Universidade de Lisboa

Faculdade de Farmácia

Evaluation of aquaporins in cancer as potential biomarkers or drug targets

António João de Almeida Lemos

Dissertação orientada pela Professora Doutora Graça Soveral e coorientada pela Professora Doutora Isabel Antolin Rivera

MESTRADO EM CIÊNCIAS BIOFARMACÊUTICAS

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Summary

Cancer is a class of diseases characterized by a generalized loss of cell growth control. There are over two hundred different varieties of cancer classified by the type of cell that is initially affected. Being among the leading causes of death worldwide, cancer has a major impact on society. In 2012, there were 14.1 million new cases and 8.2 million cancer-related deaths around the world.

Tumors can grow and interfere with the majority of the body systems, including the digestive, nervous, and circulatory ones, being also able to release hormones that alter body function. Tumors that neither invade surrounding normal tissue nor spread to distant body sites, demonstrating also limited growth are generally considered to be benign. Malignant tumors on the other hand develop when a cancerous cell is able to move throughout the organism using the blood or lymphatic systems. The induction of expression programs normally associated to developmental processes facilitate the invasion of normal tissue by cancer cells. This way, cancer cells can seed secondary tumors in any location in the body (metastasis) depending on their tissue of origin. There are some cancer risk factors that include exposure to certain chemicals like tobacco smoke, as well as the practice of certain risk behaviors like exposure to radiation. There are also some situations people cannot control like age and family history that can be relevant in cancer development. In general, cancer cells have more genetic changes, such as mutations in DNA, than normal cells.

Certain characteristics of epithelial cells, such cell-cell adhesion and absence of motility are lost during epithelial-mesenchymal transition (EMT), and gain distinctive features of mesenchymal cells, such as motility, invasiveness and apoptotic resilience. This molecular process plays a major role in embryonic development as well as in tumorigenesis. At a transcriptional level, tumor growth and invasiveness are regulated through signal transduction by important signaling pathways, such as the EGFR/ERK/p38 MAPK pathway. Studies of cell signaling pathways in normal cells and cancer cells have contributed greatly to our knowledge about the disease, revealing molecular alterations that are shared among different types of cancer and pointing to possible strategies for treatment.

Aquaporins (AQPs) are transmembrane proteins that form protein channels mostly associated to the transport of water and small solutes (such as glycerol) through the

plasma membrane. The aquaporin family can be divided into three sub-groups based on their permeability characteristics. The first sub-group (orthodox or classical AQPs) are mainly permeated by water, facilitating its movement across cell membranes. Members of the second sub-group (aquaglyceroporins) are permeated by water to varying degrees but are also permeated by other small uncharged solutes like glycerol and urea. Members of the third AQP sub-group, the S-aquaporins are thought to have a role in intracellular homeostasis, although little is known about its subcellular location and permeability. AQPs are also involved in tumor angiogenesis, invasion, metastasis and growth. Numerous studies have shown that AQPs are highly expressed in several different tumor cell types.

This thesis main objective is to set aquaporins as novel molecular targets for the development of new anti-cancer drugs and to associate the expression of *AQP1, AQP3* and *AQP5* with the expression of markers that play an important role in tumor development and progression, both in cancer cell lines and human pancreas and pancreatic tumor samples.

The relative expression of the genes in study was quantified by RT-qPCR. Complementary validation of RT-qPCR results was done by western blot analysis. After analyzing the obtained results, the major conclusions can be summarized as follows: (i) with minor exceptions, AQP3 was the most expressed isoform in both cell lines and tissues, with a possible connection with the activation of EGFR/ERK/p38 MAPK signaling pathway; (ii) a relationship between AQP's expression and the cell differentiation responses that took place was suggested, both in cell lines and tissues; (iii) among the tested AQP isoforms, *AQP3* and *AQP5* stand out as the more promising targets for developing new anti-cancer drugs due to its importance in tumor development and progression;

Key words: Aquaporins, Cancer cells, RT-qPCR, Pancreatic cancer, Cancer development

Resumo

O cancro é uma classe de doenças caracterizada por uma generalizada perda de controlo sobre o crescimento celular. Estão identificadas mais de duzentas variedades diferentes de cancro, classificadas pelo tipo de célula que foi inicialmente afetada. Situando-se dentro das principais causas de morte a nível mundial, o cancro tem um enorme impacto na sociedade. Em 2013, cerca de 14.1 milhões de novos casos foram identificados e cerca de 8.2 milhões de mortes no mundo estão associadas ao cancro. Um tumor pode crescer e interferir com a maioria dos sistemas do corpo humano, incluindo o sistema digestivo, nervoso e circulatório, sendo capaz de libertar hormonas que alteram certas funções corporais.

Tumores que não invadem o tecido à sua volta nem se propagam para locais mais distantes do corpo, demonstrando também um crescimento limitado são considerados benignos. Tumores malignos, por outro lado, desenvolvem-se quando uma célula cancerígena se torna capaz de movimentar pelo organismo através do sistema sanguíneo ou linfático.

A indução de mecanismos de expressão que são normalmente associados a processos de desenvolvimento celular facilitam a invasão de tecido normal por parte das células cancerígenas. Desta forma, as células cancerígenas podem enraizar-se e formar tumores secundários em qualquer localização do corpo (metástases), dependendo do seu tecido de origem.

Existem alguns fatores de risco associados ao cancro, que incluem a exposição do organismo a determinados agentes químicos como o fumo do tabaco, assim como a prática de determinados comportamentos de risco como a exposição exagerada a radiação. Existem também certas situações que não são controláveis como o envelhecimento e o histórico familiar e que são igualmente relevantes para o desenvolvimento tumoral. No geral, as células cancerígenas apresentam mais variações genéticas do que as células normais, como por exemplo mutações no ADN.

Determinadas características das células epiteliais como a cell-cell adhesion ausência de motilidade são perdidas durante a transição de célula epitelial para mesenquimal (EMT) e ganham certas características distintivas das células mesenquimais, como motilidade celular, capacidade invasiva e resiliência apoptótica. Este processo molecular desempenha um papel importante no desenvolvimento

embriológico e na tumorigénese. O nível transcricional, o crescimento tumoral e a capacidade invasiva são regulados através de transduções de sinais em mecanismos de sinalização como o mecanismo EGFR/ERK/p38 MAPK, por exemplo. O estudo de vias de sinalização celular em células normais e tumorais contribuiu muito positivamente para o desenvolvimento do conhecimento sobre esta doença, revelando alterações moleculares que são partilhadas entre os diferentes tipos de cancro e apontando para possíveis estratégias de tratamento.

Aquaporinas (AQPs) são proteínas transmembranares que formam canais proteicos, e são maioritariamente associadas ao transporte de água e pequenos solutos (como o glicerol) através da membrana plasmática. A família das aquaporinas pode ser dividida em três subgrupos baseados nas suas características em relação à permeabilidade. O primeiro subgrupo (AQPs ortodoxas ou clássicas) são maioritariamente permeadas por água, facilitando o seu movimento através da membrana. Os membros do segundo subgrupo (aquagliceroporinas) são permeadas por água mas também permitem a passagem de outros pequenos solutos de carga neutra como o glicerol e a ureia. Membros do terceiro subgrupo das aquaporinas, as S-aquaporinas, estão possivelmente associadas a homeostase intracelular, apesar de haver pouca informação sobre a sua localização subcelular e sobre a sua permeabilidade. AQPs estão também envolvidas na angiogénese tumoral, na invasão, metástase e crescimento de tumores. Vários estudos evidenciam a elevada expressão das AQPs em vários tipos de células tumorais.

Esta dissertação tem como principal objetivo postular as aquaporinas como sendo novos alvos terapêuticos moleculares para o desenvolvimento de novos fármacos contra o cancro, e também para associar a expressão das *AQP1, AQP3* e *AQP5* com a expressão de marcadores com um papel importante no desenvolvimento e progressão tumoral, tanto em linhas celulares de cancro como em amostras humanas de pâncreas e de cancro de pâncreas.

A expressão relativa dos genes estudados foi quantificada por RT-qPCR e posteriormente validada por western blot. Após a análise dos resultados obtidos, as principais conclusões podem ser resumidas nos seguintes pontos: (i) com pequenas exceções, a AQP3 foi a isoforma mais expressa tanto nas linhas celulares como nos tecidos, havendo uma possível ligação destes níveis de expressão com a ativação da via de sinalização EGFR/ERK/p38 MAPK; (ii) a existência de uma relação entre a

expressão das diferentes AQPs e as respostas de diferenciação celular que ocorreram tanto nas linhas celulares como nos tecidos. (iii) a AQP3 e a AQP5 são, das três isoformas estudadas, as que se destacam como os alvos mais promissões para o desenvolvimento de novos fármacos anti-cancro devido à sua importância no desenvolvimento e progressão tumoral.

Palavras-chave: Aquaporinas, células cancerígenas, RT-qPCR, Cancro do pâncreas, Desenvolvimento tumoral

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List of Abbreviations

AP-1	Activation protein-1
AQP0	Aquaporin-0
AQP1	Aquaporin-1
AQP2	Aquaporin-2
AQP3	Aquaporin-3
AQP4	Aquaporin-4
AQP5	Aquaporin-5
AQP6	Aquaporin-6
AQP7	Aquaporin-7
AQP8	Aquaporin-8
AQP9	Aquaporin-9
AQP10	Aquaporin-10
AQP11	Aquaporin-11
AQP12	Aquaporin-12
ATP	Adenosine triphosphate
b-Actin	Beta-actin
BCA	Bicinchoninic acid assay
BCL-2	B-cell lymphoma 2
bFGF	Basic fibroblast growth factor
Bp	Base pairs
BSA	Bovine serum albumin
cDNA	Complementary DNA
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DNTP	Deoxyribonucleoside triphosphate
E-Cad	E-Cadherin
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinase
FBS	Foetal Bovine Serum
FGF-2	Fibroblast growth factor-2

GFR	Growth factor receptor
glpF	Glycerol uptake facilitator protein
His	Histidine
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
kDa	Kilodalton
MAPK	Mitogen-activated protein kinase
MIP	Major intrinsic protein
MMLV	Moloney murine leukemia virus
mRNA	Messenger RNA
NPA	Asparagine–proline–alanine
NPC	Asparagine–proline–cysteine
NPT	Asparagine–proline–threonine
NSCLC	Non-small-cell lung carcinoma
PDA	Pancreatic ductal adenocarcinoma
PI3K	Phosphoinositide 3-kinase
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rpm	Revolutions per minute
RT-PCR	Reverse transcriptase-polymerase chain reaction
RT-qPCR	Real time quantitative PCR
SCC	Squamous-cell carcinoma
TAD	Transactivation domain
TBE	Tris/borate/EDTA
TBS-T	tris-buffered saline with tween-20
Tm	Melting Temperature
TNM	Tumor-nodes-metastasis
VEGF	Vascular endothelial growth factor
Vim	Vimentin
XIAP	X-linked inhibitor of apoptosis protein

1 Introduction

1.1 Cancer development

Cancer is a class of diseases characterized by a generalized loss of cell growth control. There are over two hundred different varieties of cancer classified by the type of cell that is initially affected (Shurin, 2012).

Cancer harmfulness to the body happens when altered cells divide uncontrollably to form lumps or masses of tissue called tumors. Tumors can grow and interfere with the digestive, nervous, and circulatory systems, and they can release hormones that alter body function. Tumors that stay in one spot, neither invading surrounding normal tissue nor spreading to distant body sites and demonstrate limited growth are generally considered to be benign (Bruce R. Zetter, 1998).

More dangerous (malignant) tumors develop when a cancerous cell is able to move throughout the organism using the blood or lymphatic systems. The induction of expression programs normally associated to developmental processes facilitates the invasion of normal tissue by cancer cells, and this invasion can occur as epithelial sheets or as single cells. This way, cancer cells can seed secondary tumors in any location in the body (metastasis) depending on their tissue of origin (Yilmaz, Christofori, & Lehembre, 2007).

Only malignant tumors are properly referred to as cancers, and it is their ability to invade and metastasize that makes cancer so dangerous (Cooper, 2000). Whereas benign tumors can usually be removed surgically, the spread of malignant tumors to distant body sites frequently makes them resistant to such localized treatment.

Tumors induce blood vessel growth (angiogenesis) by secreting various growth factors, such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) that can induce capillary growth into the tumor (D. Li et al., 2016; Verheul & Pinedo, 2000; Yilmaz et al., 2007) supplying required nutrients for the tumor to expand. Large solid tumors contain cells that release this two angiogenic factors (Ucuzian, Gassman, East, & Greisler, 2010).The expansion of the primary tumor and metastasis to distant organs depend critically on the formation of new blood vessels that provide increased availability of oxygen and nutrients to the tumor as well as the most important route of exit from the primary tumor into the blood stream (Bruce R. Zetter,

1998). A large number of tumor cells can be shed daily into the angiogenic blood vessels that have been recruited to the tumor (Robert, 2013).When a tumor successfully spreads to other parts of the body and grows, invading and destroying other healthy tissues, it is said to have metastasized. This process itself is called metastasis, and the result is a serious condition that is very difficult to treat (Qian, Mei, & Zhang, 2017).

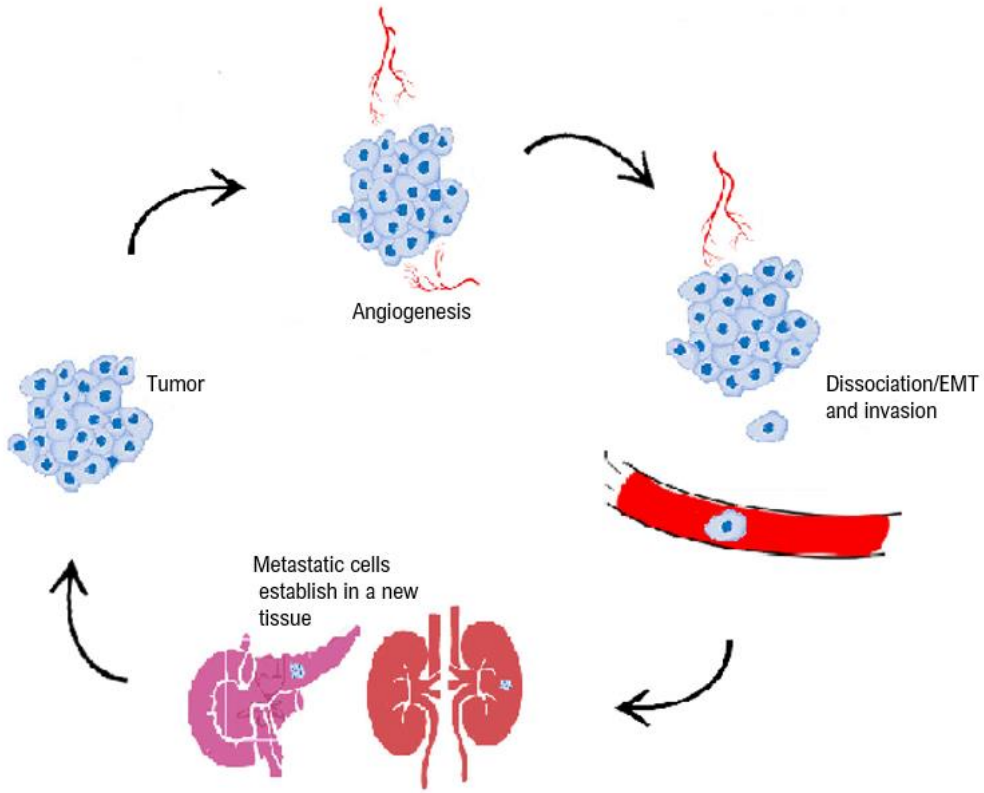

Figure 1. Representation of the steps in cancer metastasis, favored by cell migration from the primary tumor to distant organs through blood or lymph systems. Loss of cell-cell adhesion is one of the phenotypic changes that primary tumor cells experience, enabling cells to dissociate from primary tumor and enter in circulation. Circulating tumor cells extravasate and seed at secondary sites at where the process can reoccur.

