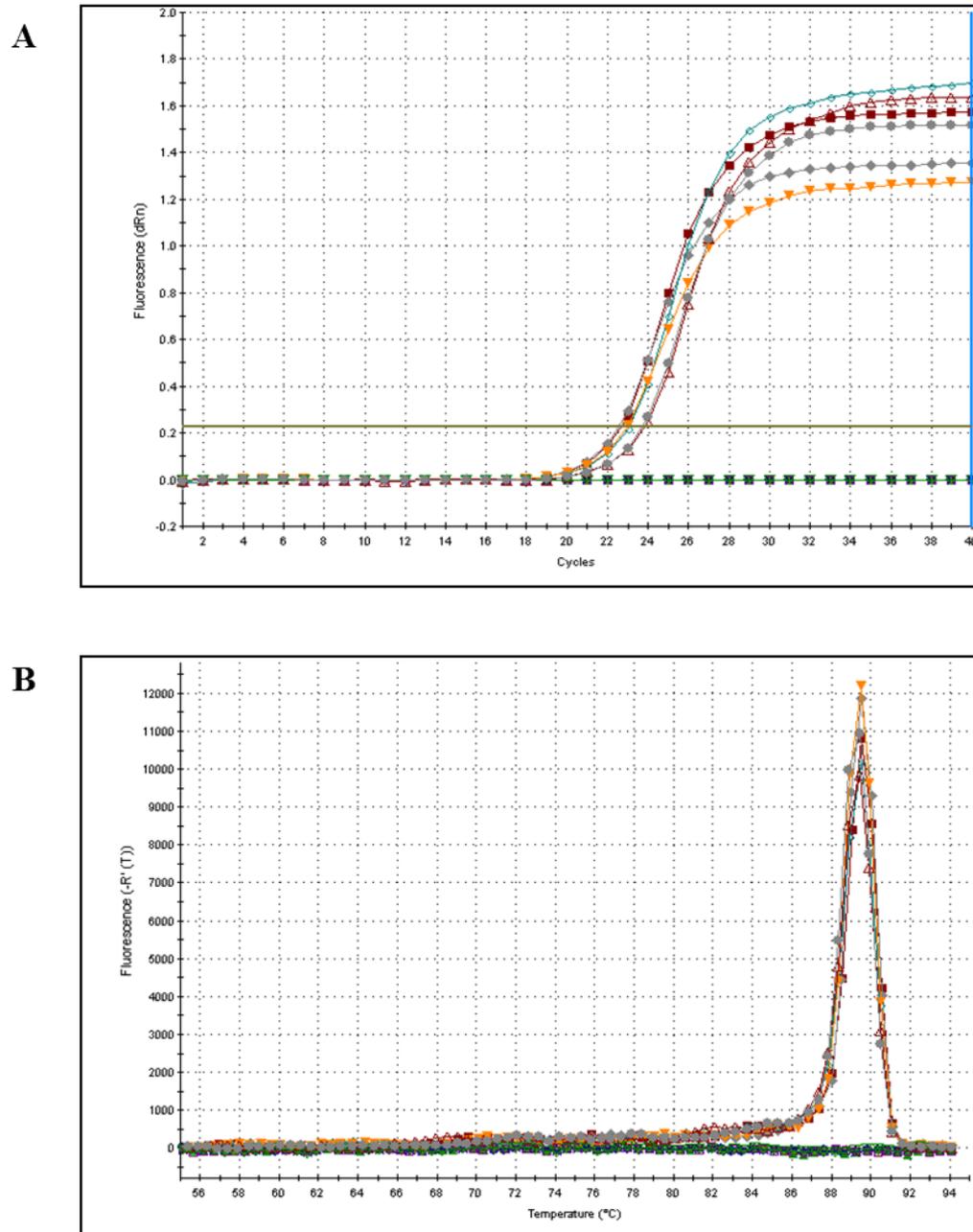
Figure 4.1   SYBR® Green real-time PCR analysis of beta actin gene expression.

Gene expression of beta actin was assessed in diluted cDNA samples of MDA-MB-231 cells. (A) Amplification curves showed that the mean Ct value for beta actin in these cells was 20.61. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 89°C suggesting excellent primer specificity. n=3.
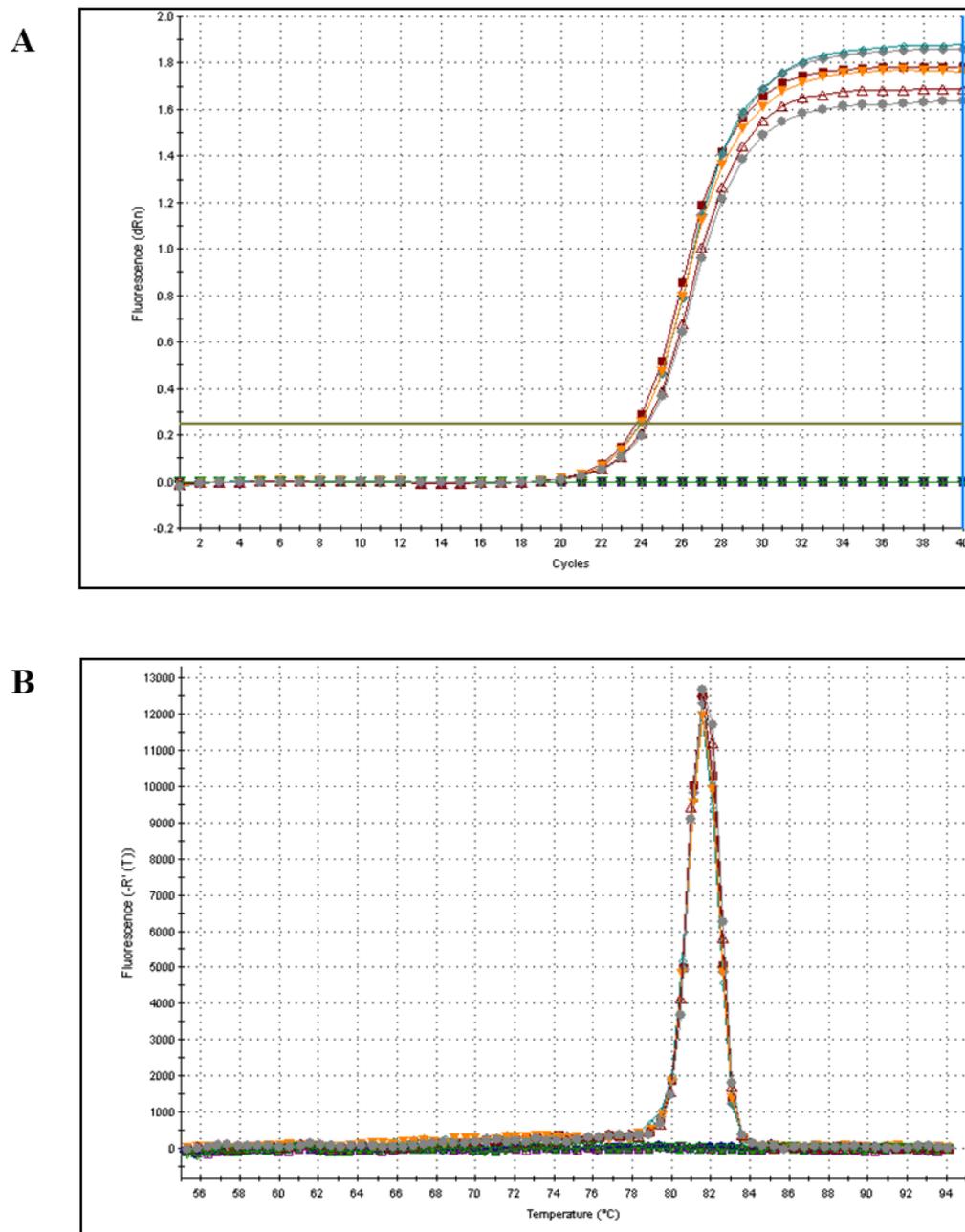
Figure 4.2 SYBR® Green real-time PCR analysis of YWHAZ gene expression.

Gene expression of YWHAZ was assessed in diluted cDNA samples of MDA-MB-231 cells. (A) Amplification curves showed that the mean Ct value for YWHAZ in these cells was 21.28. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 81°C suggesting excellent primer specificity; n=3.
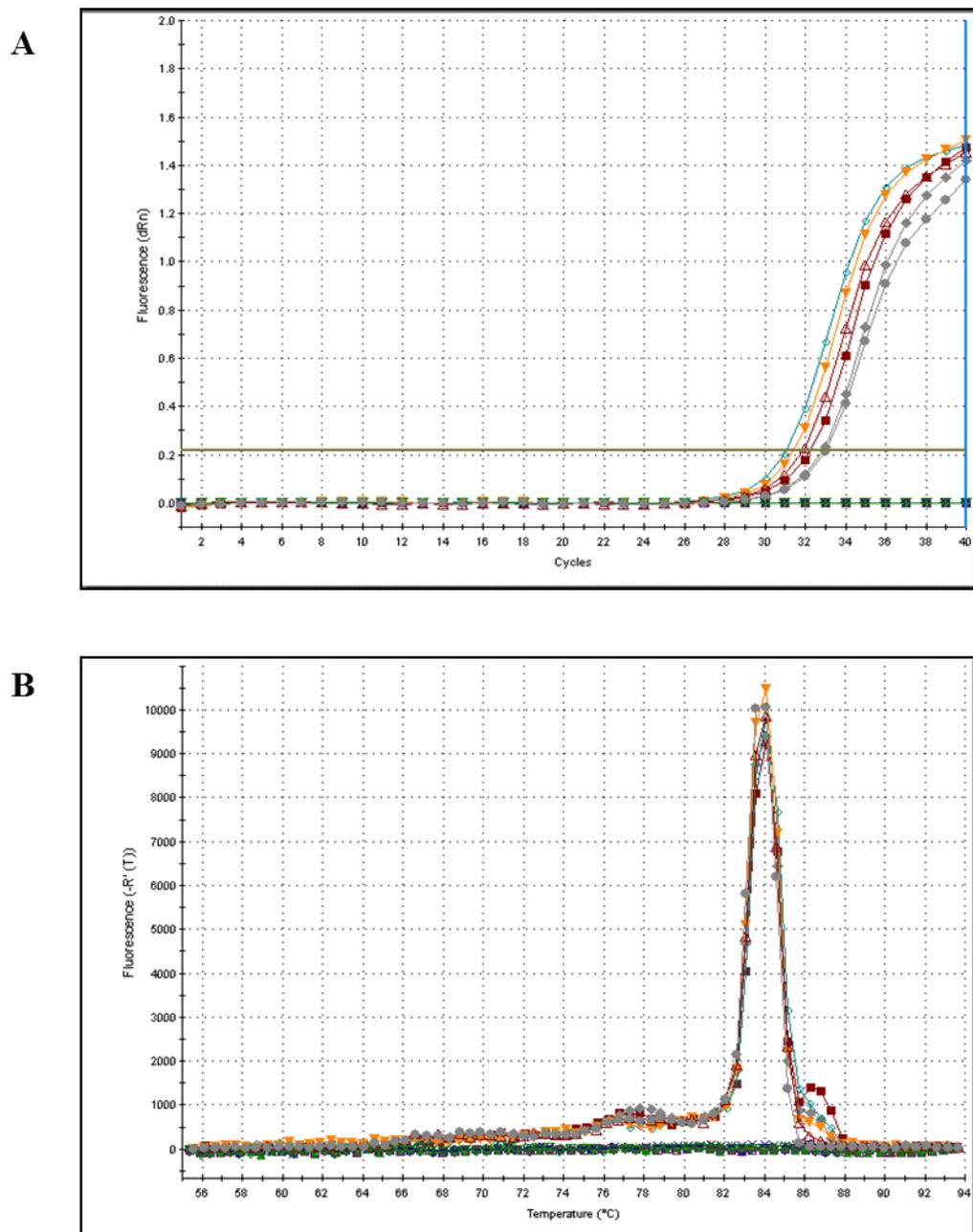
Figure 4.3   SYBR® Green real-time PCR analysis of AQP0 gene expression.

Gene expression of AQP0 was assessed in diluted cDNA samples of MDA-MB-231 cells using pre-validated primers. (A) Amplification curves showed that the mean Ct value for AQP0 in these cells was 30.02. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 84°C suggesting excellent primer specificity; n=3.
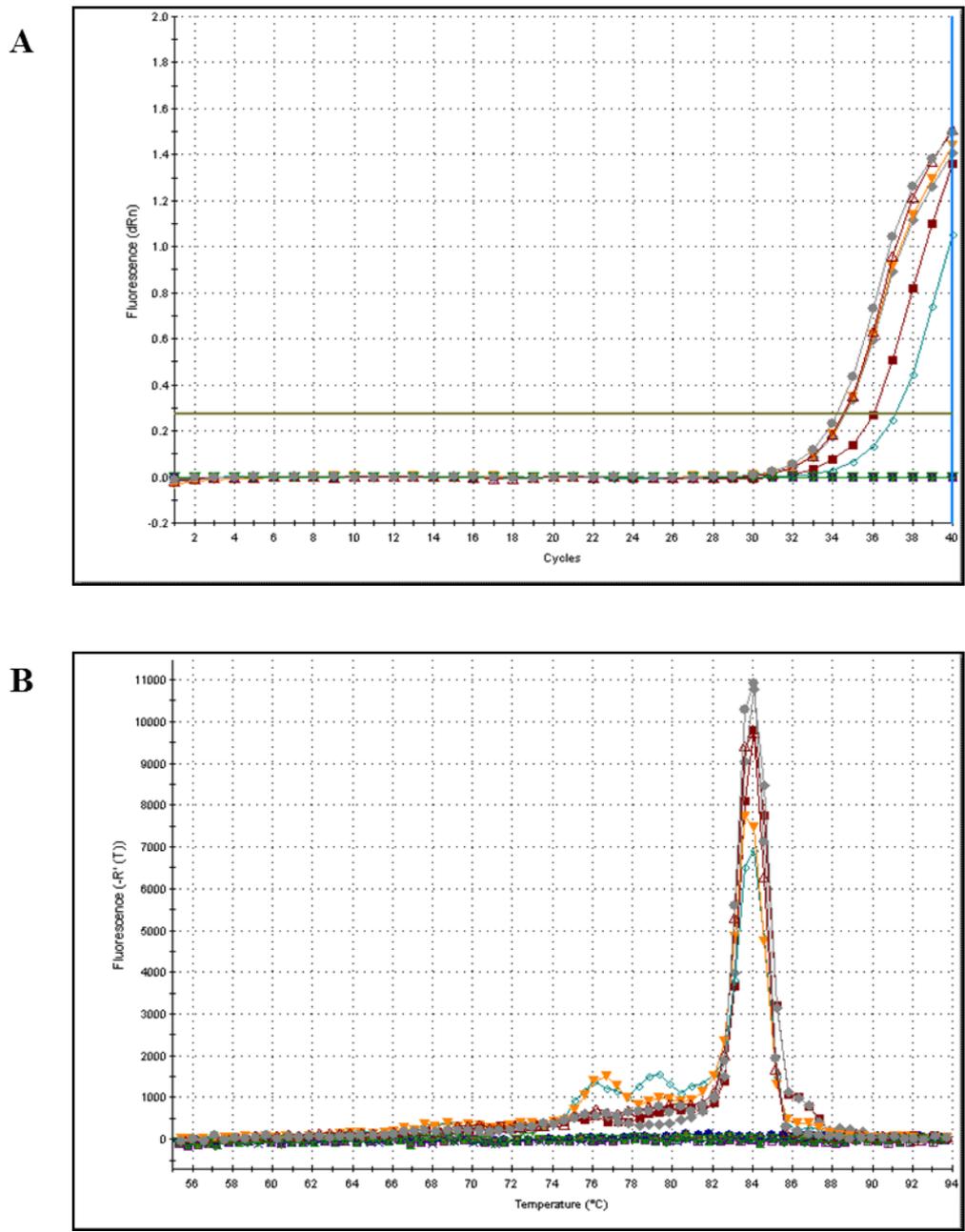
Figure 4.4   SYBR® Green real-time PCR analysis of AQP1 gene expression.

Gene expression of AQP1 was assessed in diluted cDNA samples of MDA-MB-231 cells using pre-validated primers. (A) Amplification curves showed that the mean Ct value for AQP1 in these cells was 32.81. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 84°C suggesting excellent primer specificity. n=3.
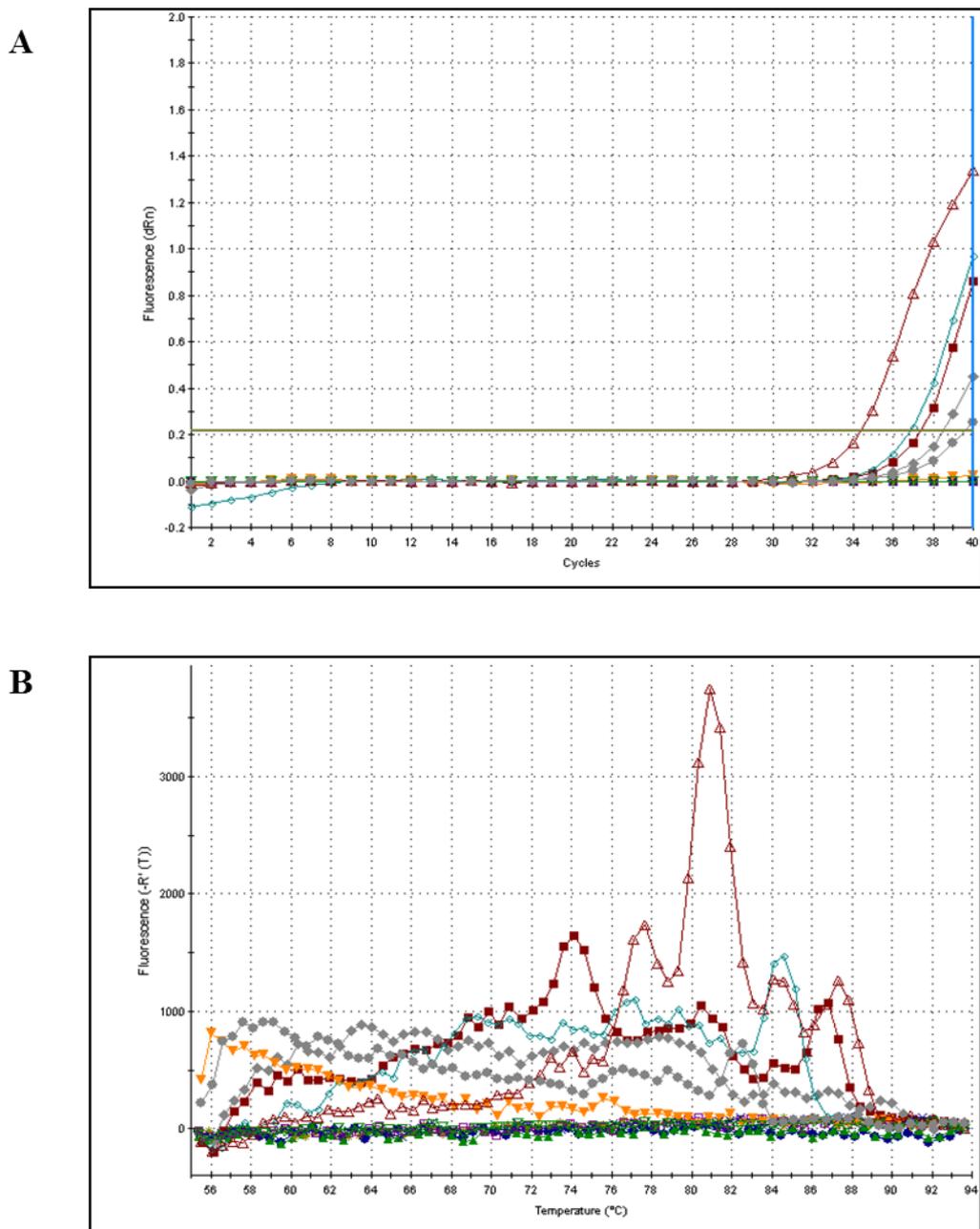
Figure 4.5   SYBR® Green real-time PCR analysis of AQP2 gene expression.

Gene expression of AQP2 was assessed in diluted cDNA samples of MDA-MB-231 cells using pre-validated primers. (A) Amplification curves showed that the mean Ct value for AQP2 in these cells was 36.05. (B) Dissociation curve analysis showed that the amplicon from the PCR reaction dissociated at range of temperatures suggesting nonspecific primer binding; n=3.
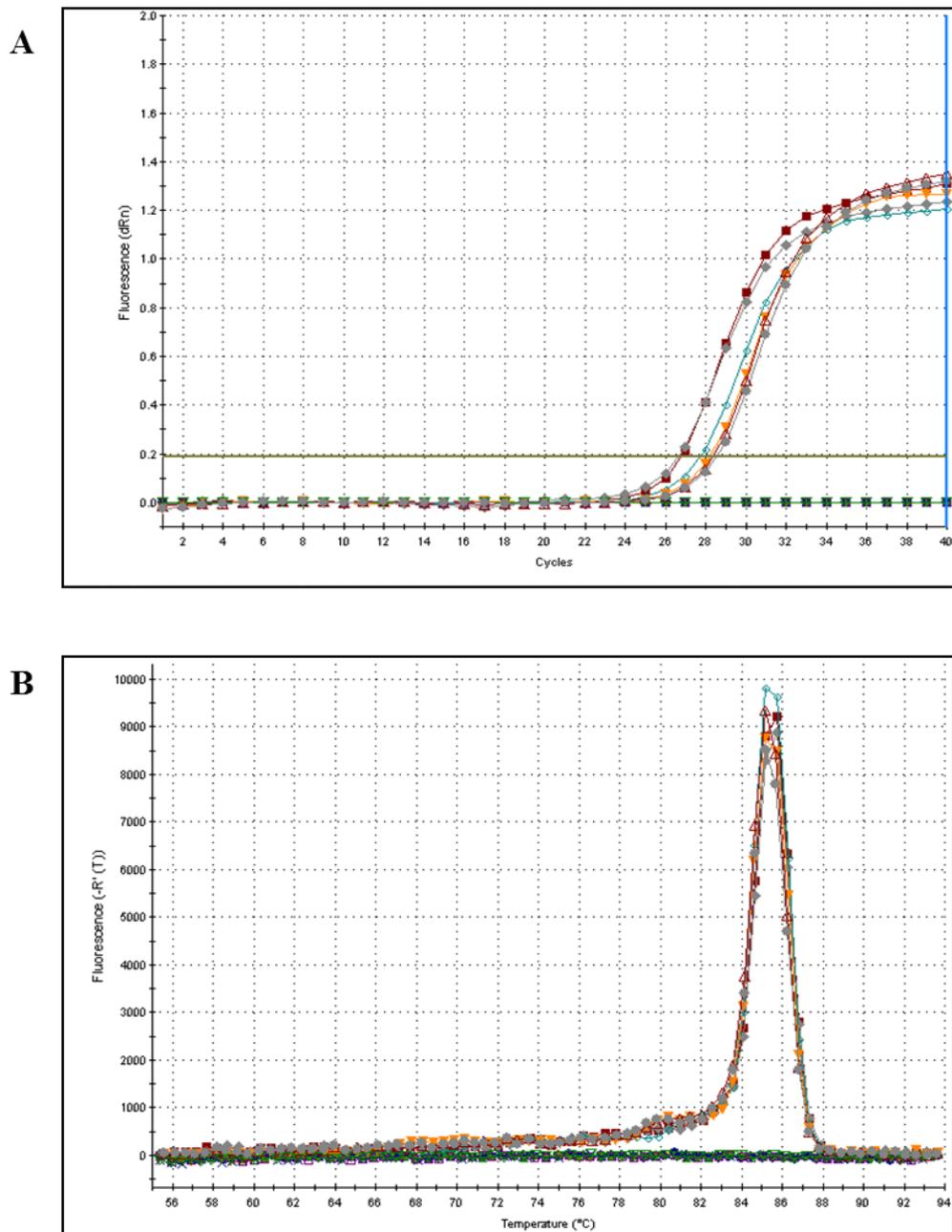
Figure 4.6   SYBR® Green real-time PCR analysis of AQP3 gene expression.

Gene expression of AQP3 was assessed in diluted cDNA samples of MDA-MB-231 cells using pre-validated primers. (A) Amplification curves showed that the mean Ct value for AQP3 in these cells was 26.00. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 85°C suggesting excellent primer specificity; n=3.
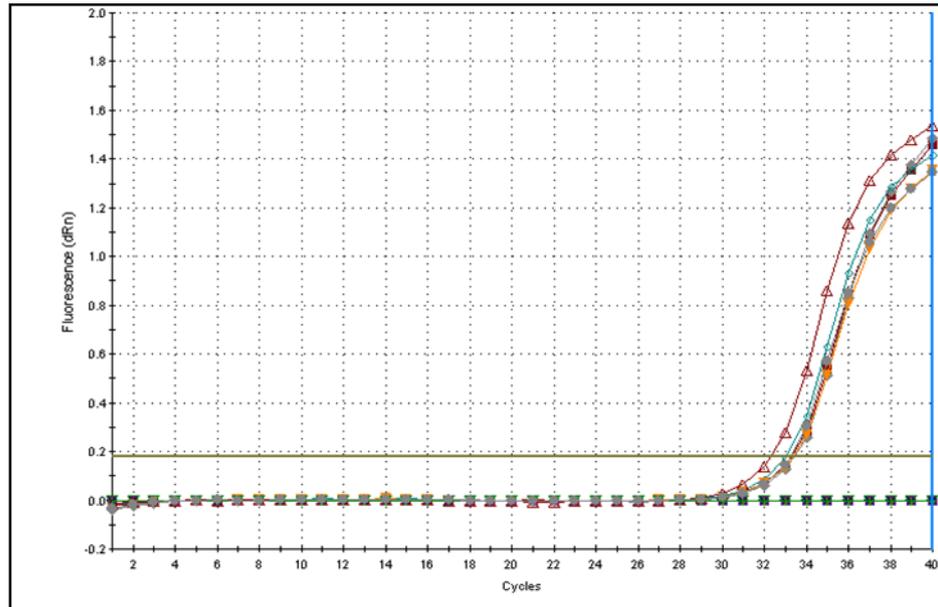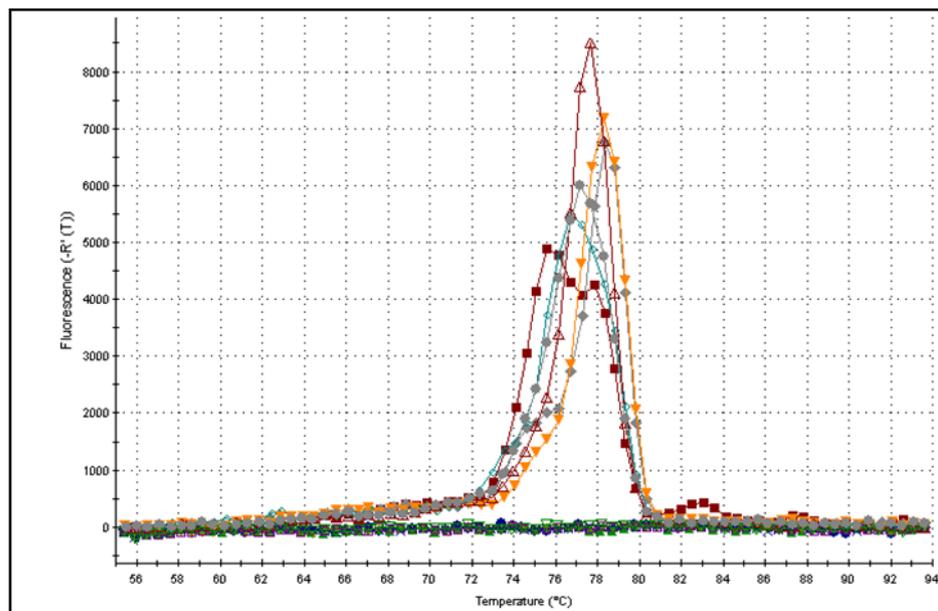
Figure 4.7   SYBR® Green real-time PCR analysis of AQP4 gene expression.

