Universidade de Lisboa
Faculdade de Medicina de Lisboa

Role of PLCγ1 in the Resistance Mechanism to Anti-EGFR Therapy in Metastatic Colorectal Cancer

Raquel Sofia Cruz Duarte

Tese orientada por:
Doutora Marta Sofia Alves Martins

Dissertação especialmente elaborada para obtenção do grau de Mestre em Oncobiologia

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Abstract

Tumor metastases are responsible for approximately 90% of all cancer-related deaths. Cetuximab (Cetx) is a monoclonal antibody targeting the epidermal growth factor receptor (EGFR), which was recently approved for the treatment of metastatic colorectal cancer (mCRC). However, Cetx effectiveness is only about 20% due to the existence of multiple resistance mechanisms downstream of EGFR. *KRAS* mutations are recognized as a predictor of resistance to anti-EGFR treatment, nevertheless, 54% of wild-type *KRAS* patients still do not respond to this therapy. Therefore, there is a clear need for new biomarkers capable of accurately predict response to therapy. PLCγ1 is activated by direct binding and phosphorylation by EGFR and has been implicated in oncogenic signaling downstream of this receptor. PLCγ1 catalyzes the hydrolysis of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3), involved in diverse cellular processes, such as cell proliferation, differentiation and motility.

In this thesis, we investigate the contribution of PLCγ1 for the resistance mechanism to Cetuximab, in an *in vitro* and a clinical approach. Overall, our results show that PLCγ1 is highly expressed in Cetuximab-resistant colon cancer cell lines. PLCγ1 knockdown in resistant cell lines (CACO-2 and HT-29) was able to sensitize them to Cetx. Furthermore, PLCγ1 overexpression in the most sensitive cell line (SW48) confers increased Cetuximab resistance. Additionally, SW48 cell line that was continuously exposed to Cetx for five months shows a slightly increase in PLCγ1 expression when compared with parental control. Finally, immunohistochemical analysis of PLCγ1 in human CRC samples shows an association between increased PLCγ1 expression and poor progression-free survival of patients under Cetx treatment.

Taking together, our results show a correlation between PLCγ1 expression levels and Cetuximab resistance, suggesting that PLCγ1 could be a predictive biomarker of EGFR resistance, helping selecting patients more likely to respond to this therapy.

Keywords: Metastatic Colorectal Cancer, EGFR-Target Therapy, Cetuximab, Phospholipase Cγ1, Therapy Resistance.

Resumo

O cancro colo-rectal é o terceiro cancro mais incidente a nível mundial, com a quarta maior taxa de mortalidade. A elevada mortalidade associada a este tipo de cancro é essencialmente devida à acrescida dificuldade no tratamento da doença metastática. Nos últimos anos, o desenvolvimento e utilização de novos fármacos, como os anticorpos monoclonais dirigidos contra o recetor do fator de crescimento epidérmico (EGFR), têm aumentado a eficácia das terapêuticas convencionais em tumores metastáticos. No entanto, existe ainda um grande número de doentes que não responde a estas terapêuticas ou que acaba por desenvolver resistência às mesmas, após um período inicial de tratamento. Dado este cenário, torna-se cada vez mais urgente a procura de novos biomarcadores, mais sensíveis e específicos, que possam indicar com maior clareza quais os pacientes que beneficiam destas terapêuticas.

O EGFR está implicado no desenvolvimento e progressão de múltiplos tumores, nomeadamente nos casos de cancro colo-rectal. A ativação deste recetor conduz à ativação de várias vias de sinalização celulares implicadas no controlo da sobrevivência celular, progressão do ciclo celular, angiogénese, migração e invasão/metastização. O Cetuximab (Cetx) é um anticorpo monoclonal direcionado especificamente contra o EGFR, que se liga à sua porção extracelular com uma afinidade superior à dos seus ligandos endógenos. Desta forma, o Cetx bloqueia a ligação dos ligandos ao EGFR, impedindo a ativação do recetor, o que se traduz na inibição das vias intracelulares e dos processos por elas regulados. Além disso, o Cetx induz a internalização do EGFR levando à diminuição dos recetores disponíveis na superfície celular. Finalmente, o Cetx permite ainda o reconhecimento das células tumorais pelas células efetoras imunitárias citotóxicas, desencadeando o processo de citotoxicidade mediada por células dependentes de anticorpo.

Infelizmente, apenas um pequeno número de pacientes responde eficazmente a esta terapêutica. Mutações ativadoras no gene *KRAS*, que codifica para uma proteína a jusante na via de sinalização do EGFR, estão já identificadas como fortes indicadores de resistência ao Cetuximab, uma vez que ativam constitutivamente as vias intracelulares, de forma independente do recetor. Ainda assim, 54% dos doentes que não apresentam mutações neste gene desenvolvem resistência (intrínseca ou adquirida) a esta terapêutica. A ativação constitutiva de outros efetores intracelulares, tais como BRAF, PI3K e PLCγ, pode também constituir um mecanismo de resistência à terapia anti-EGFR, sendo que a PLCγ nunca foi anteriormente estudada neste contexto. Existem duas isoformas da PLCγ, a PLCγ1 e a PLCγ2, sendo que a primeira é amplamente expressa, estando

presente em quase todos os tecidos, e a segunda é expressa essencialmente em células do sistema imune. Ambas as PLCγ são diretamente ativadas por recetores do tipo tirosina cinase, dos quais fazem parte o EGFR, e, quando ativas, são responsáveis pela conversão do fosfolípido de membrana fosfatidilinositol 4,5-bifosfato (PIP_2) em dois mensageiros secundários, o diacilglicerol (DAG) e o inositol trifosfato (IP_3). Estes mensageiros secundários são essenciais para a regulação de múltiplos processos celulares como proliferação, diferenciação, migração e angiogénese. Diversos estudos demonstraram que a PLCγ1 possui um papel importante no desenvolvimento e progressão tumoral, nomeadamente ao nível da migração celular. Neste sentido, o *knockdown* da expressão da PLCγ1 numa linha celular de carcinoma mamário (MDA-MB-231) inibiu o desenvolvimento de metástases pulmonares em modelo animal de ratinho. Por outro lado, a sobre-expressão de PLCγ1 foi observada em diversos tipos de tumores, incluindo colo-rectais, quando comparados com tecidos normais adjacentes, e foi associada a um pior prognostico e a um risco aumentado de desenvolvimento de metástases à distancia. Para além deste facto, mutações no gene *PLCG1* foram recentemente associadas com o desenvolvimento de angiossarcomas e linfomas cutâneos de células T. Finalmente, apesar de pouco estudada no contexto de resistência à terapêutica, mutações no gene da *PLCG2* (isoforma maioritariamente expressa em células hematopoiéticas) foram associadas ao mecanismo de resistência ao Ibrutinib, um inibidor da tirosina cinase de Bruton, no tratamento de leucemia linfocítica crónica.

Tendo em conta o papel da PLCγ1 na regulação de processos celulares como a migração, invasão e progressão tumoral, e a sua estreita relação com o recetor EGFR, o principal objetivo deste trabalho é explorar a hipótese do envolvimento da PLCγ1 no mecanismo de resistência à terapêutica anti-EGFR. Para testar esta hipótese começou-se por avaliar a resposta de um painel de cinco linhas celulares de cancro colo-rectal (todas *KRAS wild-type*) ao tratamento com Cetuximab e correlacionar essa resposta com o nível de expressão da PLCγ1. Os nossos resultados mostram que os níveis de expressão da PLCγ1 estão aumentados nas linhas celulares mais resistentes ao Cetuximab, quando comparados com os níveis de expressão das linhas mais sensíveis. De seguida, foi realizada a sobre--expressão da PLCγ1 na linha mais sensível (SW48), enquanto que na linha mais resistente (CACO-2) foi realizado o *knockdown* da expressão da PLCγ1. O *Knockdown* da expressão da PLCγ1 na linha CACO-2 permitiu sensibilizá-la de forma significativa (p=0,0289) ao tratamento com Cetx. Por outro lado, a sobre-expressão da PLCγ1 na linha SW48 fez com que esta aumentasse a resistência ao tratamento com Cetuximab. A sobre-expressão de um mutante PLCγ1 constitutivamente ativo na sua função lipase (ΔSA), não mostrou diferenças na resposta ao Cetuximab quando comparada com

o controlo. Estes resultados indicam um possível mecanismo de resistência independente da função lipase da PLCγ1. Simultaneamente, uma linha celular com sensibilidade intermedia ao Cetx (HT-29) mas com mutação ativadora do gene *BRAF*, foi também selecionada para se realizar o *knockdown* da expressão da PLCγ1. Mais uma vez, o *knockdown* da expressão da PLCγ1 permitiu também aumentar a sensibilidade das células ao Cetx (p=0,0222), mesmo na presença de uma mutação ativadora no gene do *BRAF* (V600E). Por fim, a linha SW48 foi também continuamente exposta a elevadas concentrações de Cetuximab por um período de cinco meses, a fim de avaliar um possível envolvimento da PLCγ1 na resistência adquirida ao Cetx. Neste caso, foi possível ver um aumento de expressão da PLCγ1, comparativamente com as células parentais não tratadas. De uma forma geral, os nossos resultados sugerem que o aumento da expressão desta proteína poderá estar associado não só a um mecanismo de resistência inato ao tratamento, mas também a um mecanismo adaptativo de resistência ao Cetx.