It is usually not possible to know exactly why one person develops cancer and another does not, but research has shown that certain risk factors may increase the chances of developing cancer (Institute of Medicine (US) Committee on Cancer Control in Low- and Middle-Income Countries, 2007). Cancer risk factors include exposure to certain chemicals as well as the practice of certain risk behaviors. There are also some situations people cannot control like age and family history that can be relevant in cancer development. A family history of certain cancers, which represents about 5% of

all cancers (Rahner & Steinke, 2008) can be a sign of a possible inherited cancer syndrome such as Li-Fraumeni Syndrome . The World Health Organization estimates that, worldwide, there were 14 million new cancer cases and 8.2 million cancer-related deaths in 2012 (their most recent data) (World Health Organization, 2018).

Cancer is a genetic disease - that is, it is caused by changes to genes that control the way our cells function, especially how they grow and divide. Genetic changes responsible for cancer development can be inherited from our parents (Hodgson, 2008). They can also arise during a person's lifetime as a result of errors that occur during cell division or because of damage to DNA caused by certain environmental exposures (Parsa, 2012). Cancer-causing environmental exposures include substances, such as the chemicals in tobacco smoke, and radiation, such as ultraviolet rays from the sun. In general, cancer cells have more genetic changes, such as mutations in DNA, than normal cells.

Chemical species, like the reactive oxygen species (ROS) have been detected in almost all cancers at elevated levels, showing its involvement in many aspects of tumor development and progression and participating in cell growth/proliferation, differentiation, protein synthesis, glucose metabolism, cell survival and inflammation (Liou & Storz, 2010). At the same time, tumor cells express increased levels of antioxidant proteins to detoxify from ROS, which suggests that a fragile balance of intracellular ROS levels is essential for the function of cancer cells. Also, the generated radical and its generation location, as well as the local concentration are important for ROS cellular functions in cancer (Liou & Storz, 2010). Reactive oxygen species, particularly hydrogen peroxide, can also act as second messengers in cellular signaling (Forman, Maiorino, & Ursini, 2010).

Activation protein-1 (AP-1) is a family of transcription factors associated with the stimulation of extracellular signal-regulated kinases (ERKs). The activation of AP-1 is a nuclear event induced by growth factors. In the case of c-Fos, a member of AP-1 family, the activation of ERK leads to an enhanced expression of c-Fos mRNA. It has been recently shown that ERK phosphorylates multiple residues within the carboxyl-terminal transactivation domain (TAD) of c-Fos, augmenting its transcriptional activity. However, the way ERK-dependent phosphorylation coordinates the function of c-Fos is still not fully understood (P. Monje, Hernandez-Losa, Lyons, Castellone, & Gutkind, 2005).

Certain characteristics of epithelial cells, such cell-cell adhesion and absence of motility are lost during a complex molecular process called Epithelial-Mesenchymal Transition (EMT), and gain distinctive features of mesenchymal cells, such as motility, invasiveness and apoptotic resilience (Polyak & Weinberg, 2009). This molecular process plays a major role in embryonic development as well as in tumorigenesis (Kyprianou, 2010; Thiery, Acloque, Huang, & Nieto, 2009). Some molecular alterations take place during EMT, including the decrease of E-cadherin levels and consequent increase of N-cadherin and vimentin levels, leading to defective cell-cell adhesion and loss of cell-cell junctions, contributing to metastatic dissemination and invasion of tumor cells, as well as acquired therapy resistance (Imbert et al., 2012; Tiwari, Gheldof, Tatari, & Christofori, 2012).

Mitogen-activated protein kinases (MAPKs) play a significant role in differentiation, proliferation and apoptosis of various cells, and as a members of the MAPKs family, ERK is mainly responsible for proliferative responses (Du et al., 2007; Wen et al., 2010).

Raf/MEK/ERK cascade often regulates two ERK molecules: ERK1 and ERK2. Mutations and overexpression of upstream molecules such as epidermal growth factor receptor (EGFR) are often the causes for this pathway activation in certain tumors. Dependent on the stimulus and cell type, this pathway can transmit regulatory signals upon apoptosis and cell cycle progression (Steelman, Bertrand, & McCubrey, 2004; Steelman, Pohnert, et al., 2004).

Little is known about the different in vivo targets of ERK1 and ERK2. However, according to Mazzuchelli (Mazzucchelli et al., 2002), ERK1 has anti-proliferative effects while ERK2 has been postulated to have pro-proliferative effects in mice brains.

Epidermal growth factor receptor (EGFR), which is a tyrosine kinase receptor, can be overexpressed in a wide variety of cells resulting in cell proliferation and invasion with downstream effects (Butowski & Chang, 2006). At a transcriptional level, tumor growth and invasiveness are regulated through signal transduction by important signaling pathways, such as the EGFR/ERK/p38 MAPK pathway (Singh, Schneider, Knyazev, & Ullrich, 2009; Thomson, Petti, Sujka-Kwok, Epstein, & Haley, 2008). Recent studies suggest that one of the main mediators of tumor cells proliferation and migration through this pathway is AQP5 (Xu & Xia, 2014; J. Yang et al., 2017). Some intervenients in this pathway include ERK1/2 and p38 (members of the MAPKs

family), and EGFR, fundamental in differentiation, proliferation and apoptotic responses (Butowski & Chang, 2006; J. Yang et al., 2017).

1.2 Oncogenic Signaling Pathways

The genetic changes that contribute to cancer tend to affect three main types of genes: proto-oncogenes, tumor suppressor genes, and DNA repair genes. Proto-oncogenes are involved in normal cell growth and division. Conversion, or activation, of a proto-oncogene into an oncogene generally involves a gain-of-function mutation, allowing cells to grow and survive when they shouldn't (Lodish H, 2000).

Mutated proto-oncogenes become genes that stimulate excessive division, and mutations in tumor suppressor genes inactivate these genes, eliminating the critical inhibition of cell division that normally prevents excessive growth (Alberts B, 2002). Collectively, mutations in these two categories of genes account for much of the uncontrolled cell division that occurs in human cancers.

Most proto-oncogenes code for proteins that are involved in molecular pathways, that receive and process growth-stimulating signals from other cells in a tissue (Chial, 2008). Usually, such signaling begins with the production of a growth factor, a protein that stimulates division. These growth factors slip through the gaps between cells and attach to specific receptor proteins located on the neighboring cells surface (Bafico A, 2003). This will emit a signal to proteins in the cytoplasm, which will transmit a stimulatory signal to other proteins in the cell until the division-promoting message reaches the cell's nucleus and activates a set of genes that help move the cell through its growth cycle (National Institutes of Health (US), 2007).

Oncogenes cause the proteins involved in these growth-promoting pathways to be overactive and for the cell to proliferate much faster than it would in a non-mutated condition. Some oncogenes cause cells to overproduce growth factors, which stimulate the growth of neighboring cells, but they also may drive excessive division of the cells that just produced them (Abelev & Eraiser, 2008).

To become cancerous, cells must also escape from the inhibitory stimuly that normally counteracts these growth-stimulating pathways. Normal cells experience a flow of inhibitory messages to its nucleus, but when this flow is interrupted, the cell can disregard the substantially strong inhibitory messages at its surface (Baba AI, 2007).

Scientists are still trying to identify the normal functions of many known tumor suppressor genes. Some of these genes code for proteins that operate as parts of specific inhibitory pathways. When a mutation causes such proteins to be inactivated or absent, these inhibitory pathways no longer function normally (Lodish H, 2000). Other tumor suppressor genes appear to block the flow of signals through growth-stimulating pathways; when these genes no longer function properly, such growth-promoting pathways may operate without normal restraint (Sever & Brugge, 2015). Mutations in all tumor suppressor genes, however, apparently inactivate critical tumor suppressor proteins, depriving cells of this restraint on cell division.

In addition to the controls on proliferation afforded by the coordinated action of proto-oncogenes and tumor suppressor genes, cells also have at least three other systems that can help them avoid runaway cell division (Sancar, Lindsey-Boltz, Ünsal-Kaçmaz, & Linn, 2004). The first of these systems is the DNA repair system. This system operates in virtually every cell in the body, detecting and correcting errors in DNA (Clancy, 2008). Across a lifetime, a person's genes are under constant attack, both by carcinogens imported from the environment and by chemicals produced in the cell itself. Errors also occur during DNA replication. In most cases, such errors are rapidly corrected by the cell's DNA repair system (Clancy, 2008). Should the system fail, however, the error (mutation) becomes a permanent feature in that cell and in all its descendants.

The system's normally high efficiency is one reason why many years typically must pass before all the mutations required for cancer to develop occur together in one cell. Mutations in DNA repair genes themselves, however, can undermine this repair system in a particularly devastating way by damaging the cell's ability to repair errors in its DNA (Lodish H, 2000).

A second cellular back-up system forces a cell to commit suicide (apoptosis) if some essential component is damaged or its control system is deregulated (Sancar et al., 2004). This observation suggests that tumors arise from cells that have managed to evade such death. One way of avoiding apoptosis involves the p53 protein, which activity is tightly controlled and influenced by a series of quantitative and qualitative events that influence the outcome of p53 activation. In its activated form, this protein not only pauses cell division, but also induces apoptosis in abnormal cells. In many types of cancer, p53 gene is inactivated (Fridman & Lowe, 2003).

A third back-up system limits the amount of times a cell can divide and makes sure that cells can't duplicate endlessly (National Institutes of Health (US), 2007). This system is governed by a counting mechanism that involves the DNA segments at the ends of chromosomes, the telomeres. These segments shorten each time a chromosome replicate. Once the telomeres are shorter enough, they trigger an internal signal that causes the cell to stop dividing. If the cells continue dividing, further shortening of the telomeres eventually causes the chromosomes to break apart or fuse with one another, a genetic crisis that is inevitably fatal to the cell (Shay, 2016).

Early observations of cancer cells grown in culture revealed that, unlike normal cells, cancer cells can proliferate indefinitely. It has been discovered that the molecular basis for this characteristic is an enzyme called telomerase, that systematically replaces telomeric segments that are trimmed away during each round of cell division. Telomerase is virtually absent from most mature cells, but is present in most cancer cells, where its action enables the cells to proliferate endlessly (National Institutes of Health (US), 2007).

To understand how cancer develops and progresses, researchers first need to investigate the biological differences between normal cells and cancer cells. That work focuses on the mechanisms that underlie fundamental processes such as cell growth, the transformation of normal cells to cancer cells, and the spread, or metastasis, of cancer cells.

Knowledge gained from such studies deepens on our understanding of cancer and produces insights that could lead to the development of new clinical interventions (Sanchez-Vega et al., 2018). For example, studies of cell signaling pathways in normal cells and cancer cells have contributed greatly to our knowledge about the disease, revealing molecular alterations that are shared among different types of cancer and pointing to possible strategies for treatment (Sever & Brugge, 2015).

The last few decades of basic research in cancer biology have created a broad base of knowledge that has been critical to progress against the disease. In fact, many advances in the prevention, diagnosis, and treatment of cancer would not have occurred without the knowledge that has come from investigating basic questions about the biology of cancer.

1.3 Aquaporins

The existence of proteins channels that intervened in the specific transportation of water through the membrane was hypothesized for several decades, assumed by biophysical measurements of membrane permeability in red blood cells and in epithelial cells of the renal proximal tubule. Until the discovery of a red-blood-cell protein that led to the description of aquaporin-1 (AQP1) as the first molecular water channel by Peter Agre, these proteins were unknown (Brown, 2017; Moon, Preston, Griffin, Jabs, & Agre, 1993). After the recognition of the unique properties of aquaporins, there was a change in our consideration of membrane permeability, being now known that water transport across the membrane can be regulated independently of solute transport.

AQPs are members of the Major Intrinsic Protein (MIP) family and often referred to as water channels. In mammals and plants, they are present in almost all organs and tissues and their function is mostly associated to the transport of water and small solutes (such as glycerol) through the plasma membrane, determined by osmotic gradients or by the concentration gradient of the solute (A. S. Verkman, Mariko Hara-Chikuma, & Marios C. Papadopoulos, 2008b).

The aquaporin family can be divided into three sub-groups based on their permeability characteristics, which generally coincide with specific amino-acid-sequence patterns (C. Li & Wang, 2017). The members of the first sub-group (orthodox or classical AQPs) are mainly permeated by water, facilitating its movement across cell membranes, according to osmotic and pressure gradients, and this group includes AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8. AQP6 and AQP8 are in this group based on primary sequence analysis, although AQP6 is permeated by anions and AQP8 might be permeated by water and urea. Members of the second sub-group (aquaglyceroporins), which includes AQP3, AQP7, AQP9 and AQP10, are permeated by water to varying degrees, but are also permeated by other small uncharged solutes, in particular, glycerol and urea (Ishibashi, Morishita, & Tanaka, 2017; C. Li & Wang, 2014).

More recently two new AQP members were reported in mammals, being named AQP11 and AQP12, and the most remarkable feature of these two new AQPs is their unusual sequence of the first NPA motif: NPC in AQP11 and NPT in AQP12. Based on this discovery, a new AQP sub-group was defined, the non-orthodox or S-aquaporins.

AQP11 is localized intracellularly, suggesting a role in intracellular homeostasis (Yakata et al., 2007).

1.4 Structure, selectivity and regulation

Since the first AQP was identified, thirteen human AQPs have been discovered. Structural results for several family members have established that AQP channels share a common structural architecture. Together with biochemical studies, these structural data revealed that the functional AQP unit is a homotetramer (Fig. 2C) and that each AQP monomer is composed of six transmembrane α-helices connected by alternating intracellular and extracellular loops (Tornroth-Horsefield, Hedfalk, Fischer, Lindkvist-Petersson, & Neutze, 2010) (Fig. 2B). The transmembrane domains form a right-handed bundle around the central pore of each AQP monomer through which water/solute transport occurs.