Gene expression of AQP4 was assessed in diluted cDNA samples of MDA-MB-231 cells using pre-validated primers. (A) Amplification curves showed that the mean Ct value for AQP4 in these cells was 31.43. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 79°C showing adequate primer specificity. n=3.
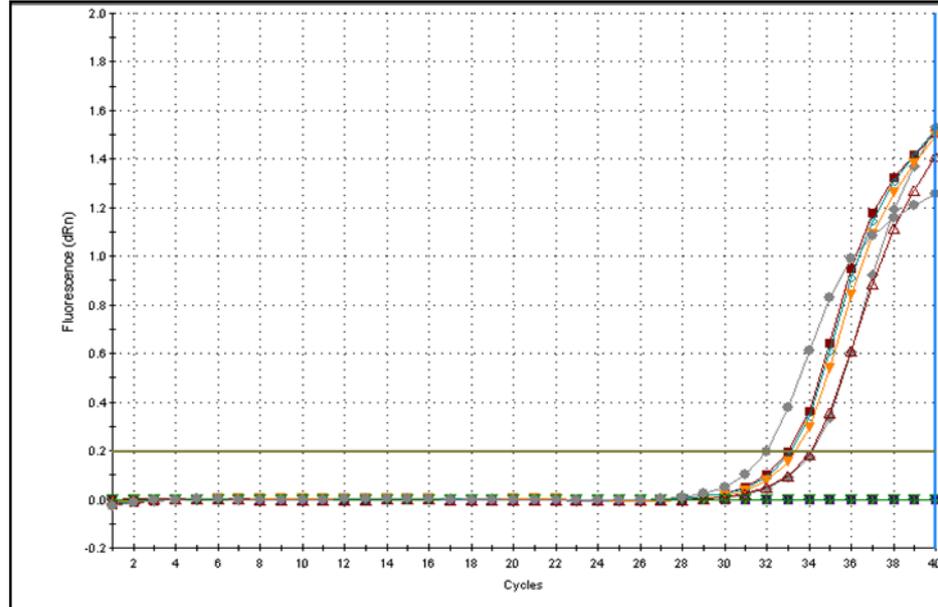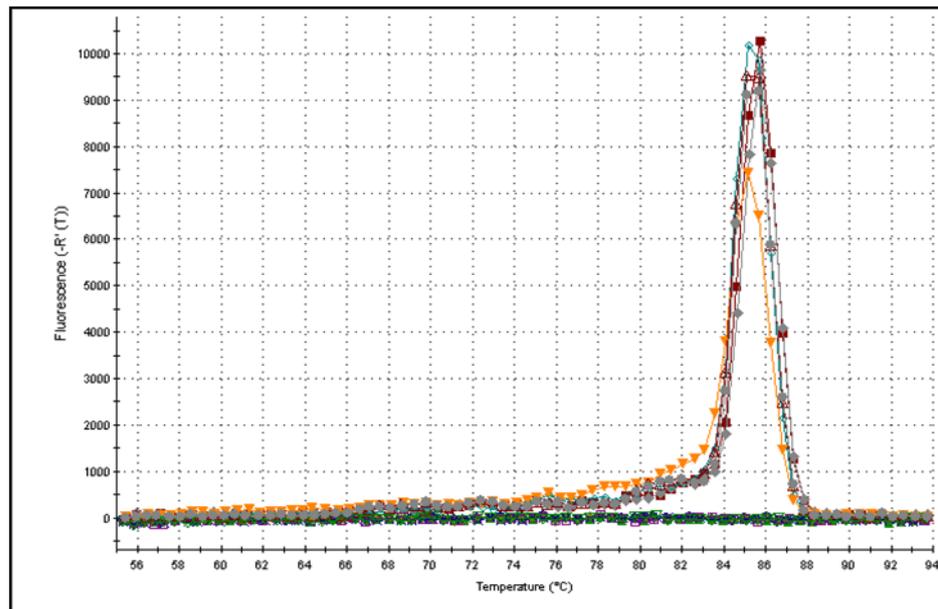
Figure 4.8   SYBR® Green real-time PCR analysis of AQP5 gene expression.

Gene expression of AQP5 was assessed in diluted cDNA samples of MDA-MB-231 cells using pre-validated primers. (A) Amplification curves showed that the mean Ct value for AQP5 in these cells was 31.34. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 85°C suggesting excellent primer specificity; n=3.
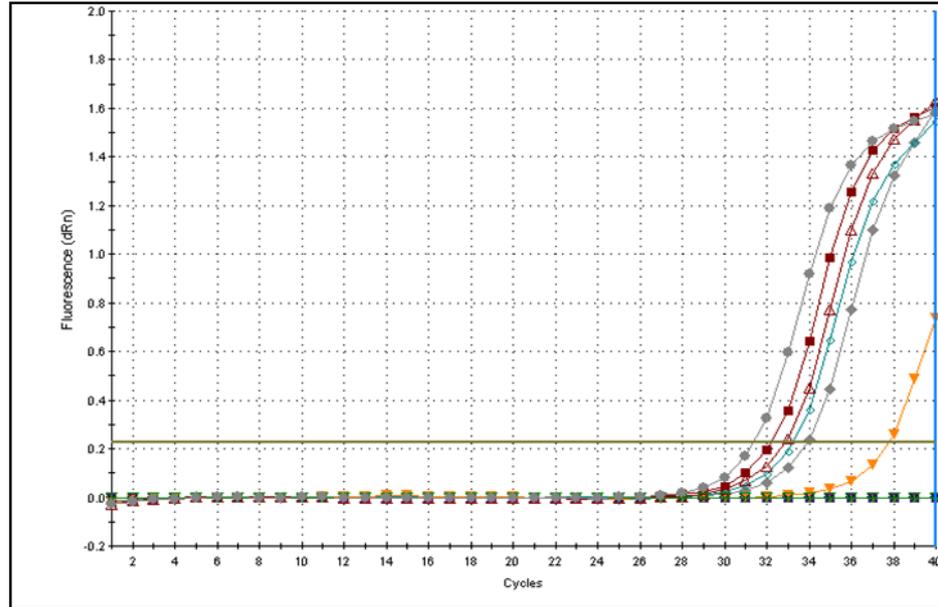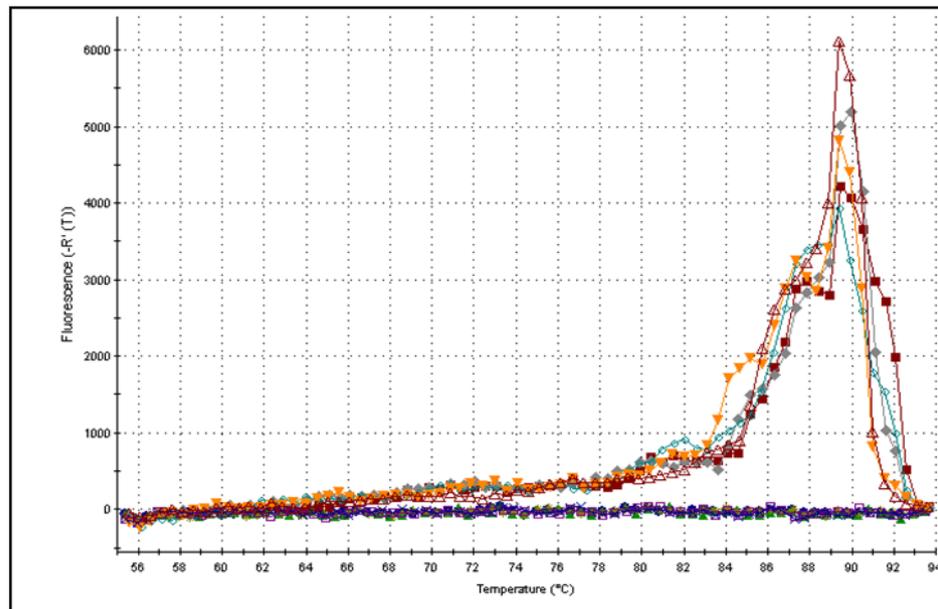
Figure 4.9   SYBR® Green real-time PCR analysis of AQP6 gene expression.

Gene expression of AQP6 was assessed in diluted cDNA samples of MDA-MB-231 cells using pre-validated primers. (A) Amplification curves showed that the mean Ct value for AQP6 in these cells was 30.57. (B) Dissociation curve analysis showed that the amplicon from the PCR reaction dissociated at approximately 89°C suggesting adequate primer specificity; n=3.
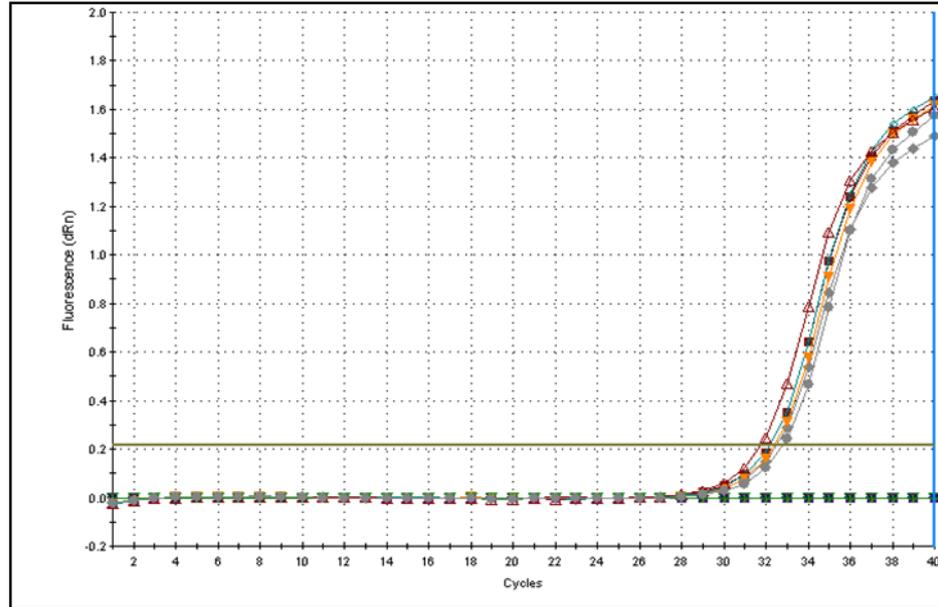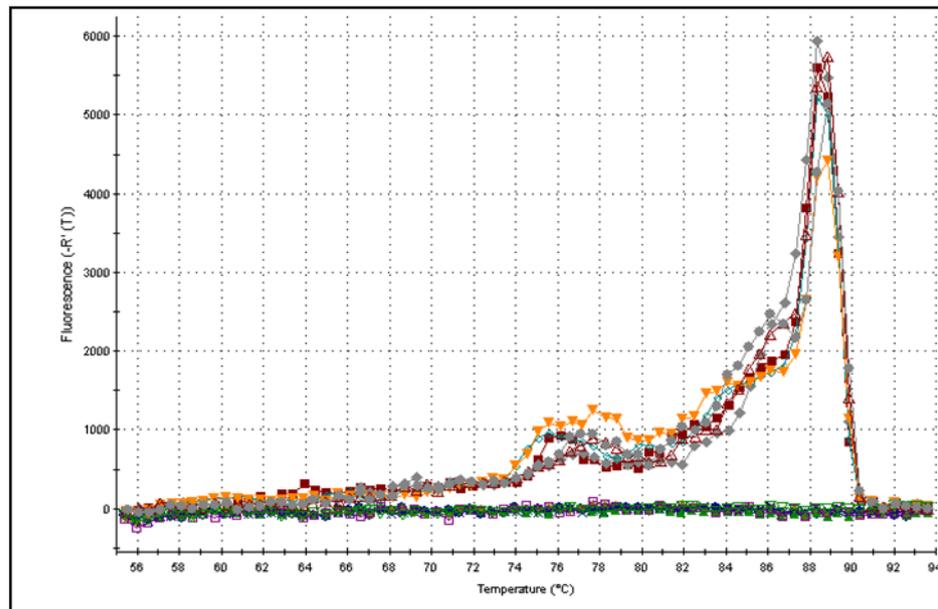
Figure 4.10   SYBR® Green real-time PCR analysis of AQP7 gene expression.

Gene expression of AQP7 was assessed in diluted cDNA samples of MDA-MB-231 cells using pre-validated primers. (A) Amplification curves showed that the mean Ct value for AQP7 in these cells was 30.30. (B) Dissociation curve analysis showed that the amplicon from the PCR reaction dissociated at approximately 89°C suggesting excellent primer specificity; n=3.
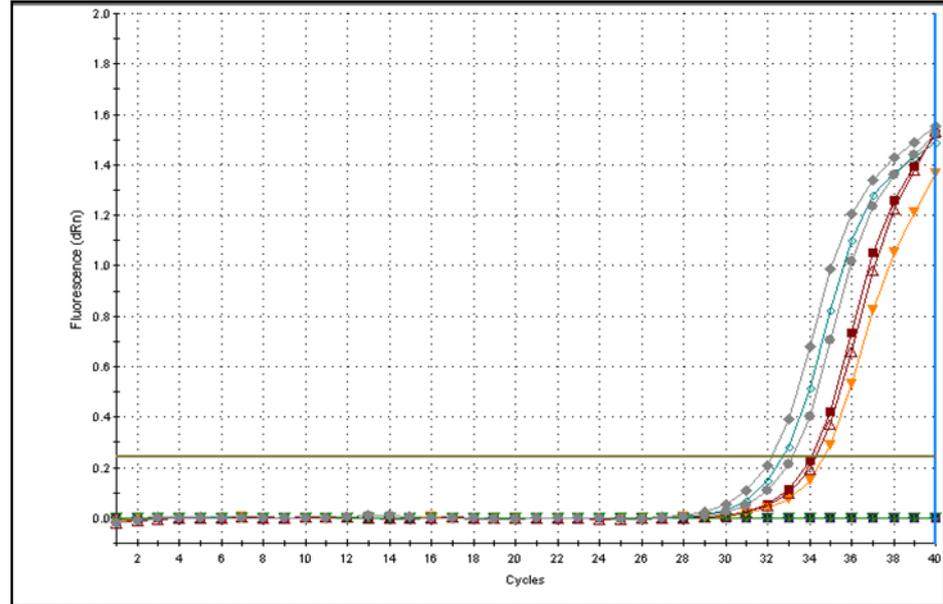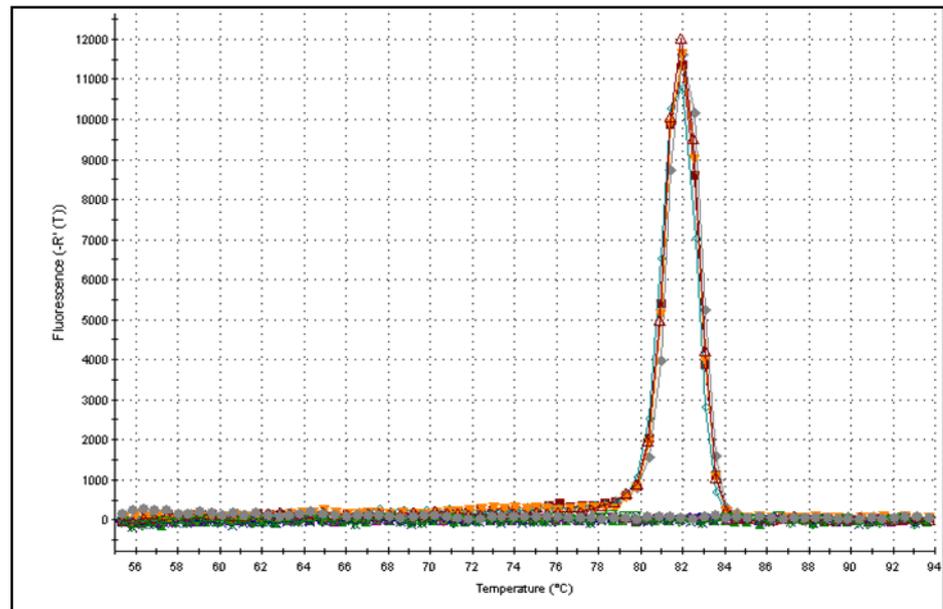
Figure 4.11   SYBR® Green real-time PCR analysis of AQP8 gene expression.

Gene expression of AQP8 was assessed in diluted cDNA samples of MDA-MB-231 cells using pre-validated primers. (A) Amplification curves showed that the mean Ct value for AQP8 in these cells was 31.31. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 82°C suggesting excellent primer specificity; n=3.
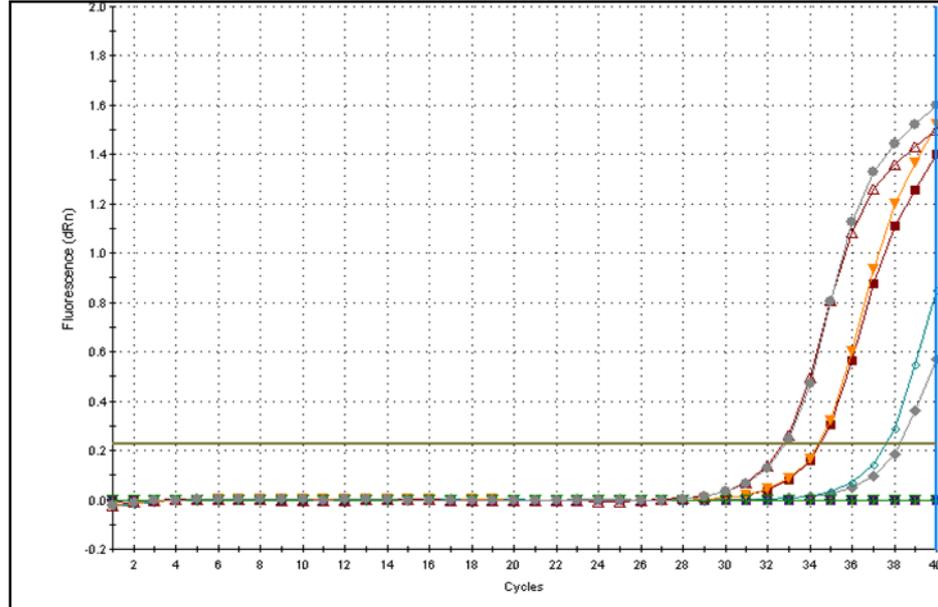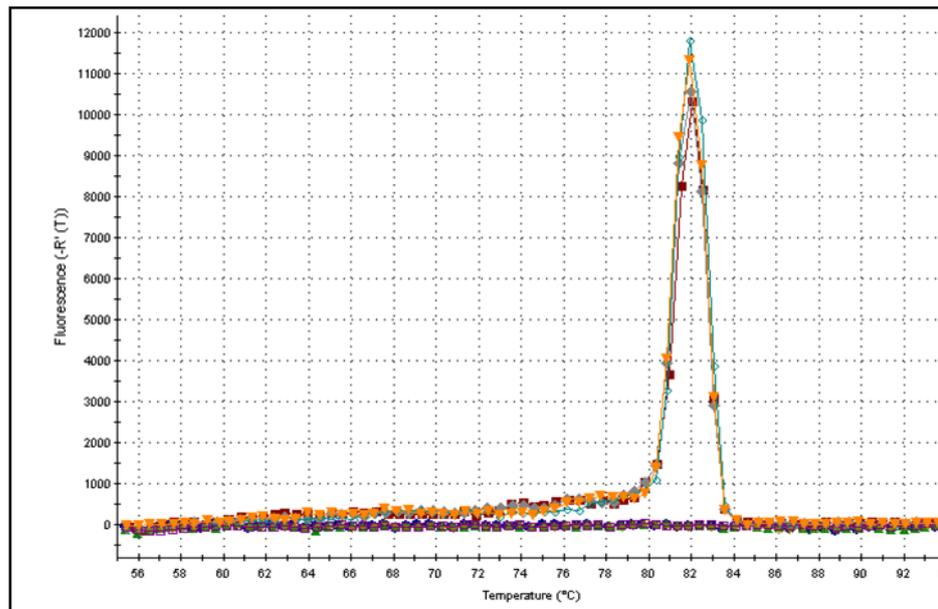
Figure 4.12   SYBR® Green real-time PCR analysis of AQP9 gene expression.

Gene expression of AQP9 was assessed in diluted cDNA samples of MDA-MB-231 cells using pre-validated primers. (A) Amplification curves showed that the mean Ct value for AQP9 in these cells was 32.89. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 82°C suggesting excellent primer specificity; n=3.
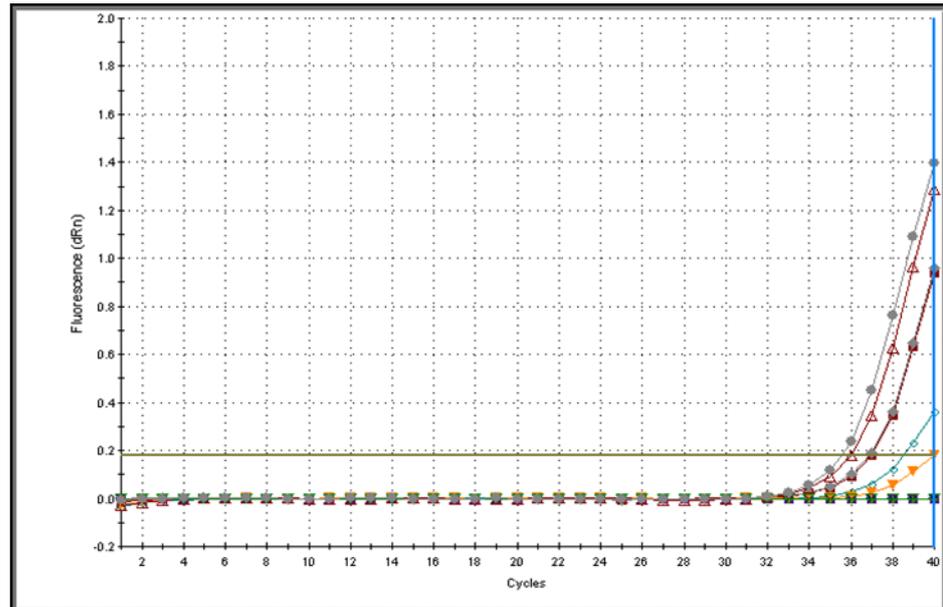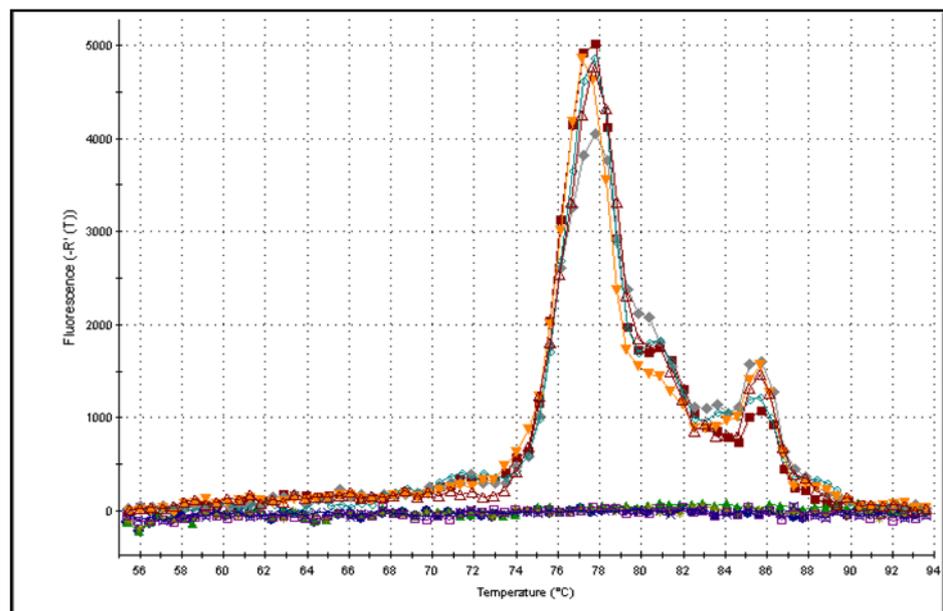
Figure 4.13   SYBR® Green real-time PCR analysis of AQP10 gene expression.

Gene expression of AQP10 was assessed in diluted cDNA samples of MDA-MB-231 cells using pre-validated primers. (A) Amplification curves showed that the mean Ct value for AQP10 in these cells was 35. (B) Dissociation curve analysis showed that the amplicon from the PCR reaction dissociated at approximately 78°C showing adequate primer specificity; n=3.
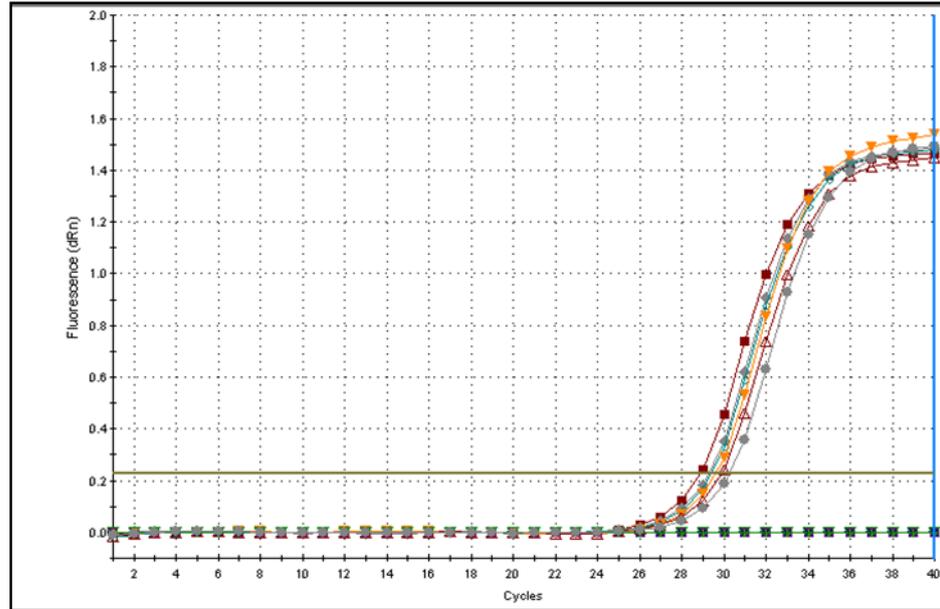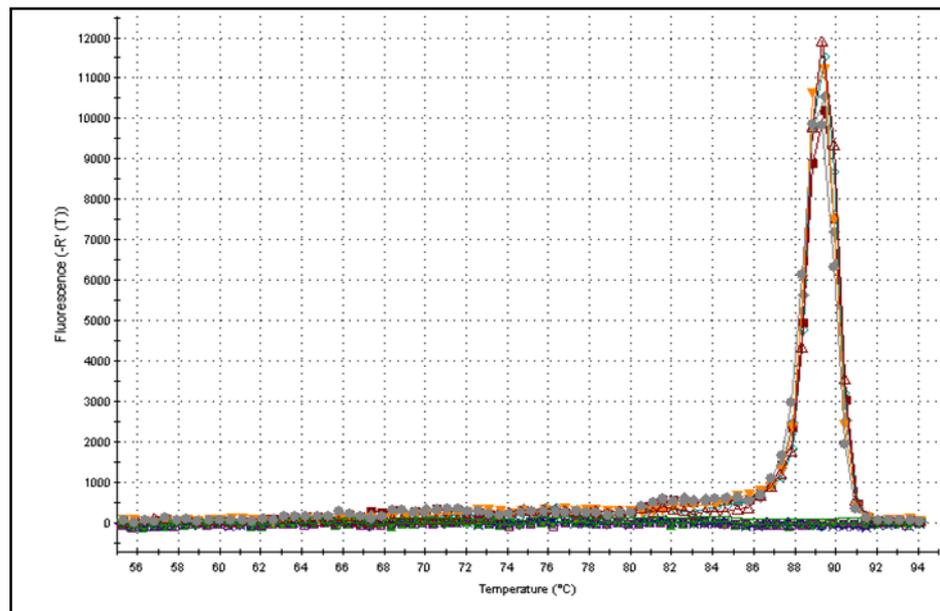
Figure 4.14   SYBR® Green real-time PCR analysis of AQP11 gene expression.