Por fim, a expressão da PLCγ1 foi avaliada num grupo retrospetivo de amostras (n=25) de casos de carcinoma colo-rectal, provenientes do serviço de anatomia patológica do Hospital de Santa Maria. Estas amostras correspondem a amostras de tumores primários de doentes tratados com Cetuximab em contexto da doença metastática. A expressão da PLCγ1 foi avaliada por imunohistoquímica e os resultados foram analisados por um médico patologista, que realizou um *score* de intensidades de marcação. A elevada expressão da PLCγ1 foi significativamente associada (p=0,0460) a uma diminuição no tempo de sobrevivência livre de progressão, em doentes sob tratamento com Cetx. Observou-se também uma tendência entre maiores níveis de expressão de PLCγ1 e uma diminuição da sobrevivência global, sem, no entanto, existir significância estatística.

Em conclusão, os resultados obtidos neste trabalho sugerem uma associação negativa entre os níveis de expressão da PLCγ1 e a resposta ao Cetuximab. Esta relação foi observada nos estudos *in vitro* e na avaliação de amostras de pacientes. O aumento da expressão da PLCγ1 pode também estar associado ao desenvolvimento de resistência adquirida ao Cetuximab. Demonstrar o envolvimento da PLCγ1 em mecanismos de resistência ao Cetuximab, e possivelmente a outras terapias anti-EGFR, poderá ter um grande impacto clínico no tratamento do cancro colo-rectal metastático, não só como potencial biomarcador preditivo de resposta à terapêutica, mas também como um possível novo alvo terapêutico.

Palavras-chave: Cancro Colo-Rectal Metastático, Terapêutica Anti-EGFR, Cetuximab, Fosfolipase Cγ1, Resistência Terapêutica

Abbreviations

^{3}H	Tritium
γSA	PLCγ specific array
aa	Amino acids
AKT	v-akt murine thymoma viral oncogene homolog
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BRAF	B-Raf proto-oncogene
Ca^{2+}	Calcium
Cetx	Cetuximab
cDNA	Complementary deoxyribonucleic acid
CHLN-HSM	Centro Hospitalar Lisboa Norte - Hospital Santa Maria
CI	Confidence interval
CLL	Chronic lymphocytic leukemia
CO_2	Carbon dioxide
CRC	Colorectal carcinoma
CT	Threshold cycle
DAB	3,3-Diaminobenzidine
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMA	European Medicines Agency
ERK	Extracellular regulated kinase
ESMO	European society for medical oncology
FBS	Fetal bovine serum
FDA	Food and drug administration
FFPE	Formalin-fixed, paraffin-embedded

FGFR	Fibroblast growth factor receptor
FLWT	Full-length wild-type
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GEF	Guanine nucleotide exchange factor
h	Hours
H-score	Histoscore
HB	Heparin-binding
HCL	Hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
HNSCC	Head and neck squamous cell carcinoma
HR	Hazard ratio
HRP	Horseradish peroxidase
IHC	Immunohistochemistry
IP_3	Inositol 1,4,5-trisphosphate
JAK	Janus kinase
KRAS	Kirsten rat sarcoma viral oncogene homolog
LiCl	Lithium chloride
MAPK	Mitogen activated protein kinase
mCRC	Metastatic colorectal cancer
min	Minutes
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NRAS	Neuroblastoma RAS viral oncogene homolog
NRG	Neuregulin
NSCLC	Non-small cell lung cancer
OS	Overall survival
ORR	Overall response rate
OSCC	Oral squamous cell carcinoma
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
Pen/Strep	Penicillin-streptomycin

PFS	Progression-free survival
PH	Pleckstrin domain
PI3K/PIK3CA	Phosphatidylinositol 4,5-bisphosphate 3-kinase
PIKE	PI3K enhancer
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositol 3,4,5-trisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PLCγ/PLCG	Phospholipase C gamma
PLD_2	Phospholipase D 2
PTB	Phosphotyrosine binding
PTEN	Phosphatase and tensin homolog
RA	RAS-binding domain
Rac1	Ras-related C3 botulinum toxin substrate 1
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Room temperature
RT-qPCR	Quantitative reverse transcription PCR
RTK	Receptor tyrosine kinase
SEM	Standard error of the mean
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH2/SH3	Src homology 2/3
shRNA	Short hairpin RNA
SOS 1	Son of sevenless homolog 1
SRE	Serum response element
STAT	Signal transducer and activator of Ttanscription
TBS	Tris-buffered saline
TCEP	Tris(2-carboxyethyl)phosphine hydrochloride
TGF-α	Transforming growth factor α
TRP3	Transient receptor potential cation channel 3
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor Receptor

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1. Introduction

Cancer is a widespread health problem which incidence has been increasing year by year, severely threatening human wellbeing and lives. Colorectal cancer (CRC) is the third most frequent cancer worldwide and the fourth leading cause of cancer-related death. In 2012, 1.4 million new cases of CRC were identified globally, with an overall incidence of 17,2% and 693.933 deaths[1,2]. Based on GLOBOCAN prediction, 1.7 million cases of CRC are expected to be diagnosed in 2020, when 853.550 people will die from this disease[3,4]. In Portugal 7.129 new cases were diagnosed in 2012, corresponding to the second most incident cancer, with approximately 3.797 deaths[5,6].

The high mortality associated to CRC is mainly due to the increased difficulty in the treatment of advanced metastatic disease[7]. However, over the past decade novel therapeutic options have been introduced for the treatment of metastatic CRC (mCRC), such as EGFR-targeted specific antibodies and inhibitors, which have been improving the clinical outcome of patients. Nevertheless, there is still a large number of patients who don't benefit from these therapies[8]. Therefore, there is an urgent need for highly sensitive and specific predictive biomarkers, as well as new molecular targets of more efficient therapies.

1.1. EGFR Signaling

Epidermal growth factor receptor (EGFR) has long been recognized as an important target of therapy since its expression is deregulated in many cancer types[9]. EGFR up-regulation, gene amplification and mutations have been demonstrated to occur in several carcinomas, including colorectal, being in this way involved in the pathogenesis and progression of these malignancies[10,11].

EGFR is a transmembrane receptor belonging to the ErbB tyrosine kinase family which consists of four related proteins: EGFR (ErbB1/HER1), HER2/neu (ErbB2), HER3 (ErbB3) and HER4 (ErbB4)[12]. All family members contain an extracellular ligand-binding domain with two cysteine-rich regions, a single membrane-spanning region and a cytoplasmatic tyrosine kinase domain[12,13]. Under normal physiological conditions, activation of ErbB receptors is controlled by the presence of their specific ligands that are produced by the same cells that express ErbB receptors (autocrine secretion) or by surrounding cells (paracrine secretion)[14]. This family of ligands is characterized by the presence of an EGF-like domain that consists of six cysteine residues, which confers binding specificity, and can be divided into three groups[15]. The first group includes EGF-like ligands,

transforming growth factor-α (TGF-α) and amphiregulin, which bind specifically to EGFR. The second is composed by betacellulin, heparin-binding growth factor (HB-EGF) and epiregulin, which show dual specificity by binding both EGFR and ErbB4. The third group includes neuregulins (NRGs) and can be divided in two subgroups based on their capacity to bind ErbB3 and ErbB4 (NRG-1 and NRG-2) or only ErbB-4 (NRG-3 and NRG-4)[15,16]. None of these ligands bind to ErbB2[17] (Figure 1).