Biochemical analyses of AQP1 revealed that the 28-kDa polypeptide that was evident on immunoblots represented the monomeric form of the protein, but that AQP1 (as well as other aquaporins) is present as a tetramer in the cell membrane. Recent studies have showed some evidence about the details of the aquaporin structure that direct this tetrameric arrangement, as well as into those features that dictate the permeability characteristics of the water channel (Agre et al., 2002).

A common fold shared by the aquaporin family is given by a monomeric structure formed by six transmembrane helices and two half-helices, with their N-terminal ends located in the center of the pore. Each of the half-helixes contains a sequence of three-amino-acids, Asn-Pro-Ala (NPA), considered the signature sequence motif of the members of aquaporin family (Ishibashi et al., 2017) (Fig. 2D). Mutational analysis of residues around the conserved NPA motifs led to predictions of an hourglass-like structure, with two loops, the intracellular loop B and the extracellular loop E, folding into the membrane to form the pore. Several structural studies, which include cryo-electron microscopy of human red-blood-cell AQP1 and X-ray crystal structures of Escherichia Coli GlpF and bovine AQP1, have confirmed the predicted hourglass structure (King, Kozono, & Agre, 2004).

Aquaporins are present in the membrane as tetramers, but, unlike ion channels, the channel for water permeability does not reside at the center of the tetramer. Instead, each monomer contains a channel. Structural studies have provided insights into the

apparent requirement for tetramer formation. The helices of each AQP1 monomer that are positioned on the outside face of the tetramer are hydrophobic, whereas those that are placed towards the center of the tetramer are hydrophilic (Verkman, 2013).

Structural studies also revealed that the restriction of AQP1 permeability to water arises from two principal mechanisms. First, the channels narrow to a diameter of 2.8 Å approximately 8 Å above the center of the bilayer, which physically limits the size of molecules that can pass through them. A highly conserved arginine residue provides a fixed positive charge at this constriction site in each channel. This size restriction filter is named ar/R (aromatic arginine) selectivity filter (King et al., 2004). The narrowest part of an E. coli GlpF channel is ~1 Å wider than in AQP1, and this increased diameter is sufficient to allow glycerol to pass through GlpF channels. Such channel widening is predicted to occur in all aquaporin homologues that are permeated by small solutes like glycerol or urea. The second mechanism involves the orientation of a pair of dipoles at the NPA motifs, behaving as a charge selectivity filter (Hub & de Groot, 2008). These dipoles interact with individual water molecules and prevent them from hydrogen bonding to adjacent water molecules. The functional separation of water molecules eliminates the possibility of H^+ transfer through a channel. The combination of size and charge filters provide the basis for the unique permeability characteristics of the aquaporins (King et al., 2004).

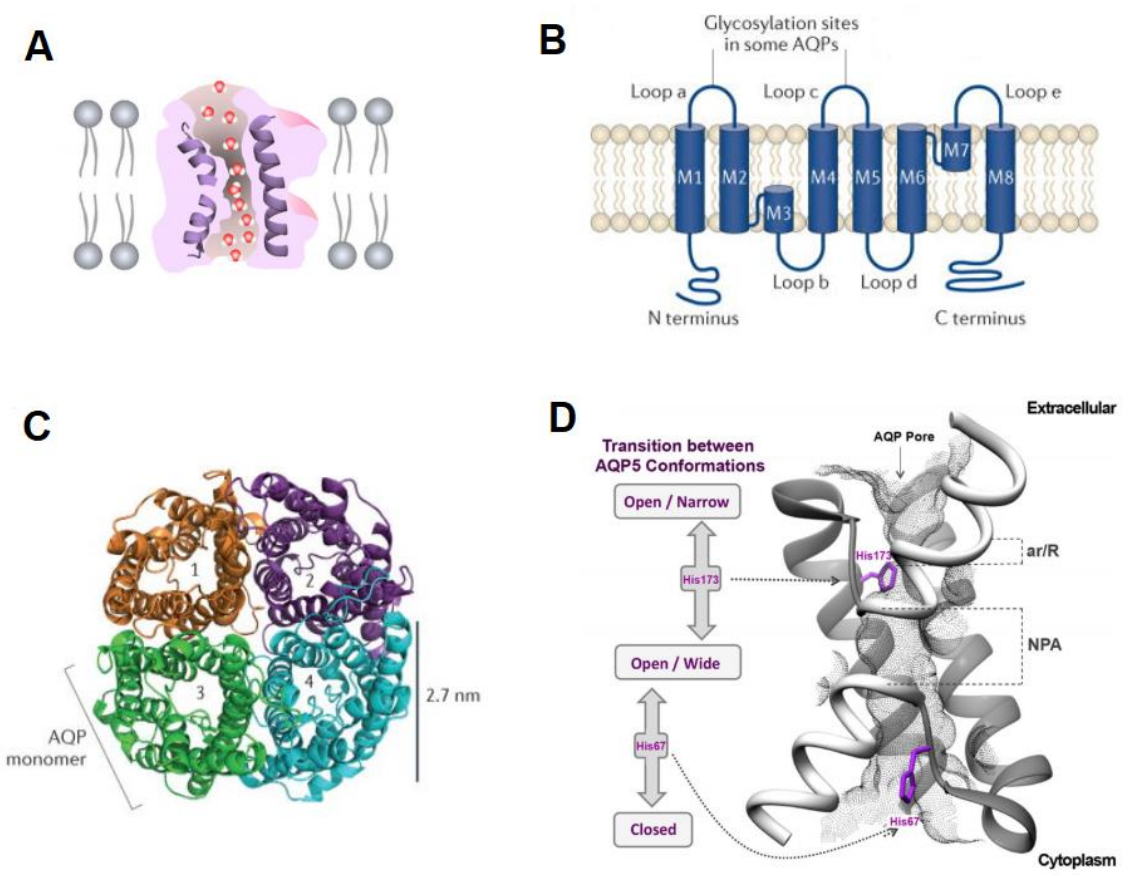

Figure 2. **A** | Diagram illustrating how water molecules permeate through AQP pore. **B** | A schematic of AQP membrane topography. **C** | A top view of the extracellular face of an aquaporin 1 (AQP1) homotetramer, with monomers labelled 1–4, based on the X-ray structure of bovine AQP1 (Protein Data Bank (PDB) code: 1J4N). **D** Detailed view of AQP5 pore and schematic representation of the proposed AQP5 gating mechanism. The two half-helices are depicted in white and the positioning of ar/R and NPA selectivity filters is indicated. The grey mesh represents the residues lining AQP5 pore. Key histidine residues involved in AQP5 gating are highlighted: His67, which controls the transition between closed and open conformations, and His173, controlling the transition between wide and narrow states. Structures were generated with Chimera (http://www.cgl.ucsf.edu/chimera) and are based on AQP5 X-ray structure (protein data bank code: 3D9S).

(Adapted from Direito, I., Madeira, A., Brito, M.A. et al. Cell. Mol. Life Sci. (2016) 73: 1623. And Verkman AS, Anderson MO, Papadopoulos MC. Aquaporins: important but elusive drug targets. *Nat Rev Drug Discov*. 2014;13(4):259-77).

AQPs are widely distributed in human tissues and are generally preserved in mammals including rodents and humans. In some organs, such as the kidney, several AQPs are expressed and play a major role in normal function. For example, in response to antidiuretic hormone vasopressin, AQP2 (which is found in intracellular vesicles) becomes expressed in the apical plasma cell membrane of collecting duct epithelial cells and increases the reabsorption of urine by the kidney. Humans with AQP2 mutations have congenital nephrogenic non-X-linked diabetes insipidus thus confirming the key role of AQP2 in water reabsorption by the kidney.

Defects in urine concentration were reported in mice that lack AQP2, AQP3 or AQP4, suggesting that inhibition of these AQPs would result in a response similar to that produced by vasopressin V_2 receptor antagonists (Ma et al., 2000; Ma et al., 1997; B. Yang, Gillespie, Carlson, Epstein, & Verkman, 2001; B. Yang, Zhao, & Verkman, 2009).

AQP5 is expressed in salivary and airway sub-mucosal glands, and it was reported that mice that lack AQP5 had abnormal secretion of airway mucus and saliva. Inhibition of AQP5 could reduce salivation and airway mucus production during anesthesia (Ma et al., 1999; Y. Song & Verkman, 2001).

Secretion of cerebrospinal fluid by the choroid plexus and of aqueous fluid by the ocular ciliary epithelium is facilitated by AQP1 (Oshio, Watanabe, Song, Verkman, & Manley, 2005; D. Zhang, Vetrivel, & Verkman, 2002), and therefore, AQP1 inhibition might reduce intraocular pressure in glaucoma and intracranial pressure in brain trauma or stroke.

AQP4 is the principal water channel expressed in astrocytes throughout the central nervous system (CNS) (Rash, Yasumura, Hudson, Agre, & Nielsen, 1998). Evidence showing AQP4 involvement on water transport in the brain and spinal cord, as well as in neuroexcitation and astrocyte migration following injury suggests that AQP4 modulators are potential therapeutic targets in the treatment of brain edema, epilepsy and neural regeneration (Papadopoulos & Verkman, 2008).

AQP1 is highly expressed in tumor-associated microvascular endothelial cells (Endo, Jain, Witwer, & Brown, 1999) and in several different tumor cell types. Mice lacking AQP1 have reduced growth of implanted and spontaneously generated tumors as a consequence of defective tumor angiogenesis (Esteva-Font, Jin, & Verkman, 2014; Saadoun, Papadopoulos, Hara-Chikuma, & Verkman, 2005). Also, implanted AQP1-

expressing tumors have greater local invasiveness and more metastases than tumors lacking AQPs (Hu & Verkman, 2006).

AQP3 is expressed in the basal layer of proliferating epidermal keratinocytes. It has been reported that hydration of the stratum corneum and reduced skin elasticity was reduced in mice lacking AQP3 (Ma, Hara, Sougrat, Verbavatz, & Verkman, 2002). This AQP3 deficiency leads to reduced glycerol permeability of epidermal cells, causing a reduction in stratum corneum and epidermis of glycerol content.

Cell volume regulation is a necessary mechanistic component of AQP-mediated transcellular water flow. It comprises regulatory volume decrease, usually in response to hypotonicity-induced cell swelling, and regulatory volume increase, usually in response to hypertonicity-induced cell shrinkage. The molecular mechanisms underlying these responses are not yet fully understood, but it is unlikely that there is a single common mechanism. The signaling pathways associated with cell volume regulation appear to be cell-type dependent. Nonetheless, the end results of these varied pathways are similar, as regulatory volume decrease relies on osmolyte (Huxtable, 1992) (potassium chloride and taurine) and water efflux from the cell to reduce cell volume whereas regulatory volume increase is achieved by osmolyte and associated water influx via import of sodium to the cell. Although the rapid regulatory volume increase process following cell shrinkage involves inorganic ions, after hours of prolonged hypertonic exposure, animal cells often replace the ions with non-perturbing organic osmolytes (Day et al., 2014). The mechanisms for this include external transport into the cell, down-regulation of organic degradation and up-regulation of organic synthesis. These mechanisms facilitate the homeostasis of osmolality within the cell (Day et al., 2014).

Regulation of AQPs is critical to osmoregulation and water homeostasis in microorganism and in mammalian organs involved in fluid transport. Eukaryotic water selective AQPs are frequently regulated post-translationally either by gating, controlling the channels flux rate, or by trafficking, whereby AQPs are shuttled from intracellular storage sites to the plasma membrane. Gating of AQPs has been described for several cell systems. The gating behavior or mammalian, plant and yeast AQPs has been reported to be affected by a variety of factors, including pH, solute gradients, membrane tension, temperature, and phosphorylation (Chaumont, Moshelion, & Daniels, 2005; Maurel, 2007; Soveral, Macey, & Moura, 1997; Soveral, Madeira, Loureiro-Dias, & Moura, 2008; Tornroth-Horsefield et al., 2010).

1.4.1 Aquaporins in cell migration and proliferation

In addition to the well-established role of AQPs in maintaining tissue water balance, other roles of AQPs include facilitating cell migration, cell proliferation and cell adhesion. An unanticipated role for AQPs in facilitating cell migration was first suggested by Loitto et al. (Loitto, Forslund, Sundqvist, Magnusson, & Gustafsson, 2002) who studied AQP9 in neutrophils. Subsequently, Saadoun et al. (Saadoun, Papadopoulos, Davies, Bell, & Krishna, 2002a) showed that several AQPs facilitate cell migration in different cell types including AQP1 in aortic endothelial cells and AQP4 in astrocytes. The overall conclusion from several studies *in vitro* and in vivo is that AQP expression enhances cell migration towards a chemotactic stimulus. The exact mechanism remains unclear but may involve targeted water entry into the leading edge of a migrating cell, which enhances formation of the lamellipodium (a flattened protrusion at the leading end of a migrating cell, which is essential for cell motility). The idea that AQPs facilitate formation of the lamellipodium is consistent with the polarization of AQPs to the leading end of migrating cells. It has been suggested that AQPs also facilitate the rapid changes in cell shape that take place as a migrating cell squeezes through the sinuous extracellular space. Such changes in cell volume are likely to require rapid flow of water into and out of the cell. Some authors have recently suggested that cells may utilize directed water permeation mediated by AQPs to create a net inflow of water and ions at the cell leading edge and a net outflow of water and ions at the trailing edge leading to net cell displacement (Stroka et al., 2014). This mechanism may allow cell migration through confined micro-spaces without the need for actin depolymerization–polymerization or myosin II-mediated contractility. It is important to note that lack of AQPs does not entirely inhibit cell migration but provides a migratory motion towards a chemotactic stimulus less efficient. This may explain why AQP-null mice develop normally in utero even though cell migration is an important component of embryogenesis. AQP3, which is expressed in the epidermis, enhances the proliferation rate of basal keratinocytes (H. Qin et al., 2011). AQP3 null mice have impaired wound healing, due to reduced glycerol and ATP content in the keratinocytes, which are required for biosynthesis. There is direct and indirect evidence that AQP3 and AQP5 play a role in tumor cell proliferation. AQP5 might interact with the Ras pathway in colon cancer (Woo et al., 2008). Ras activation switches on other proteins that

ultimately turn on genes involved in cell growth, differentiation and survival. Another study showed AQP5-facilitated lung cancer cell proliferation and migration, possibly through activation of the EGFR/ERK/p38 MAPK signaling pathway (Z. Zhang et al., 2010). These AQP5–oncogene interactions may represent novel AQP functions, which are unrelated to water transport. AQP3 null mice are remarkably resistant to the development of skin tumors following exposure to the tumor initiator and promoter, phorbol ester. Glycerol supplementation corrected the reduced proliferation in AQP3 deficiency, with cellular glycerol, ATP, and proliferative ability being closely correlated (Hara-Chikuma & Verkman, 2008). There is, therefore, an established link between AQP3 expression in the epidermis and skin cancer. AQP3 expression is high in non-small cell lung cancer and, in a mouse model, AQP3 knockdown suppressed tumor growth and reduced angiogenesis in human non-small cell lung cancer xenografts (Xia et al., 2014).