Gene expression of AQP11 was assessed in diluted cDNA samples of MDA-MB-231 cells using pre-validated primers. (A) Amplification curves showed that the mean Ct value for AQP11 in these cells was 27.52. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 89°C suggesting excellent primer specificity; n=3.
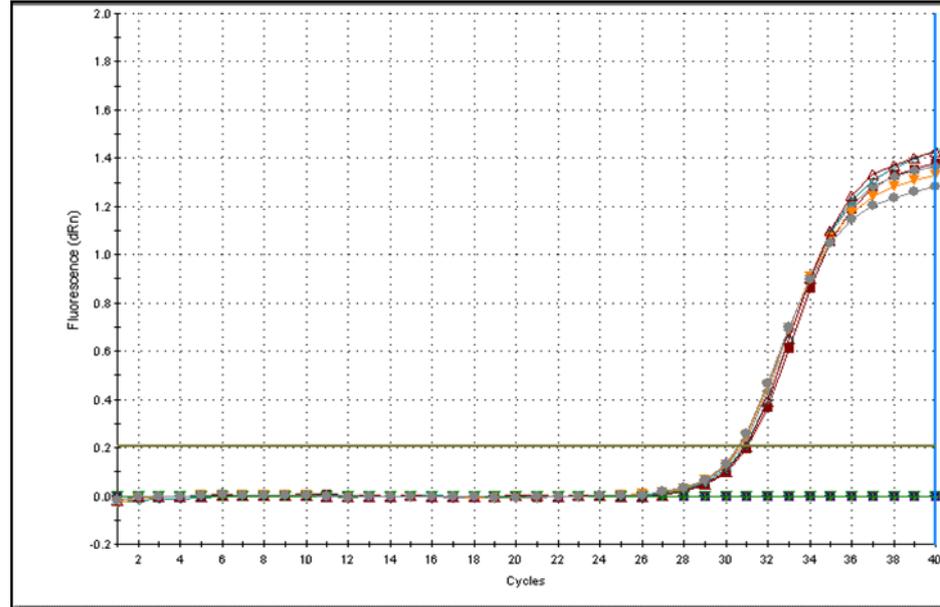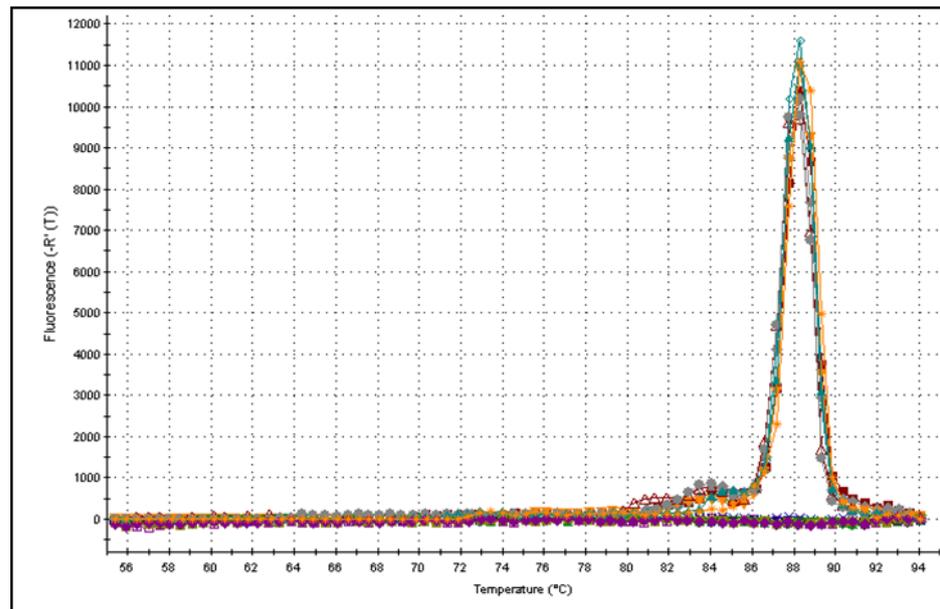
Figure 4.15   SYBR® Green real-time PCR analysis of AQP12 gene expression.

Gene expression of AQP12 was assessed in diluted cDNA samples of MDA-MB-231 cells using pre-validated primers. (A) Amplification curves showed that the mean Ct value for AQP12 in these cells was 28.89. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 88°C suggesting excellent primer specificity; n=3.

Table 4.1   Mean C_t values from real-time PCR reactions for housekeeping genes and all AQPs in MDA-MB-231 cells.

Gene	Mean C_t values
Actin	20.61
YWHAZ	21.28
AQP0	30.02
AQP1	32.81
AQP2	36.05
AQP3	26
AQP4	31.43
AQP5	31.34
AQP6	30.57
AQP7	30.3
AQP8	31.31
AQP9	32.89
AQP10	35
AQP11	27.52
AQP12	28.89
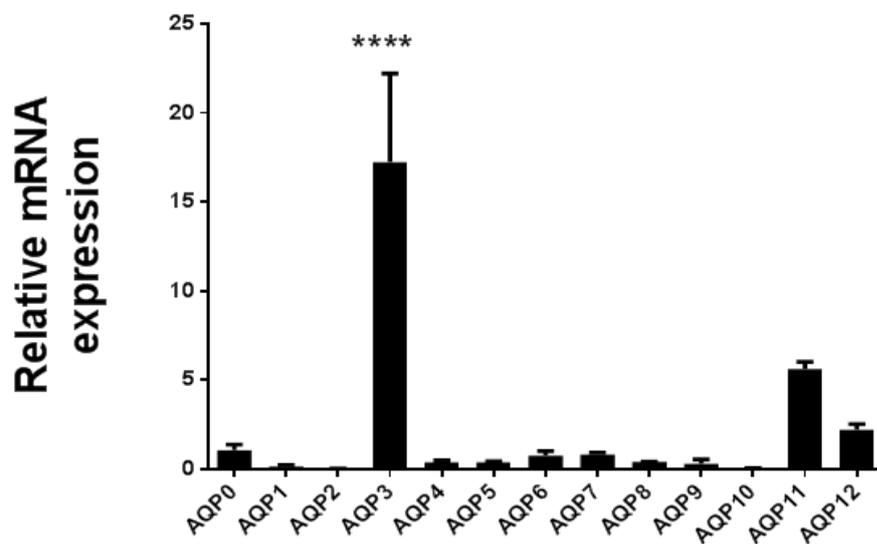
Figure 4.16   Relative AQP mRNA Expression in MDA-MB-231 cells.

The expression of AQPs was normalised to beta actin and YWHAZ housekeeping genes. Relative expression of all AQPs was compared to AQP0, calculated by $2^{-\Delta\Delta Ct}$ by using Ct values shown in Table 4.1. The mRNA expression of AQP3 was significantly higher than all other AQPs. Data are shown as mean ± SEM; n = 3, ****p <0.0001.

4.3.2 Relative AQP3 mRNA expression in MCF-7 and MDA-MB-231 cells

Real-time PCR reaction was performed to further investigate the AQP3 gene expression in MCF-7 (ER and PR positive cell line) and MDA-MB-231 (TNBC cell line) cells. The expression of AQP3 was normalised to beta actin and YWHAZ housekeeping genes. Relative expression of AQP3 in MCF-7 and MDA-MB-231 cell lines was compared using the $2^{-\Delta\Delta Ct}$ method. The mRNA expression of AQP3 was significantly reduced in MCF-7 cells (mean fold value 1.40, p <0.05) than MDA-MB-231 cells. Statistical significance was assessed by Student's t test (see Figure 4.17).
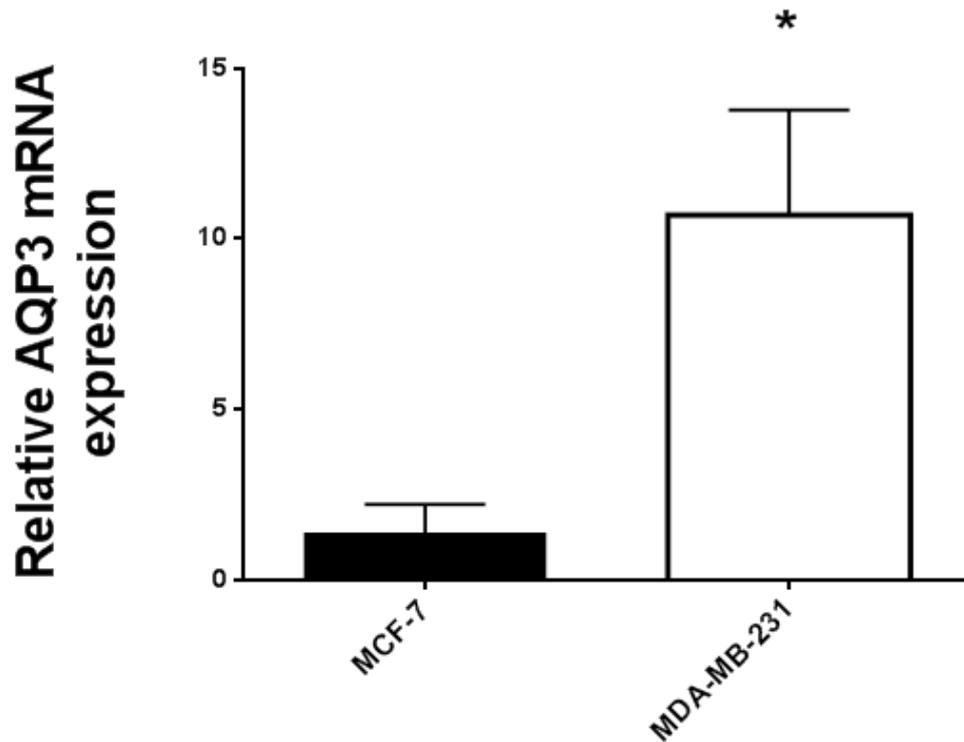
Figure 4.17   Expression of AQP3 mRNA in MCF-7 and MDA-MB-231 cells.

qPCR analysis of AQP3 mRNA expression in MCF-7 and MDA-MB-231cells showed that AQP3 expression level in MDA-MB-231 cells was significantly higher than in MCF-7 cells. Bar graph displaying the relative fold difference of AQP3 in both cell lines internally normalised to housekeeping genes. Data represent mean ± SEM; *p = 0.043, n=3.

4.3.3   Expression of AQP3 by Immunocytochemistry in MDA-MB-231 cells

To examine the AQP3 protein expression in MDA-MB-231 cells, immunofluorescence staining of AQP3 peptide was performed. The cells were stained with rabbit anti-AQP3 antibody and viewed on a Leica DMI4000B inverted microscope by using GFP (for DyLight488) and A4 (for DAPI) filter cubes. The results demonstrated the AQP3 expression in cytoplasm as well as on the cell membrane of MDA-MB-231 cells (see Figure 4.18). The result further confirmed the expression of AQP3 in MDA-MB-231 cells. This finding also suggested that MDA-MB-231 cells mostly express AQP3 in cytoplasm which might be translocated to the cell membrane like most AQPs.
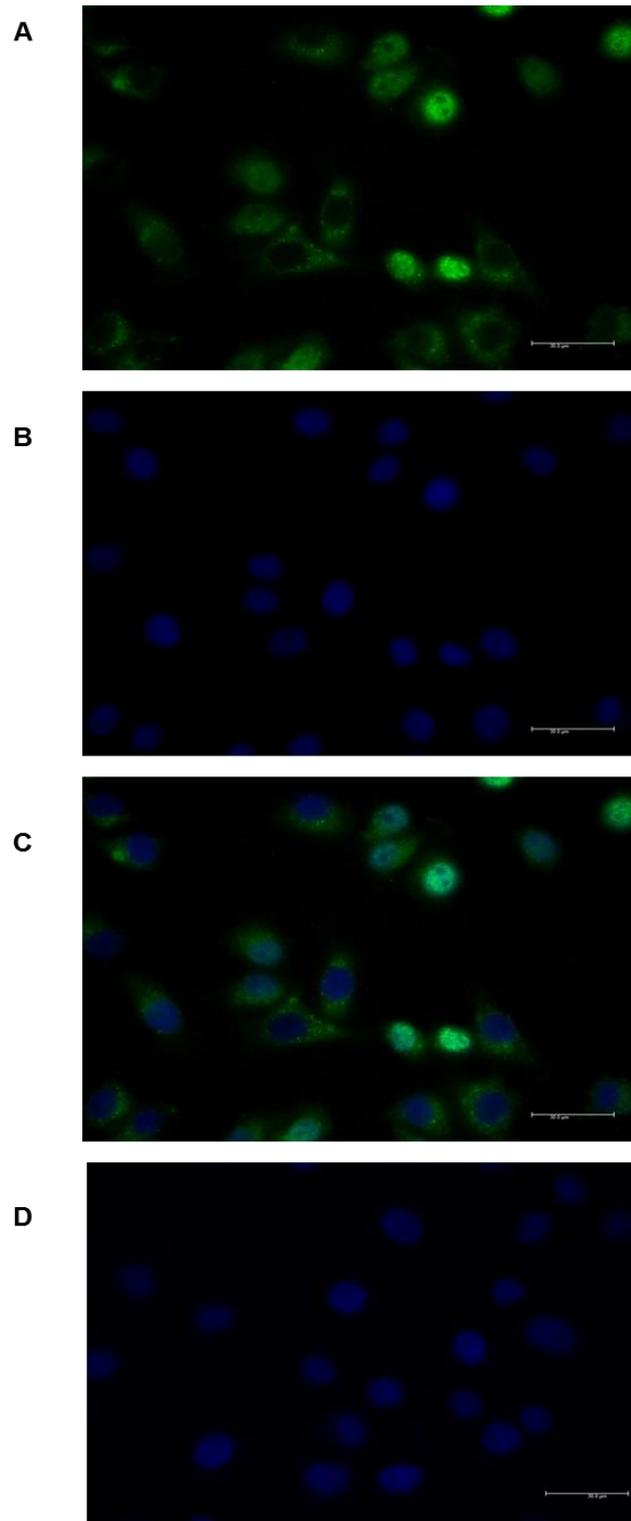
Figure 4.18   Fluorescence Immunocytochemistry for AQP3 in MDA-MB-231 cells

Immunocytochemical characterisation of AQP3 protein expression in MDA-MB-231 cells stained with (A) DyLight488 dye-conjugated AQP3 antibody for AQP3 protein and (B) DAPI for nucleus. (C) Overlaid image of A and B. (D) Negative control showing DAPI staining only. Bar =30 μm. Image is representative of n=5 experiments.

4.3.4　Puromycin Sensitivity

As described in chapter 2 the AQP3-silencing shRNA plasmid contained a puromycin selection marker therefore puromycin was used to select cells that stably expressed the vector. Consequently, it was necessary to detect the optimal concentration of puromycin needed to kill the MDA-MB-231 cells without transfected vector. The effect of puromycin on MDA-MB-231 cell sensitivity was investigated over 24, 48 and 72 h (see Figures 4.19 - 4.21).
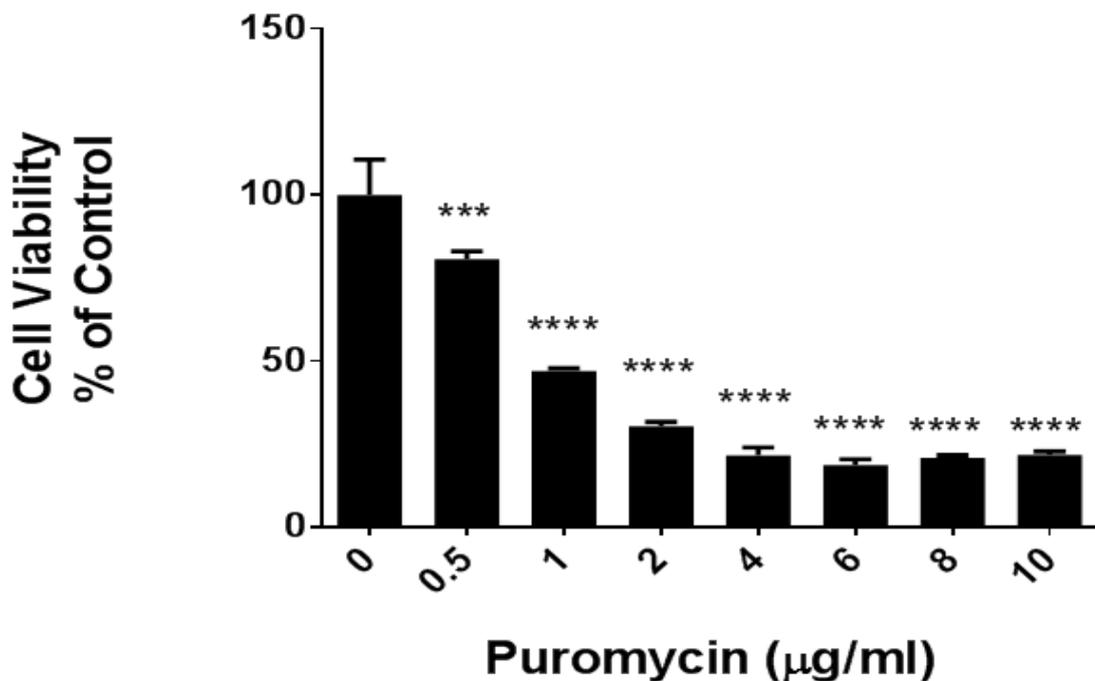
Figure 4.19　Puromycin Sensitivity after 24 h in MDA-MB-231 cells.

MDA-MB-231 cells were seeded at 20,000 cells/well in a 96 well plate and incubated for 24 h in the presence of indicated concentrations of Puromycin in triplicate. Data show that puromycin reduces the cell viability of cells. Results are expressed as percentage of non-treated control. Data are shown as mean ± SEM, n = 3, ***p <0.001, ****p <0.0001.
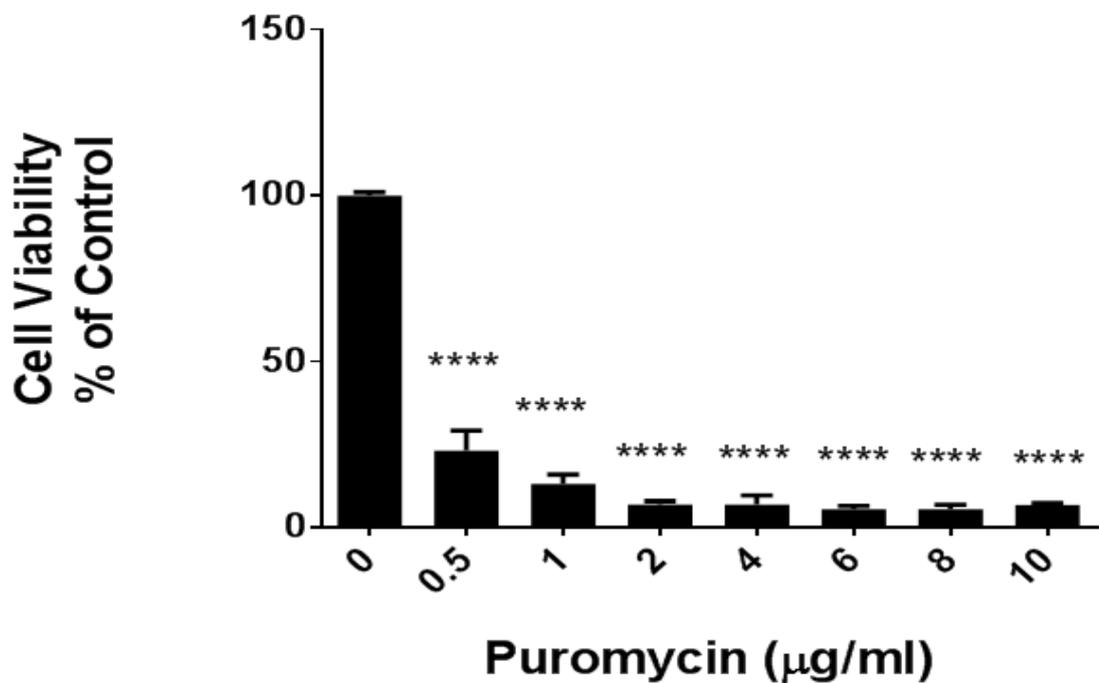
Figure 4.20   Puromycin Sensitivity after 48 h in MDA-MB-231 cells.

MDA-MB-231 cells were seeded at 20,000 cells/well in a 96 well plate and incubated for 48 h in the presence of indicated concentrations of Puromycin in triplicate. Data show that puromycin reduces the cell viability of cells. Results are expressed as percentage of non-treated control. Data are shown as mean ± SEM, n = 3, ****p <0.0001.
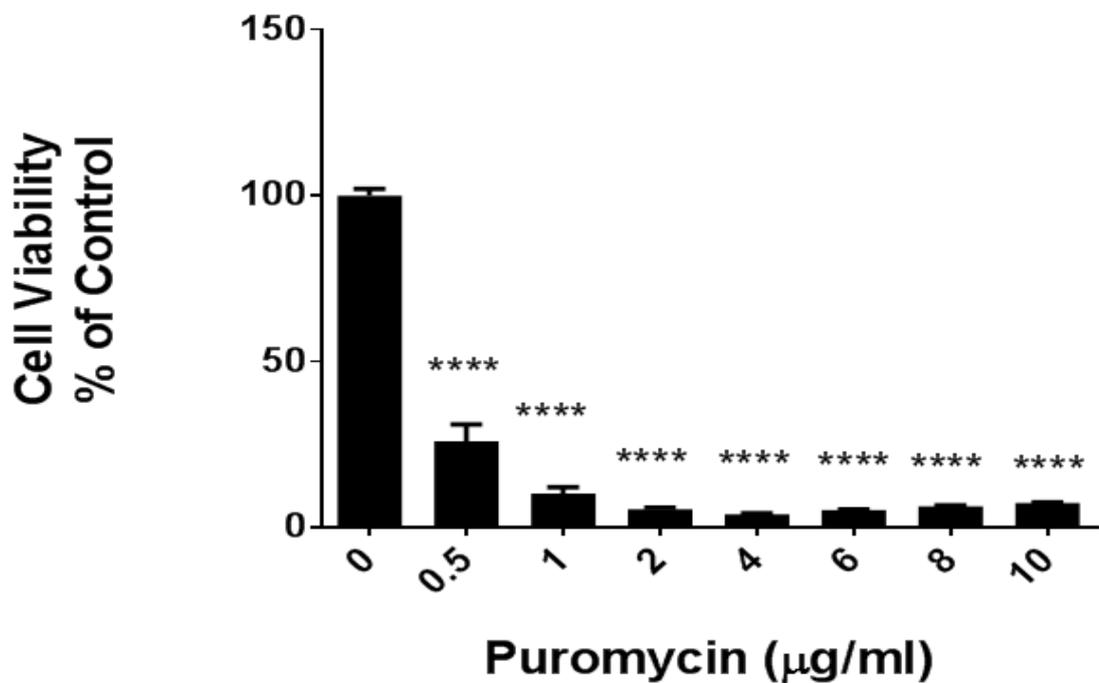
Figure 4.21   Puromycin Sensitivity after 72 h in MDA-MB-231 cells.

MDA-MB-231 cells were seeded at 20,000 cells/well in a 96 well plate and incubated for 72 h in the presence of indicated concentrations of Puromycin in triplicate. Data show that puromycin reduces the cell viability of cells. Results are expressed as percentage of non-treated control. Data are shown as mean ± SEM, n = 3, ****p <0.0001.

4.3.5 Real-time PCR analysis of AQP3 mRNA expression in selected AQP3 knockdown clones.

Insertion of AQP3 shRNA constructs and scrambled sequence into MDA-MB-231 cells produced transfected cells. Cells were imaged 24 h post-transfection (see Figures 4.22 to 4.24) as described in chapter 2 showing transfected cells under fluorescence microscope. Transfected cells were allowed to grow in the presence of 2 μg/mL puromycin till single cell colonies appeared. A single colony (see Figure 4.25) was picked and further serial diluted to generate single cell clones as mentioned in chapter 2. Stable cell lines were generated for all constructs (see Figure 4.26) including 841, 844 and SC clones.

Real-time PCR reaction was performed for all clones to confirm the level of knock down of AQP3. The amplification curves of WT, SC 841 and 844 for AQP3 expression illustrated in Figure 4.27A. The expression of AQP3 was normalised to beta actin and YWHAZ housekeeping genes. Relative expression of AQP3 in knockdown and scrambled control cell lines was compared to WT using the $2^{-\Delta\Delta Ct}$ method. The mRNA expression of AQP3 was significantly reduced in 844 cells with mean fold difference of 0.922 and 0.852 as compared to WT and SC respectively. Similarly, the mRNA expression of AQP3 was significantly reduced in 841 cells with mean fold difference of 0.792 and 0.722 as compared to WT and SC respectively (see Figure 4.28).
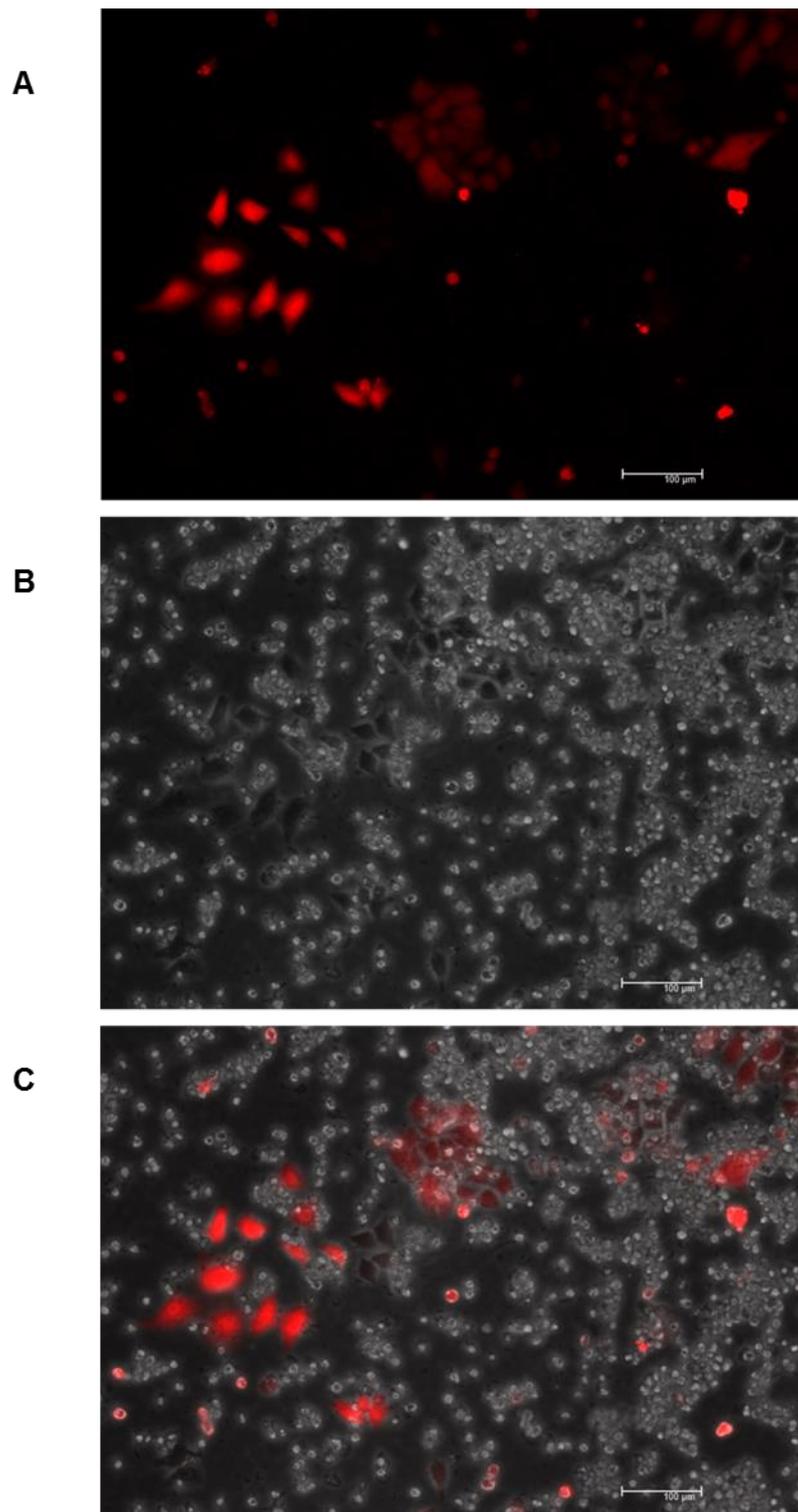
Figure 4.22   24 h Post Transfection Images of SC (015).