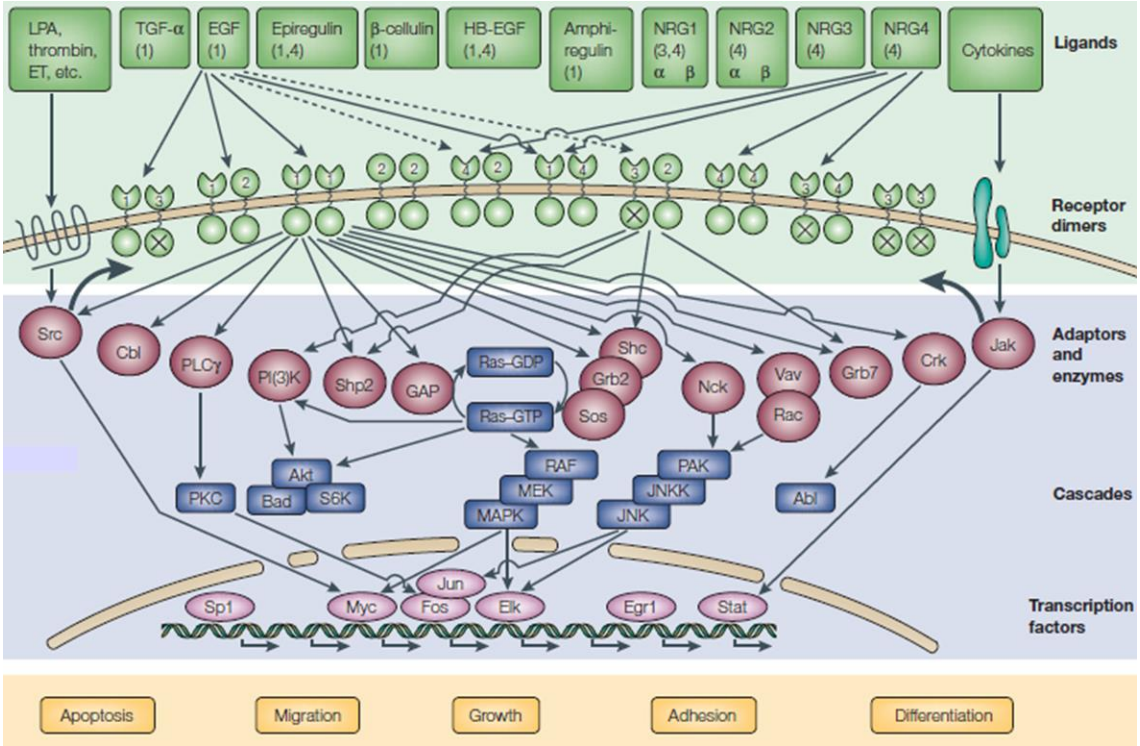

Figure 1: EGFR signaling. Ligands and the ten dimeric receptor combinations (numbers in each ligand block indicate the respective high-affinity ErbB receptors). No ligand for HER2 has been identified. Each receptor contains an extracellular ligand-binding domain, a transmembrane domain and an intracellular tyrosine kinase domain. Ligand binding induces dimerization, autophosphorylation and causes activation of downstream signaling pathways, that regulate multiple cellular processes. Adapted from Yarden *et al*., 2001[9].

Binding of ligands to the extracellular domain of ErbB receptors induces major conformational changes that lead to receptor homo or heterodimerization and subsequent activation of the intrinsic tyrosine kinase domain, which causes autophosphorylation of specific tyrosine residues within the cytoplasmic tail of each dimer pair[12]. These phosphorylated residues serve as docking sites for proteins containing Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains, that further propagate multiple signal transduction pathways[14] (Figure 1). ErbB activation also leads to receptor internalization by endocytosis that enables specific signaling pathways from intracellular sites and is thought to initiate termination of the signal[18,19].

Different ErbBs preferentially modulate specific signaling pathways, due to the ability to bind specific effector proteins. The specificity and potency of intracellular signals are determined by the identity of the ligand and heterodimer composition, that regulates which sites are autophosphorylated and, therefore, which signaling proteins are engaged and activated[9,12] (Figure 2). Two of the main pathways activated by these receptors are mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)-AKT pathways. However other important pathways are activated by ErbB signaling like JAK/STAT, SRC tyrosine kinase and PLCγ1/PKC[9,14]. The activation of different signaling pathways leads to different cellular processes that range from proliferation and migration to adhesion, differentiation, transformation and apoptosis.

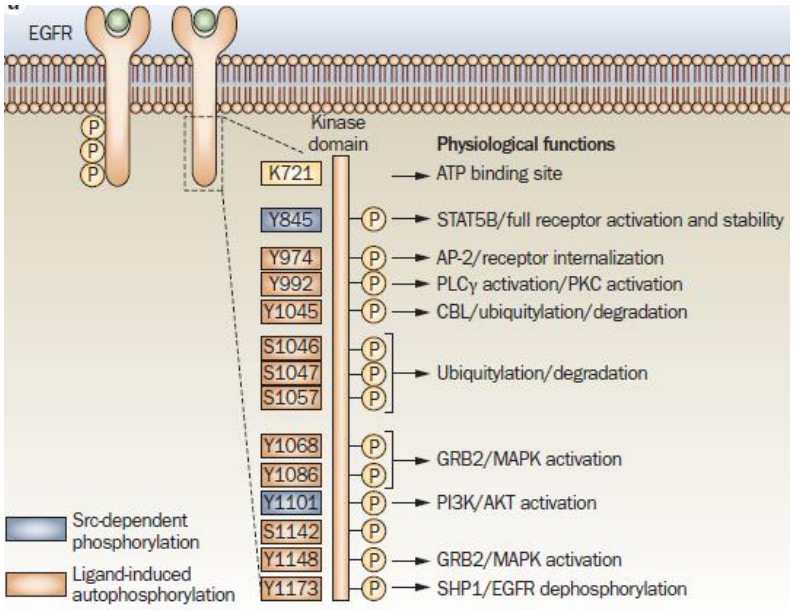

Figure 2: EGFR biology. Ligand binding to EGFR causes receptor dimerization that leads to autophosphorylation of the cytoplasmic tail tyrosine residues. Lysine 721 (K721) is the critical site for ATP binding and kinase activity of EGFR. Tyrosine phosphorylation in the C-terminus includes Y974, Y992, Y1045, Y1068, Y1086, Y1148 and Y1173. Biological effects of phosphorylation of each tyrosine are indicated. Adapted from Wheeler *et al*., 2010[21].

Patients with tumors that have alterations in ErbB receptors tend to have a more aggressive disease, associated with poor prognosis and poor clinical outcome, that define a subgroup of early-relapsing patients[14].

1.2. EGFR Target Therapy

Since EGFR pathways are hyperactivated in a wide range of human cancers and are generally responsible for more aggressive tumors, they are excellent targets for selective anticancer therapies. A large variety of EGFR-targeting agents are currently approved or in advanced clinical development for the treatment of various cancer types[20]. Two classes of anti-EGFR drugs are in clinical use: anti-EGFR monoclonal antibodies and small-molecule EGFR tyrosine kinase inhibitors[21]. Tyrosine kinase inhibitors like Gefitinib and Erlotinib are low molecular weight synthetic molecules that block the magnesium-ATP-binding pocket of the intracellular tyrosine kinase domain, inhibiting EGFR autophosphorylation and, consequently, downstream signaling[20]. Monoclonal antibodies, such Cetuximab and Panitumumab, recognize and bind to the extracellular domain of EGFR when it is in the inactivate configuration, occulting the ligand-binding region and therefore blocking EGFR activation and further signaling propagation[13,20]. Anti-EGFR monoclonal antibodies recognize EGFR exclusively and are therefore highly selective for this receptor[20].

Cetuximab (Cetx) is a human-mouse chimeric immunoglobulin G1 monoclonal antibody that specifically targets EGFR and has a mean half-life of approximately 112h in circulation in the human body[21]. Cetx was approved by the Food and Drug Administration (FDA) and by European Medicines Agency (EMA) for the treatment of mCRC in 2004 based on the improvement of overall survival (OS), progression-free survival (PFS), and overall response rate (ORR)[13,20]. Binding of Cetx to EGFR results in inhibition of cell growth (G1 phase arrest), induction of apoptosis and enhances receptor internalization and degradation[21,22]. Cetuximab also induce antibody-dependent cell-mediated cytotoxicity due to their ability to recruit immune effectors cells, such macrophages and monocytes, to the tumor, through the binding of the antibody constant Fc domain to specific receptors in these cells[23]. Finally, Cetuximab also potentiates antitumor activity of cytotoxic drugs and enhances antitumor effects of radiation[20,21].

1.2.1. Biomarkers of Anti-EGFR Therapy Resistance

Unfortunately, only a small number of patients respond positively to EGFR target therapies and Cetuximab effectiveness is only about 20% due to the existence of multiple resistance mechanisms downstream to this receptor[21,24].