AQPs have a very well determined role in cell-cell adhesion. The expression of AQP4 in L-cells that lack endogenous adhesion molecules lead to the formation of a bundle of cells, which indicates its role in cell-cell adhesion. On the other hand, recent experiments in two different AQP4 isoforms suggested a role in cell adhesion for one of them but not for the other, as one can bound to adhesion complexes of the extracellular matrix while the other polarizes to the leading edge of the cell, being involved in cell migration (Smith, Jin, Ratelade, & Verkman, 2014). Also, the expression of AQP0 in lens fiber cells in the eye is well determined. Studies in AQP0 null mice demonstrated the important role of AQP0 in maintaining the structure of interlocking protrusions that have a critical role in maintaining the lens transparency and integrity (Kumari & Varadaraj, 2009).

1.4.2 Mechanisms of aquaporins involvement in cancer

Two mechanisms have been proposed to explain how AQPs facilitate cell migration: AQPs facilitate rapid water flow across the plasma membrane into the front end of migrating cells, driven by changes in osmolality produced by transmembrane ion flux and actin depolymerization, explaining why AQPs tend to polarize to the leading edge of migrating cells (Papadopoulos, Saadoun, & Verkman, 2008). The second theory postulates that cells migrating through the irregularly shaped extracellular space

undergo rapid changes in their volume and experience rapid changes in transmembrane water fluxes. It was recently discovered a remarkable phenotype in AQP3 null mice: the resistance to the development of skin tumors. In mammalian skin, AQP3 is expressed strongly in plasma membranes of the basal epidermal cell layer. Phenotype analysis of AQP3 null mice showed dry skin and delayed barrier recovery after removal of the stratum corneum (Verkman et al., 2008b). These defects were attributed to the absence of AQP3-facilitated glycerol transport, resulting in reduced stratum corneum and epidermal cell glycerol content, slowing cutaneous wound healing.

The function of AQP1 in vascular endothelial cells was established using an in vivo tumor angiogenesis assay in which wild-type and AQP1-null mice were subcutaneously implanted with B16F10 melanoma cells (Saadoun et al., 2005). Tumor growth was reduced in AQP1-null mice due to impaired tumor angiogenesis causing extensive tumor necrosis. Cultured aortic endothelial cells from wild-type and AQP1 null mice had comparable morphology, growth, and adherence to different surfaces but showed remarkable impairment in their migration. These findings suggested that the strong AQP1 expression observed in tumor microvascular endothelial cells facilitates their migration, an essential component of tumor angiogenesis, depending on a water influx into the cells, with consequent expansion of their lamellipodia (13).

A recent study has shown that AQP1 plays a crucial role in estrogen-induced tubulogenesis of vascular endothelial cells (Zou et al., 2013), providing fresh new insight into the molecular mechanisms underpinning the angiogenic effects of estrogen. The authors found that expression of AQP1 in blood vessels of human breast carcinoma tissues were significantly higher than controls, confirming the observations of several earlier studies.

The up-regulation of AQP5 in lung cancer cells activates EGFR, which is known to trigger the RAS/MAPK as well as phosphatidylinositol-3- kinase (PI3K)/AKT signal pathways (McCubrey et al., 2015). PI3K activates AKT that in turn blocks caspase-9, ultimately blocking apoptosis in AQP5 expressing cancer cells.

In colon cancer cells, AQP5 is also associated with the p38 MAPK pathway, which is activated in response to stress signals, like chemotherapy-damaged DNA. Activation of p38 MAPK pathway leads to the expression of multidrug resistance proteins responsible for tumor drug resistance (Direito, Madeira, Brito, & Soveral, 2016).

1.4.3 Aquaporins as cancer biomarkers

In the last decade, an increasing number of reports showed that AQP5 is abundantly expressed in different tumors and could serve as biomarker with prognostic value of cancer aggressiveness.

Recent publications suggest that this isoform may enhance cancer cell proliferation, migration and survival in a variety of malignancies, with strong evidences pointing to AQP5 as a promising drug target and as a novel biomarker for cancer aggressiveness with high translational potential for therapeutics and diagnostics.

Interestingly, co-expression of AQP5 and AQP3 is related with invasion depth, lymph node involvement, metastasis and a poorer survival rate (Zhengcai Zhu et al., 2018), which suggests that the combined detection of these two isoforms may be useful prognostic biomarkers for esophageal squamous-cell carcinoma (SCC). Although the correlation between AQP5 overexpression and oral cancer requires validation, the available data indicate that AQP5 could be a drug target and/or a useful biomarker for this disease.

An association between AQP5 expression and clinicopathological variables for lung cancer patients is still controversial. AQP5 overexpression was found to be associated with the histological tumor type, TNM (tumor-nodes-metastasis) staging and lymph node metastasis (T. Song et al., 2015). A positive correlation was also reported between AQP5 overexpression and worst clinical outcomes, with higher rates of tumor recurrence, early disease progression and decreased survival rates, suggesting that AQP5 could be a novel prognostic marker in non-small-cell lung carcinoma (NSCLC). However, these observations were not confirmed in a posterior study (Machida et al., 2011) thus rendering necessary additional investigation.

AQP5 overexpression relates with clinicopathological variables, such as tumor size and histological type as well as with tumor malignancy and the risk of recurrence after curative surgery, pointing that it could constitute a basis for selecting an appropriate postoperative treatment.

There is a great translational potential in AQP5-based therapeutics and diagnostics. In view of the wide range of cancer malignancies in which AQP5 is implicated, the potential of AQP5 as a biomarker for cancer detection and prognosis should be explored. Further pathophysiological investigation is required to establish AQP5 as cancer drug target and as a biomarker for cancer detection and follow up.

AQP3 was found as a critical and necessary factor for the migration of human breast cancer cells induced by fibroblast growth factor-2 (FGF-2) (Cao et al., 2013). FGF-2 could increase AQP3 expression and cell migration through EGFR-PI3K or GFR-ERK signaling pathways, which was blocked by the deletion of AQP3. AQP3 expression in breast cancer cells was increased by the stimulation with 5′-deoxy-5-fluoropyrimidine nucleosides (5′-DFUR), which was used in the chemotherapy of solid tumors. It was proposed that AQP3 might be a limiting factor in the pharmacological effects of 5′-DFUR, since the deletion of AQP3 reduced the efficacy of the drug. It seemed that AQP3 can be considered as a chemotherapeutic target to develop the combining strategy for cancer treatment. However, it should be questioned whether AQP3 can act as a breast cancer-specific diagnostic biomarker or therapeutic target.

1.4.4 Colorectal cancer

Fischer et al. developed a study that was designed to detect additional genes altered during colorectal carcinogenesis. One of the genes found, AQP8, was expressed in all normal colon samples but not, or to a less extent, in the colorectal tumors. Those results suggest that the expression of AQP8 is a marker of normal proliferating colonic epithelial cells and that these cells are involved in fluid transport in the colon (Fischer, Stenling, Rubio, & Lindblom, 2001).

On the other hand, Moon et al. used a reverse transcriptase-polymerase chain reaction (RT-PCR) analysis to see the expression of different AQPs in several colon and colorectal cell lines, and found that AQPs 1, 3, and 5 were expressed in those cell lines, and a western blot analysis confirmed their expression in four of these. In situ hybridization demonstrated that during colorectal carcinogenesis, the expression of AQP1 and AQP5 was induced in early-stage disease (early dysplasia) and maintained through the late stages of colon cancer development, suggesting that multiple AQP expression may be advantageous to colorectal carcinogenesis (Moon et al., 2003).

The relationship between the overexpression of AQP5 and the stage of tumor differentiation is still arguable and requires further investigation. In fact, the overexpression of AQP5 was associated with decreased tumor differentiation and a more significant tumor aggressiveness which translates into a poorer prognosis (Shi et al., 2014).

1.4.5 Breast cancer

Very recently, Qin et al. reported that AQP1 was localized dominantly in the cytoplasm of cancer cells of invasive breast cancer patients and cytoplasmic AQP1 was an independent prognostic factor, and the high expression of AQP1 was an indicator of a shorter survival. They also state that the cytoplasmic expression of AQP1 was further validated in both primary cultured breast cancer cells and AQP1 over-expressing cell lines, in which the functional importance of cytoplasmic AQP1 was confirmed *in vitro*. This study provides evidence of cytoplasmic expression of AQP1 promoted breast cancer progression and it could be a potential prognostic biomarker for breast cancer (F. Qin et al., 2016).

Breast cancer cells invasiveness and loss of apical membrane domain polarity in benign epithelial cells was observed when AQP5 was up-regulated. AQP5 is diffused intracellularly in cancer cells, eminently in situations where there is lymph node metastasis. This isoform subcellular localization may influence the activation of pathways associated to proliferation and drug-resistance mechanisms. By knocking down AQP5 in MCF7 breast cancer cell line, Jung et al. (Jung, Park, Jeon, & Kwon, 2011) showed a diminution in cell migration and proliferation, which indicates that this isoform plays a major role in tumor spread, although the specific pathways involved remain unknown.

1.4.6 Pancreatic Cancer

Recently, a study conducted by Direito et al. (Direito, Paulino, Vigia, Brito, & Soveral, 2017) suggested that AQP3 and AQP5 are involved in pancreatic ductal adenocarcinoma (PDA) development and progression, pointing out AQP3's involvement with late and more aggressive stages of PDA, and AQP5 as a potential novel histological marker in earlier stages of PDA. It was also proposed that AQP5 overexpression is related with tumor differentiation independently of AQP3, suggesting that AQP5 may be useful as a therapeutic target due to its involvement in PDA development. These findings emphasize the usefulness of AQP5 in early PDA diagnosis, although the mechanisms underlying the different expressions of AQP3 and AQP5 in PDA tumorigenesis require further clarification.

In another study, Huang et al. (Huang, Huang, & Shao, 2017) determined the expression level of 12 members of the AQP family and not only revealed that AQP3 had the relative higher level of expression of all isoforms in PDA, but also provided evidence supporting that AQP3 has a critical role in the malignant phenotypes of pancreatic cancer by silencing AQP3 (small interfering-mediated knockdown of AQP3), which resulted in cell growth arrest and increased cell apoptosis.

1.4.7 Melanoma

According to Lao et al. (Gao et al., 2012) AQP3 is expressed in most melanoma cell lines, and the overexpression of this isoform protects these cells from arsenite-induced apoptosis by increasing the expression levels of the anti-apoptotic proteins BCL-2 and XIAP (X-linked inhibitor of apoptosis protein).

Nicchia et al. (Nicchia et al., 2013) confirmed the role of AQP1 in sustaining an active endothelium during angiogenesis using a mouse model of melanoma. Their results showed that AQP1 silencing caused an increase in Vascular Endothelial Growth Factor (VEGF) levels, which stimulates blood vessel formation. This information opens the possibility that the inhibition of both AQP1 and VEGF at the same time could be a valid choice for optimal therapeutic results, validating AQP1 as a pro-angiogenic protein, relevant for the therapy of cancer and other angiogenic-related diseases.

Verkman et al. demonstrated that tumor growth was diminished due to impaired tumor angiogenesis, which led to extensive tumor necrosis in AQP1-null mouse model injected subcutaneously with melanoma cells (Saadoun et al., 2005). Also, Hu et al. found that microvessels in the tumors of AQP1-null mice had lower density (Hu & Verkman, 2006). Contrastingly, in human and rat proliferating tumors, microvessels had AQP1 being greatly expressed (Endo et al., 1999; Saadoun, Papadopoulos, Davies, Bell, & Krishna, 2002b). Additionally, Hu et al. found that tumor cell migration and metastatic potential greatly increased in two mouse tumor cell lines with AQP1 expression as compared to the same cell lines without AQP1 expression (Hu & Verkman, 2006).

2 Aims

An emerging amount of evidence linking AQPs to cancer development and several processes related to tumor proliferation, edema formation, migration and angiogenesis opened the way for the discovery of AQP inhibitors that can be useful anti-cancer drugs. In this study:

1) we postulated on the variability of aquaporins (*AQP1*, *AQP3* and *AQP5*), cell differentiation markers (*E-Cad* and *Vim*) and markers involved in tumor signaling pathways (*EGFR*, *ERK1* and *ERK2*) gene expression across different tumor cell lines.

2) we hypothesized that different patterns of aquaporins expression exist between human pancreas and pancreatic tumors, being this expression related to the tumor's type, stage and degree of differentiation.

3) ultimately, the goal was to set aquaporins as novel molecular targets for the development of new anti-cancer drugs and to associate the expression of this aquaporins with the expression of markers that play an important role in tumor development and progression.

3 Materials and Methods

3.1 Human pancreatic tumors and corresponding normal pancreas tissues

Samples from various histological types of pancreatic tumors were gathered by a clinical team from Hospital Curry Cabral, Centro Hospitalar de Lisboa Central, Lisbon, Portugal. For the majority of patients, the histological diagnosis was made only after resection of the specimen.

Two types of biopsy were collected from the fresh specimen, immediately after its surgical removal: a sample of the tumor itself or in its edge in the case of very small nodes (sample t-tumor), and a sample of normal pancreatic tissue (sample p-pancreas) for comparative purposes. Immediately after collection, samples were preserved in an RNA stabilization solution (RNAlater, Sigma) and stored at -80ºC.

3.2 Cell Cultures

Human cell lines HCT116 (colorectal carcinoma), MCF7 (breast adenocarcinoma), MNT1 (melanoma) and HaCaT (keratinocytes) were cultured in DMEM (Sigma Aldrich, Missouri, USA) supplemented with 10% (v/v) Fetal Bovine Serum (FBS), 100 U/mL penicillin/streptomycin, and 4 mM L-glutamine (Sigma) in an incubator at 37°C with 5 % $C0_2$.

3.3 Total RNA extraction

Human cultured cell lines HCT116 (colorectal carcinoma), MCF7 (breast adenocarcinoma), MNT1 (melanoma) and HaCaT (keratinocytes), as well as human pancreatic tumors and corresponding normal pancreas tissues were collected in a 1.5 mL microtube and 1 mL of Trizol (Invitrogen) was added to each tube. If the RNA extraction was not done right away, the samples would be stored at -20°C. Samples previously stored were placed at room temperature for 10 minutes before RNA extraction occurs. To separate the homogenate into different phases, 200 µL of chloroform were added followed by 8 to 10 gentle inversions of the tube. Samples were then incubated at room temperature for 2-3 minutes and then centrifuged at 12,000 rpm at 4 °C for 15 minutes. The upper phase was then transferred to another tube and 500 µL

of isopropanol (VWR Chemicals) were added, followed by shaking and incubation at room temperature for 10 minutes. The tubes were then centrifuged at 10,000 rpm for 10 minutes at 4 °C. The supernatant was discarded, and the pellet washed two times with 500 μL 75% ethanol, followed by centrifugation at 7,500 rpm for 5 minutes at 4 °C. The pellets were then dissolved in diethylpyrocarbonate (DEPC)-treated water.