MDA-MB-231 cells were transfected with RFP (red) tagged SC shRNA vector by using GenJet™ transfection reagent. The cells were visualized 24 h post transfection by Leica DMI4000 B inverted microscope with (A) Bright Field imaging, (B) DsRred imaging and (C) Overlay image. Bar =100 µm
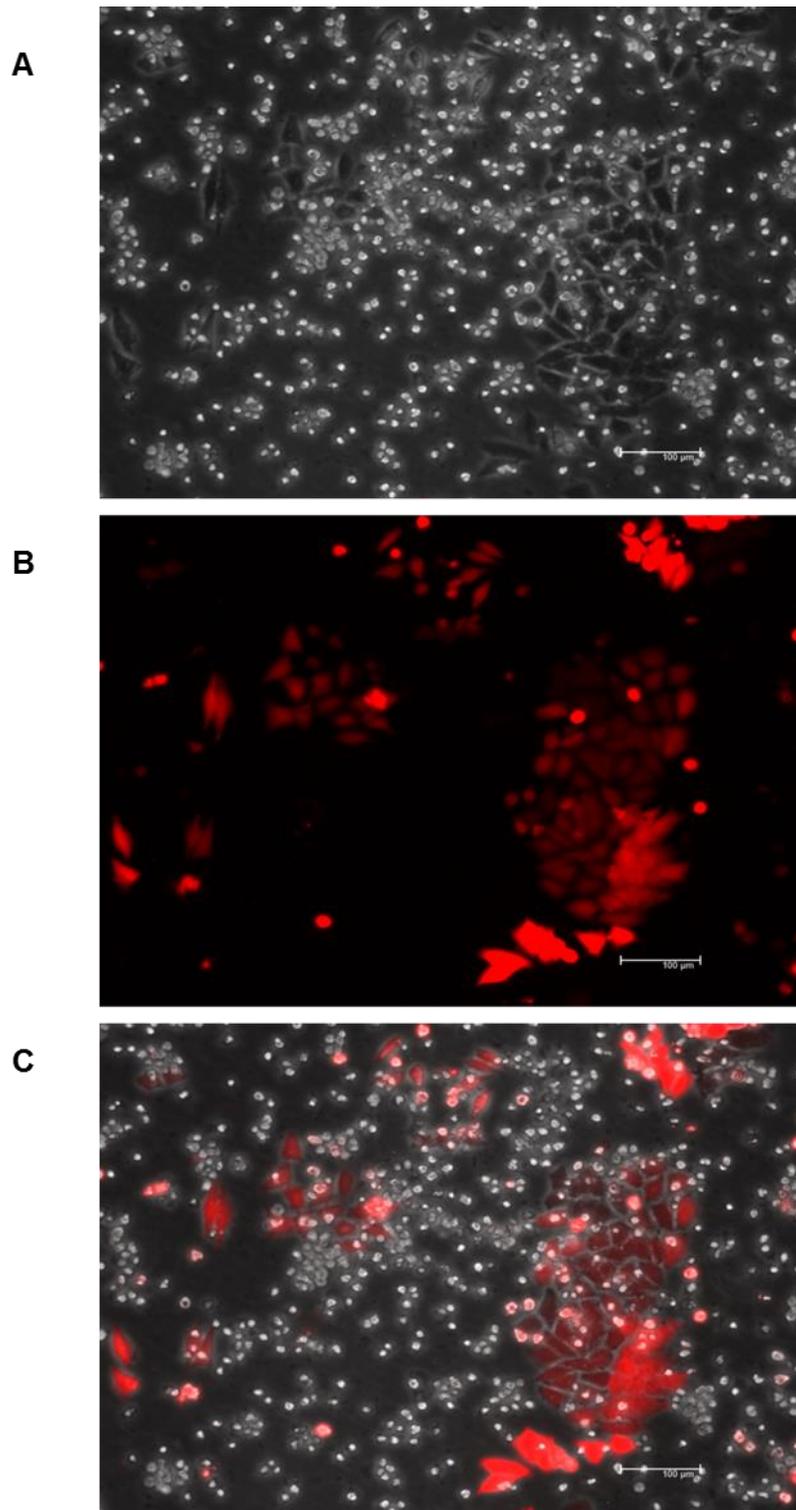
Figure 4.23   24 h Post Transfection Images of 841

MDA-MB-231 cells were transfected with RFP (red) tagged AQP3 shRNA (841) vector by using GenJet™ transfection reagent. The cells were visualized 24 h post transfection by Leica DMI4000 B inverted microscope with (A) Bright Field imaging, (B) DsRred imaging and (C) Overlay image. Bar =100 µm
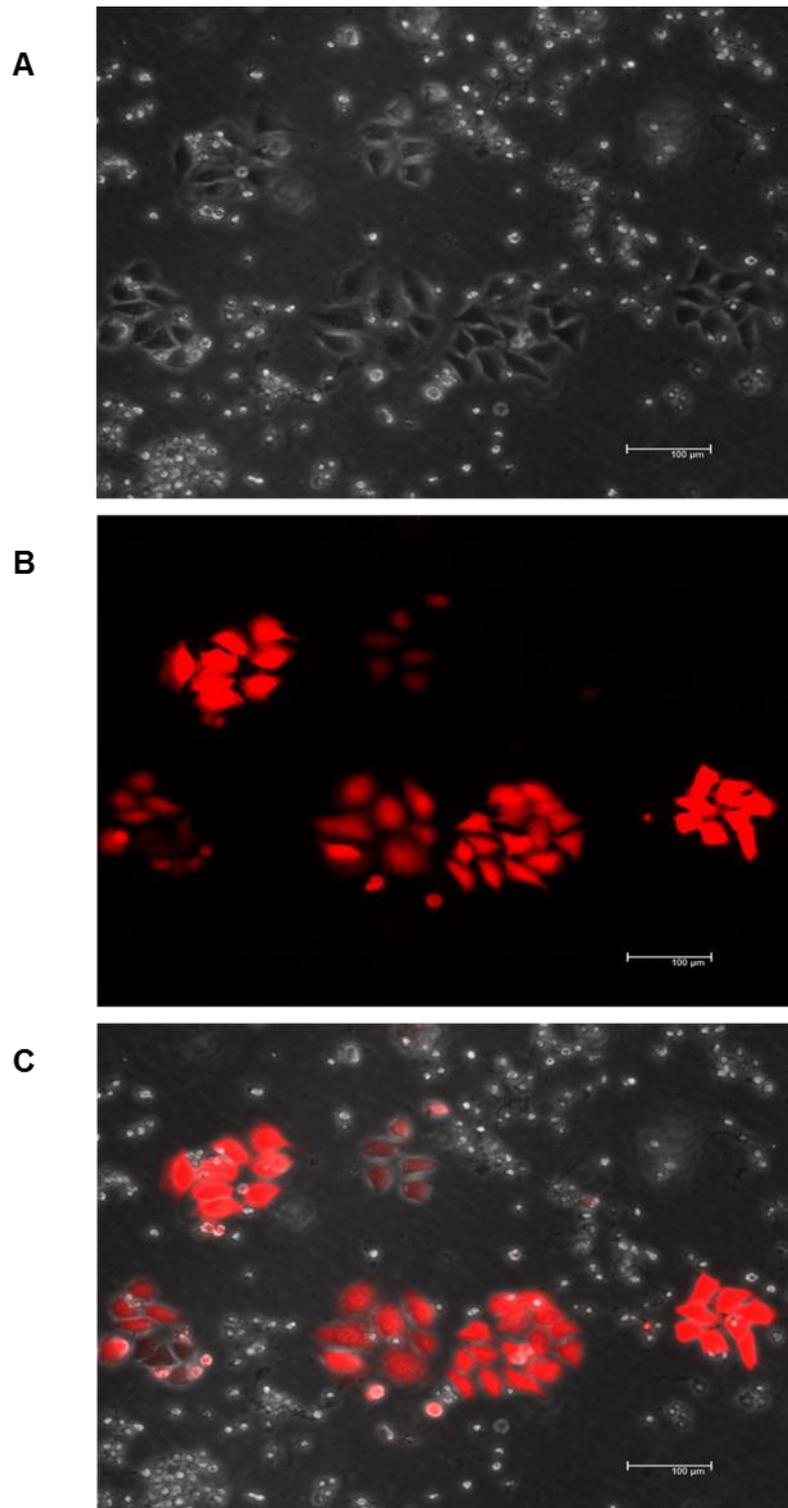
Figure 4.24   24 h Post Transfection Images of 844

MDA-MB-231 cells were transfected with RFP (red) tagged AQP3 shRNA (841) vector by using GenJet™ transfection reagent. The cells were visualized 24 h post transfection by Leica DMI4000 B inverted microscope with (A) Bright Field imaging, (B) DsRred imaging and (C) Overlay image. Bar =100 µm
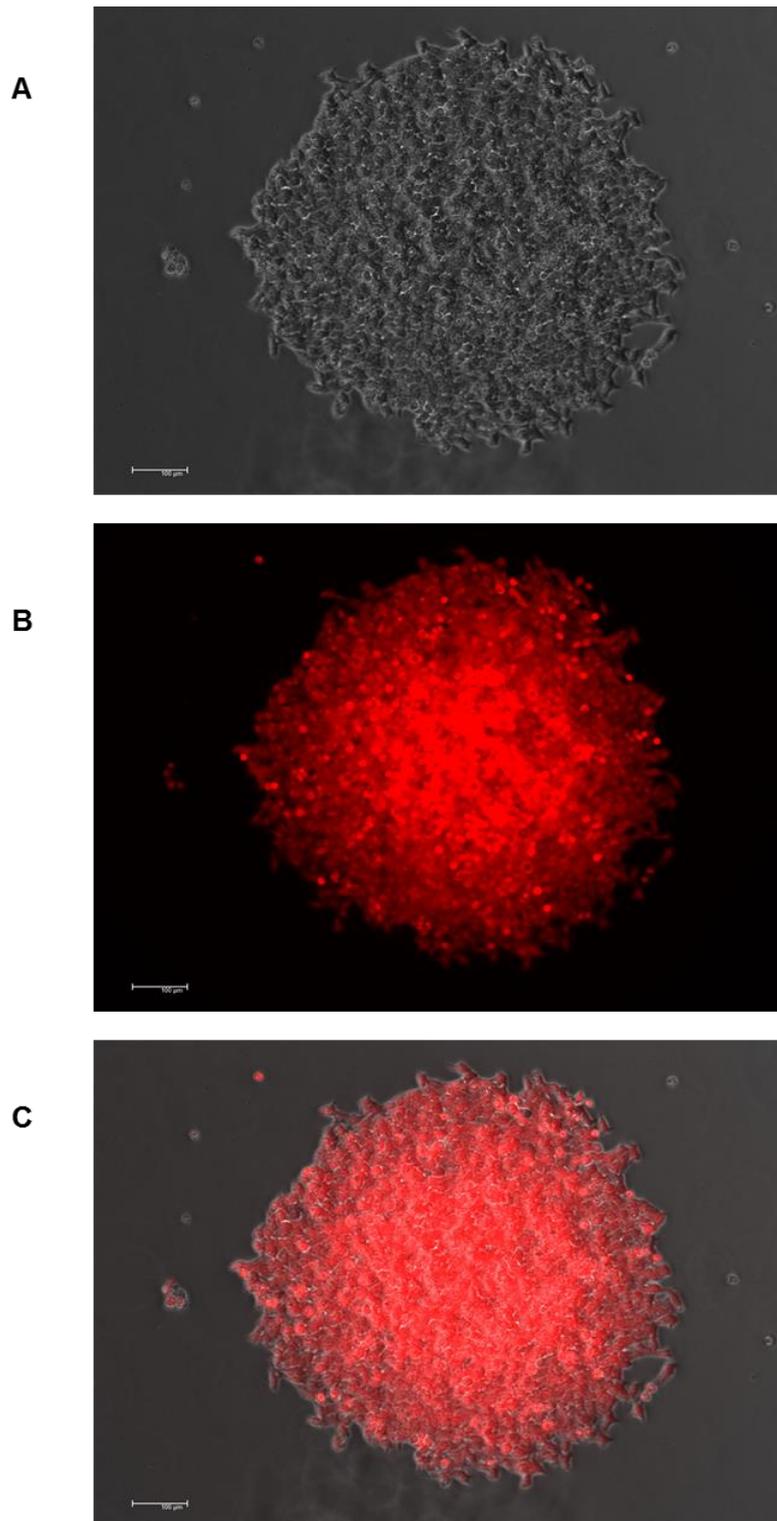
Figure 4.25   Typical example of the appearance of colonies used for clonal selection.

Bright field microscopy demonstrating typical appearance of clones growing pre-selection. Colonies of transfected cells derived from MDA-MB-231 cells using 2 µg of AQP3 shRNA vectors were selected with 2 µg/mL puromycin for two weeks. Bar =100 µm.
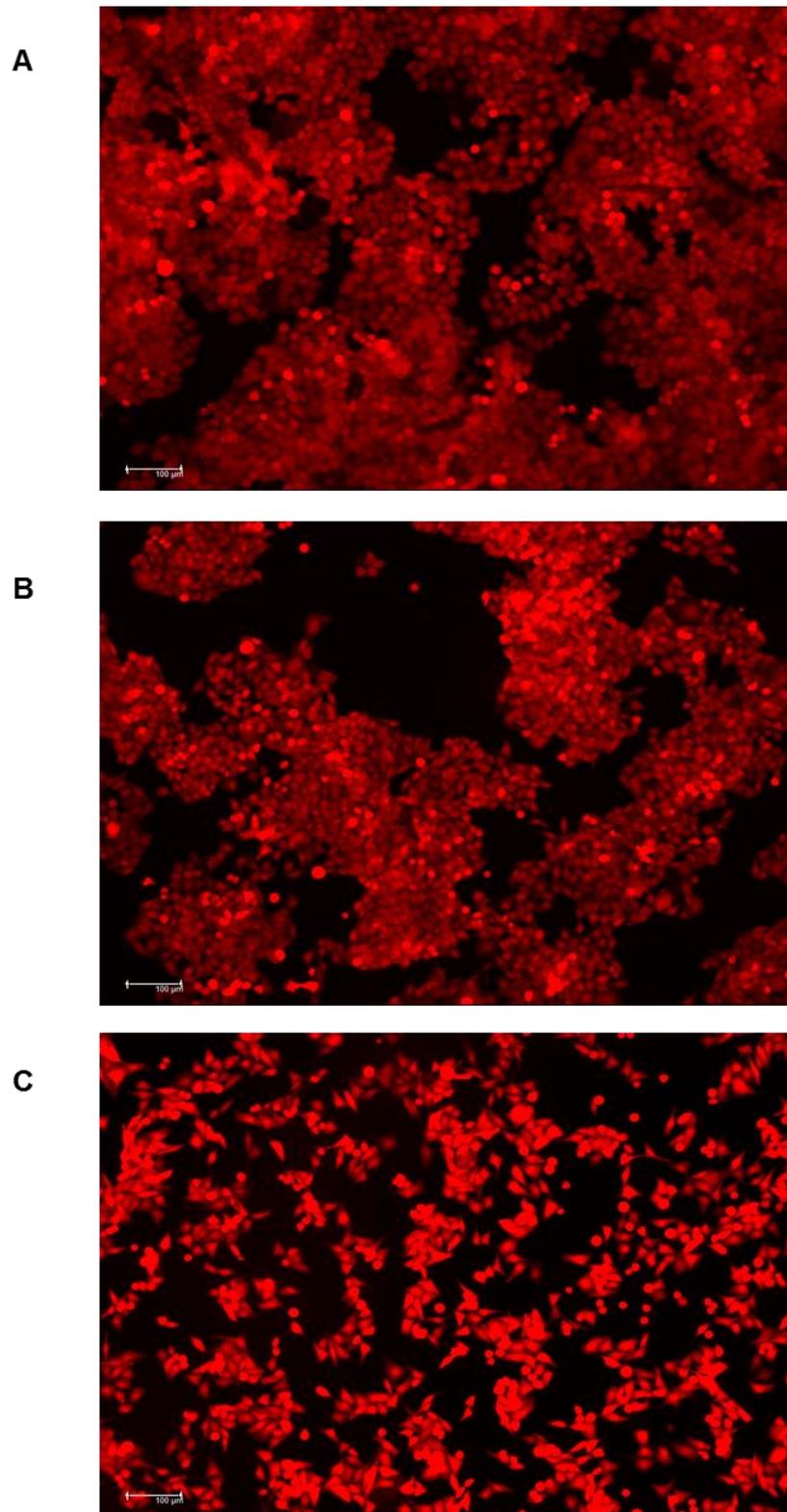
Figure 4.26   Generation of Stable SC, 841 and 844 Cell Lines

Cells transfected with 2 µg of shRNA construct were selected with 2 µg/mL puromycin for two weeks. Clones were picked and expanded for an additional 1 month and stable cell lines were generated (A) negative control SC (015), (B) AQP3 shRNA construct 841and (C) AQP3 shRNA construct 844. Bar =100 µm.
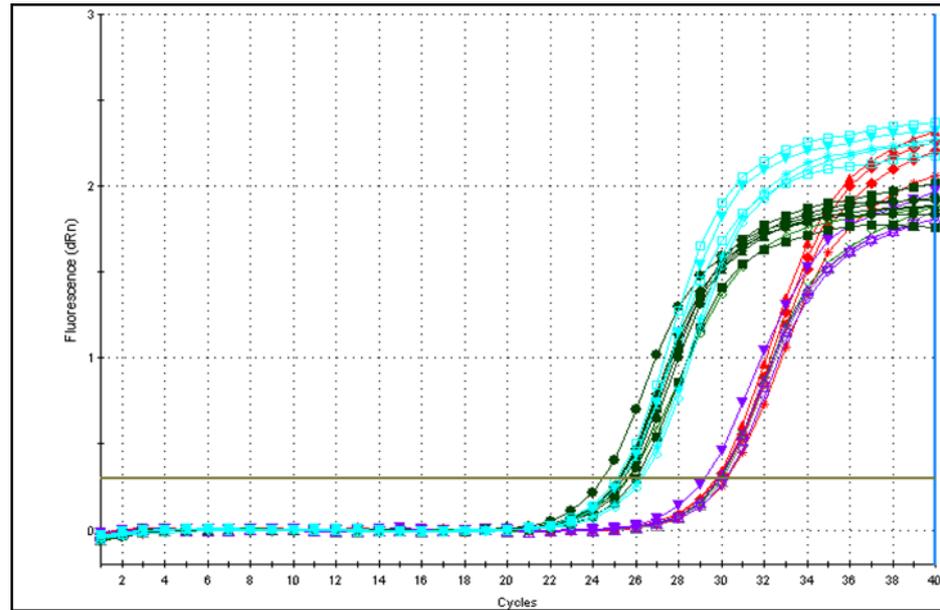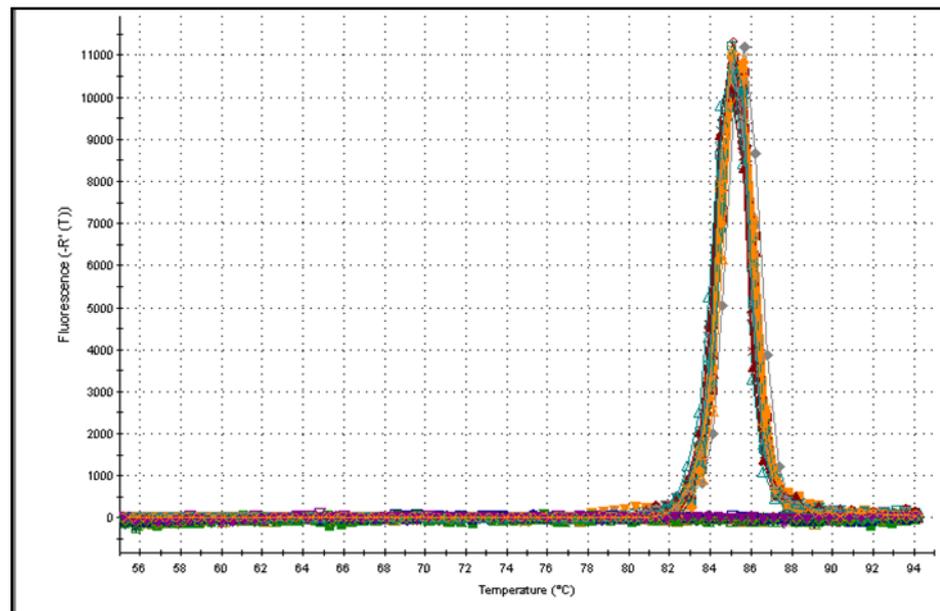
Figure 4.27   SYBR® Green real-time PCR analysis of AQP3 gene expression.

Gene expression of AQP3 was assessed in diluted cDNA samples of WT, SC, 841 and 844 cells using pre-validated primers. (A) Amplification curves generated for AQP3. Blue and green curves represent WT and SC respectively whereas red and purple curves represent 841 and 844 respectively. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 85°C suggesting excellent primer specificity; n=3.
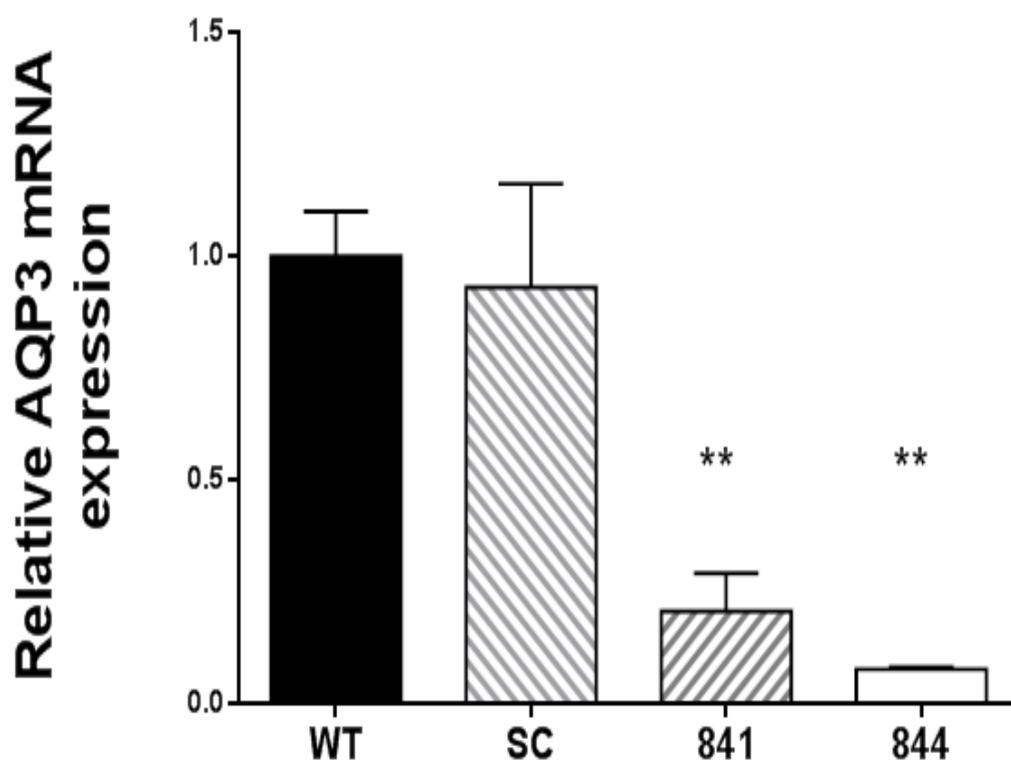
Figure 4.28   Real-time PCR analysis of AQP3 gene expression.

qPCR analysis of AQP3 mRNA expression in WT, SC, 841 and 844 samples showed that AQP3 mRNA expression was significantly reduced in 841 and 844 cells as compared to WT and SC cells. Bar graph displaying the corresponding relative fold difference internally normalised to housekeeping genes. Relative fold expression was calculated by comparing the normalised fold difference of SC, 841 and 844 to WT. The mRNA expression of AQP3 was significantly reduced in both 841 (mean fold difference 0.792) and 844 (mean fold difference 0.922) cells. Data represent mean ± SEM with **p value <0.01; n=3. Significance was tested by one way of ANOVA with Dunnett's post-hoc test.