Somatic *KRAS* activating mutations, which occur in approximately 40-45% of patients with CRC, are an example of intrinsic resistance to Cetx[25,26]. In 2006, Lièvre *et al.*[27] reported that *KRAS*

mutations in codons 12 or 13 were predictive of resistance to Cetuximab, given that activating mutations in this EGFR effector resulted in EGFR-independent activation of the MAPK pathway. Several consequent studies and clinical trials confirmed this correlation and shown that also codons 59, 61, 117 and 146 of *KRAS* and 12, 13, 59, 61, 117 and 146 of *NRAS* were significantly associated with resistance to Cetuximab therapy[21,28]. Taken together, these results revealed that activating mutations in RAS isoforms are strong predictors of resistance to EGFR-targeted therapy and evaluation of *KRAS* and *NRAS* mutation status has emerged as an important predictive biomarker that enables improved selection of patients more likely to respond to this therapy[20,29]. Currently, and accordantly with ESMO consensus guidelines, RAS testing is mandatory for mCRC patients before treatment with EGFR-target monoclonal antibodies and should include codons 12, 13, 59, 61, 117 and 146 of both *KRAS* and *NRAS* (extended RAS testing)[8].

On the other hand, 54% of wild-type RAS patients do not respond or eventually develop acquired resistance to EGFR monoclonal antibodies[20]. Current data suggests that constitutive activation of other downstream effectors of EGFR, such as *BRAF* and *PIK3CA* can contribute to the resistance mechanism to EGFR-targeted therapy[30,31]. *BRAF* V600E mutation is found in 8-12% of patients with mCRC and is known to be mutually exclusive *KRAS* mutations[32]. Although some studies suggest that this mutation correlates with poor response to EGFR target antibodies, there is still unclear evidence to support this correlation and *BRAF* testing is not recommended in the clinical practice[8,33]. *PIK3CA* and *PTEN* alterations can co-occur with *KRAS* or *BRAF* mutations and may predict resistance to EGFR target therapy but, once again, there is insufficient evidence for their use as predictive biomarkers[8,34].

EGFR expression determined by immunohistochemical methods was the first biomarker investigated as a potential predictor of response to Cetuximab[35]. However, further studies have failed to show any relationship between EGFR expression and the clinical activity of anti-EGFR drugs[36]. EGFR increased copy number evaluated by *in situ* hybridization was pointed as another possible biomarker of response[37]. Even so, further studies do not confirm the predictive value of this biomarker to be used in clinical practice for selection of patients[38]. Finally, specific alterations of EGFR gene, including somatic gain-of-function mutations, are not associated with response to EGFR specific antibodies[39].

Despite rapid advances in EGFR target therapies have been achieved over the past decades, more studies are essential for an improved efficacy of this treatments. Since only a subgroup of patients with mCRC have a clinical benefit from treatment with anti-EGFR inhibitors, there is an

urgent need for identification and clinical validation of more useful biomarkers that allow a better selection of patients[21]. Study of alternative pathways that are activated following EGFR signaling and that may bypass or evade inhibition of EGFR is one area of investigation. One possible mechanism of resistance, neglected so far in this context, is the constitutive activation of PLCγ1 proteins. PLCγ1 is a direct EGFR downstream effector involved in the regulation of a variety of cellular functions such as cell motility, growth and differentiation[40].

1.3. Phospholipase C

Phospholipase C family members are key elements in signal transmission networks that link almost all types of cell surface receptors to downstream components, being, in this way, involved in the direct and indirect regulation of a variety of cellular functions such as cell motility, growth and differentiation[41,42]. In response to extracellular stimuli such as hormones and growth factors, all PLCs catalyzes the hydrolysis of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) into two second messengers: diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3)[40,43] (Figure 3). DAG remains in the membrane where activates a variety of enzymes such as the protein kinase C (PKC) and GTPases regulating proteins. It also stimulates the activity of structural proteins by binding to a conserved C1 domain and is the substrate for synthesis of phosphatidic acid, which is also a regulatory molecule *per se*[40,42]. IP_3 is a major regulator of intracellular levels of Ca^{2+} by binding to its receptors at the endoplasmic reticulum and releasing Ca^{2+} into the cytoplasm[40]. Ca^{2+} is itself the center of a major regulatory network, being involved in the activation of Calmodulin pathway, regulation of apoptosis and cytoskeleton proteins[44]. In addition, IP_3 is the rate-limiting substrate for the synthesis of inositol polyphosphates, which stimulates multiple protein kinases, transcription and mRNA processing[40]. Finally, PIP_2 although being the substrate for phosphatidylinositol 4,5-triphosphate (PIP_3) synthesis is also a signaling molecule by itself, regulating ion channels and components of the actin cytoskeleton [42,45].

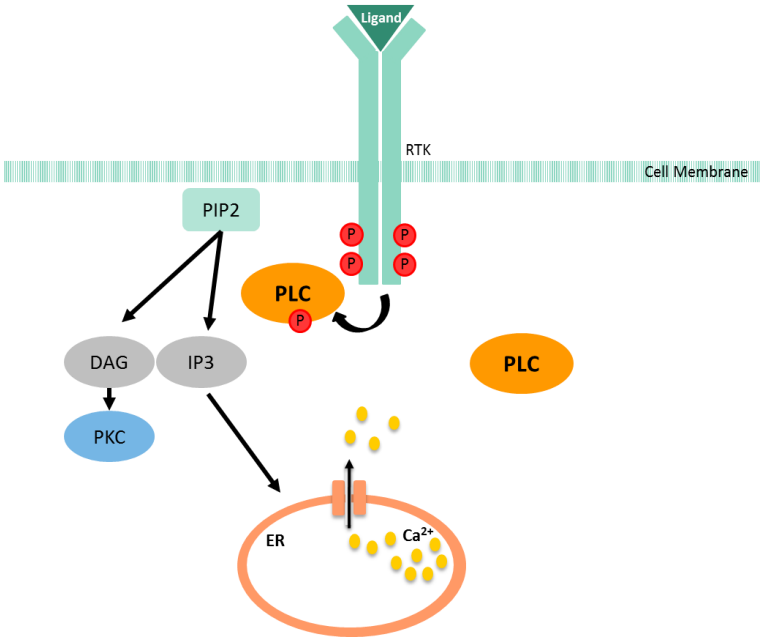

Figure 3: PLC Signaling. PLC enzymes are activated by receptor tyrosine kinases (RTK) or G Protein-coupled receptors. In this case, PLCγ1 is activated by direct binding to RTK. Activated PLC hydrolyses PIP_2 that creates two new signaling molecules, DAG and IP3. DAG activates a variety of enzymes such as the protein kinase C (PKC). IP_3 is a major regulator of intracellular levels of Ca^{2+} by binding to its receptors at the endoplasmic reticulum and releasing Ca^{2+} into the cytoplasm.

Thirteen mammalian PLCs are classified into six families (β, γ, δ, ε, η, ζ), according to their structure (Figure 4). Different families differ in their expression pattern and regulatory mechanisms[40]. Four PLCβ isozymes are activated mainly downstream of G protein-coupled receptors. While PLCβ1 is highly expressed in the cerebral cortex and hippocampus, PLCβ2 is mainly expressed in hematopoietic cells, PLCβ3 is broadly expressed and PLCβ4 expression is enriched in the cerebellum and in the retina[42,45]. A single isoform of PLCε exists. It is ubiquitously expressed with highest levels found in heart, liver, and lung. PLCε incorporates a RAS-binding domain (RA), which allows the binding of RAS family members that activates its lipase domain. Furthermore, PLCε also incorporates a guanine nucleotide exchange factor (GEF) domain that can activate RAS family members itself. PLCε enzyme activity can also be stimulated by subunits of heterotrimeric G proteins[40,46]. PLCδ and PLCη are activated by intracellular calcium mobilization and, therefore, are considerate secondary PLCs[45]. PLCδ family members (PLCδ1, PLCδ3, PLCδ4) show broad tissue distribution but differ in cellular localization. PLCδ1 is mainly a cytoplasmic protein, whereas PLCδ3 is detected in membrane fractions. PLCδ4 is principally located in the nucleus, where its expression is directly linked with the cell cycle[42,43]. Both PLCη isoforms (PLCη1 and PLCη2) are expressed in neuron-enriched regions of the brain, suggesting a role of these proteins in neuronal development[42].

PLCζ exists as a gamete-specific PLC, only expressed in spermatids. It is the smallest PLC isozyme, and is the only one that lacks an N-terminal PH domain. The activation mechanism of PLCζ remains to be elucidated[42].