To exclude possible DNA contamination, treatment with DNase I (AMPD1 SIGMA) was performed following the manufacturer's protocol.

3.4 Measurement of total RNA concentration and quality

The RNA concentration was determined spectrophotometrically at 260 nm using the NanoDrop1 ND-2000c (Thermo Fisher Scientific, Waltham, USA). The ratios 260/280 nm and 260/230 nm were determined to assess the purity of RNA samples and dismiss the presence of contaminants. The 260/280 nm and 260/230 nm ratios correspond to protein and solvent presence, respectively, and the samples would proceed to the cDNA synthesis step if these values were comprised between 1.8 - 2.2.

3.5 cDNA synthesis

Complementary DNA (cDNA) was synthesized from total RNA samples (1 μg each sample). The reverse transcription reaction was performed in a final volume of 20 μL containing using GRS One-Step RT-PCR Kit (GRISP). The synthesis conditions were: 37°C for 60 min, 95°C for 5 min and 4°C after termination. The final cDNA samples were diluted to 1:3 with DEPC-treated water and stored at -20°C.

3.6 Primers design

Specific primers for real time quantitative PCR (RT-qPCR) were designed for seven genes (*b-Actin, HPRT, EGFR, ERK1, ERK2, E-Cadherin, Vimentin, c-Jun* and *c-Fos*). The mRNA sequences of the human (*Homo sapiens*) genes were obtained from GenBank (accession numbers listed in Table 1) and then submitted to the Primer3 bioinformatic tool (http://primer3.ut.ee/) to generate the primers according to a specific set of parameters in order to obtain the best pair of primers in size (19-25 bp), melting temperature (55-65ºC), % GC (50-60%) and product size range (75-250 bp). The

characterization of the selected genes used in RT-qPCR is described in Table 1.

Table 1. Gene specific primer sequences used for RT-qPCR

Gene	Full gene name	GenBank accession no.	Forward/reverse Primers	Product size (bp)
HPRT1	Hypoxanthine-guanine phosphoribosyltransferase 1	NM_000194.2	F: 5' ACTGAACGTCTTGCTCGAGATG '3 R 5' AGCAGGTCAGCAAAGAATTTATAGC '3	101
EGFR	Epidermal growth factor receptor	NM_201284.1	F: 5'GAAATCCTCGATGAAGCCTACGTG '3 R 5' GTCTTTGTGTTCCCGGACATAGTC '3	150
ERK1	extracellular signal–regulated kinase 1	NM_002746.2	F: 5' AAGATCAGCCCCTTCGAACATC '3 R 5' CTTGTACAGGTCAGTCTCCATCAG '3	180
ERK2	extracellular signal–regulated kinase 2	NM_138957.2	F: 5' TACACCAACCTCTCGTACATCG '3 R 5' CATGTCTGAAGCGCAGTAAGATT '3	169
E-Cad	E-Cadherin	NM_004360.4	F: 5' TCGACACCCGATTCAAAGTG '3 R 5' GTCCCAGGCGTAGACCAAGA '3	101
Vim	Vimentin	NM_003380.4	F: 5' TGCCCTTAAAGGAACCAATGAG '3 R 5' AGGCGGCCAATAGTGTCTTG '3	102
c-Jun	Jun Proto-Oncogene, AP-1 Transcription Factor Subunit	NM_002228.3	F: 5' GTATCCTGCCCAGTGTTGTTTG '3 R 5' GCAGAAAAGAGGTTAGGGGAGTAC '3	167
c-Fos	Fos Proto-Oncogene, AP-1 Transcription Factor Subunit	NM_005252.3	F: 5' CCGGGGATAGCCTCTCTTACT '3 R: 5' CCAGGTCCGTGCAGAAGTC '3	93

3.7Real time Quantitative PCR primers validation and optimization

Each pair of primers must be validated prior to the RT-qPCR run to achieve the most accurate template quantification. For each reaction, only a simple product must be amplified, and the amplification efficiency must be independent from template concentration. Primer efficiency (90-110%) and specificity (a single melting temperature) were evaluated. When these conditions were not achieved, primer optimization was attempted by varying the annealing temperature (55-65 ºC) and/or primer concentration (200-300 nM) individually.

Table 2 and Table 3 depict the optimized amplification conditions for the genes targeted in this project.

Table 2. Optimized conditions to quantify *HPRT, EGFR, ERK1, ERK2, Vim, E-Cad, c-Jun and c-Fos* mRNA expression levels by RT-qPCR.

	Temperature (°C)	Time	Cycles
Initial denaturation	95	10 minutes	1
Denaturation	95	15 seconds	39
Annealing/extension	60	1 minutes	

Table 3. Amplification conditions to quantify b-Actin, AQP1, AQP3 and AQP5 mRNA expression levels by RT-qPCR using TaqMan assay.

	Temperature (°C)	Time	Cycles
Initial denaturation	95	10 minutes	1
Denaturation	95	15 seconds	44
Annealing/extension	60	1 minutes	

3.8 Real time Quantitative PCR (RT-qPCR)

To examine the expression of our genes of interest, RT-qPCR assay was performed using the CFX96™ Real-Time PCR Detection System C1000 (BioRad, California, USA).

For TaqMan assay, primers and probes mix (*b-Actin, AQP1, AQP3* and *AQP5)* were obtained from Applied Biosystems, Inc. (Carlsbad, CA, USA). The reaction

mixture was prepared for a final reaction volume of 20 µL, containing 10 µl of TaqMan Universal Master Mix II with UNG, 1 µL of TaqMan Gene Expression Assay and 9 µL of cDNA template (dilution 1:9), and was amplified as follows: denaturation at 95°C for 10 min and 44 cycles at 95°C for 15 s and 60°C for 1 min. Direct detection of PCR products was monitored by measuring the fluorescence produced by the result of TaqMan probe hydrolysis after every cycle.

For SYBR green assay, primers were designed specifically for our genes of interest (Table 1). The reaction mixture was prepared for a final reaction volume of 25 µL, containing 12.5 µL of Xpert Fast SYBR™ SYBR® Master Mix (GRiSP Research Solutions, Porto, Portugal) 3.75 µL of template cDNA, 2.5 µL of each forward and reverse primers and 3.75 µL DEPC-treated water. The reaction consisted of an initial denaturation step at 95 °C for 10 minutes, 39 cycles of denaturation at 95 °C for 15 seconds and annealing/extension for 1 minute. A dissociation stage was added to determine the melting temperature (Tm) of a single nucleic acid target sequence as a quality and specificity measure.

Relative expression levels were normalized to reference genes (*HPRT* for *EGFR, ERK1, ERK2, E-Cadherin, Vimentin, c-Jun and c-Fos; β-actin* for *AQP1, AQP3* and *AQP5*) and calculated using a variation of the Livak method (Livak & Schmittgen, 2001), corrected for variation in amplification efficiency, as described by Fleige and Pfaffl (Fleige & Pfaffl, 2006; Fleige et al., 2006):

$$\text{Relative expression} = \frac{E^{CT}(Housekeeping\ Gene)}{E^{CT}(Target\ Gene)} \qquad \text{E = 1 + efficiency of the reaction}$$

3.9 SDS-PAGE for nucleic acid detection

For electrophoretic analyses, 10% polyacrylamide gels (100 mm × 150 mm × 1 mm) were prepared with a tris-borate (TBE) buffer (2 mM EDTA, 89 mM Tris and 89 mM Boric acid, pH 8.0). The ratio of acrylamide to Bis was 19 : 1. Sample Buffer (Laemmli SDS sample buffer, reducing 4x) was mixed with each sample (1:4). A molecular weight marker (DNA Ladder VI NZYTech, Lisboa, Portugal) was loaded in the first lane and samples were loaded in each of the following lanes in the polyacrylamide gels. Ethidium bromide (Sigma Aldrich, Missouri, USA) was the dye used to identify and visualize nucleic acid bands in the

gel. Imaging of the gel was done with ChemiDoc (ChemiDoc XRS+ system, BioRad, California, USA).

3.10 Western Blot Analysis

3.10.1 Total protein harvest

On collection day, cells grown in 75 cm^2 culture flasks were washed twice with 1x PBS followed by the addition of 500 uL RIPA Buffer buffer (Sigma Aldrich, Missouri, USA). Cell lysate was homogenized by pipetting and transferred to a 1.5 mL microcentrifuge tube, where it was kept for 5 minutes at 4 °C. The mixture was then centrifuged at 12.000g for 15 minutes at 4°C and the supernatant was then transferred to a new tube to be stored at -20°C.

3.10.2 Protein Quantification

Protein was quantified using the Pierce™ BCA Protein Assay Kit. The samples were diluted 1:5 in milli-Q water. A mix for each sample with BCA working reagent was prepared in a final volume of 200 µL (1:8) and then plated in a 96 multi-well plate. After 30 min of incubation at 37ºC, absorbance was read in Biochrom Asys Expert Plus Microplate Reader at 550 nm. The standard curve ranging from 125 to 1000 µg/mL was prepared using known concentrations of Bovine Serum Albumin (BSA) solution.

3.10.3 Western Blot

An equivalent amount of protein (50µg) from each was separated by 12% polyacrylamide gel electrophoresis. Protein separation was carried out, at room temperature, at 25 mA for 40 minutes for migration in the concentration gel, followed by 40 mA until the dye front got to the bottom of the separation gel. In every gel, a molecular weight standard (NZYColour Protein Marker II, NZYTech) was included to allow molecular weight estimation. Once protein separation was complete, proteins

were transferred to a PVDF membrane (Amersham Hybond 0.45 PVDF, GE Healthcare) during 50 min at 300 mA. To diminish the heat produced during the transference, ice was incorporated into the buffer tank. The membranes were then stained with Ponceau S reagent to confirm equal protein loading in each sample lane.

After membrane blocking with 5% milk in Tris-Buffered Saline Tween (TBS-T; 50 mM Tris-HCl, pH 8; 154 mM NaCl and 0.1% Tween 20) for 2 hours at room temperature and under continuous stirring, membranes were washed 4 times for 10 min each with TBS-T. Membranes were then incubated with primary antibody, selected according to the protein of interest, overnight at 4ºC, under continuous stirring. All primary antibodies were prepared in 5% milk in TBS-T (listed in Table 4). Membranes were washed 4 times for 10 min with TBS-T after incubation, followed by incubation with secondary antibody for 2 hours at room temperature under continuous stirring. All secondary antibodies were prepared in TBS-T (1:5000). Finally, membranes were washed again and incubated with ECL Prime Western Blotting Detection Reagent (Amersham, GE Healthcare) for 2 minutes at room temperature. Chemiluminescence was evaluated using the ChemiDoc XRS+ system, BioRad, California, USA). Densities of each band were calculated with ImageJ software.

Table 4. List of primary antibodies used in Western Blot protein analysis.

Marker	Dilution	Host Species	MW (kDa)	Catalog Number	Manufacturer
b-Actin	1:1000	Rabbit	41.7	A2066	Sigma-Aldrich
AQP1	1:100	Mouse	28.5	sc-25287	Santa Cruz Biotechnology
AQP3	1:200	Goat	31.5	sc-9885	Santa Cruz Biotechnology
AQP5	1:200	Rabbit	28.3	ab92320	Abcam
EGFR	1:1000	Rabbit	134.3	ab52894	Abcam
Vimentin	1:.1000	Rabbit	53.6	ab92547	Abcam
E-Cadherin	1:10000	Rabbit	97.5	ab40772	Abcam

4 Results

4.1 AQPs gene expression in cancer cell lines

Several reports suggest an important role for AQPs in cancer (Ribatti, Ranieri, Annese, & Nico, 2014; Verkman, 2012; Wang et al., 2015), manly supported by AQPs overexpression in a considerable variety of tumors (Direito et al., 2017; Guo et al., 2013; Kusayama et al., 2011; A. Li et al., 2013; Liu et al., 2007; Machida et al., 2011). *AQP1* (Fig. 4A), *AQP3* (Fig. 4B) and *AQP5* (Fig. 5C) gene expression was quantified using real-time quantitative PCR (RT-qPCR) in three different human tumor cell lines: HCT116 (colorectal carcinoma), MCF-7 (breast adenocarcinoma) and MNT1 (melanoma), as well as in a normal human skin cell line: HaCaT (keratinocytes), and the results are displayed in Figure 4.

It is clear that MNT1 predominantly expresses the three AQP isoforms over the other cell lines. *AQP1* and *AQP3* expression have been reported in melanocytes (Boury-Jamot et al., 2006) but, to our knowledge, there are no previous report of *AQP5* expression in these cells neither in melanoma cells, suggesting that the tumorigenesis associated with this cell line might have led to *AQP5* overexpression. The expression levels of *AQP3* and *AQP5* in HaCaT are also worth mentioning, as *AQP3* has been reported to be abundantly expressed in keratinocytes (Sougrat et al., 2002), and yet this cell line presents a much lower *AQP3* expression level than the other studied cells. On the other hand, *AQP5* presents a higher expression level that would be expected, as there are no clear indications in previous reports that this AQP isoform is highly expressed in keratinocytes.

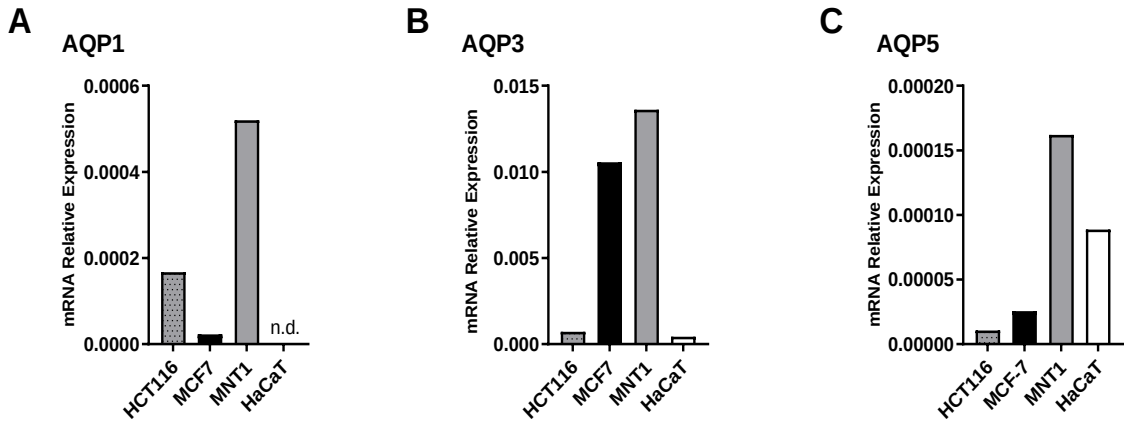

Figure 3. Differences in relative expression levels of aquaporins (AQPs) in HCT116, MCF7, MNT1 and HaCaT cell lines. *AQP1* (A), *AQP3* (B) and *AQP5* (C).