4.3.6 Confirmation of AQP3 protein levels in selected shRNA clones by Western blotting

Cell lysates of WT and transfected cells were run on a SDS-PAGE gel and analysed using Western blotting in order to assess the knockdown efficiency. Western blotting showed reduction in AQP3 protein expression in 844 and 841 cells as compared to WT and SC cells (see Figure 4.29A). Here band intensity was quantified using Image J software and normalised to relevant tubulin expression from the same stripped blots. 844 cells exhibited approximately 73% and 69% significant reduction in AQP3 expression as compared to WT and SC ($p < 0.01$) respectively as shown in Figure 4.29B. Similarly 841 cells inhibited AQP3 protein expression approximately 66% and 61% as compared to WT and SC ($p < 0.01$) respectively.
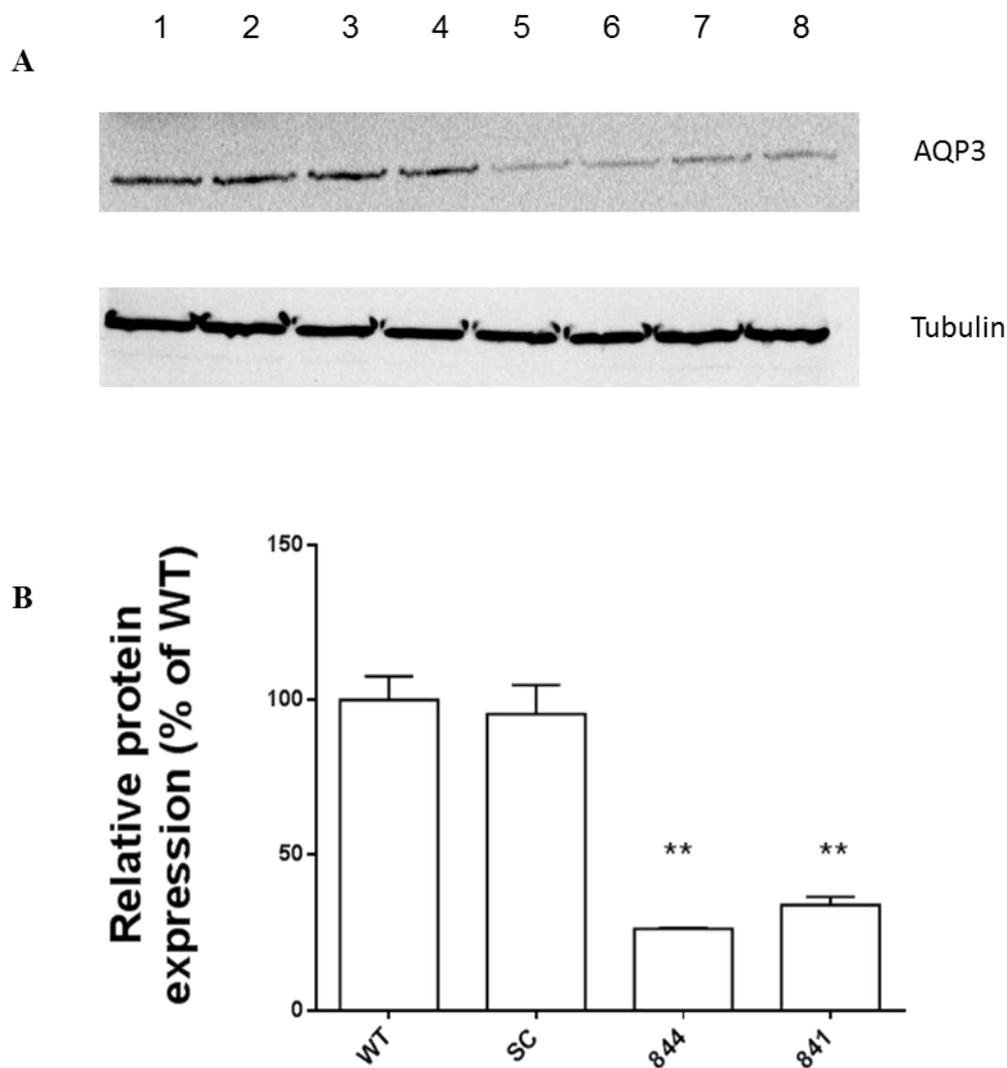
Figure 4.29   Western Blot analysis of AQP3 protein in MDA-MB-231 cells

Western blot analysis of AQP3 protein expression in whole cell lysates of WT, SC, 844 and 841 showed reduced expression of AQP3 in 844 and 841 as compared to WT and SC cell lysates. (A) Representative Western blots of bands generated for AQP3 and tubulin protein expression. Bands in lane 1and 2 represent WT, lane 3 and 4 represent transfected SC, lane 5 and 6 represent AQP3-targeted shRNA (844) and lane 7 and 8 represent AQP3-targeted shRNA (841). (B) Graph displaying relative levels of AQP3 protein expression in WT, SC, 844 and 841 samples. Relative density of AQP3 bands was normalised with corresponding density of tubulin bands. 844 and 841 cells demonstrated reduced level of AQP3 protein expression to 26.27% and 33.88% respectively; ** p <0.01.

4.3.7   mRNA expression profile of AQPs in WT and 844 cells

As 844 cells showed minimum level of AQP3 expression for mRNA and protein, therefore these cells were selected for further work in the following chapters. The expression profile for all AQPs was investigated in 844 cells to confirm that other AQPs did not compensate for the loss of AQP3 in the knockdown cell line. SYBR® Green qPCR was performed to investigate the gene expression of all AQPs in WT and 844 cells. AQP3 knockdown in 844 cells did not significantly change the expression levels of other AQPs compared to WT. The expression of AQPs was normalised to beta actin and YWHAZ housekeeping genes. Relative expression of all AQPs was compared to AQP0 using the $2^{-\Delta\Delta Ct}$ method. Statistical significance was assessed by Student's t test.
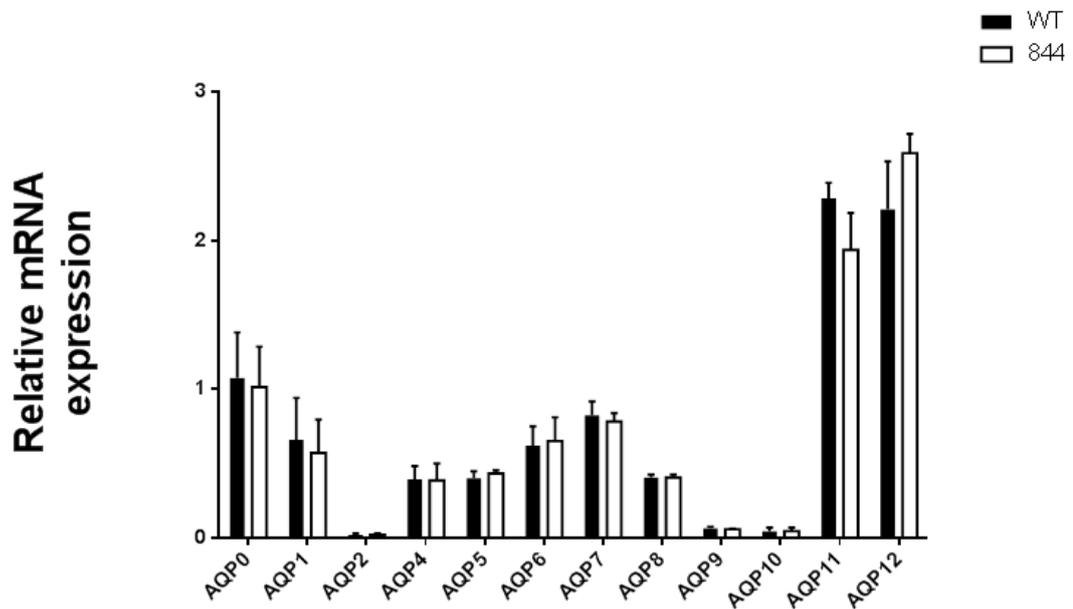
Figure 4.30   Relative AQPs mRNA expression apart from AQP3 in WT and 844 cells.

Relative expression of all AQPs was compared to AQP0 for both WT and 844 cells. No significant difference was observed in the expression levels of any other AQPs between WT and 844 cells. Data are shown as mean ± SEM; n = 3.

4.4 Discussion

The main objectives of this chapter were to knockdown AQP3 in MDA-MB-231 cellular model and to manipulate AQP3 expression for future work on cellular function via generation of stable cell lines. Analysis of AQP family mRNA expression in MDA-MB-231 cells showed that the most abundant transcript in these cells was AQP3 (see Figure 4.16). The higher expression of AQP3 in MDA-MB-231 cell line used as a test model in the present study further confirmed the finding of higher expression of AQP3 in BC tissues as compared to the adjacent normal tissues reported in chapter 3. Overexpression of AQP3 was reported previously in WalBC breast cancer cell line, which was established from a primary breast cancer in a female Tammar Wallaby. In this study gene expression profiling using a microarray was performed on the WalBC cell line has identified a profile similar to human basal-like breast cancer (Sharp *et al.*, 2008). Similarly gene expression analysis in human breast tissue samples also indicated the expression of AQP3 in some cases of inflammatory breast cancer (Dressman *et al.*, 2006). Very recently at the time of writing this thesis, Cao and colleagues reported AQP3 protein expression induced by FGF-2 in MDA-MB-231 and Bcap-37 cell lines (Cao *et al.*, 2013). This study further validated the findings presented here that AQP3 is overexpressed in BC cell lines.

As expression of AQP3 was found to be high, and taking the previously published findings mentioned above into consideration, it was decided that producing cell clones that had a knocked-down expression of AQP3 would provide an excellent model to study the role of AQP3 in breast cancer cells. Two clones that exhibited reduced levels of AQP3 expression were produced (841 and 844 clones) and a negative control scrambled sequence clone was also produced (clone 015 referred to as SC). Alongside the gene-silencing technique, this study originally included an overexpression model to investigate whether further increasing AQP3 expression had functional consequences. In this series of experiments AQP3 was inserted into MDA-MB-231 cells using methods listed in chapter 2. Unfortunately, after some initial experiments listed in the following

chapters, a critical loss of liquid nitrogen led to loss of all stored AQP3 over-expressing cell clones. This led to an immediate cessation of over-expression work due to time limitations.

Previous studies have employed similar vector-based shRNA methodologies to inhibit the expression of AQPs in different cell types with functional consequences. For example, modulation of AQP1 expression using shRNA constructs was shown to cause a 55% reduction in protein expression in glioma cells (with a resulting 70% reduction in cell migration) (McCoy and Sontheimer, 2007). Transfection of the AQP3 siRNA expression vector into human lung adenocarcinoma (CL3 cells) has been shown to decrease AQP3 protein expression by approximately half which resulted in lower arsenic accumulation in arsenic resistant cells (Lee *et al.*, 2006). It has also been previously reported that knockdown of AQP3 by specific siRNA caused suppression of squamous cell carcinoma (SCC) of the tongue (Kusayama *et al.*, 2011). The role of AQP3 in freeze tolerance in human prostate cancer cells was also reported by using siRNA. The knock down AQP3 protein expression resulted in increased sensitivity of cancer cell to cryoinjury compared with untreated cells (Ismail *et al.*, 2009). Similarly recently, it is reported (as mentioned before) that transfection of one construct shRNA inhibited the expression of AQP3 by 65% and 75% and second construct 54% and 63% in MDA-MB-231 and Bcap37 cells, respectively (Cao *et al.*, 2013).

Taken together these studies demonstrated that RNAi can generally achieve approximately 50-75% inhibition of AQP protein expression with few exceptions where more than 90% knockdown was achieved. In the present study, the AQP3 protein expression in 841 and 844 cell lines was reduced to approximately 66% and 73% respectively although the mRNA expression was knockdown by over 90%. The complexity of protein folding mechanisms and other cellular factors could be reason of achieving lesser reduction of protein expression. Nevertheless as mentioned above, many studies have reported inhibition of protein expression in AQPs by RNAi only to 50-75% and only rarely few studies achieved over 90% AQP protein inhibition. Therefore reduction of AQP3 expression in present study correlates well with others. As 844 cells showed highest inhibition of AQP3

expression at both mRNA and protein levels as compared to 841 cells, therefore 844 was selected for further experimental work in the following chapters. Although most experiments were performed for 841 cell line as well (at least once) to ensure that the effects of AQP3 down regulation are not artefacts (data not shown).

In summary AQP3 down-regulated stable cell lines (841 and 844) and a scrambled sequence (represented as SC) were derived from MDA-MB-231 (WT) cells using shRNA mediated cell silencing in a fashion that has previously been used in other published studies to investigate cell function in response to gene manipulation.

Chapter 5

Functional Consequences of AQP3 Gene Knockdown in MDA-MB-231 Cells

5.1 Introduction

Numerous changes may occur in a cancerous cell which ultimately define its biological phenotype (Calabrese et al., 2004). The overall ability of a cancer cell to produce a tumour or a metastatic process are governed by many individual factors including the rate of cellular proliferation, the migratory potential of the cell, the ability of the cell to invade through the basement membrane, cellular adherence, evasion of immune cells and the ability to develop new blood vessels (angiogenesis). These key characteristics of cancer cells provide multiple targets for therapeutic intervention and form the basis of most anti-cancer drugs. For example, proliferation, the key characteristic of a cancer cell is targeted by most chemotherapeutic agents (Ricci and Zong, 2006).

In light of this, obtaining a greater understanding of the biological factors that are involved in regulating these cellular properties is essential in improving our understanding of cancer. Previous studies have shown that AQPs are involved in a number of these cellular functions (Hara-Chikuma and Verkman, 2006a; Levin and Verkman, 2006; Huang *et al.*, 2013) which has increased interest in AQPs as potential therapeutic targets. The present study focussed on AQP3 as it was shown to be the most abundant AQP transcript in MDA-MB-231 cells and increased in clinical BC samples. Using the shRNA-mediated AQP3 knock-down cells described in chapter 4, cellular proliferation, migration, invasion, adherence and resistance to chemotherapeutic agent were assessed.

The aims of this part of the study were to investigate the effects of AQP3 downregulation on cellular migration, proliferation, invasion, adhesion and resistance to chemotherapeutic agents in MDA-MB-231 cells.

5.2 Material and Methods

All methods for functional analysis of AQP3 protein expression in cellular proliferation, migration, invasion, adhesion and effect of 5-FU on cell viability were performed for WT, SC and 844 cells as described in Chapter 2.

Statistical analysis of data was performed using GraphPad Prism 6 software. One-way ANOVA with a Dunnett's post-hoc test for comparisons of > 3 groups or Student's t-test for comparisons of < 3 groups were performed for the statistical analysis between experimental conditions and p values < 0.05 were considered significant.

5.3 Results

5.3.1 AQP3 knockdown reduces MDA-MB-231 cellular proliferation

AQP3 has been identified as a positive regulator of cellular proliferation in several cell types (Levin and Verkman, 2006; Hara-Chikuma and Verkman, 2008a; Nakahigashi *et al.*, 2011). The present study investigated the effects of silencing AQP3 in MDA-MB-231 cells on cellular proliferation. Results showed that AQP3 knockdown in MDA-MB-231 cells produced a significant anti-proliferative effect. Analysis of total cell number 72 h post-seeding showed that AQP3-knockdown cells (844) had a 28% reduction ($\pm$ 9%) in total cell number compared to WT and SC cells (p <0.0001, Figure 5.1). Repeated counting at 24, 48 and 72 h time points was used to calculate the doubling time of cells which were as follows: WT = 30.98 h, SC = 31.24 h and 844 = 39.27 h. AQP3 silencing therefore significantly (p <0.05) decreased the rate of cellular proliferation in MDA-MB-231 cells resulting in an increased doubling time. These data show that AQP3 expression levels are able to regulate cell proliferation in MDA-MB-231 cells.
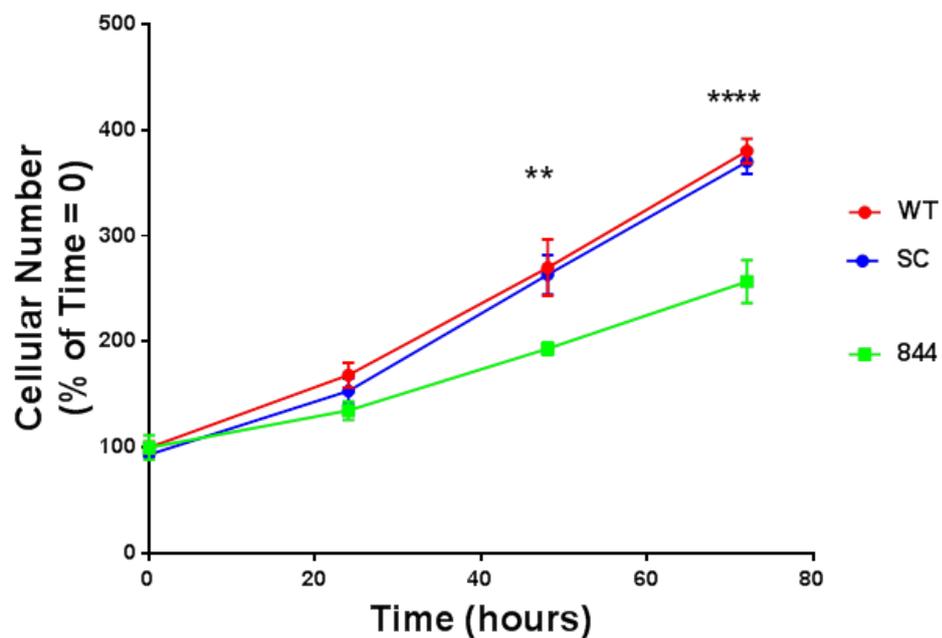
Figure 5.1   Effect of AQP3 knockdown on cellular proliferation of MDA-MB-231 cells.

The effect of knocking down AQP3 was measured in MDA-MB-231 cells over a period of time. AQP3-knockdown cells (844) demonstrated a 28% reduction (± 9%) in cell number after 72 h compared to WT and SC cells. The doubling time of cells was calculated as follows: WT = 30.98 h, SC = 31.24 h and 844 = 39.27 h; n = 3, ** p <0.01, **** p <0.0001).

5.3.2 Reduction of AQP3 impairs cellular migration in MDA-MB-231 cells

The role of AQP3 in cell migration and wound healing is well established (Hara-Chikuma and Verkman, 2008a; Ji *et al.*, 2008; Li *et al.*, 2013a). The present study therefore assessed the impact of AQP3 gene silencing on MDA-MB-231 cells, a cell line known to have migratory potential (Cazet *et al.*, 2009). In order to assess cell migration, cells were subjected to two methodologies; a wound scratch assay, an established measure of MDA-MB-231 cell migration (Adams *et al.*, 2010; Du *et al.*, 2010) and analysis using the Cell-IQ® system. The Cell-IQ® system was used not only to assess the migratory rate by inhibiting proliferation but also to further confirm the results of scratch assay.

For the wound scratch assay, images of WT, SC, and 844 cells were captured at 0, 24, 48 and 72 h (see Figure 5.2) and data represented in graphical form as shown in Figure 5.3. Data acquired in % (as described in chapter 2) through the T-scratch software. The images were analysed by the software on time-series basis therefore 0 h for all respective cell types was used as a control and analysis was done comparing 0 vs 24, 0 vs 48 and 0 vs 72 h. WT, SC and 844 cells showed 56.29% ($\pm$ 3.7%), 65.18% ($\pm$ 1.8%) and 78.75% ($\pm$ 2.0%) percentage of open wound after 48 h respectively. After 72 h, WT, SC and 844 cells showed a space of open wound of around 42.88% ($\pm$ 2.5%), 50.47% ($\pm$ 1.4%) and 74.50% ($\pm$ 2.6%) respectively.

Using the Cell-IQ® system, in conjunction with mytomycin C treatment to inhibit cellular proliferation, WT cells showed a 38.2% ($\pm$ 5.833) wound closure after 24 h compared to 844 cells which showed 20.96% ($\pm$ 1.708) (see Figure 5.4). These data demonstrate that AQP3 is an important regulator of MDA-MB-231 cell migration, and that cell migration can be potently inhibited *in vitro* by targeting AQP3 expression. The results also show that this observation is not due to the effect of AQP3 silencing on proliferation described previously.
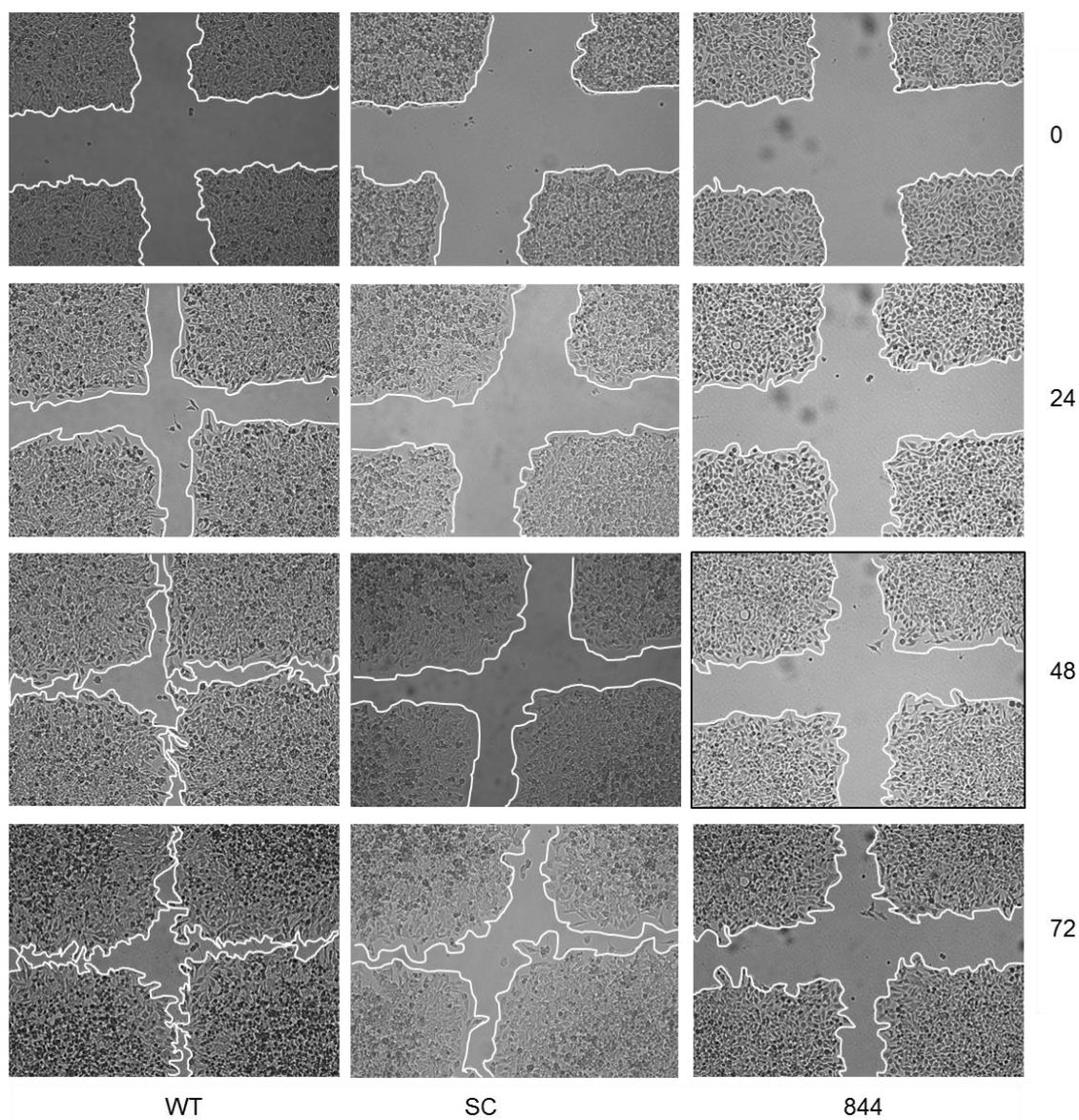
Figure 5.2   Effect of AQP3 silencing on cellular migration: WT, SC and 844 cells.

The effect of AQP3 down regulation was observed in MDA-MB-231 cells by wound scratch assay. WT and AQP3 knockdown 844 cells showed differential migratory rate. 844 knockdown cells showed less migration and were slow to close the wound as compared to WT. Images were captured at 0, 24, 48 and 72 h. The edges of the wounds were marked white to show the distance covered by the cells. Image is representative of n=9 repeats.
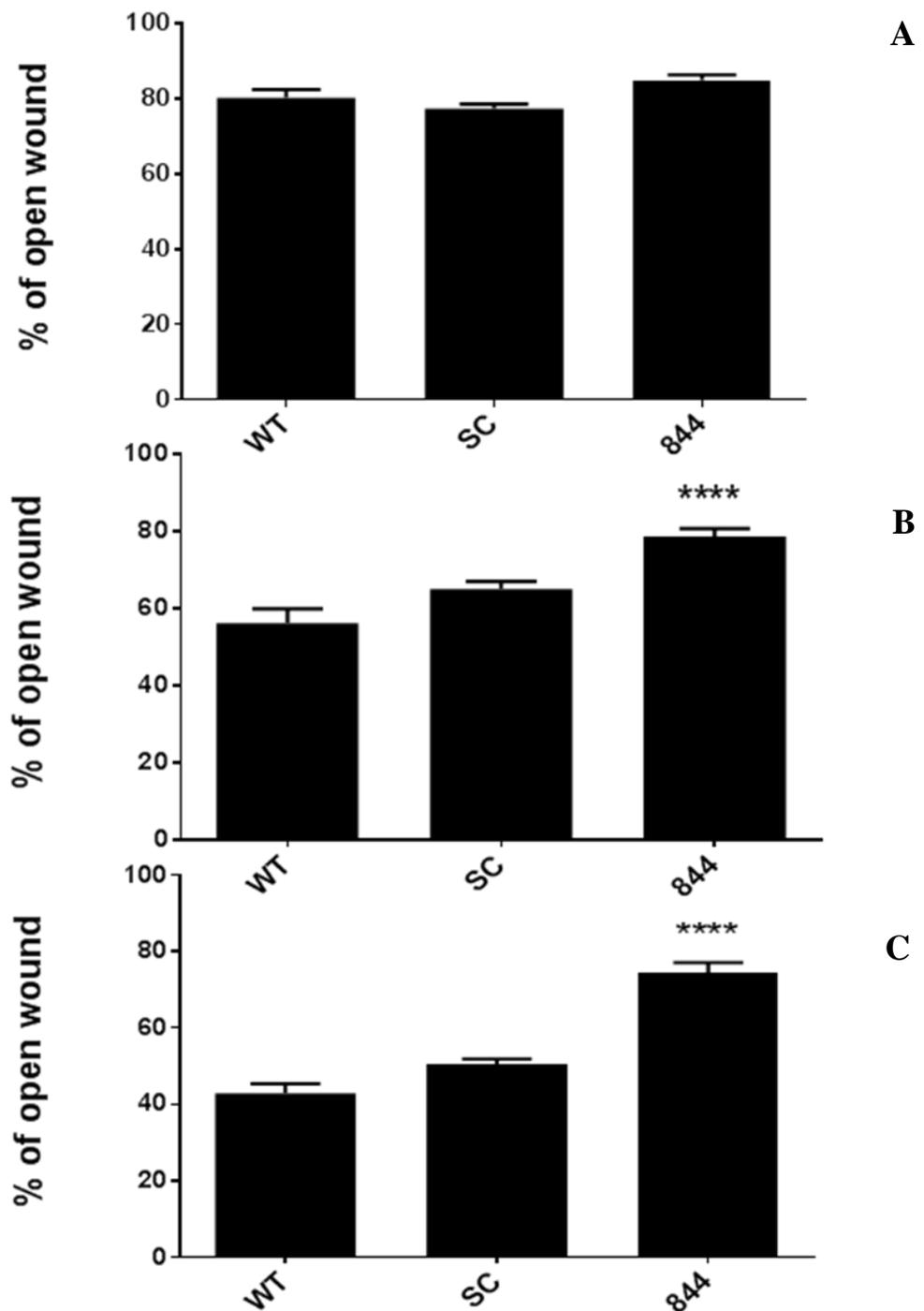
Figure 5.3   AQP3 knockdown cells exhibit reduced migration (wound scratch assay).