All families of PLC share a conserved core region, essential for their catalytic activity, and domains specific to each family[42]. The core enzyme is composed of an N-terminal pleckstrin domain (PH), four tandem EF motifs, a TIM barrel domain and a C-terminal C2 domain[40] (Figure 4). The PH domain is important for the binding of various lipids and proteins[47]. The EF motifs, which are Ca^{2+} binding motifs, bind to calcium ions and are important to enhance PLC enzymatic activity[42]. The C2 domain is involved in membrane traffic and interacts with both EF motifs and TIM barrel[46]. The catalytic TIM barrel domain is the most conserved domain among all PLC isoforms, both structurally and functionally, and include the active site and all catalytic residues[40,46]. This domain is interrupted by an auto-inhibitory insert that is central for the regulation of the activity of all PLC and divides the TIM barrel domain into X and Y domains, and is therefore named X-Y linker[40,47]. The N-terminal half (X-box) is the more conserved and contains all catalytic residues. The C-terminal half (Y-box) has an important role in substrate recognition[40,46].

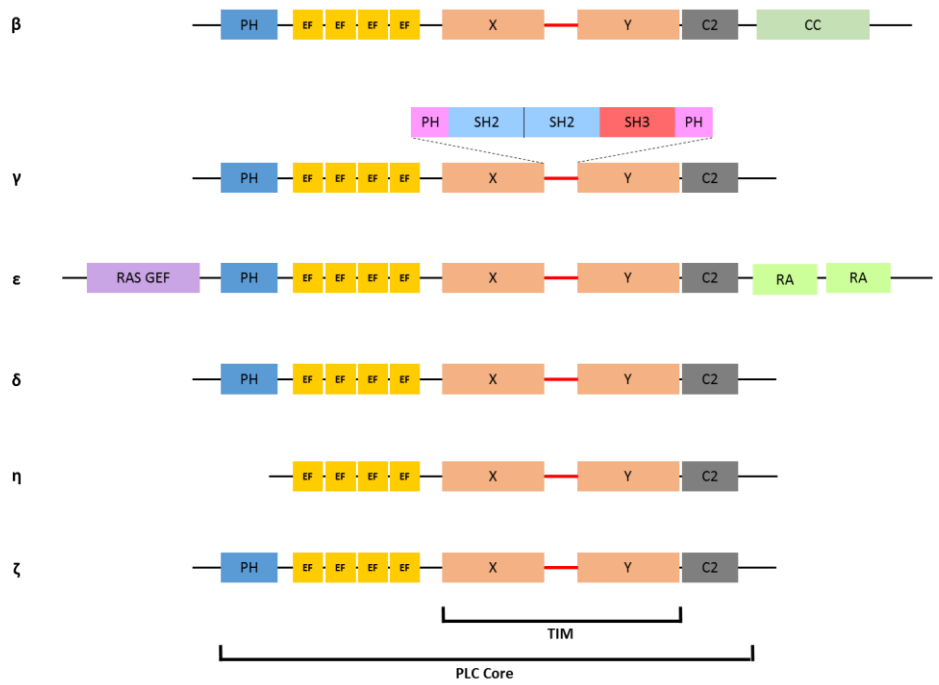

Figure 4: Phosphoinositide family domain organization. Domain organization of PLCβ, PLCγ, PLCε, PLCδ, PLCη and PLCζ enzymes highlights their common and unique features. The four domains that comprise PLC core: PH domain (blue), EF-hands (yellow), the catalytic TIM barrel domain (orange), that incorporate regions of high sequence similarity X and Y, and the C2 domain (gray) are found in all PLC families, except for PLCη, which lacks the N-terminal PH domain. The unique regions in PLCβ, PLCε and PLCγ are show.

1.3.1. PLCγ1

Two isoforms of PLCγ have been identified in humans: PLCγ1 and PLCγ2 (encoded by *PLCG1* and *PLCG2* genes, respectively)[41]. Ubiquitously expressed, PLCγ1 is mainly activated downstream of growth factor stimulation, such as EGF stimuli, and is important for the control of cell growth and differentiation, whereas PLCγ2 is predominantly expressed in hematopoietic cells where it is activated by immune cell receptors such as B cell and Fc receptors and modulates more acute responses[40,41]. The exception is the T cell receptor activation which is linked to PLCγ1, not to PLCγ2[42]. However, many cells express both isoforms, that exert non-overlapping functions and one enzyme generally cannot compensate for depletion of the other[48]. Nevertheless, both PLCγ1 and PLCγ2 are similar in structure and regulation in most cases[40].

Structure

The two PLCγ isoforms are structurally characterized by a large and highly structured multidomain insert in the X-Y linker, the γ specific array (γSA), that consists in a split PH domain, two SH2 domains (nSH2 and cSH2) and a SH3 domain[49] (Figure 5). PH domains mediates interactions with phosphatidylinositol-3,4,5-trisphosphate (PIP_2) and directly interact with the calcium-related transient receptor potential cation channel 3 (TRP3), providing a direct coupling mechanism between PLCγ and agonist-induced calcium entry[41,50]. The SH2 domains recognize phosphotyrosine residues, being important for membrane recruitment, interaction with the receptor and tyrosine phosphorylation of PLCγ1[51]. SH3 domain mediates interactions with proline-rich sequences and is involved in formation of multiprotein complexes that contain both upstream regulators and downstream effectors. Targets of the SH3 domain, include adaptor proteins (SOS1)[52], cytoskeleton components (dynamin-1)[53], and diverse signaling proteins (PLD_2, AKT, PIKE)[54–56].

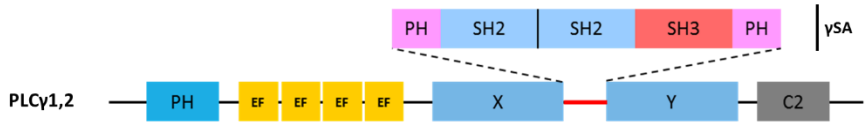

Figure 5: PLCγ1 Structure. PLCγ1 and PLCγ2 have the same domain organization and share high sequence identity across all domains. They incorporate a core set of domains shared by all PLC isozymes: an N-terminal PH domain, EF-hands, TIM-barrel-like fold and a C2 domain. Uniquely for PLCγ1, the linker between the two halves (X and Y boxes) of the TIM-barrel is highly structured and consists of a 'split' PH domain, two src homology 2 (SH2) domains (nSH2 and cSH2), and one SH3 domain.

Regulation

PLCγ1 is mainly activated downstream of receptor tyrosine kinases (RTKs) in response to agonist binding. After receptor dimerization and autophosphorylation, PLCγ1 is recruited to the plasma membrane where directly binds to phosphotyrosine docking sites by its nSH2 domain (Figure 3)[46]. Depending on the cell and the stimulus, PLCγ1 phosphorylation and consequent activation can be catalyzed by diverse RTK, such as epidermal growth factor receptor (most common), platelet-derived growth factor receptor (PDGFR), nerve growth factor receptor, or fibroblast growth factor receptor (FGFR)[42,57]. Specifically, autophosphorylation of Tyr992 in EGFR[58] (figure 2), Tyr766 in FGFR1[59], and Tyr1021 in PDGFR[60] confer a high-specificity interaction with the nSH2 domain of PLCγ1.

Following receptor association, PLCγ1 is phosphorylated at three known sites: Y783, Y771 and Y1244 with diverse effects, but it was shown that phosphorylation of Y783 is both necessary and sufficient for stimulation of lipase activity[41,61]. Y783 lies between the cSH2 and SH3 domains. Phosphorylation of Tyr783 results in high affinity interaction with the cSH2 domain, which in turn results in a conformational change responsible for removal of autoinhibition[42]. Additional phosphorylation depends on cell type and stimulus and its effects can include interaction and recruitment of other signaling proteins, but in many cases this functions are not clear[40].

PLCγ1 is also activated downstream of receptors that lack intrinsic tyrosine kinase activity, including T cell receptors, cytokine receptors, angiotensin II and bradykinin receptors[42].

Physiology

PLCγ1 has been implicated in many growth factor induced cell signaling processes such as cell proliferation, differentiation, receptor endocytosis, cell motility and angiogenesis. Vascular endothelial growth factor receptor (VEGFR) activates PLCγ-PKC pathway that activate MAPK and DNA synthesis in endothelial cells, leading to endothelial cell proliferation and migration[62]. In several studies, PLCγ1-knockout mice die at early embryonic stages due to impaired vasculogenesis and erythropoiesis by loss of both erythroid progenitors and endothelial cells, necessary for both processes[63,64]. A zebrafish model also suggests that VEGF signaling through PLCγ1 govern the formation of the arterial system and modulates cardiac contractility[62,65].