4.2 Epithelial to mesenchymal transition (EMT) and cell proliferation in cancer cells

All cell lines displayed a similar expression profile of the three tested AQP isoforms, being *AQP3* the most expressed. Evidence suggesting that *AQP3* fulfills a major role in tumor development and metastasis is rapidly increasing (Jensen, Login, Koffman, Kwon, & Nejsum, 2016; M. C. Papadopoulos & S. Saadoun, 2015; Ribatti et al., 2014; Verkman, 2012; A. S. Verkman, M. Hara-Chikuma, & M. C. Papadopoulos, 2008a; Wang et al., 2015), which strengthens the possibility that *AQP3* expression levels, and the possible downstream signaling pathways stimulated by this isoform, can be used as a drug target.

To assess the possible relationship between *AQP1, AQP3* and *AQP5* expression and specific cellular responses such as cell differentiation, proliferation, migration and apoptosis, which can be associated with metastatic dissemination and invasion of tumor cells, the expression of specific genes that are directly and indirectly responsible for these cellular responses was determined by RT-qPCR and is illustrated in Figure 5.

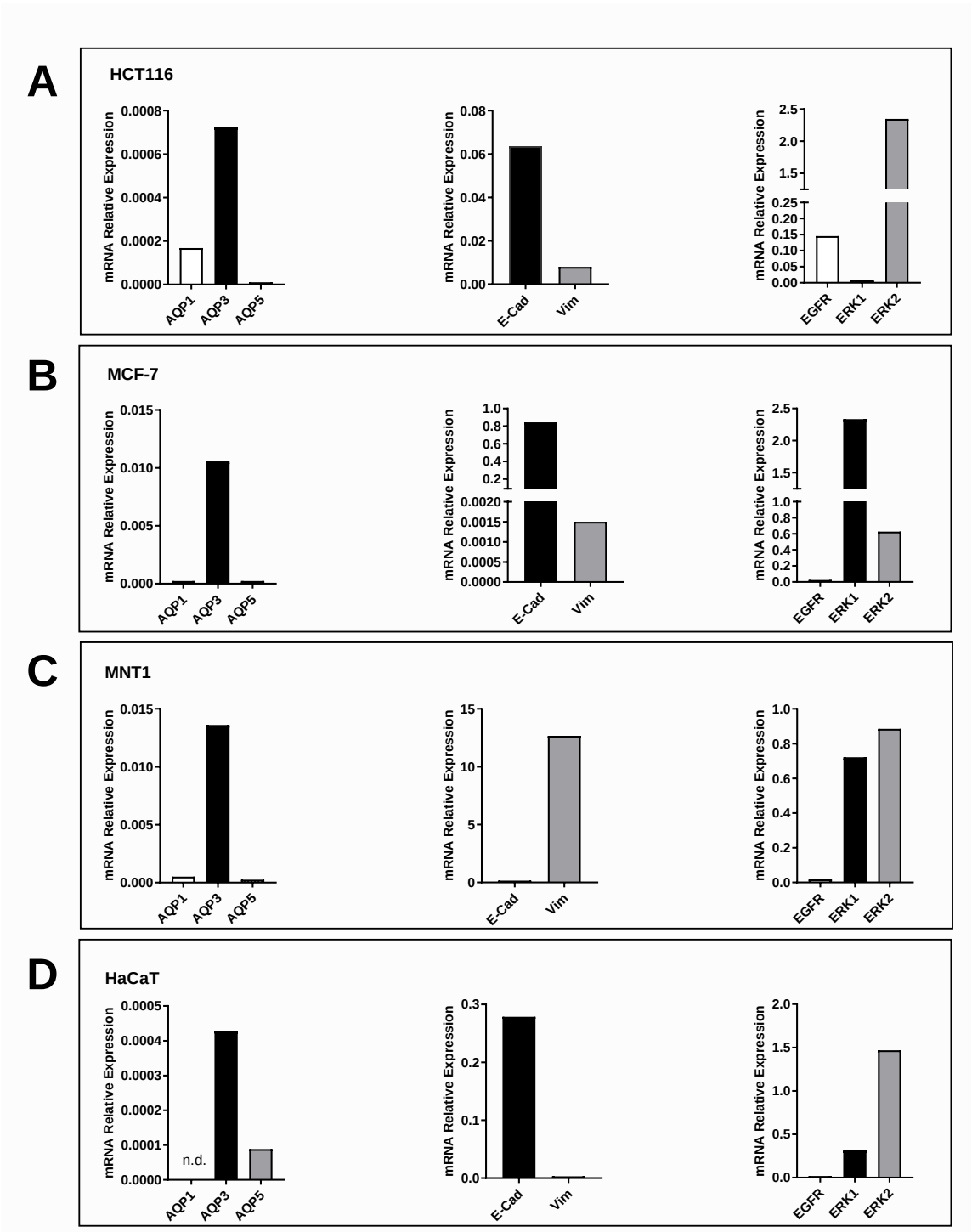

Figure 4. Relative expression levels of aquaporins (AQPs), markers of cell differentiation (E-Cad and Vim*)* and signaling markers of the EGFR/ERK/p38 MAPK pathway (EGFR, ERK1 and ERK2) in HCT116 (A), MCF-7 (B), MNT1 (C) and HaCaT (D).

During EMT, epithelial cells acquire a mesenchymal phenotype. Cancer cells that have undergone EMT are more aggressive, displaying increased invasiveness, stem-like features, and resistance to apoptosis. Increased Vimentin expression has been noted in many human carcinomas, such as colon, breast and prostate cancers, and is generally indicative of aggressive tumor behavior and poor prognosis (Andreolas, Kalogeropoulou, Voulgari, & Pintzas, 2008; Armstrong et al., 2011; Katz et al., 2011).

Figure 6 illustrates the expression levels of two EMT-related genes, E-Cadherin (Fig. 6A) and Vimentin (Fig. 6B), in the four cell lines used in this study. In MCF-7, *E-Cad* level is substantially higher than in the remaining cell lines, followed by HaCaT, HCT116 and MNT1, respectively. Remarkably, *Vim* expression levels in MNT1 are much higher compared to the other cell lines. Also, *E-Cad* levels for this cell line are very low, suggesting that EMT has occurred in a more extended way compared to the other cells in the study, indicating that MNT1 generally has characteristics of a more aggressive and invasive tumor.

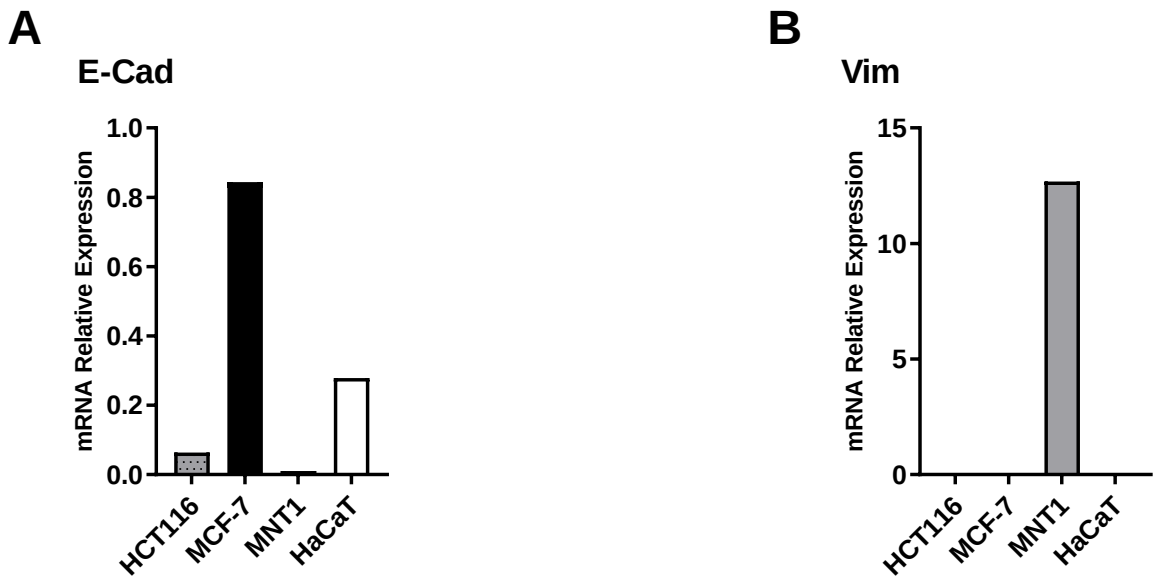

Figure 5. Relative expression levels of two epithelial to mesenchymal transition (EMT)-related genes, E-Cadherin (A) and Vimentin (B), in HCT116, MCF-7, MNT1 and HaCaT.

Activation of important signaling pathways, such as the EGFR/ERK/p38 MAPK pathway, are associated with tumor growth and invasiveness. *EGFR* gene is overexpressed in a wide variety of cells, resulting in cell proliferation and invasion. *ERK1*, *ERK2* and *EGFR* mRNA expression was assessed in our cancer cell lines in order to establish a connection between *EGFR* expression and a variation in *ERK1* and *ERK2* levels. The results are illustrated in Figure 7.

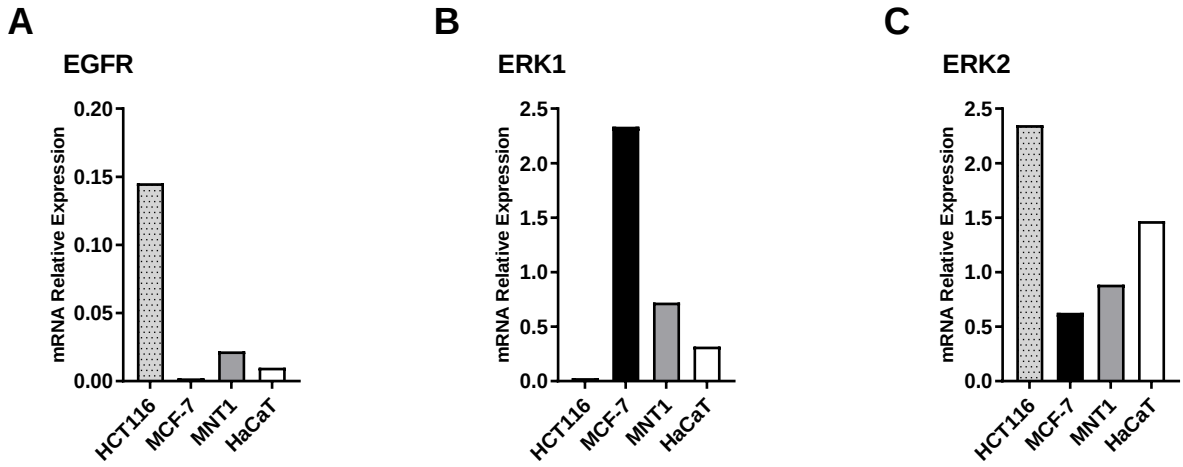

Figure 6. Relative expression levels of signaling markers involved in EGFR/ERK/p38 MAPK pathway: *EGFR* (A), *ERK1* (B) and *ERK2* (C) in HCT116, MCF-7, MNT1 and HaCaT.

EGFR mRNA expression is notably higher in HCT116 compared with the other cell lines (Fig 7A). This colorectal carcinoma cell line also presents the lowest level of *ERK1* mRNA expression and the highest expression level of *ERK2* mRNA (Fig 7B and 7C). Such different expression levels between *ERK1* and *ERK2*, associated with an apparent overexpression of *EGFR* mRNA, what might suggest that an intensified proliferative response is occurring. On the other hand, the opposite situation happens with MCF-7, where the *EGFR* mRNA expression level is the lowest of the four cell lines and *ERK1* mRNA expression level appears as the highest one. Although *ERK2* mRNA expression level in MCF-7 does not differ from the other cell lines, at least as significantly as *ERK1* does, it is still the lowest expression level of them all. Similarly to HCT116, in HaCat the *ERK2* mRNA expression level is considerably higher than the one of *ERK1*.

4.3 AQPs gene expression in human pancreas and pancreatic tumors

In order to screen the expression profile of *AQP1*, *AQP3* and *AQP5* in human pancreatic tumors and corresponding normal pancreas tissues, RT-qPCR was performed using 10 samples from 5 different patients with pancreatic cancer (5 pancreatic tumor samples and 5 normal pancreas tissue samples), and the results are illustrated in Figure 8.

When comparing the variation of *AQP*s expression between normal and tumoral samples from each individual, it is important to know at what extent can we consider each *AQP* isoform as a tumor marker and drug target. Regarding patient 1, *AQP1* (Fig. 8A) and *AQP3* (Fig. 8B) expression levels are considerably lower in the pancreatic tumors in comparison with the normal pancreas. As for *AQP5* (Fig. 8C), its expression level is higher in the pancreatic tumor.

For patient 2, *AQP1* expression is also significantly lower in the pancreatic tumor, but *AQP3* has slightly higher expression than in the normal pancreas. Patient 3 shows no significant difference in *AQP1* expression between the two conditions but has a slightly higher expression for *AQP3* in the pancreatic cancer tissue. *AQP5* expression is significantly higher in the pancreatic cancer tissue than in the normal pancreas and it's the highest *AQP5* expression between all the patients. Patient 4 show a moderate decrease in *AQP1* expression in the pancreatic cancer tissue in comparison with the normal pancreas, and a significantly high decrease in *AQP3* expression in the pancreatic cancer comparing with the normal pancreas tissue. *AQP5* expression is significantly increased in the pancreatic tumor. Patient 5 has similar expression levels of *AQP1* in both conditions, but it is slightly higher in the pancreatic tumor tissues. *AQP3* and *AQP5* expression levels are significantly higher in pancreatic cancer tissues in comparison with the normal pancreas.