Wound closure assay showed reduction in cellular migration of 844 cells as compared to WT and SC cells. Wound closure for each cell line was compared with its time of 0 h and shown as (A) 24 VS 0 h, (B) 48 VS 0 h and (C) 72 VS 0 h. WT, SC and 844 cells showed a wound closure of 57.12%, 49.53% and 25.50% after 72 h respectively. Data are shown as mean % ± SEM; n = 9, ****p <0.0001, ***p <0.001.
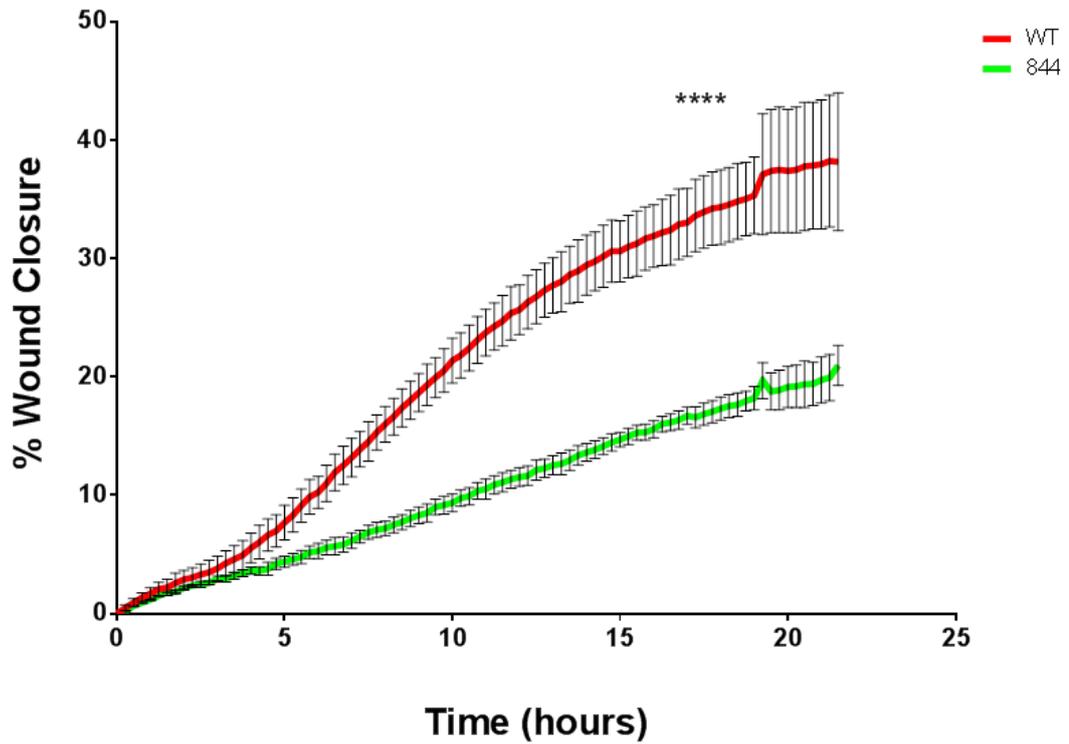
Figure 5.4   AQP3 knockdown cells exhibit reduced migration (Cell-IQ® assay).

Wound closure of WT and 844 was observed in Cell-IQ® automated system for 24 h. WT cells showed wound closure about 38.2% whereas 844 cells showed wound closure about 20.96%. Graph represents the average of three experiments. Data shown as mean % ± SEM; n=3. ****p <0.0001

5.3.3 Reduction of AQP3 expression in MDA-MB-231 cells impairs *in vitro* cellular invasion

The process of metastasis, the spread of cancer cells from a primary site to distant parts of the body, is initiated by cellular invasion through the basement membrane and is considered a pivotal aspect of cancer mortality (Liu *et al.*, 2013a). In order to assess the levels of invasiveness of cells in which AQP3 had been knockdown, the present study employed proprietary BME-based and collagen IV based cellular invasion assays, whereby cells were challenged in either BME coated or collagen coated Transwell® dishes through which they were required to invade in order to reach towards a chemoattractant (FBS) in a bottom chamber. After a 48 h incubation period, 844 cells showed significantly reduced level of invasion compared to WT cells. The 844 cells showed mean difference of 24.14% (±7.09%) and 22.89% (±7.09%) of cellular invasion levels across BME as compared to WT and SC respectively. Similarly, 844 cells showed mean difference of 22.66% (±5.10%) and 13.19% (±4.68%) of cellular invasion across Collagen as compared to WT and SC respectively (see Figure 5.5, n=5).
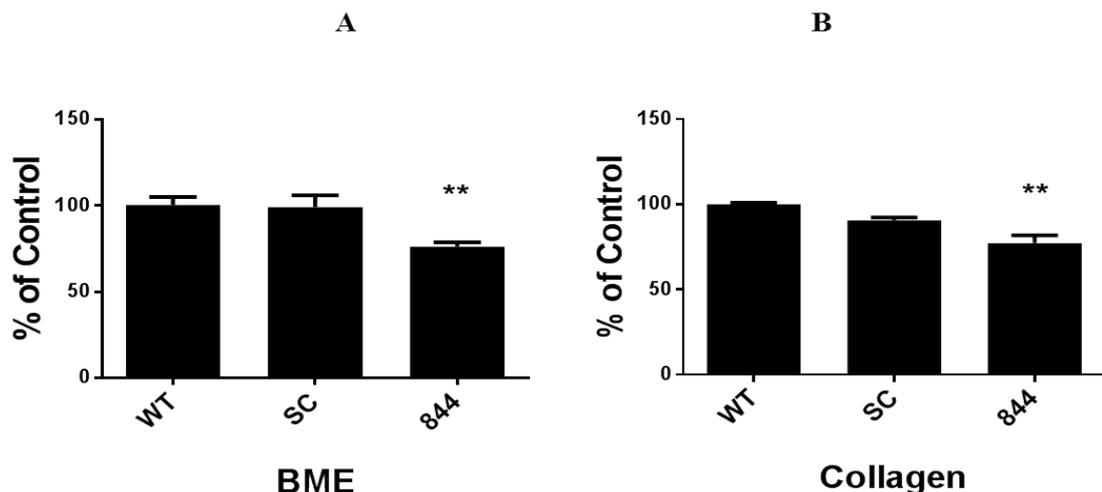
Figure 5.5   AQP3 knockdown reduces cellular invasion in MDA-MB-231 cells.

Disruption of AQP3 by shRNA impaired the cellular invasion into Cultrex® BME and Collagen IV in MDA-MB-231 cells. (A) Cellular invasion across BME reduces to 76.24% in 844 cells. (B) Cellular invasion across Collagen barrier reduces to 77.37% in 844 cells. Data are shown as mean % of control ± SEM, n = 5; **p <0.01.

5.3.4 AQP3 gene knockdown does not affect cellular adhesion

To explore the role of AQP3 function in cellular adhesion in MDA-MB-231 cells, cellular adhesion assay was performed. Cellular adhesion plays an important part in cancer progression and metastasis (Bendas and Borsig, 2012) with circulating cancer cells ultimately interacting with endothelial cells, leading to extravasation (Earley and Plopper, 2008). Also cellular migration depends on the adhesion and retraction of the cell from the substratum (Ridley *et al.*, 2003). WT, SC and 844 cells were seeded into fibronectin coated plates for 4 h to assess levels of cellular adhesiveness. Analysis of Calcein-AM staining in each well after this period showed that there was no significant difference in the levels of cellular adhesion between the three cell types (p =0.787, Figure 5.6; n=8 wells) suggesting that AQP3 does not have a role in MDA-MB-231 cell adhesion.
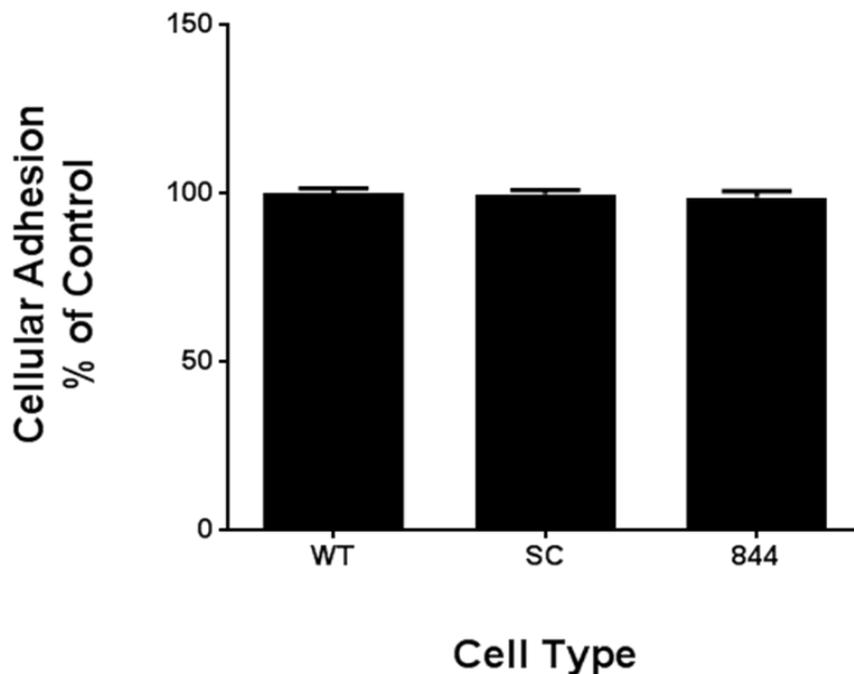
Figure 5.6   Cellular adhesion in MDA-MB-231 cells is not altered by AQP3 down-regulation.

Adhesion assay was performed to evaluate the effect of AQP3 in MDA-MB-231 cells. No effect of silencing AQP3 on adhesion of MDA-MB-231 to fibronectin was observed. Values are average of three independent experiments (mean % of control ± SEM); n=9, p = 0.787.

5.3.5   AQP3 silencing potentiates 5-FU induced cell death

5-FU is a fluoropyrimidine chemotherapy drug commonly given to treat bowel, breast or stomach cancer. 5-FU inhibits cell division during S-phase by preventing purines or pyrimidines to attaching to DNA/RNA (Longley *et al.*, 2003). The role of AQP3 in cell death was investigated in the present study by exposing WT, SC and 844 cells to a range of 5-FU concentrations for 24 and 48 h and subsequently cell viability was measured using PrestoBlue® (Invitrogen, UK) cell viability reagent. 844 cells showed significantly lower (see Table 5.1) levels of cell viability at all tested doses of 5-FU when compared to WT or SC cells after 48 h, suggesting that AQP3 is required by MDA-MB-231 cells to show resistance against 5-FU based treatment (see Figure 5.7).

Table 5.1   Reduction in cell viability of 844 cells as compared to WT and SC cells after 48 h of toxicity induced by a range of 5-FU doses.

Table show mean and standard deviation (SD) for each tested dose of 5-FU on WT, SC and 844 cells. The statistical calculation for p values was based on difference in cell viability % between WT, SC and 844 cells. Significance was determined through ANOVA with a Dunnett's post-hoc multiple comparisons test

5-FU in µM	WT		SC		844		*P* value
	Mean	SD	Mean	SD	Mean	SD	
0	100	12.80	100	10.17	100	17.38	> 0.999
100	73.21	18.63	74.59	16.09	55.95	19.02	0.011
250	63.85	15.29	66.11	15.62	49.97	10.75	0.006
500	62.40	12.14	61.85	16.80	44.24	8.63	0.0004
1000	60.15	18.92	61.46	19.23	44.31	9.61	0.011
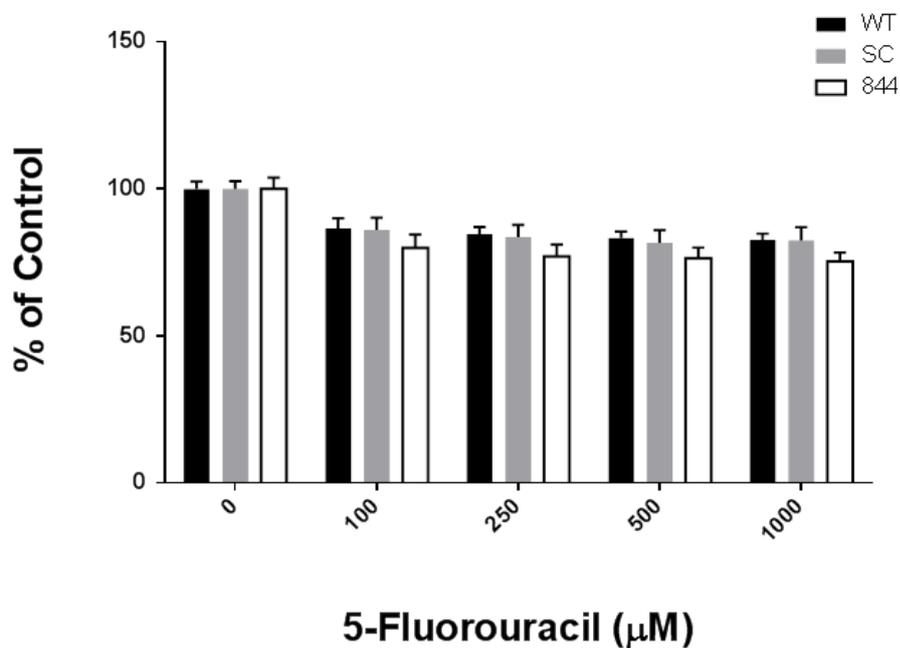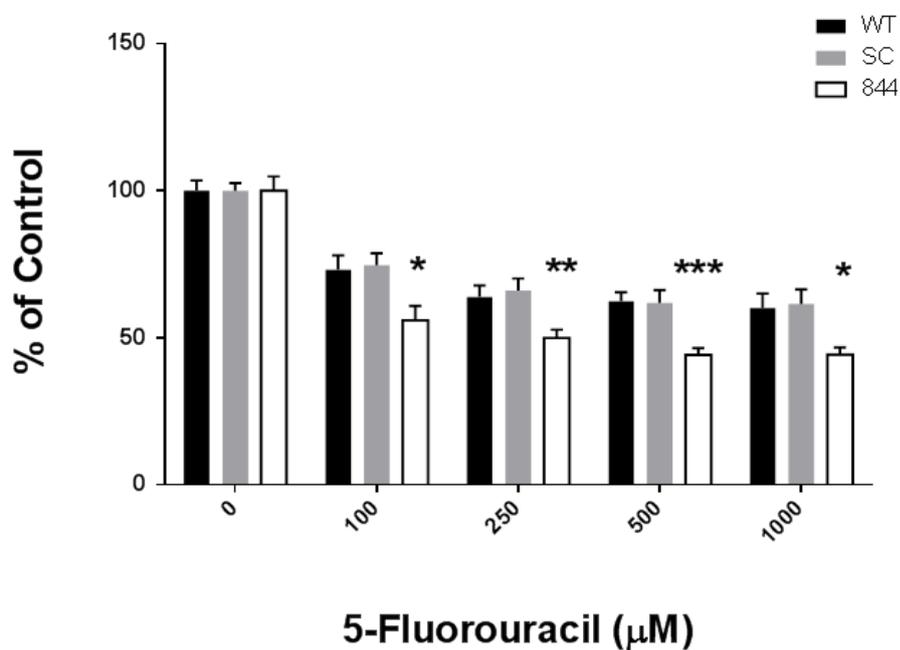
Figure 5.7   AQP3 reduction increases sensitivity to 5-FU in MDA-MB-231 cells.

MDA-MB-231 cells were treated with various concentrations of 5-FU for (A) 24 h and (B) 48 h. AQP3 knockdown synergistically sensitises MDA-MB-231 cells to all tested doses of 5-FU after 48 h but not after 24 h. Data are shown as mean % of control ± SEM; n=9, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.01$.

5.4   Discussion

The work described here demonstrates the wide effects that down-regulation of AQP3expression in MDA-MB-231 cells has on cellular phenotype. As stated earlier, the main objectives of this part of the study were to compare the different aspects of MDA-MB-231 cell biology to ascertain what impact was seen after down-regulating AQP3 expression. The first and possibly key finding presented here is that down-regulation of AQP3 expression produced a significant reduction in cellular proliferation. Previous evidence has suggested that AQP3 is involved in epithelial cell proliferation (Levin and Verkman, 2006), although no evidence of this effect in breast epithelial cells has been reported at the time of writing. Targeting the proliferation rate of cancerous breast epithelial cells is of great importance as cellular proliferation (as measured by Ki-67 expression or mitosis counting) has been significantly correlated with disease prognosis in numerous studies (van Diest *et al.*, 2004; Urruticoechea *et al.*, 2005). Based on this, it is possible that targeting cellular proliferation by either pharmaceutical down-regulation or inhibition of AQP3 expression or function might be associated with a better prognosis in BC. The level of proliferation reported here was relatively modest (<30%) but this reduction occurred over a short time-frame (72 h) and with a corresponding ~73% reduction in AQP3 levels. It would be interesting in future studies to investigate what longer-term implications there are of down-regulating AQP3 on cell growth and/or tumour formation. Additionally, the model that was used here had an approximate 73% reduced AQP3 expression level, it would also therefore be desirable to assess what effect greater reductions, or complete ablations, of AQP3 expression has on cellular proliferation. These experiments fall outside the scope of the present study but would be of great interest in future studies.

Metastasis is often considered the worst case scenario in many cancers and most deaths occur due to metastatic aspect of BC (Lu *et al.*, 2009). Metastasis is dependent on both the migration and invasion of cancer cells and the present study therefore compared the capacity of WT, SC and 844 cell lines to migrate and to invade through substrates. To observe cellular migration both wound scratch assay technique and Cell-IQ® system was applied. Scratch assays have been used to study

cell polarisation, matrix remodelling and cellular migration (Yarrow *et al.*, 2004). There are some disadvantages related to *in vitro* scratch assay such as no involvement of a chemotaxis gradient and the relatively long time required to perform the experiment (Liang *et al.*, 2007). However many researchers still use this technique as it is relatively easy to do and is cost effective with reliable results. Thus, scratch wound assay was the initial method used in the present study to investigate cellular migration.

In present study, it was observed that knockdown of AQP3 resulted in a significant decrease in cellular migration demonstrated by both scratch wound assay and Cell-IQ® system. These findings are supported by one previous study which employed an *in vitro* scratch wound assay in AQP3 deficient keratinocytes that reported that wound closure was delayed compared to control cells (Hara-Chikuma and Verkman, 2008a). It also measured migration using a Transwell®-based assay and showed impaired migration in AQP3-knockdown (siRNA) human keratinocyte cultures and keratinocyte cultures from AQP3 knockout (KO) mouse (Hara-Chikuma and Verkman, 2008a). This defect was corrected by adenoviral infection of either AQP1 or AQP3 in keratinocyte cultures from AQP3 null mice and restored cell membrane water permeability. As AQP1 transports water only and AQP3 can allow both water and glycerol, this study concluded that the absence of AQP3-facilitated water transport in the AQP3 null keratinocytes is responsible for their migration defect (Hara-Chikuma and Verkman, 2008a). Similarly, another study using AQP4 as the target also used a wound scratch assay to measured cellular migration in glioblastoma LN229 cells and demonstrated that AQP4 reduction again delayed cellular migration (Ding *et al.*, 2011). These findings suggest that AQPs are possibly commonly involved in cellular migration.

The mechanism by which AQP3 may influence cellular migration remains to be elucidated, but as mentioned above previous studies have suggested that AQPs can regulate the membrane protrusion of cells via their ability to regulate water flow (Karlsson *et al.*, 2013). As the wound scratch assay employed here was not performed in the presence of a mitotic inhibitor it cannot be said with certainty that the reduction in wound closure observed in 844 cells compared to WT was solely due

to migration as 844 cells also proliferate at a slower rate. In the present study therefore, cellular migration was also measured for 24 h using the live cell imaging Cell-IQ® system in the presence of the mitotic inhibitor mitomycin C to control for proliferation of cells. Data obtained using this system confirmed the inhibitory effect of AQP3 down-regulation (see Figure 5.4) thus confirming the results of scratch wound assay. Taken together these data suggested that AQP3 facilitates cellular migration in MDA-MB-231 cells. The mechanism for this role of AQP3, whether it involves water or glycerol passage, requires clarification and is an excellent area for future research.

Cellular invasion was also investigated in this study and AQP3 knock down cells demonstrated a reduction in invasion through both BME and collagen substrates compared to WT cells. This suggests that a high expression of AQP3 facilitates invasion through basement membrane substrates in these cells, and that targeted down-regulation of AQP3 can mitigate this to a significant degree. It has been previously reported that reduction of AQP4 in glioblastoma LN229 cells led to reduced invasion (Ding *et al.*, 2011). This observation, in conjunction with the data presented here, is important as it suggests that targeted reduction in AQP levels reduces the ability of cancer cells to break through a basement membrane and spread to distant sites.

Cellular adhesion is also an important factor deciding the fate of metastatic cells *in vivo*. Cell adhesion was investigated in WT, SC and 844 cells by the use of fluorescent staining of fibronectin coated plates after 4 h. Experiments showed that there was no significant difference observed between WT cells and 844 cells. This suggests that AQP3 is not an essential molecule for cellular adhesion in these cells. Other published data have suggested that AQP3 does have a role on cellular adhesion (Kusayama *et al.*, 2011) in one lung cancer cell line but not in the other. The data presented here also showed no association of AQP3 with cellular adhesion in breast cancer-cell line thus partly agreed with the previous study. This suggests that cellular adhesion is likely to be a cell-specific phenomenon.

The effect of AQP3 gene knockdown on sensitivity to the chemotherapic drug 5-FU was also investigated in the present study. Figure 5.7 shows that 844 cells were more sensitive to the drug compared to WT and SC at 48 h. This finding suggests that AQP3 has a possible role in chemotherapeutic resistance at a cellular level in MDA-MB-231 cells. A regulatory role for AQP3 in chemotherapy-induced breast cancer cell death has previously been shown that AQP3 silencing reversed the cytotoxicity induced by 5-FU in MCF-7 cells. (Trigueros-Motos *et al.*, 2012b). The study showed increase in AQP3 mRNA expression in MCF-7 cells after treating cells with different doses of 5-FU (5, 25 and 75 μM) for 90 minutes however the AQP3 mRNA expression was reduced at 250 and 500 μM and it was concluded that AQP3 expression is needed for cytotoxic activity of 5-FU (Trigueros-Motos *et al.*, 2012b). On the contrary, the present study showed increase in 5-FU sensitivity in AQP3 knockdown MDA-MB-231 cells (844 cells) at all doses (100, 250 and 500 μM) after 48 h. The difference could possibly because of the relative expression levels of AQP3 in MCF-7 and MDA-MB-231 cell types. Experiments carried out early in this study showed that there was significantly lower expression of AQP3 in MCF-7 cells than in MDA-MB-231 cells (see Figure 4.17) and it is possible that reducing this level further induced a different effect to that reported here. Additionally in the present study, knocking down AQP3 levels did not alter the mRNA expression of any other AQP levels in 844 cells (see Figure 4.30); it is unclear whether the same can be said of the MCF-7 cells used in the published study. Furthermore, the incubation time (90 minutes) used in the published study was contrary to present study in which effects of 5-FU on MDA-MB-231 cells were tested for 48 h with a range of concentrations were treated as described previously (Sims et al., 2009).

The main aim of the present study was to test the hypothesis that AQP3 plays an important role in controlling some of the essential aspects of BC cell biology including cellular proliferation, migration, invasion, adhesion and response to chemotherapy. In the present study, AQP3 was down-regulated in MDA-MB-231 invasive BC cells, and experimental results showed that AQP3 knockdown exhibited significant impairment in proliferation, migration and invasion assays in vitro. Previous studies have shown that it is altered water flow through AQP3 that is likely the

mechanism by which AQP3 silencing can reduce migration and invasion in other cells, but further studies are necessary to confirm whether this is the case in MDA-MB-231 cells. Furthermore, data presented here indicates that reducing AQP3 expression potentiated cell death induced by 5-FU. Chemotherapy is the most significant option in TNBC as hormonal therapy and immunotherapy cannot be used and MDA-MB-231 cell line represents a cellular model of TNBC. Axillary lymph node metastases remains an important prognostic factor for breast cancer, and therefore any intervention that can reduce the capacity of primary breast cancer cells to spread to distant sites is of significant clinical interest. Understanding of the molecular mechanisms involved in BC metastasis is essential for the development of novel therapeutic treatments that can improve survival rates in human populations. Any improvement in cancer cells response to traditional chemotherapeutic agents is welcome and of the results presented here suggest that targeted suppression of AQP3 may be of clinical value.

To conclude, this study suggests that AQP3 is involved in MDA-MB-231 cell proliferation, migration and invasion, and may be a therapeutic target molecule for prevention of malignant infiltration.

Chapter 6

The Role of AQP3 in MDA-MB-231 Cell Bioenergetics

6.1 Introduction

Two of the most important pathways involved in energy homeostasis are glycolysis and oxidative phosphorylation (Zheng, 2012). Under physiological conditions, normal mammalian cells depend on cellular respiration. The energy stored in nutrients, such as glucose, is transferred to ATP molecules so that it can be used in metabolic functions. Both cytosolic and mitochondrial processes generate energy *via* oxidation of glucose, fatty acids and amino acids. (Barbosa *et al.*, 2012). However, glycolysis is the first process which produces ATP generally by non-oxidative breakdown of glucose followed by oxidative breakdown of pyruvate (Verschoor *et al.*, 2013). Cancer cells are thought to rely mainly on glycolysis as their primary source of energy but under stress conditions have the ability to use mitochondrial respiration to survive. However, commonly a balance between ATP production and consumption within cells is always maintained thus supplying necessary ATP. The mRNA levels of aquaglyceroporins have been reported to be modulated by insulin secretion and suggested to have an important role in glucose metabolism (Lee *et al.*, 2009). AQP3 is an aquaglyceroporin as discussed earlier and thus can transport glycerol into the cell as well as water. Glycerol is used by cells as an important metabolite for energy production during starvation (Brisson *et al.*, 2001).

The aim of this part of the study is to determine the effects of down-regulation of AQP3 expression on the bioenergetics of MDA-MB-231 cells. This was assessed by measuring the glucose levels in conditioned medium of WT, SC and 844 cells and ATP content in WT, SC and 844 cell types. Glycerol-induced changes in cell viability were also assessed in WT, SC and 844 cells.

6.2 Materials and Methods

All methods performed in this chapter to analyse the involvement of AQP3 in cellular bioenergetics of WT and 844 cells were described in Chapter 2 in detail. All experiments (experiments which showed significant difference observed between WT and 844) were also performed at least once on 841 clone as well (data not shown). Briefly glucose uptake by WT, SC and 844 cells from media was measured. ATP content and glycerol-induced changes in cell viability were also assessed in WT, SC and 844 cells. A glycolysis stress test was performed on WT, SC and 844 cells by using a SeaHorse flux bioanalyser. mRNA expression of enzymes involved in glycerol metabolism, glycerol kinase (GK) and Glyceraldehyde 3-phosphate dehydrogenase (G3PDH), was investigated using real-time PCR in WT, SC and 844 cell types.

Statistical analysis of data was performed using GraphPad Prism 6 software. One-way ANOVA with a Dunnett's post-hoc test for comparisons of > 3 groups or Student's t-test for comparisons of < 3 groups were performed for the statistical analysis between experimental conditions and p values < 0.05 were considered significant.