PLCγ1 is also involved in growth factor-induced mitogenesis[66]. Microinjection of purified PLCγ1 into quiescent NIH-3T3 cells (mouse fibroblast cells) induced DNA synthesis and

microinjection of PLCγ1 antibody into the same cells blocks PDGF-induced DNA synthesis[67]. Microinjection of a polypeptide corresponding to the non-catalytic γSA domain of PLCγ1 blocked growth factor-induced S-phase entry, therefore the mechanism of action of PLCγ1 in growth factor-induced mitogenesis is likely to be through the activation of PKC[66]. Other studies also show that PLCγ1 catalytic activity is necessary for Ras-mediated induction of DNA synthesis in NIH-3T3 fibroblasts[66].

Recent studies have shown that PLCγ1 is involved in much broader cell signaling processes than previously revealed and not exclusively resulting from its lipase activity[68,69]. Actually, most of the recently identified interactions of PLCγ1 with other proteins are mediated by its SH3 domain. PLCγ1 SH3 domain acts as a guanine nucleotide exchange factor for dynamin-1 in an EGF-dependent manner[53]. This GEF activity regulates the influence of dynamin-1 upon EGFR endocytosis, accelerating this process and therefore upregulating activation of ERK and serum response element (SRE) dependent transcriptional activity[53]. PLCγ1 SH3 domain also acts as a GEF for Rac1 upon EGF stimulation and this interaction play an important role in EGF induced cytoskeleton remodeling and cell migration[70]. Ye *et al.*[71] describe that PLCγ1 acts as a GEF for PIKE (PI3K enhancer) that is a nuclear GTPase that mediates the physiological activation by nerve growth factor of nuclear PI3K activity. Finally, PLCγ1 directly interacts with AKT proline rich motifs by its SH3 domain, resulting in PLCγ1 S1248 phosphorylation by AKT and enhanced EGF-stimulated cell motility[54].

PLCγ1 in Cancer

PLCγ1 is known to contribute to oncogenic signaling downstream of EGFR by playing an important role in cell migration and invasion, being in this way, involved in tumor progression. Furthermore, several studies have showed that PLCγ1 plays a critical role in both cytoskeletal changes and migration associated with metastatic process[72–75]. PLCγ1 plays an important role in growth factor induced cell motility since it is activated by multiple receptor tyrosine kinases and by integrin engagement to the extracellular matrix[72–74]. Moreover, PLCγ1 activity is required for the early steps of cell migration, in particular for the actin polymerization[76–78]. PLCγ1 knockdown inhibits the early actin polymerisation, which results in lack of cell spreading and in a rounded and poorly motile phenotype[75,79]. Additionally, PLCγ1 also interacts with Rac1, an important protein in cytoskeleton rearrangement and actin polymerization[68].

PLCγ1 was found to be overexpressed in different malignances when compared to normal adjacent tissues. Initial studies indicated that increased levels of PLCγ1 occurred in malignances of

the breast when compared to normal tissue[80]. Other reports also found that PLCγ1 is overexpressed in colorectal carcinoma tissues and that expression of PLCγ1 increases from normal mucosa to adenoma and finally to carcinoma, progressively[81–83]. Overexpression of PLCγ1 was latter reported in head and neck squamous cell carcinoma (HNSCC)[84], non-small cell lung cancer (NSCLC)[85], prostate cancer[86], oral squamous cell carcinoma (OSCC)[87,88] and gastric cancer[89,90]. Accordingly, downregulation of PLCγ1 expression in MDA-MB-231 cell line (a human breast cancer cell line) inhibits the development of lung metastasis in nude mice[72,86]. In a clinical setting, higher expression of PLCγ1 was associated with worse clinical outcome in terms of poor overall survival, disease-free survival and distant metastasis-free survival in OSCC, breast cancer and gastric cancer[91,92].

More recently, acquired mutations in the PLCG genes have been identified by whole-genome sequencing in angiosarcoma, cutaneous T cell lymphoma and chronic lymphocytic leukemia (CLL). *PLCG1* activating mutations R707Q and S345F were associated with development of angiosarcoma and cutaneous T-cell lymphoma, respectively. These mutations are expected to cause a conformational change that activates constitutively PLCγ1 lipase activity, resulting in increased cell migration, survival and invasiveness, that leads to overactive angiogenesis and enhanced T cell receptor signaling[93–96]. Finally, mutations in *PLCG2*, the predominantly isoform expressed in hematopoietic cells, were shown to be responsible for the mechanism of resistance to Bruton's tyrosine kinase inhibitor, Ibrutinib, used for the treatment of CLL[97]. *PLCG1* Activating mutation R707Q was also found in a patient with hepatic angiosarcoma with acquired resistance to Sunitinib, a RTK inhibitor, and was associated with resistance to this target therapy[98].

Therefore, these studies suggest an important role of PLCγ1/2 in tumor progression and a possible involvement of this proteins in the resistance mechanisms to inhibitors of tyrosine kinases receptors, such as EGFR. Being able to establish a correlation between PLCγ1 and resistance to EGFR-targeted therapy in metastatic CRC will have great clinical implications for the treatment and quality of life of CRC patients.

2. Objectives

Despite that new target therapies, such as anti-EGFR specific antibodies, have improved the efficacy of conventional chemotherapy in metastatic CRC over the last decade, a large number of patients still do not benefit from these treatments. *KRAS* mutations are recognized as strong predictors of resistance to anti-EGFR therapies, however, 54% of wild-type *KRAS* patients do not respond or develop acquired resistance to this therapy. Therefore, there is an urgent need for highly sensitive and specific predictive biomarkers, as well as new molecular targets of more efficient therapies. Since PLCγ1 is a direct downstream effector of EGFR and is involved in the regulation of multiple cell processes related to oncogenesis and tumor progression, we decided to evaluate the possible contribution of PLCγ1 activation to the resistance mechanism to EGFR-target therapy in *KRAS* wild-type metastatic CRC. Our specific objectives are:

1. In an *in vitro* approach:
    a. Correlate the expression levels of PLCγ1 with the intrinsic sensitivity of a panel of colon cancer cell lines to Cetuximab.
    b. Access the effect of PLCγ1 knockdown and overexpression in the sensitivity of colon cancer cell lines to Cetuximab.
    c. Determine the possible involvement of PLCγ1 in the development of acquired resistance to Cetuximab treatment.
2. In the clinical setting:
    a. Evaluate the expression levels of PLCγ1 in *KRAS* wild-type primary CRC human samples and correlate with responses to Cetuximab.

3. Methods

3.1. Cell Culture

Human colon cancer cell lines CACO-2, HT-29 and SW48 and COS-7 cells (from *Cercopithecus aethiops* kidney) were purchased from American Type Culture Collection (ATCC, Virginia, USA). COLO320DM and LIM1215 cell lines were gently provided by collaborators. Cells were cultured in either DMEM (Gibco) or RPMI-1640 (Gibco) media supplemented with 10% or 20% Fetal Bovine Serum (FBS; Gibco), 1% Penicillin-Streptomycin (Pen/Strep; Gibco), 200mM L-Glutamine (Gibco) and other cell line specific supplements listed in Table 1 at 37˚C in a humidified 5% carbon dioxide (CO_2) atmosphere.

Table 1: Summary of cell lines culture conditions.

Cell Lines	Growth Medium	% FBS	Antibiotics	Amino acids	Supplements	Cryopreservation
CACO-2	DMEM	20	Streptomycin, Penicillin	200mM L-glutamine	10mM Hepes 1x Non-Essential aa	Complete Growth Medium 5% DMSO
COLO320DM	RPMI1640	10	Streptomycin, Penicillin	200mM L-glutamine	-	Complete Growth Medium 5% DMSO
COS-7	DMEM	10	Streptomycin, Penicillin	200mM L-glutamine	-	Complete Growth Medium 5% DMSO
HT-29	DMEM	10	Streptomycin, Penicillin	200mM L-glutamine	-	Complete Growth Medium 5% DMSO
LIM1215	RPMI1640	10	Streptomycin, Penicillin	200mM L-glutamine	25mM HEPES 0,6g/mL Insulin 1µg/mL Hydrocortisone 10µM 1-Thioglycerol	FBS 10% DMSO
SW48	DMEM	10	Streptomycin, Penicillin	200mM L-glutamine	-	Complete Growth Medium 5% DMSO

3.1.1. Cell Viability Assay

For determination of Cetx sensitivity, 10 000 cells/well (CACO-2, COLO320DM, HT-29, LIM1215 and SW48) were seeded in 96-well plates with specific growth medium and treated with 0; 0,01; 0,1; 1 and 10 µg/ml Cetx for 72h. Following treatment, cell viability was assessed by the AlamarBlue™ assay (Invitrogen), according to the manufacturer's instructions. Briefly, 10µl of AlamarBlue™ reagent was added do each well containing 200µl of growing medium and incubated for 3h at 37˚C and 5% CO_2. After this period, the fluorescence was measured in the Infinite 200 Plate Reader (Tecan) at an excitation wavelength of 560nm, an emission of 590nm and a manual gain of 90. The relative rate of cell growth for each cell line was calculated by dividing the average values

of fluorescence intensity of the treated cells by the average values of fluorescence intensity of untreated cells. Cetuximab (Erbitux®, Merk) was kindly provided by the Pharmacy of the CHLN-HSM.