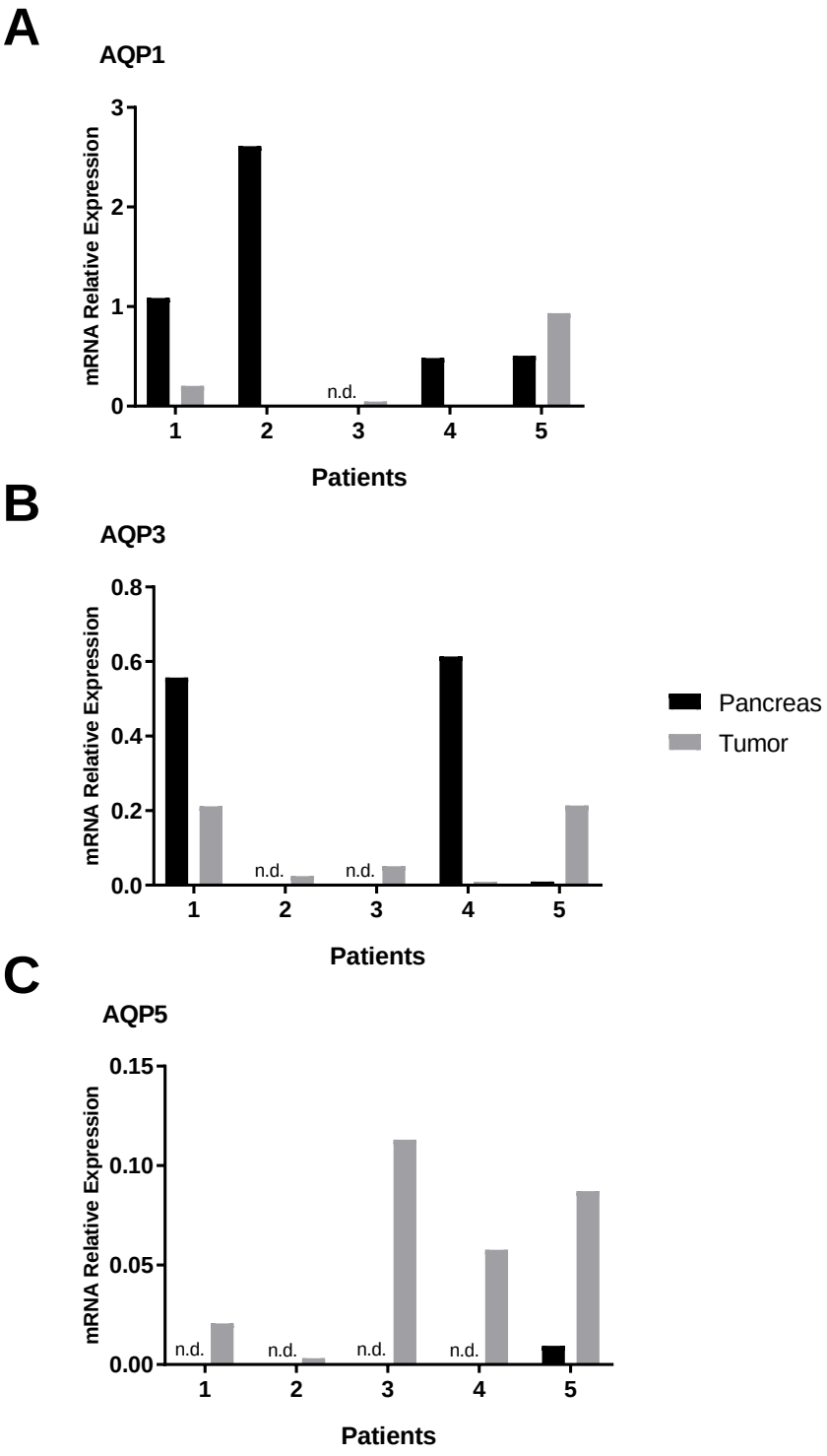

Figure 7. Relative expression levels of *AQP1* (A), *AQP3* (B) and *AQP5* (C) in human pancreatic tumors (tumor, gray bars) and corresponding normal pancreas (pancreas, black bars) tissues collected from 5 consecutive patients.

4.4 Epithelial to mesenchymal transition (EMT) and cell proliferation in human pancreas and pancreatic tumors

Variation in the expression levels of the EMT-related genes, *E-Cad* and *Vim* was determined. The obtained results are illustrated in Figure 9.

Patient 1 has a much higher expression level of *E-Cad* (Fig. 9A) in pancreatic cancer in comparison with the normal pancreas tissue. At the same time, the expression level of *Vim (Fig. 9B)* in this patient is extremely higher in pancreatic cancer. Patient 2 has a similar *E-Cad* and *Vim* expression profile as patient 1, with both markers being significantly higher in pancreatic cancer in comparison with the normal pancreas tissues. Patient 3 has a higher *E-Cad* expression in pancreatic cancer, but it is not very significant. *Vim* expression in this patient is very low and similar in both conditions. The same situation happens with patient 3, as *E-Cad* and *Vim* expression levels are very low for both conditions. Patient 5 has a similar *E-Cad* expression in both conditions and has a moderately higher expression of *Vim* in pancreatic cancer tissues.

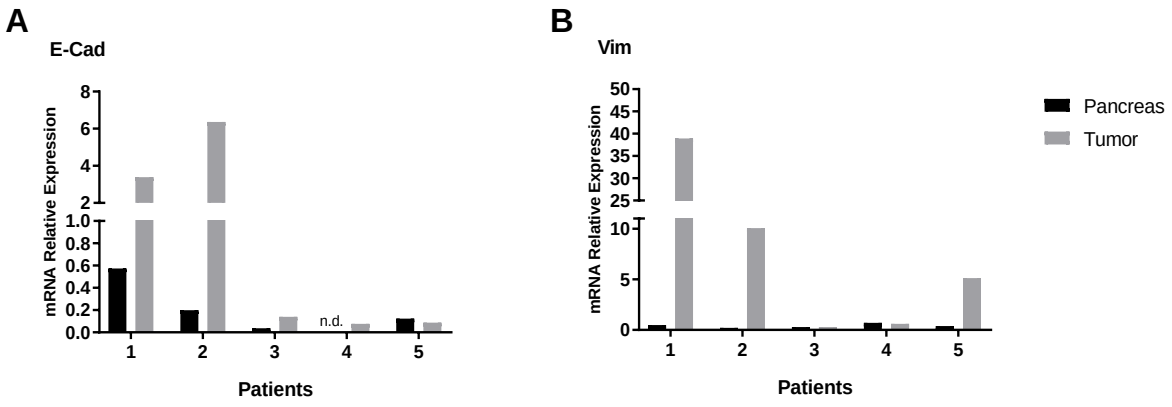

Figure 8. Relative expression levels of *E-Cad* (A) and *Vim* (B) in human pancreatic tumors (tumor, gray bars) and corresponding normal pancreas (pancreas, black bars) tissues collected from 5 consecutive patients.

The expression levels of *EGFR, ERK1* and *ERK2* (signaling markers of the EGFR/ERK/p38 MAPK pathway) were also determined in the same normal and pancreatic cancer tissues. Results are illustrated in Figure 10.

In normal pancreas tissues, *EGFR* expression (Fig 10A) was only detected in patient 5, with a nearly undetectable expression level. In pancreatic cancer tissues, patients 1 and 2 present a significant increase in *EGFR* expression levels, contrasting

with the low expression in patients 3 and 4 and the non detectable expression in patient 5. Patient 1 presents also an extremely high *ERK1* expression (Fig. 10B) in normal pancreas in comparison with pancreatic cancer tissue. Although patient 2 shows relatively high expression levels of *ERK1*, no significant variation between the two conditions was observed. Both patient 1 and 2 presented a significant increase in mRNA expression levels for *ERK2* (Fig 10C) in pancreatic cancer tissues in comparison to the normal pancreas. Patient 5 also displayed an increase in *ERK2* expression in pancreatic cancer tissue although not as accentuated as in patients 1 and 2.

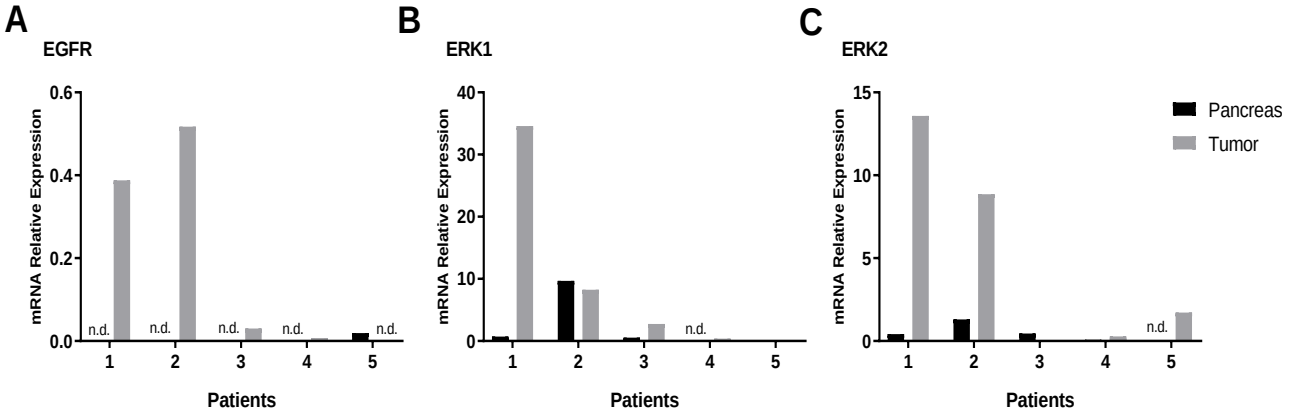

Figure 9. Relative expression levels of *EGFR* (A), *ERK1* (B) and *ERK2* (C) in human pancreatic tumors (tumor, gray bars) and corresponding normal pancreas (pancreas, black bars) tissues collected from 5 consecutive patients.

4.5 c-Fos and c-Jun gene expression variation in human pancreas and pancreatic tumors

The transcription factors c-Jun and c-Fos are members of the activation protein-1 (AP-1) family which play an important role in the stimulation of ERKs. It is postulated that the increase of *c-Fos* mRNA expression can occur due to the activation of ERK (Paula Monje, Hernández-Losa, Lyons, Castellone, & Gutkind, 2005), also suggesting that the EGFR/ERK/p38 MAPK pathway might also be activated.

The expression levels of *c-Jun* and *c-Fos* were assessedand the results are illustrated in Figure 11. The mRNA expression levels of *c-Fos* (Fig. 11A) were significantly increased in patient 1 comparing to the corresponding normal pancreatic tissue. Patients 2 and 5 also had a slight increase in *c-Fos* expression level in the pancreatic cancer tissues comparing to the normal ones, although not that accentuated as in patient 1.

The same overexpression occurred for *c-Jun* (Fig. 11B) in tumor tissue derived from patient 1, being the increase in mRNA expression level far superior in comparison with the other patients. A small decrease was observed in patients 2 and 4 and a similar increase was seen in patient 5, while patient 3 maintained the same relative expression levels of *c-Jun* both in normal and cancer pancreas tissues.

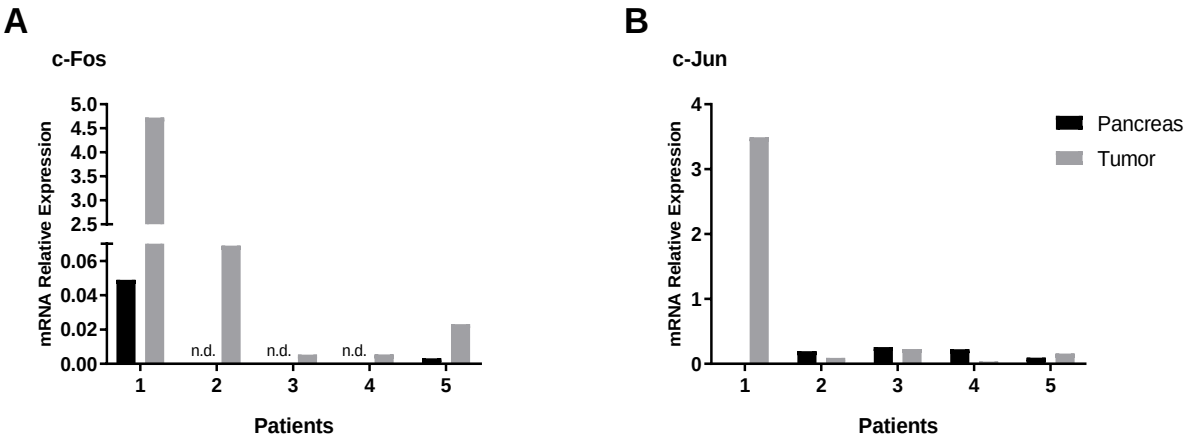

***Figure* 10.** Relative expression levels of *c-Fos* (A) and *c-Jun* (B) in human pancreatic tumors (tumor, gray bars) and corresponding normal pancreas (pancreas, black bars) tissues collected from 5 consecutive patients.

4.6 Western Blot analysis of AQP3, E-Cad, Vim, EGFR, p38 and p-p38 expression in human cell lines

In order to validate RT-qPCR results for AQP3, EMT-related markers and EGFR, western blot analysis was performed in HCT116, MCF7, MNT1 and HaCaT cells. Also, since p38 and its phosphorilated form intervene in EGFR/ERK/p38 MAPK signaling cascade, we decided to assess the expression profile of this mitogen-activated protein kinases. The expression values presented are normalized to β-actin expression level in each cell line. Protein bands aquired in all cell lines are represented in Figure 12. The expression levels of our proteins of interest in all cell lines are illustrated in Figure 13.

AQP3 protein expression levels (Fig. 13A) are higher in MNT1 than in the other cell lines, followed by MCF7, HCT116 and HaCat, respectively. E-Cad protein expression level (Fig. 13B) is higher in MCF7 followed by HaCaT, HCT116 and MNT1 respectively, this last one having a very low protein expression of this epithelial marker. Vim protein expression levels (Fig. 13C) are clearly favored in MNT1 cell line, as it

displays the highest expression. Concerning HaCaT, MCF-7 and HCT116 cell lines, they present almost vestigial levels of Vim expression when compared to MNT1.These results corroborate the mRNA expression patterns evaluated by RT-qPCR in the same cell lines, serving as strong indicator for their acceptance and validation.

Regarding EGFR, the protein expression levels (Fig. 13D) in these cell lines are different from the ones antecipated from RT-qPCR, where HCT116 was the cell line with the highest mRNA expression level, considerably above all other cells. The cell line that shows the highest protein expression levels in the western blot analysis is HaCaT.

Protein expression levels of p38 and p-p38 (Fig. 13E) show different profiles amoung the different cell lines. Although in three out of the four cell lines p38 has a higher expression than p-p38, only MCF-7 shows a very significant difference. Both HCT116 and MNT1 have relatively similar protein expression levels between this two MAPKs, being p38 the one with a slightly higher protein expression level. HaCaT on the other hand has a higher expression of p-p38 compared with p38. Interestingly, p38 protein expression in HaCaT is also the second highest p38 expression of the four cell lines.