6.3 Results

6.3.1 Determination of glucose levels

Indirect glucose uptake was investigated in WT, SC and 844 cell cultures after 24 and 48 h by measuring the amount of glucose remaining in cell culture media after the specified times. The starting concentration of glucose in the medium was 2000 µg/mL. 844 cells demonstrated a significantly higher glucose content of media after both 24 and 48 h suggesting a decrease in glucose uptake as compared to WT (Figure 6.1). After 24 h of cell culture the glucose levels in the media were WT: 1281.83 ± 39.74 µg/mL, SC: 1254.09 ± 27.17 µg/mL and 844: 1805.48 ± 33.06 µg/mL (p <0.0001). After 48 h, the glucose content of the media was WT: 823.85 ± 8.32 µg/mL, SC: 831.73 ± 19.22 µg/mL and 844: 1573.45 ± 32.61 µg/mL. Statistical significance was determined through one-way ANOVA with a Dunnett's post-hoc multiple comparisons test.
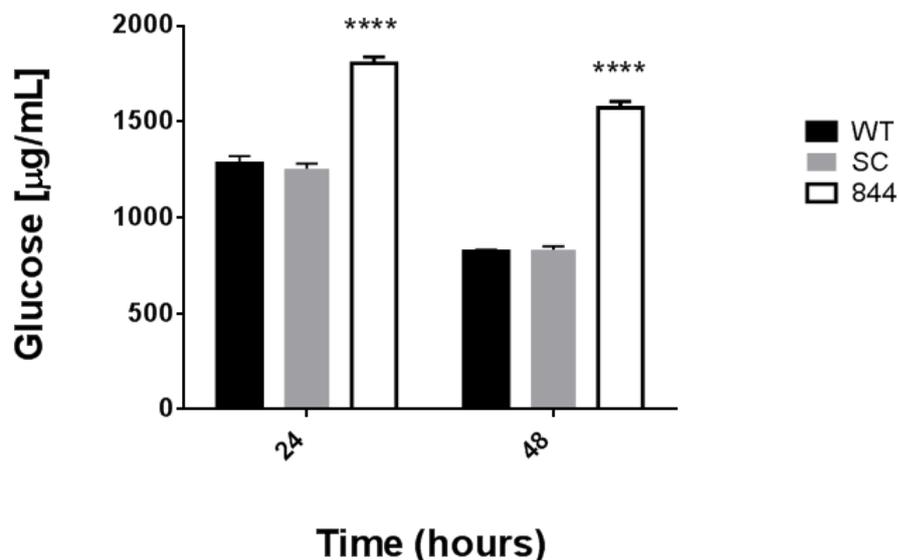
Figure 6.1   Glucose content of cell culture media.

Glucose levels were measured in the media after 24 and 48h post culturing cells. Glucose uptake assay showed that glucose content of media collected from 844 cells was significantly higher than glucose media content of both WT and SC cells after 24 and 48 h. Results are expressed as mean µg/mL ± SEM; n=9. ****p <0.0001

6.3.2 Glycolytic stress test

The rate of glycolysis measured using the SeaHorse bioanalyser flux assay is represented as ECAR. Glycolysis and total glycolytic capacity in WT and 844 cells were observed by exposure to oligomycin and 2-deoxyglucose (2-DG) as ATP and glucose inhibitors respectively. As a result, oligomycin decreased the ATP production and 2-DG (a glucose analogue) inhibited glucose consumption by glycolysis. ECAR decreases to a greater extent in both WT and 844 cells with addition of 2-DG (see Figure 6.2A). This result showed that glycolysis is essential for both WT and 844 bioenergetics and reduction in AQP3 expression has no significant effect on glycolysis (see Figure 6.2B). Glycolytic ability in WT and 844 cells during the stress test was not deemed significant.
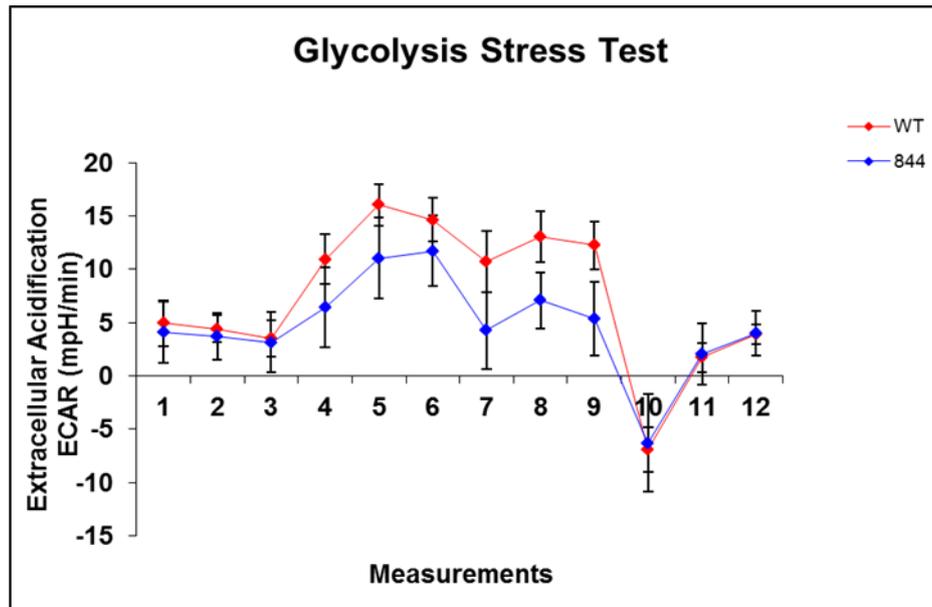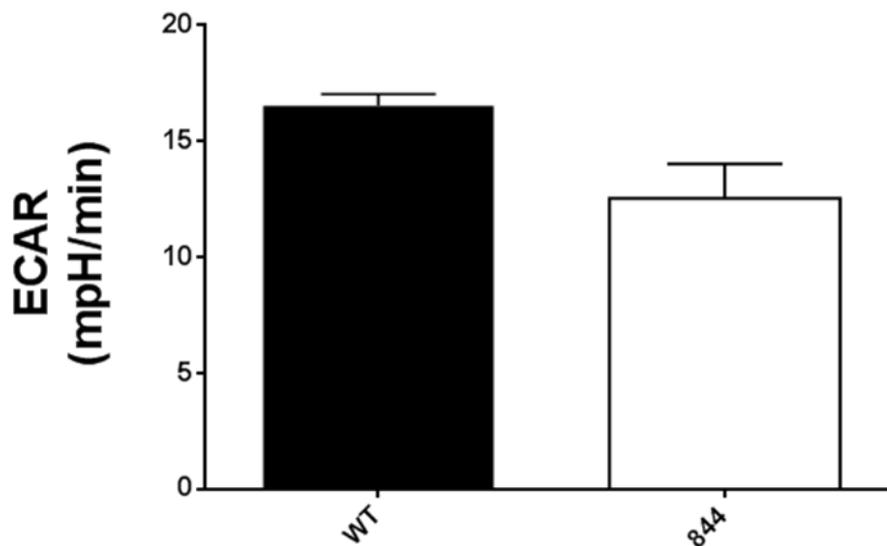
Figure 6.2   Glycolysis in MDA-MB-231 is not altered by AQP3 down-regulation.

Glycolysis was measured by using average ECAR for both WT and 844 cells. (A) Graph shows measuring points at x-axis to measure ECAR. The first three points measure the ECAR of both cell types when cells were incubated in the glycolysis stress test medium (without glucose). Then glucose was injected through first port into the media which was used by the cells resulted in increasing the ECAR shown at points 3-6. Oligomycin (ATP inhibitor) was injected from the second port due to which there was a slight dip in the ECAR of the cells at points 6-9 and final injection of 2-DG decreased the ECAR to a greater extent in both WT and 844 cells at points 9-12. (B) Bar graph showed that overall there was no significant effect of AQP3 knockdown on glycolysis in MDA-MB-231 cells. Data are expressed as mean ± SEM of three independent experiments, n= 10; p = 0.127

6.3.3 Glycerol induced cell death

Previous report showed that glycerol can suppress proliferation of cancer cells (Sugiyama *et al.*, 2002). Extracellular glycerol transported in excess amounts can cause cell damage and death due to changes in osmotic potential. Since AQP3 is known to transport glycerol, the effect of exposure to a concentration range of glycerol on cell viability was investigated in WT, SC and 844 cells. Glycerol-induced cell death was investigated over 24, 48 and 72 h. 844 cells demonstrated a significantly reduced level of cell death when challenged with 2.5% and 5% glycerol over the three day period compared to control cells (see Figures 6.3 - 6.5). Statistical significance was observed by one way ANOVA with a Dunnett's post-hoc test for comparisons.
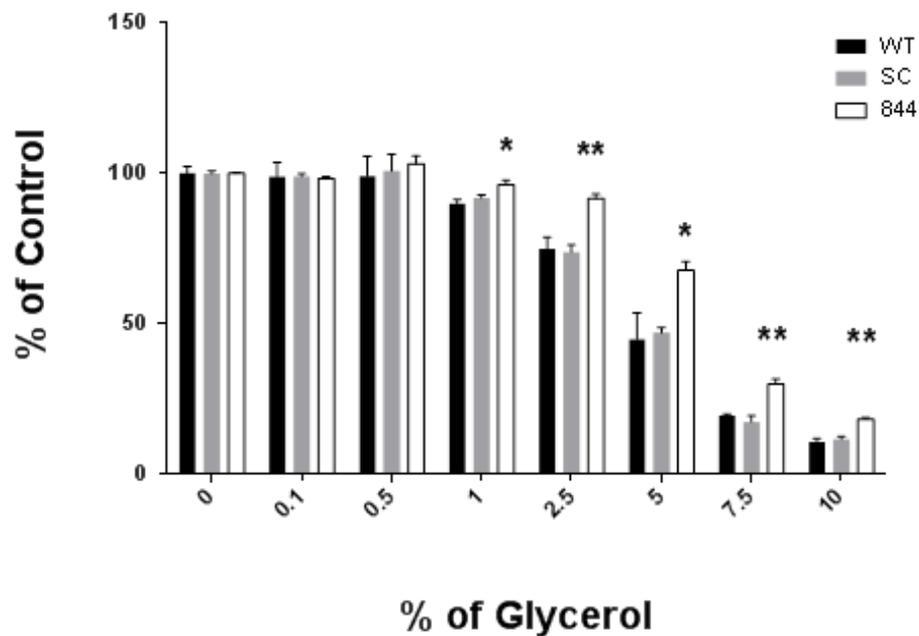
Figure 6.3   Glycerol sensitivity after 24 h in MDA-MB-231 cells.

WT, SC and 844 cells were for 24 h in the presence of indicated concentrations of glycerol in triplicate. Results are expressed as percentage of non-treated control. Data are shown as mean % of control ± SEM, n = 3, **p <0.01, *p <0.05.
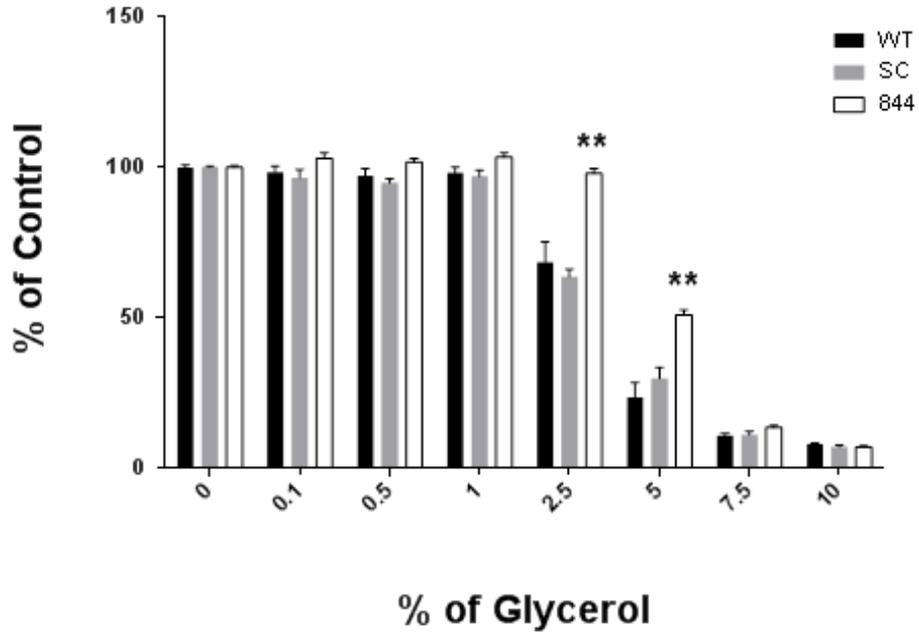
Figure 6.4   Glycerol sensitivity after 48 h in MDA-MB-231 cells.

WT, SC and 844 cells were for 48 h in the presence of indicated concentrations of glycerol in triplicate. Results are expressed as percentage of non-treated control. Data are shown as mean % of control ± SEM, n = 3, **$p < 0.01$.
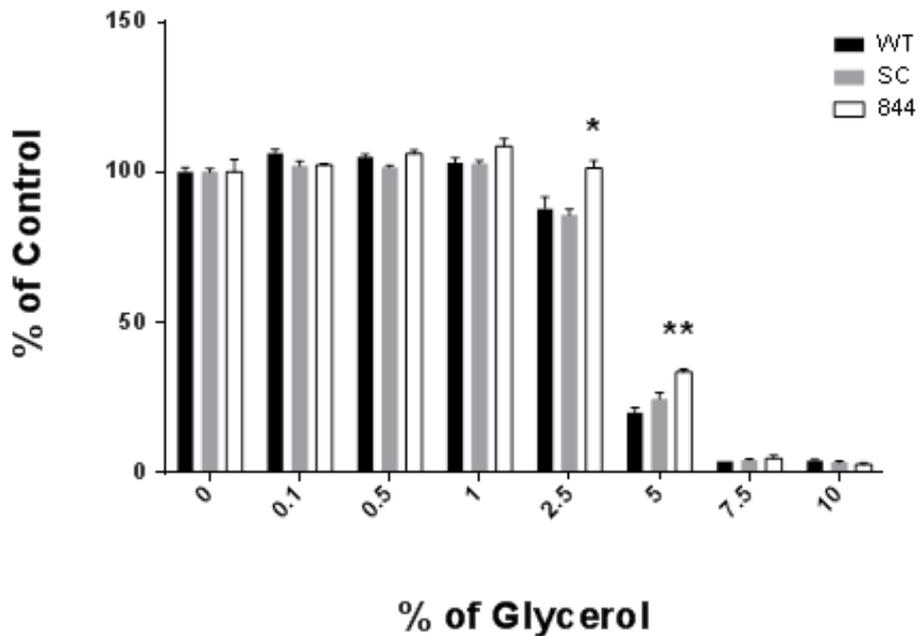
Figure 6.5   Glycerol sensitivity after 72 h in MDA-MB-231 cells.

WT, SC and 844 cells were for 72 h in the presence of indicated concentrations of glycerol in triplicate. Results are expressed as percentage of non-treated control. Data are shown as mean % of control ± SEM, n = 3, **$p < 0.01$, *$p < 0.05$.

6.3.4   Gene expression of glycerol metabolic enzymes

To examine the expression of GK and G3PDH, the two important enzymes involved in glycerol metabolism, in WT, SC and 844 cells, qPCR was performed. The relative mRNA expression of GK and G3PDH was measured using $2^{-\Delta\Delta CT}$ method. The relative fold difference of both GK and G3PDH enzymes was measured in 844 cells by comparing it to WT (see Figures 6.7 - 6.10). The statistical significance was examined by unpaired t test. No significant difference in GK or G3PDH mRNA expression was seen between WT and 844 cells.
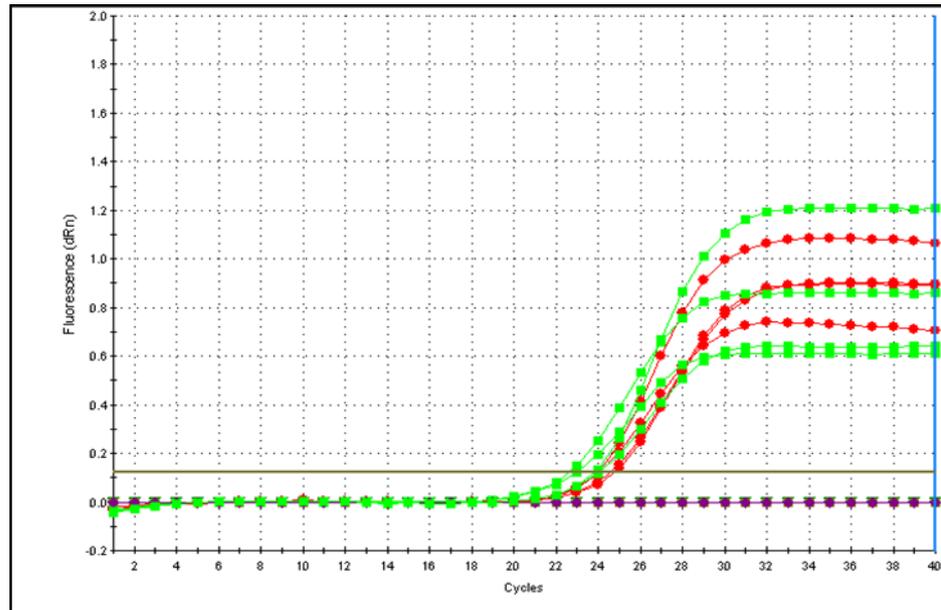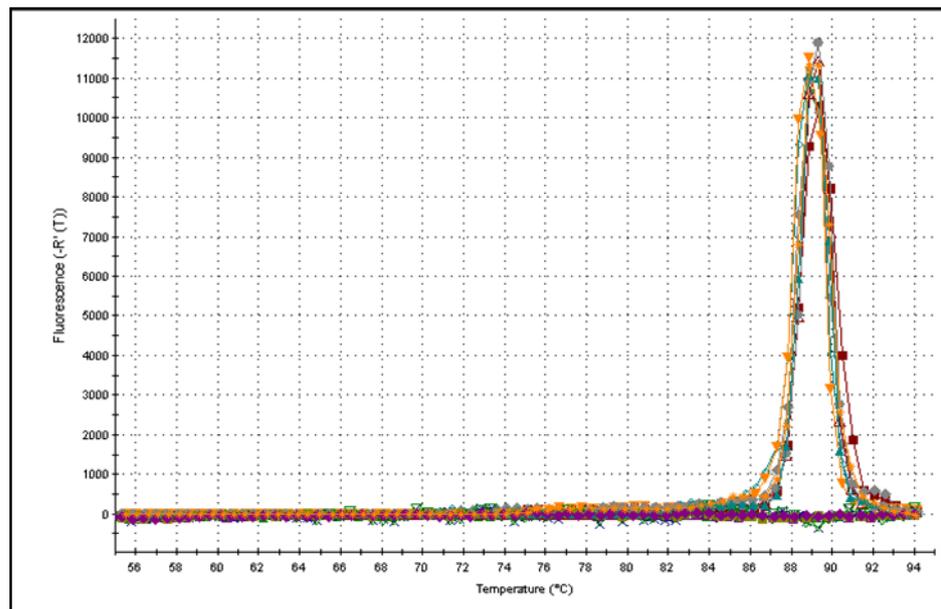
Figure 6.6   SYBR® Green real-time PCR analysis of GK gene expression.

Gene expression of GK was assessed in diluted cDNA samples of MDA-MB-231 cells using pre-validated primers. (A) The amplification curves for GK were obtained after qPCR. Red and green curves represent WT and 844 cells respectively. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 89°C suggesting excellent primer specificity; n=3.
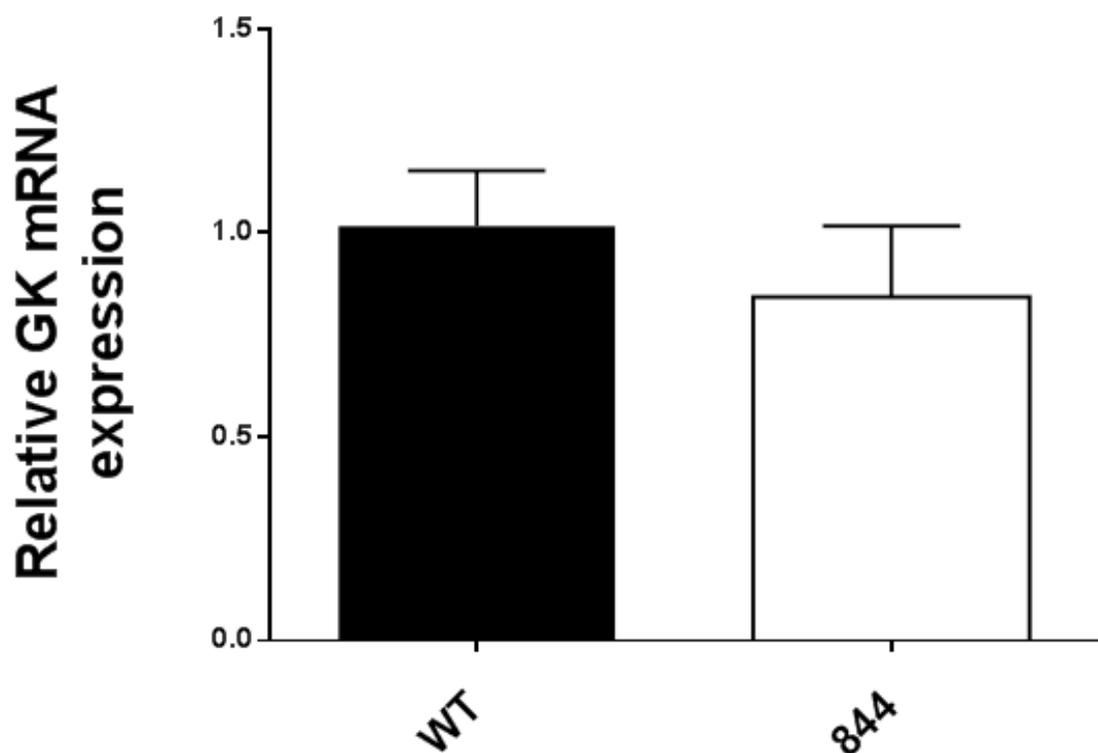
Figure 6.7   Expression of GK mRNA in MDA-MB-231 cells.

Gene expression of GK was assessed in WT and 844 cells. Bar graph shows the corresponding results of qPCR as relative fold difference internally normalised to β-actin and YWHAZ genes. Relative fold expression was calculated by considering WT samples as control. The mRNA expression of GK level was statistically not deemed significant. The mean fold expression of WT and 844 was 1.078 and 0.8427 respectively with p = 0.4708, n=3.
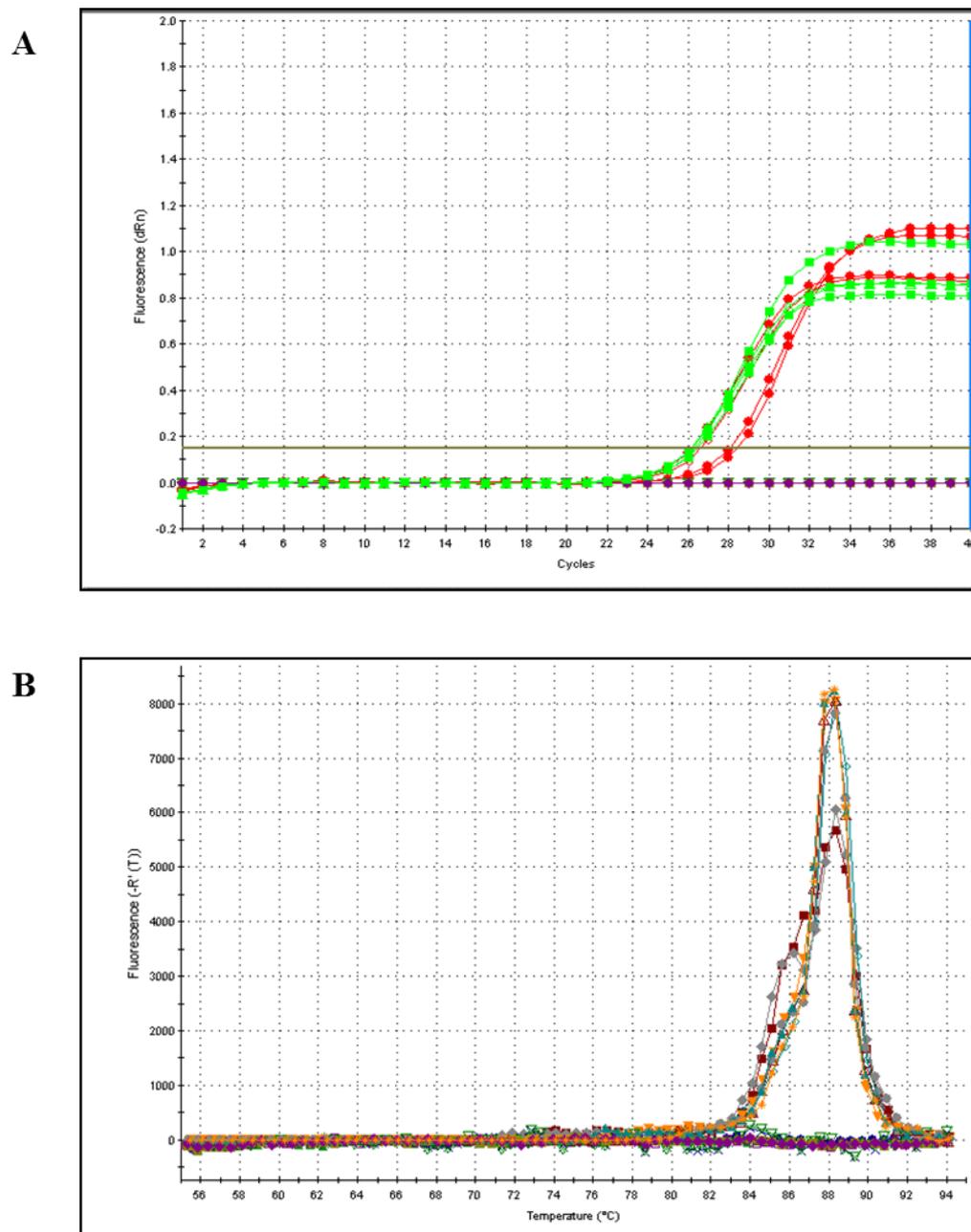
Figure 6.8 SYBR® Green real-time PCR analysis of G3PDH gene expression.

Gene expression of G3PDH was assessed in diluted cDNA samples of MDA-MB-231 cells using pre-validated primers. (A) The amplification curves for G3PDH were obtained after qPCR. Red and green curves represent WT and 844 cells respectively. (B) Dissociation curve analysis showed one dissociation peak that dissociated at approximately 88°C suggesting excellent primer specificity; n=3.
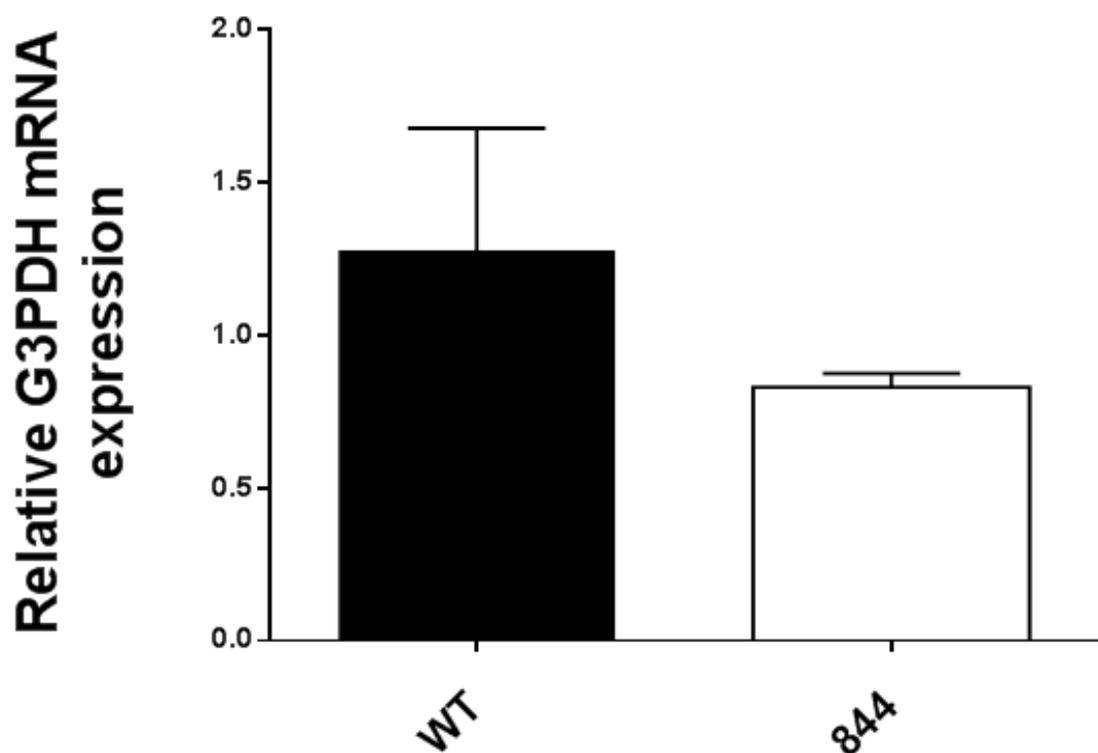
Figure 6.9   Expression of G3PDH mRNA in MDA-MB-231 cells.