3.1.2. Establishment of Cell Lines with Acquired Resistance to Cetx

Over a five month period, SW48 cell line was continuously exposed to a fixed concentration of 10µg/ml Cetuximab, establishing the SW48-Cetx resistant cell cultures.

3.1.3. PLCγ1 Overexpression

Full-length human PLCγ1 and mutant construct ΔSA (deletion of amino acids 488-933) cloned into pTriEx4 vector (Novagen) were gently provided by collaborators. For PLCγ1 overexpression, SW48 cell line was seeded approximately 12h before transfection in 24-well plates at a density of $4x10^4$ cells/well. Cells at 70-90% confluence, were transfected with both constructs using Lipofectamine®2000 transfection reagent (ThermoFisher) following manufacture instructions. Briefly, Lipofectamine®2000 reagent (3µL/transfection) and plasmid DNA (500ng/transfection) were diluted in Opti-MEM® Medium (Gibco) and incubated for 20min at room temperature to create DNA-lipid complexes that were added to the cells in a medium without serum or antibiotics. For parental control, cells were incubated with Lipofectamine®2000 reagent for the same time. Roughly, 5h after initial transfection, the medium was replaced by fresh complete growth medium with or without different Cetuximab concentrations (0,1; 1; 10µg/mL). Following a 72h treatment, cell viability was assessed by the AlamarBlue™ assay, as described before in section 3.1.1. PLCγ1 overexpression was confirmed by Western Blot.

3.1.4. PLCγ1 Knockdown

CACO-2 and HT-29 cells were plated approximately 24h before infections. 50-60% confluent dishes were infected with shRNA lentiviral particles (PLCγ1 and scrambled control) purchased from Santa Cruz Biotechnology together with 5µg/mL of polybrene® (Sigma). Puromycin (Sigma) selection started 2 days after infection with an amount of 10µg/mL for CACO-2 and 3µg/mL for HT-29 cells. Medium with puromycin was replaced every 2-3 days during at least 10 days. PLCγ1 knockdown was confirmed by real-time quantitative PCR (qPCR) and Western Blot.

3.1.5. PLC Activity Assay

$2x10^5$ COS-7 cells were seeded into 6-well plates. In the following day, cells were transfected with 2,5µg of plasmid DNA using Lipofectamine®2000. 48h after initial seeding, the cells were washed twice with inositol-free DMEM (USBiological) without serum and incubated for 24h in 1,5ml of the same medium supplemented with 0,25% fatty acid free bovine serum albumin (BSA, Sigma) and 1,5µCi/ml myo-[2-^{3}H]inositol (PerkinElmer Life Sciences). After a further 24h, the cells were incubated in 1,2ml of inositol-free DMEM without serum containing 20mM LiCl (Sigma) with or without stimulation with 100 ng/ml EGF (Calbiochem®) for 1h. The cells were then lysed by addition of 1,2ml 4,5 % perchloric acid (Fluka) and incubated on ice for 30min. Samples were centrifuged for 20min at 4000rpm and supernatants and pellets were separated. The supernatants were neutralized by addition of 3ml of 0,5M potassium hydroxide/9mM sodium tetraborate (both from Sigma) and centrifuged for a further 20min at 4000rpm. Supernatants were loaded onto an Anion exchange AG1-X8 200–400 columns (Bio-Rad) that had been converted to the formate form by addition of 2M ammonium formate/0,1M formic acid (both from Sigma) and equilibrated with water. The columns were washed three times with 5ml of 60mM ammonium formate/5mM sodium tetraborate, and inositol phosphates were eluted with 5ml of 1,2M ammonium formate/0,1M formic acid. 5ml Ultima-Flo scintillation fluid (PerkinElmer Life Sciences) was added to the eluates and the radioactivity quantified by liquid scintillation counting. The values represent total inositol phosphates. The pellets from the first centrifugation were resuspended in 100µl of water and 375µl of chloroform/methanol/HCL (200:100:15) (chloroform and HCL from Sigma and Methanol from Merck) was added. The samples were vortexed, and an additional 125µl of chloroform and 125µl of 0,1m HCL were added. After further vortexing, the samples were centrifuged at 2500rpm for 10min. 20µl of the lower phase were placed in a scintillation vial with 2ml of Ultima-Flo scintillation fluid and the radioactivity quantified by liquid scintillation counting. The obtained values correspond to radioactivity in total inositol lipids. PLC activity is expressed as the total inositol phosphates formed relative to the amount of [^{3}H]myo-inositol in the phospholipid pool.

3.2. Western Blotting

For Western Blot analysis, total protein extracts were prepared by lysing cells in 200-400µl of lysis buffer containing 25mM Tris pH 7.5 (Sigma), 500mM EDTA (Sigma), 1% Triton X-100™ (VWR), 25nM TCEP (Sigma) and in the presence of protease inhibitor cocktail (Roche) and phosphatase-

inhibitor cocktail 2 (Sigma). After 10min incubation on ice, the extracts were centrifuged at 12000rpm for 10min at 4˚C. Supernatants were transferred to a new tube and concentrations quantified using Quick Start™ Bradford Protein Assay (Bio-Rad), measuring the absorbance at 562nm. Concentration was determined by comparing to a standard curve of known BSA concentrations. 4x SDS-PAGE Sample Buffer was added to 30-70μg total protein extract and denatured for 5 min at 90˚C. Samples were loaded into 7-10% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes using the iBlot® Dry Blotting System (Life Technologies). Membranes were blocked for 1h in 5% BSA (Santa Cruz) or 5% non-fat dry milk, in TBS 0,1% Tween20 and incubated with specific antibodies overnight at 4˚C. Specific antibodies used and respectively dilutions are listed in Table 2. Next day, membranes were incubated with horseradish peroxidase-conjugated (HRP) specific secondary antibodies (donkey anti-mouse (1:2000) or goat anti-rabbit (1:4000) both from Santa Cruz Biotechnologies) for 1h at RT. Proteins were detected using Amersham ECL Western Blotting Detection Reagent (GE Healthcare Life Sciences), according to manufacturer's instructions. Signal was detected on radiographic film (Fujifilm), using Curix60 (AGFA).

Table 2: List of antibodies used in Western Blot.

Antibody	Dilution	Antibody Dilution Buffer	Source
Anti-AKT Rabbit Polyclonal Antibody	1:1000	5% w/v Milk, 1X TBS, 0.1% Tween	Santa Cruz Biotechnology® AKT1/2/3 (H-136) SC-8312
Anti-phospho-AKT (S473) Rabbit Monoclonal Antibody	1:1000	5% w/v BSA, 1X TBS, 0.1% Tween	Cell signaling® Phospho-AKT (S473) (736E11) Rabbit mAb
Anti-β-Actin Mouse Monoclonal Antibody	1:25000	5% w/v Milk, 1X TBS, 0.1% Tween	Abcam Anti-beta Actin antibody [mAbcam 8226] (ab8226)
Anti-EGFR Rabbit Monoclonal Antibody	1:1000	5% w/v Milk, 1X TBS, 0.1% Tween	Cell signaling® EGF Receptor (D38B1) Rabbit mAb
Anti-p42/44 MAPK (ERK1/2) Rabbit Monoclonal Antibody	1:1000	5% w/v Milk, 1X TBS, 0.1% Tween	EMD Millipore™ anti-MAP Kinase 1/2 (Erk1/2), Polyclonal (06-182)
Anti-phospho- p42/44 MAPK (ERK1/2) (T202/Y204) Rabbit Monoclonal Antibody	1:1000	5% w/v BSA, 1X TBS, 0.1% Tween	Cell signaling® Phospho-p44/42 MAPK (Erk1/2) (T202/Y204) (197G2) Rabbit mAb
Anti-phospho- PKC (pan) (βII Ser660) Rabbit Monoclonal Antibody	1:1000	5% w/v Milk, 1X TBS, 0.1% Tween	Cell signaling® Phospho-PKC (pan) (βII Ser660) Antibody 9371
Anti-PLCγ1 Rabbit Monoclonal Antibody	1:1000	5% w/v Milk, 1X TBS, 0.1% Tween	Cell signaling® PLCγ1 Rabbit mAb (D9H10)
Anti-S6 Ribosomal Protein Mouse Monoclonal Antibody	1:500	5% w/v Milk, 1X TBS, 0.1% Tween	Santa Cruz Biotechnology® Ribosomal Protein S6 (SC-74576)
Anti-phospho-S6 Ribosomal Protein (S240/244) Rabbit Monoclonal Antibody	1:1000	5% w/v BSA, 1X TBS, 0.1% Tween	Cell signaling® Phospho-S6 Ribosomal Protein (Ser240/244) (D68F8) rabbit mAb

3.3. RNA isolation, cDNA Synthesis and RT-qPCR

PLCγ1 and control GAPDH expression levels were evaluated by qPCR. For each sample a cell pellet was collected and RNA extraction was performed using NZY Total RNA Isolation Kit (NZYTech)

according to manufacturer's instructions. Briefly, cell pellets were lysated and loaded into a column with a silica membrane. Membranes were washed to clean impurities and treated with DNase to prevent DNA contamination. Columns were washed for three times and total RNA was eluted in RNase-free water and quantified using Nanodrop™2000 (Thermo Scientific).