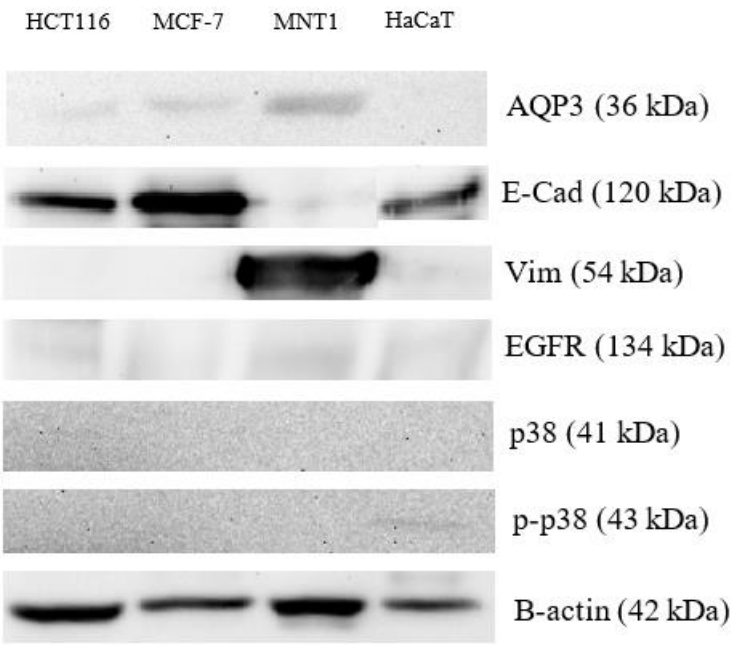

Figure 11. Western Blot analysis of AQP3, epithelial and mesenchymal proteins (E-Cad and Vim), EGFR and EGFR/ERK/p38 MAPK signaling proteins (p-38 and p-p38) in HCT116, MCF-7, MNT1 and HaCaT.

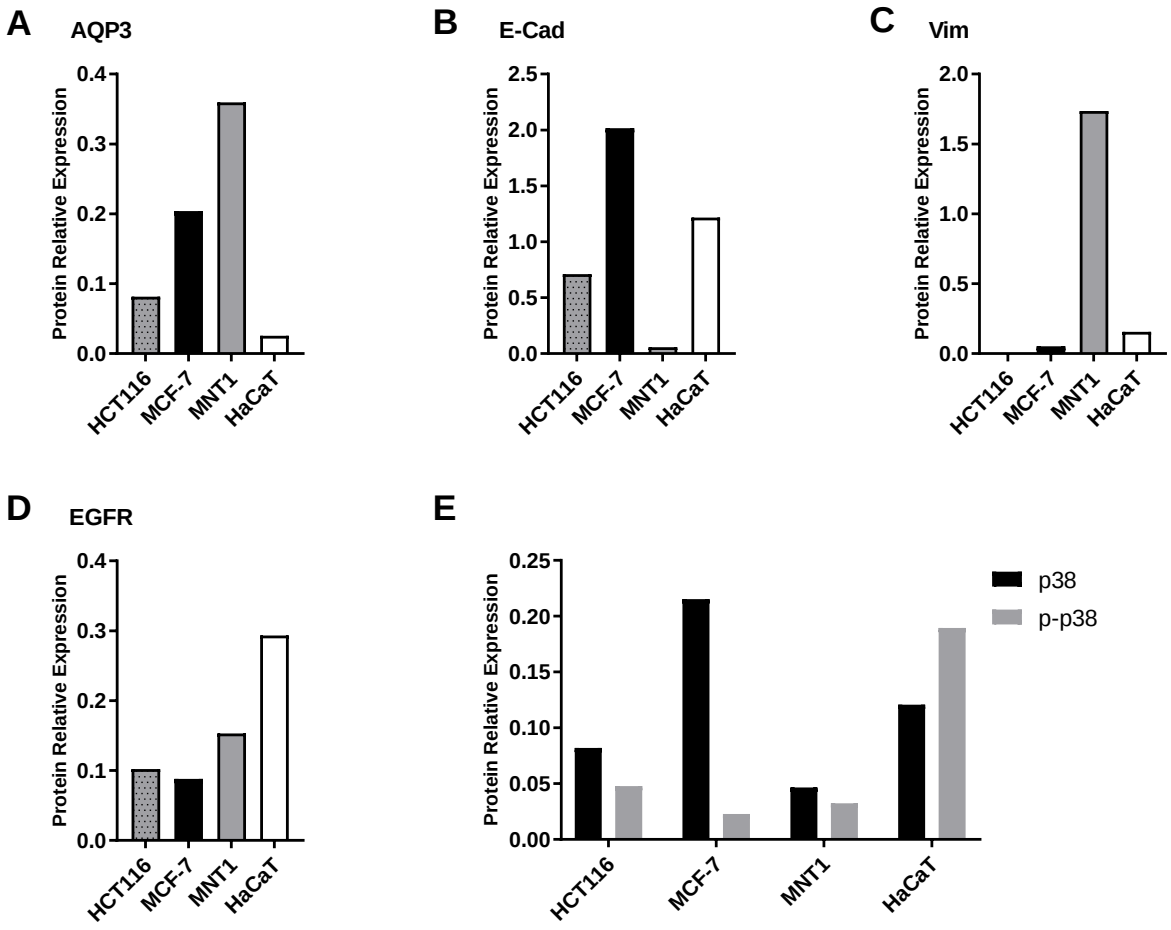

Figure 12. Protein expression levels of AQP1 (A), E-Cad (B), Vim (C), EGFR (D), p38 and phospho-p38 (E) in HCT116, MCF-7, MNT1 and HaCaT. Band densities were calculated with ImageJ.

5 Discussion and conclusions

Over the last decade, a considerable amount of evidence linking AQPs to cancer has emerged and continues to increase day after day. Several processes related to tumor proliferation, edema formation, migration and angiogenesis appear to be majorly associated with AQPs, opening the way for the discovery of AQP inhibitors that can be useful anti-cancer drugs (Marios C. Papadopoulos & Samira Saadoun, 2015).

There is direct and indirect evidence that both AQP3 and AQP5 play a significant role in tumor cell proliferation and, although it seems likely to happened, further studies are required to seal the link between some AQPs expression level and tumor cell proliferation.

The results achieved during this study support some previous statements regarding the overexpression of certain AQPs in tumor cells, as well as the relations between AQPs expression and the unfold of responses from the cell, culminating in processes connected with tumor development and progression. As the only non-tumoral cell line used in this study, HaCaT had the lowest *AQP3* relative mRNA expression of the four cell lines studied. A previous study conducted in human keratinocytes cell lines, including HaCaT, where *AQP3* expression was increased with different retinoic acid-related drugs in these cells presented the same weakly *AQP3* expression in non-treated HaCaT cells (Xing, Liao, Jiang, Xu, & Jin, 2016). On the other hand, *AQP5* presents a higher expression level that would be expected, as there are no clear indications in previous reports that this AQP isoform is highly expressed in keratinocytes. In HCT116, the expression levels of *AQP3* and *AQP5* were expected to be much higher due to a variety of reports that found a significant correlation between *AQP1*, *AQP3*, and *AQP5* expression and lymph node metastasis in colon cancer (Byung Woog Kang et al., 2014; B. W. Kang et al., 2015; S. K. Kang et al., 2008). In MCF-7 cells, *AQP3* expression level was relatively high, as predicted, and similar levels for *AQP5* expression were expected, although it did not occur. In a very recent study, *AQP3* and *AQP5* expression was notably stronger in breast carcinoma tissue compared with adjacent normal breast tissue. Overexpression of *AQP3* and *AQP5* was significantly associated with tumor size, lymph node status and metastasis (Z. Zhu et al., 2018). MNT1 cell line had the higher expression in all three tested AQP isoforms in comparison with the other cell lines. A correlation with EMT and the activation of

oncogenic signaling pathways might be a promising lead to consider these AQPs as potential tumor biomarkers and drug targets.

During EMT, epithelial cells convert to a more mesenchymal morphology. Cell–cell adhesions are weakened and disruption of polarity complexes, as well as cytoskeleton reorganization, occurs, which increases cell migration.

In a study using the gastric cancer cell lines SGC7901 and MGC803, overexpression of AQP3 correlated with down-regulation of E-cadherin expression and up-regulation of vimentin expression levels (Chen et al., 2014). In HCT116, which presented a low expression of *AQP3, E-Cad* levels are still very low in comparison to the other cell lines, but at the same time Vimentin expression is even lower, substantiating that EMT was not occurring. For MCF-7 cells, although *AQP3* expression was substantially high, *E-Cad* expression is also high, with only vestigial expression of *Vim*. In this case, we cannot assume that *AQP3* high expression influences EMT. HaCaT expression levels of *AQP3, E-Cad* and *Vim* are according to what would be expected in a situation where EMT does not occur because of the low expression of AQP3, the moderate expression of *E-Cad* and the vestigial expression of *Vim*. MNT1 on the other hand has the expression levels of *AQP3, E-Cad* and *Vim* expected in a situation where EMT is occurring or has occurred. The high expression of *AQP3* and low expression of the epithelial marker *E-Cad*, joined with the very high expression level of mesenchymal marker *Vim* are the excellent example of EMT occurrence. The characteristics of cells that underwent through EMT are those of a more malignant and invasive tumor with migratory capability, suggesting that AQP3 expression level might be considered a tumor marker in melanoma.

ERK1, *ERK2* and *EGFR* mRNA expression levels were assessed in all cell lines. *EGFR* expression level in HCT116 is significantly higher compared to the remaining cell lines. MNT1 and HaCat have a low *EGFR* expression level and in MCF7's case, *EGFR* expression is vestigial. Comparing these results with the expression levels of *ERK1* and *ERK2* in the respective cell lines, we notice that for HCT116, *ERK2* is highly expressed, contrarily to the *ERK1* expression level, which is very low. For MCF-7, *ERK1* expression is substantially high while *ERK2* expression is the lowest of the four cell lines. This opposite *ERK1/2* expression profiles might be related with *EGFR* expression level in these cells, suggesting that a higher expression of *EGFR* is related with a higher expression of *ERK2* and *a* decrease in *ERK1* expression, and a lower

expression of *EGFR* related with a higher expression of *ERK1* and a lower expression of *ERK2*.

Regarding the human pancreatic tumors and corresponding normal pancreas tissues, it is important to mention that tumors were from different types of pancreatic cancer and were in different stages, had different degrees of differentiation between individuals and were in different sites within the pancreas. The detailed information about the pancreatic cancer samples are in the database of the hospitals where the samples were extracted and will be provided to allow a comprehensive analysis of the results.

With all data available we will be able to categorize every type of pancreatic cancer according to its specificities, and we will also be able to analyze and group every individual condition according to their age and gender. The expression profile of our genes of interest will be accurately describing the type and characteristics of the pancreatic tumors we are dealing with.

Little is known about AQPs expression and significance in human pancreatic tumors, although it is thought that AQPs can be of predictive value for prognosis of pancreatic cancer based on previous studies in luminal and basolateral membranes of rat and human acinar and ductal epithelia (Burghardt et al., 2003; Itoh et al., 2005; Ko et al., 2002).

The AQP1 expression profile for patients 1, 2 and 4 is similar regarding the comparison between the normal pancreas and the pancreatic tumor tissues, as AQP1 is more expressed in normal pancreas. Patients 3 and 5 have AQP1 more expressed in the tumor than in the normal pancreas tissue, although this difference is less notorious than in the other patients.

For AQP3, patients 1 and 4 present a higher mRNAa expression level in normal pancreas tissues comparing with the tumor ones, being patient 4 the one with the highest variation in gene expression . Patients 2, 3 and 5 present the opposite expression profile, being patient 5 the one with the higher AQP3 expression difference between normal and tumoral pancreatic tissue.

In all patients, AQP5 is more expressed in tumor tissues than in normal pancreas. Patient 3 has the most prominent variation in AQP5 expression between the two conditions. This higher *AQP5* expression consistency in all five patients suggests that this AQPs expression level might be indicative of pancreatic tumor development and might be used as a tumoral marker or a drug target.

In the cases where AQP expression level was non detected, it doesn't mean that the gene is not being expressed, only shows that with the defined RT-qPCR parameters used in this experiment, those gene expression levels were so low that the sample cDNA didn't amplify on a level above the detection threshold.

E-Cad expression increased considerably in pancreatic tumors comparing to normal pancreas tissue in patients 1 and 2, a situation that was not expected to occur. Patients 3, 4 and 5 had little to no significant variation from a condition to another.

Patients 1 and 2 have significantly higher *Vim* expression levels in the pancreatic cancer tissues in comparison with the normal pancreas tissues, while patient 5 only has a moderately higher *Vim* expression in pancreatic cancer. Patient 1 has a more accentuated expression of *Vim* than patients 2 and 5, although all three patients had significant variations in *Vim* expression. Patients 3 and 4 presented no significant variations from one condition to another. Patients 1 and 5 had considerably higher *Vim* levels in comparison with their *E-Cad* expression, but patient 1 has a lower *AQP3* expression in pancreatic cancer tissues in comparison with the normal pancreas, which is not concordant with EMT occurrence. On the other hand, patient 5 has a much higher *AQP3* expression in pancreatic cancer tissues that in normal pancreas, which is concordant with the occurrence of EMT, suggesting that patient 5 pancreatic cancer cells underwent EMT.

As mentioned before, it is postulated that the increase of *c-Fos* mRNA expression can occur due to the activation of ERK, also suggesting that the EGFR/ERK/p38 MAPK pathway might also be activated. Although that it is at different scales, higher expression of c-Fos is observed in all pancreatic cancer tissues from all the patients in the study. Patient 1 is the one expressing the highest c-Fos level in pancreatic cancer tissues by far, followed by patient 2, patient 5, patient 4 and patient 3, orderly. Although all patients express c-Fos in higher levels in pancreatic cancer, only patient 1 has a significant expression to suggest a relation with ERK activation. As discussed previously, patient 1 has a significantly high expression of EGFR in pancreatic cancer tissues, supporting the idea that EGFR/ERK/p38 MAPK pathway activation might have occurred and be involved in tumor proliferation.

The results from western blot analysis were mainly as expected, which allow us to validade de previous results obtained with RT-qPCR for *AQP3, E-Cad* and *Vim. EGFR* was the only marker to have a significant variation in its expression levels, showing a higher expression level in HCT116 according to the RT-qPCR results, but a

reduced *AQP3* expression level in western blot. We intended to validade the results from the pancreatic cancer tissues and normal pancreas tissues by western blot analysis, but available sample amount was limited. Further studies are required to complement these results. Comparison of the RT-qPCR results of the cell lines used in this study with normal cells of the corresponding tumors would be of interest to assess expression variations within the same type of cells.

In summary, in this work:

1) we found differential aquaporin (*AQP1*, *AQP3* and *AQP5*), cell differentiation markers (*E-Cad* and *Vim*) and markers involved in tumor signaling pathways (*EGFR*, *ERK1* and *ERK2*) gene expression across different tumor cell lines.

2) we further complemented this study with the validation of RT-qPCR results for the cell lines by western blot analysis.

3) we established that cell differentiation responses took place and a relation with AQP's expression was suggested, in both tissues and cell lines

4) with minor exceptions, AQP3 was the most expressed isoform in both cell lines and tissues, with a possible connection to the activation of EGFR/ERK/p38 MAPK signaling pathway.

5) we concluded, that among the tested aquaporins isoforms, *AQP3* and *AQP5* stand out as the more promising targets for developing new anti-cancer drugs due to its importance in tumor development and progression.

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