Gene expression of G3PDH was assessed in WT and 844 cells. Bar graph shows the corresponding results of qPCR as relative fold difference internally normalised to β-actin and YWHAZ genes. Relative fold expression was calculated by considering WT samples as control. The mRNA expression of G3PDH level was statistically not deemed significant. The mean fold expression of WT and 844 was 1.275 and 0.829 respectively with p = 0.3307, n=3.

6.3.5 Intracellular ATP levels

Intracellular ATP levels were measured in WT, SC and 844 cell lysates using an ATP determination kit based on luciferase assay according to manufacturer's instructions. Cellular ATP content was normalised to the total protein content (nmol ATP/µg protein) in cell lysates. The calculated values of ATP content for WT, SC and 844 cells were 22.12 ± 0.99 nmol/µg, 21.92 ± 1.28 nmol/µg and 19.82 ± 0.93 nmol/µg respectively. There was no significant difference detected in cellular ATP content between the different cell-types tested. Statistical significance was observed by one way ANOVA with a Dunnett's post-hoc test for comparisons.
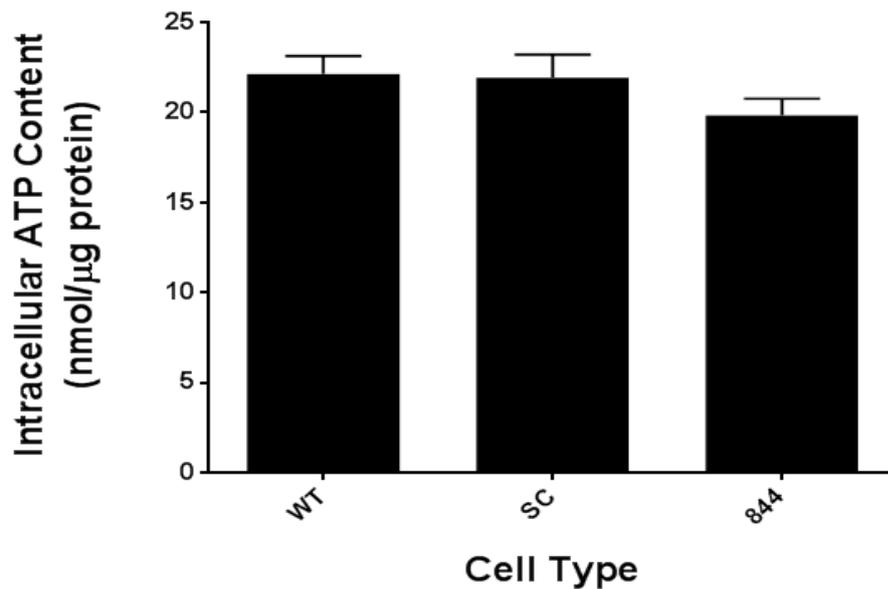
Figure 6.10   Intracellular ATP content in MDA-MB-231 cells.

Intracellular ATP levels were assessed using a luciferase based assay in WT, SC and 844 cell lysates. Data are shown as mean nmol/µg protein ± SEM; n = 5. All p values > 0.285

6.4 Discussion

The objectives of this part of the study were to determine the effects of AQP3 knockdown on glycerol transport and metabolic activity of MDA-MB-231 cells. To investigate the potential importance of AQP3 dependent metabolic activity, glycolysis stress test, ATP content, glucose uptake and mRNA expression of glycerol metabolic enzymes (GK and G3PDH) were measured. The results presented in this part indicate differential effects of AQP3 down-regulation. The present study demonstrated that knocking down AQP3 in aggressive MDA-MB-231 cells correlated with glucose uptake without effecting cellular ATP content and glycolytic capacity. Herein, it has been shown that AQP3 silencing did not disrupt the breast cancer cells ability to undergo glycolysis. Data also demonstrated that there is no change in the mRNA expression of G3PDH and GK enzymes in cells that have had AQP3 knocked down. Involvement of glycerol, glucose, G3P and ATP content in AQP3 deficient epidermal cells has previously been investigated and it has been reported that AQP3 deficient epidermal cells had significant reduction in G3P content, ATP content, glucose content and proliferative ability (Hara-Chikuma and Verkman, 2008b). The cells used in that study were non-cancerous which may in part explain the differences in results obtained in this study.

Human breast tumour tissues *in vitro* have been shown to be able to diffuse more glycerol than normal breast tissues, indicating a higher glycerol permeability (Zhong *et al.*, 2010). Also glycerol and G3P levels have been shown to be higher in BC tissue than in normal breast tissue (Brockmöller *et al.*, 2011). This increase in permeability to glycerol could be due to presence of aquaglyceroporins including AQP3 although no direct association has yet been made. Glycerol is an important nutrient, forming the backbone of phospholipids in cells, and is mainly metabolised in the liver. The present study confirms the findings that reducing AQP3 expression decreases glycerol absorption in BC cells as mentioned above. Disruption of other aquaglyceroporins (AQP7 and AQP9) has been used to demonstrate the effects of glycerol impairment on glycerol permeability in aquaglyceroporin deficient models. Severe insulin resistance coupled with higher

plasma glucose concentrations was demonstrated in AQP7 KO mice due to glycerol impairment and lack of induced GK activity was showed in AQP7 silenced 3T3-L1 adipocytes (Hibuse *et al.*, 2005). Similarly AQP9 was involved in hepatic glycerol metabolism in diabetes mellitus (Rojek *et al.*, 2007). It is possible therefore that although AQPs may have a potent metabolic role in distinct metabolic tissues, in breast cancer cells the effects are less marked.

In the present study there was no difference in the mRNA expression of GK and G3PDH enzymes between WT and 844 cells. The first enzyme used in the metabolism of glycerol is GK and it was suggested that the enzyme's activity depends on amount of available glycerol (Yeh *et al.*, 2004). The higher concentration of glycerol is required for the induction of high ATP concentrations, however at low concentrations of glycerol ATP does not activate the enzyme (Robinson and Newsholme, 1969). It has also been suggested that cellular glycerol is a key determinant of cellular ATP energy in AQP3 positive whereas AQP3 knockdown skin cells showed reduced cellular ATP content (Hara-Chikuma and Verkman, 2008b). However in the present study there was no difference in cellular ATP content between WT and 844 cells. This difference could be due to the difference in cellular types used in these two studies. The other reason could be difference in AQP3 protein expression in these knockdown cellular types as AQP3-siRNA in normal human keratinocytes reduced AQP3 protein expression by ~95% (Hara-Chikuma and Verkman, 2008b) whereas in the present study it was reduced to 73%.

It has also been demonstrated that glucose up-regulates the expression of AQP1 in cultured cells, suggesting a molecular link between glucose and aquaporins (Jenq *et al.*, 2002). Aquaglyceroporins have also been suggested to be involved in glucose homeostasis and can alter glycerol metabolism (Hara-Chikuma and Verkman, 2006b). In the present study AQP3 deficient cells showed slight decrease in glycolysis (which was deemed not significant) and significant decrease in glucose uptake by 844 cells. How AQP3 down-regulation decreases glucose uptake in breast cancer cells has not been fully clarified and warrants further investigation. There is a possibility that AQP3 might be involved in regulation of blood glucose as expression of AQP3 is controlled by

hormones (insulin and epinephrine) which control blood glucose level (Asai *et al.*, 2006). Cancer cells are known to preferentially select glycolysis as their source of energy (Gillies *et al.*, 2008; Hernandez Patino *et al.*, 2012). However in the absence of glucose, cancer cells still can survive. Indeed it has been shown that cultured cancer cells can survive in the absence of glucose after remodelling their mitochondria and mitochondrial network structurally and functionally, due to changing of the substrate, to produce ATP solely by OXPHOS (Rossignol *et al.*, 2004). The decrease in glucose uptake observed here is important as mentioned before that cancer cells mainly depend on glucose as mentioned above. The amount of glucose consumed by the body is overall related to health and wellbeing. It was observed that reduction in glucose uptake by cells enhances the overall life expectancy (Schulz *et al.*, 2007). In this regard, the present study showed encouraging results that glucose uptake was reduced in AQP3 knockdown cells. Contrary to glucose uptake, no significant difference was observed in the glycolytic capacity of WT and 844 cells. This result is surprising as the rate of glycolysis depends on the amount of glucose therefore glycolytic capacity should be lower in 844 cells. One possible explanation is that glucose uptake was measured after cells were exposed to glucose for 24 and 48 h time points whereas glycolysis stress test was performed for a maximum of 2 h time period by the XF analyser. Therefore it is possible that in the beginning there was no difference in glucose uptake between WT and 844 cells, thus showing overall no difference in glycolysis. It is also interesting to note that impairing glycolysis stimulates reactive oxygen species (ROS) formation (Kampkotter *et al.*, 2007). In fact enhanced glycolysis facilitates acidity in tumours of MDA-MB-435 cells (another invasive breast cancer cell line) predominantly by endogenous metabolism, without depending on the availability of oxygen (Schornack and Gillies, 2003). To investigate the possible mechanisms of glycolysis employed by AQP3 knockdown cells fall outside the scope of the present study but would be of great interest in future studies.

Chapter 7

Discussion, Conclusion and Future Studies

7.1   Discussion

Breast cancer is a complex disease and is one of the leading causes of cancer mortality in women worldwide. It is associated with many factors that are thought to contribute to its pathology. The ability to control the progression of breast cancer is a crucial step in controlling the disease. Therefore, identification of factors affecting cellular migration, invasion, adhesion, and proliferation in the pathophysiology of breast cancer is an important tool in identifying potential therapies against breast cancer. It is clear that the discovery of the AQP family has identified a novel area for biomedical research, extending our knowledge of both normal physiological and pathological mechanisms. The therapeutic potential of AQPs has invited much attention from the scientific community in order to understand their role in both basic biology and clinical pathologies, including cancer. Indeed, researchers have demonstrated relationships between most AQPs and cancers of various tissues as displayed in Table 1.1.

Whilst many clinical studies have revealed different prognostic factors and biomarkers for BC diagnosis there are as yet relatively few studies regarding AQPs and their possible role in BC. The first major link between AQP and breast cancer was reported in a study which showed that AQP1 expression was significantly increased in basal-like breast carcinomas (Otterbach *et al.*, 2010c). The initial experimental aim of this study was to investigate which of the AQPs were of most clinical relevance to BC. Two different strategies were used to collect clinical specimens. Fresh, snap-frozen cancer and corresponding healthy border tissues taken from eight patients and sixteen archived FFPE breast cancer tissue samples were collected as described previously. AQP3 expression was observed significantly higher in cancer tissue than in healthy border snap-frozen fresh tissues. This study therefore further confirms the recent finding that cancer tissues exhibit higher AQP3 expression as compared to border healthy tissues. However the present study shows for the first time that AQP3 expression does not vary with lymph nodal status and tumour grade in breast cancer cases. However, it should be recognised that the sample size for this arm of the study was relatively small which may have affected the data although it provides an encouraging start for

future pilot studies. Therefore AQP3 was selected as a focus for subsequent work, on the basis of initial results and also because a role for AQP3 had not yet been elucidated in breast cancer. However, recent published studies have identified AQP3 in addition to other AQPs as being of potential importance in BC. Shi *et al* (Shi *et al.*, 2012) reported the differential expression of AQPs (including AQP3) in normal and cancer tissue samples but only at the transcriptional level. Another study also reported the higher expression of AQP3 and AQP5 in MCF-7 breast cancer cell line but the group did not study AQP3 further and instead based their study exclusively on AQP5 (Jung *et al.*, 2011). These two studies though showed the potential involvement of AQP3 in breast cancer but only to a limited level.

In the present study, the human breast cancer derived invasive cell line MDA-MB-231 was used to investigate the expression of AQPs because it is a TNBC cell line. The mRNA expression of all 13 AQPs was investigated by real-time PCR which had not been utilised for the previous published work. Initial results assessing the mRNA expression profile of all 13 human AQPs in MDA-MB-231 cells showed that AQP3, a member of the aquaglyceroporin group, exhibited significantly higher gene expression as compared to all other AQPs. It was decided that, in order for this study to fully examine the role of the elevated AQP3 expression that was detected in these cells, an AQP3 gene knockdown approach should be utilised. To fully understand a role of any gene expression in cancer pathology, knockdown of the gene expression by RNAi is a relevant approach. With this rationale in mind, shRNA plasmids for silencing AQP3 were transfected into the MDA-MB-231 parental cell line to generate the AQP3 knockdown cell line 844. Several clones were generated and tested for reduction in AQP3 mRNA expression and then two stable clones 841 and 844 were further selected and AQP3 protein expression was observed. Based on minimal expression of AQP3 in these clones 844 was selected for further investigations as it showed the greatest reduction of AQP3 out of the clones generated. shRNA technology is a popular choice for silencing gene expression however not all researchers agreed with it. One main criticism is the introduction of a plasmid mediated artifact. To counter this argument, two AQP3 knockdown clones were generated plus a scrambled sequence control which showed no significant difference in AQP3 gene and

protein expression as compared to WT. Therefore it is fair to say that the results recorded, using the 844 cell line generated, during this project are unlikely to be an artifact of plasmid expression.

After a successful generation of AQP3 knockdown stable cell line (844), the next rational approach was to investigate AQP3's role in the cells in breast cancer biology. Progression of all cancers commonly depends on dysregulation of proliferation, migration, invasion and adhesion of tumour cells. To inspect if AQP3 expression played a role in these phenomena, cellular proliferation, migration, invasion and adhesion assays were performed in WT, SC and 844 cells. A number of studies have demonstrated the involvement of AQPs both *in vitro* and *in vivo* and suggested that AQPs may facilitate cell migration in different tumours of the origin. AQP3-dependent cellular migration and proliferation during has been reported previously in keratinocytes (Hara-Chikuma and Verkman, 2008a), skin fibroblasts (Cao *et al.*, 2006), corneal re-epithelialization (Levin and Verkman, 2006), gastric cancer (Jiang *et al.*, 2013), colorectal carcinoma (Li *et al.*, 2013a) pancreatic cancer, T-cell (Hara-Chikuma *et al.*, 2012) ovarian cancer (Ji *et al.*, 2008) and breast cancer (Cao *et al.*, 2013). However the involvement of AQPs in cellular migration in breast cancer is scarcely investigated. It has been shown that shRNA mediated knockdown of AQP5 expression in MCF-7 cells resulted in significantly reduced cell proliferation and migration (Jung *et al.*, 2011). Another recent study reported that FGF-2 induced migration was reduced in MDA-MB-231 cells when AQP3 expression was silenced or blocked (Cao *et al.*, 2013). This last study was reported at the time of compiling results and writing of the present study. In this regard, the present study confirms the findings that AQP3 expression is important for cellular migration of breast cancer cells. The present study demonstrated for the first time in the tested cellular model that knockdown of AQP3 gene expression effects the cellular invasion and proliferation of BC cellular model. Cellular proliferation was significantly decreased in 844 cells indicating that AQP3 has a regulatory role in breast cancer cell proliferation. Likewise cellular invasion was significantly decreased in 844 cells as compared to WT and SC cells demonstrating the potential role of AQP3 in the invasion of breast cancer. However knocking AQP3 down did not change the cellular adhesion of 844 cells. The present study has confirmed the involvement of AQP3 in cellular

migration in MDA-MB-231 cells. As migration of tumour cells requires cell volume regulation and change in shape which suggest that AQPs might be involved in development and progression of human malignancies. More importantly present study has demonstrated for the first time that AQP3 knockdown not only impaired cellular proliferation and invasion of MDA-MB-231 cells but it also increases the sensitivity of cells to 5-FU. Thus RNAi strategy can potentially be therapeutically used in combination with 5-FU based chemotherapy to slowdown the progression of BC.

Metabolic changes in cells during cancer have extensively been studied and it is now widely accepted that cancer cells undergo rapid glycolysis even in the presence of oxygen unlike normal cells. Both normal and cancer cells rely on energy to carry out cellular functions. ATP is the energy fuel used by cells to perform cellular activities. The difference in the bioenergetic profiles of cancer cells is generally different to their corresponding non-cancerous cells. Glucose and glycerol are important metabolites utilised via different metabolic pathways to generate cellular ATP. Glycerol-induced cell death was investigated over 24, 48 and 72 h. 844 cells demonstrated a significantly reduced level of cell death when challenged with 2.5% and 5% glycerol over the three day period as compared to WT and SC cells. AQP3 downregulation therefore appears to affect glycerol permeability as might be expected since it is a glycerol channel. In the present study, glucose uptake by MDA-MB-231 cells from culture medium was assessed. However, it was surprising that glucose uptake was significantly reduced. However, no significant difference in the glycolytic capacity of WT and 844 cells was recorded.

There are a few possible explanations for this slight difference. Firstly, due to the relatively low n number used the possibility of a type 2 error exists. Bioenergetic assessment using the Seahorse Bioanalyser is prohibitively expensive and therefore only an n of 3 was chosen to ensure that the experiments could be included. The p value for the t test that was performed closely approached significance with a relatively wide SEM and with more repeats it is possible that there may be a detectable difference in the rates of glycolysis between 844 and WT.

The second explanation is the difference in experimental time for the two different techniques; glucose uptake was measured over 24 and 48 hours whilst glycolysis was measured over 2-3 hours. This difference in time course may suggest that changes in glucose permeability of cells are not immediate or are additive. Therefore glucose uptake might be decreased in 844 cells due to lower metabolic requirement with the passage of time. The difference in the media content of glucose may also have been due to the significant differences observed in cellular proliferation rate, as WT cells grow faster than 844 cells. One previous study has reported that AQP3 deficient epidermal cells showed a significant reduction in G3P content, ATP content, glucose permeability and cellular proliferation (Hara-Chikuma and Verkman, 2008b) compared to normal epidermal cells, and the present study confirms several of the findings except differences in ATP content. This difference may be explained by the metabolic difference in cancer cells as compared to epidermal cells used. The other reason could be difference in reduction in AQP3 protein expression by ~95% in keratinocytes (Hara-Chikuma and Verkman, 2008b) whereas in the present study it was reduced to 73%.

From the data presented here it is therefore apparent that inhibiting or knocking down AQP3 in breast cancer cases could be a potential tool for therapeutic intervention but examining AQP3 expression in BC may not be a useful diagnostic tool. Global AQP3 KO does not result in serious phenotypic repercussions as reported by previous studies. AQP3 null mice generated have normal appearance and deletion of AQP3 is not associated with impaired development, perinatal mortality, or growth retardation (Ma *et al.*, 2000). However AQP3 null mice have been shown to exhibit polyuria, urinary hypo-osmolality and bed wetting and AQP3 KO is considered to represent a model of nephrogenic diabetes insipidus. Conversely, deletion of AQP1 and AQP5 in mice has resulted in significant perinatal mortality (Ma *et al.*, 1998; Ma *et al.*, 1999). Interestingly, AQP3 is highly expressed on erythrocytes and represent a blood group called Gil group and there are individuals identified who are AQP3 null individuals with Gil negative phenotype having no clinical syndrome (Roudier *et al.*, 2002). Thus targeting AQP3 is potentially a better option than

other AQPs due to the considerably lesser side effects. Whether these differences are significant and important in the biology of the cancer cell can be investigated in future studies.

There were some limitations of the present study which could also influence potential future approaches to further elucidate the role of AQP3 in breast cancer pathogenesis. The first constraint was the lack of an AQP3 overexpression model. At the time of commencement of the transfection work, the first targeted approach was to produce both AQP3 overexpressed and AQP3 knockdown MDA-MB-231 cellular models. A GFP tagged AQP3 overexpression vector was obtained from Dr Matt Conner, Sheffield Hallam University, and initial transfection work was carried out. After initial difficulty, successful transfection of MDA-MB-231 cells was achieved. After considerable work two AQP3 overexpression clones were isolated and stable cell lines were generated. Initially, proliferation experiments were carried out. The initial results were promising, showing that AQP3 overexpressing cells had a higher rate of proliferation than WT cells (data not shown) and possibly greater migration rates which fits well with knockdown data as well. However, a catastrophic failure of a liquid nitrogen cell storage vessel led to a total loss of all overexpressing clones. It was not possible to repeat this work due to time constraints which subsequently meant that the present study had to focus on AQP3 knockdown as a method of AQP3 regulation as inhibition appears more relevant clinically.

A further limitation of the study was that gene knockdown methodology only managed to achieve ~70% AQP3 protein knockdown which is relatively low compared to one past study which successfully achieved ~95% AQP3 knockdown of protein expression. The level of gene knockdown achieved in this study does correspond with other studies that have reported 50% - 75% knockdown of AQPs protein expression, suggesting that the 844 cells generated here shared a common level of gene knockdown with past studies. It would have been interesting to compare function and phenotype of cells that had >90% gene knockdown with 844 cells to see if greater impact would be made with greater gene knockdown.

The present study also lacked a degree of mechanistic approach which would have provided more clarity regarding the role of glycerol or water transport by AQP3 and which of these aggravates the BC phenotype. Two possible approaches were unsuccessfully used to explore the role of water and glycerol transport by AQP3 in BC biology. Phloretin, a plant derived polyphenol, and copper sulphate ($CuSO_4$) (both reportedly inhibitors of AQP3) were used in concentrations previously reported to inhibit water and glycerol transport in cells (Echevarria *et al.*, 1996; Ikarashi *et al.*, 2012a). However both these inhibitors ultimately killed all cell types tested and as there are no other non-toxic AQP3 small inhibitors available at the moment this arm of the study was halted. The second approach that was attempted to provide mechanistic insight was to overexpress a non-glycerol transporting AQP, AQP4, in the AQP3 knock down in 844 cell clone. This experiment was carried out with colleagues at the University of Birmingham but unfortunately it was ultimately not possible to transfect the 844 cells with a high enough efficiency to achieve successful AQP4 transfection and therefore see if addition of a water pore would alter the phenotype of AQP3 deficient cells.

There were also some limitations with regard to the clinical cases of breast cancer used in this study. In the present study, the clinical sample size is relatively small mainly due to time, ethical and funding constraints. Another issue was the relative quality of RNA and protein extracted from archived FFPE samples, which may have influenced the lack of significant data reported here.

The choice of AQP3 represents an exciting target for breast cancer therapy as it fulfils several criteria. First it is a membrane protein, therefore it can be used as a prognostic biomarker and a target molecule in disease treatment (Rucevic *et al.*, 2011). Second, research using AQP3 KO mice has shown that the only major phenotypic consequences of global AQP3 knockdown are limited to dry skin and reduced wound healing (Hara *et al.*, 2002; Hara-Chikuma and Verkman, 2008c; Verkman, 2009b); although a delay in wound healing is potentially an issue post-operatively, this can hypothetically be overcome by delaying any AQP3-based therapy until after wound closure.

These observations suggest that there may be only limited consequences from using a global approach to reducing either AQP3 expression or function in breast epithelial cells. The lack of potential side effects that might be associated with global AQP3 knockdown is therefore a key strength of using AQP3 as a target for novel therapeutic interventions. The precise mechanisms by which AQP3 suppression elicits the various effects reported here remains unclear. Future work is required to clarify whether it is the function of AQP3 as a water/glycerol channel, or some as yet unassigned signalling or binding role that may explain its role in these diverse cellular functions.

7.2   Future studies

The present study together with the recent published studies highlights the importance of AQP3 in the pathogenesis of breast cancer. Future studies may want to repeat some of the clinically relevant experiments that were performed here in larger groups and with other clinicopathological factors in addition to those mentioned here should be focussed to fully elucidate the role of AQP3 in breast oncology.

The genetic analysis of AQP3 in different breast tissue samples in future studies might be a better option to understand the underlying molecular mechanisms responsible for AQP3 overexpression and associated advantages. It may be essential to ablate the expression of AQP3 by generating completely silenced AQP3 breast cancer cell lines, in order to achieve more significant observations than presented here in this study. Likewise the generation of overexpressed AQP3 cell lines using both normal and cancer lines would be important to investigate the role of AQP3 in the aggression of the disease. In addition to AQP3, this study showed that AQP11 was the second highly expressed AQP in MDA-MB-231 cells. The future studies should also focus on the role of AQP11in breast cancer. Similarly future strategies must be made to block or inhibit AQP3 in breast cancer cells. Phloretin and copper sulphate were both appeared to be toxic for the tested cellular model. Recent studies indicated that some gold compounds can also inhibit AQP3. The toxic

effects of these compounds are not well documented. Nevertheless this strategy of inhibiting the transporting role of AQP3 either chemically or by using RNAi technique is important to reduce the proliferation of disease clinically. Indeed numerous RNAi based therapeutics are under clinical investigations targeting various diseases including cancer (Burnett *et al.*, 2011). It is also important to find novel natural miRNAs which can down-regulate or suppress the functions of AQP3 both in circulation and tissues of breast cancer patients without showing harmful effects.

The present study used a relatively small number of breast cancer cases to assess the mRNA expression profile all the 13 human AQPs both in snap frozen tissue and FFPE samples. Future study must be based on larger group of patients to fully explore the expression profile of AQPs in breast cancer. It is not clear whether overexpression of AQP3 in cancer tissues of breast plays an important part in tumour biology or whether it is up-regulated as a result of stimulation of other genes and clinicopathological factors. Therefore correlation with other clinicopathological markers such ER, PR and HER-2 may be beneficial to understand the underlying mechanism of AQP3 in disease pathogenesis.

Future studies might also investigate the tumorigenic capacity of the 844 cells generated here, possibly by utilising animal models such as the SCID mouse as a tool in order to observe tumour formation *in vivo*.

7.3   Conclusion

There are several key observations noted in this study which may potentially lay foundations for future larger scale studies. The research presented in this study shows for the first time that AQP3 is involved in several distinct aspects of MDA-MB-231 breast cancer cellular behaviour, including cellular proliferation and invasion. Also herein it has been shown for the first time that reduction of AQP3 expression in MDA-MB-231 cells potentiates the cytotoxic effects of the chemotherapeutic

agent 5-FU. The present study also confirms previous findings that AQP3 is involved in cellular migration in MDA-MB-231 cells. The study also reports for the first time that down-regulation of AQP3 expression in breast cancer cells line did not have any significant impact on cellular adhesion, glycolysis and ATP content. The study also showed for the first time the differential expression of AQP3 mRNA and protein between cancerous breast tissues and healthy border tissues. Furthermore the present study also demonstrates that the levels of AQP3 expression do not vary between different clinicopathological classes of breast cancer. It is clear therefore that AQP3 is involved in breast cancer cell biology, and represents a viable target for development of future pharmaceutical interventions.

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