Then, cDNA was synthesized using 1µg of total RNA and NZYM-MuLV First-Strand cDNA Synthesis Kit (NZYTech) according to manufacturer's instructions. For that, annealing reaction was performed by mixing RNA, Oligo$(dt)_{18}$ primer mix and annealing buffer. Mixture was incubated for 5min at 65˚C and then placed on ice for 1min. Reverse-transcription reaction was performed by adding NZYM 2x Master Mix (no oligos) and NZYM-MuLV RT enzyme mix to the tubes and incubated for 50min at 37˚C. The reaction was inactivated by heating at 85˚C and then chilled on ice. RNA template was degraded by incubating RNase for 20min at 37˚C.

Transcript levels of individual genes were assayed by qPCR, using Power SYBR® Green PCRMaster Mix (Applied Biosystems) in Corbett Rotor-Gene 6000 (QIAGEN Rotor-Gene Q), according to the manufacturer's instructions. Reactions were run in triplicate. Cycling conditions were the following: holding at 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 seconds, 55˚C for 40 seconds and 70˚C for 30 seconds. Relative mRNA expression levels were normalized to endogenous GAPDH and calculated using the $2^{-\Delta\Delta CT}$ method. Specific primers used were Human PLCG1 (PPH00710A-200) and Human GAPDH (PPH00150E-200) both from QIAGEN.

3.4. Immunohistochemistry

Expression of PLCγ1 was evaluated by immunohistochemistry (IHC) in a cohort of formalin-fixed paraffin-embedded (FFPE) samples from human primary colorectal carcinomas (n=25) from pathology service of Hospital de Santa Maria-CHLN. For all samples, mutation status of codons 12, 13, 59, 61, 117 and 146 of both *KRAS* and *NRAS* and codon 15 of *BRAF* were evaluated by Sanger sequencing. Only patients with wild-type *KRAS* and *NRAS* were enrolled in this study. All patients signed an informed consent and the use of these samples was previously approved by the Ethics Committee of HSM-CHLN.

Protocol optimization was performed in a random selected sample from CRC cohort which was used as positive control of all experiments. Deparaffinization and antigen retrieval was performed in PT Link Pre-Treatment Module for Tissue Specimens (Dako), using Antigen Retrieval pH6 solution (Dako), at 94˚C for 20min. Activity of endogenous peroxidase was blocked with

Blocked Endogenous Peroxidase Solution (Dako) for 15min at RT, and total protein was blocked by incubation with Protein Block Solution (Dako), for 30min at RT. Incubation with primary antibody (rabbit anti-PLCγ1 (D9h10) from Cell Signaling), diluted 1:100 was performed overnight at 4˚C. The visualization system Dako REAL™ EnVision™ Detection System, peroxidase/DAB+, rabbit/mouse (Dako) was used according to manufacturer's instructions, with 2min of incubation with DAB. Slides were counterstained with Harris hematoxylin (Sigma), dehydrated and diaphonized. Sections were mounted with Quick-D mounting medium (Klinipath) and visualized in a bright field microscope (Leica DM2500). Negative control was performed by the omission of primary antibody (replaced by protein block solution).

Samples were analyzed by a Medical Pathologist according to the Histoscore (H-score) method, which reads both the intensity of staining and the percentage of stained cells. Firstly, staining intensity was classified for each cell from 0 to 3: (0) absence of staining, (1) weak, (2) moderated and (3) strong staining. Then, the percentage of cells at each staining intensity level is calculated, giving a final score that ranges from 0-300. Dichotomization between high and low levels of PLCγ1 was done using the average of the H-score values as a cut-off.

3.5. Statistical Analysis

GraphPad Prism version 6.01 for windows (GraphPad Software) was used to perform statistical analysis.

In vitro resistance assays were performed in quadruplicates and error bars in graphs represent the standard error of the means (SEM). Multiple comparisons of means were done with repeated-measures one-way ANOVA or paired t-test, as appropriate. The level of statistical significance was set at $*p<0,05$ and $**p<0,01$. Experiments were repeated at least for three times to ensure reproducibility of the assays.

In regard to the clinical CRC samples, clinicopathological and therapeutic features were analyzed in correlation to PLCγ1 levels using Fisher's exact test or Chi-squared test, when appropriate. Kaplan–Meier plots were used to illustrate the progression-free survival (PFS, defined as the time from first Cetx treatment to disease progression) and overall-survival (OS, defined as the time from first Cetx treatment to patient dead). Univariate differences between survival rates were tested for significance using the log-rank test. Cox regression model was applied to evaluate hazard ratio (HR). Significance was defined as $*p<0,05$.

4. Results

4.1. Involvement of PLCγ1 in the Resistance to Cetuximab *in vitro*

4.1.1. Determination of Sensitivity of Colon Cancer Cell Lines to Cetuximab

In an initial approach to explore the sensitivity of different colon cancer cell lines to Cetuximab, we investigated the response of a panel of five CRC cell lines to increased concentrations of Cetuximab (0,01; 0,1; 1; 10 µg/mL) for 72h. Cell viability was, therefore, measured by AlamarBlue™ assay. With exception of HT-29 cell line that has *BRAF* V600E and *PIK3CA* P449T mutations, all selected cell lines are *KRAS*, *BRAF*, *PIK3CA* and *PTEN* wild-type. As shown in Figure 6, our results reveal a broad range of intrinsic sensibilities to Cetuximab treatment. Similarly to what was previously described by Ashrafa *et al*.[99], maximal effect of Cetx was observed in the viability of SW48 cell line, whereas minimal response was observed in CACO-2 cell line (Figure 6). Although harboring a *BRAF* activating mutation, HT-29 cell line showed an intermediate sensitivity to this monoclonal antibody treatment.

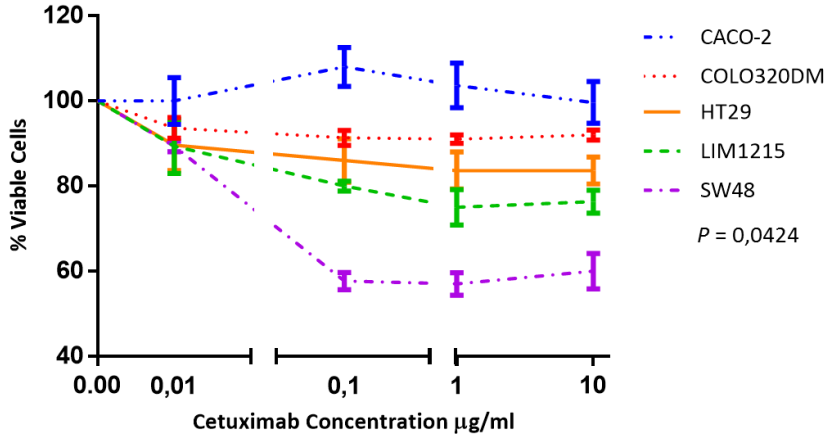

Figure 6: Differential sensitivity of colon cancer cell lines to 72-hours Cetuximab treatment. The AlamarBlue™ assay was used to determine the growth response of CRC cell lines to Cetuximab (0,01 – 10 µg/mL) (Three independent experiments are represented, n=3). Data are present as means ± SEM; *p* value was calculated by One-Way ANOVA.

4.1.2. PLCγ1 Protein Expression Correlates with Cetuximab Sensitivity in Colon Cancer Cell Lines

Ligand binding to EGFR results in direct activation of PLCγ1[40]. Furthermore, previous reports have shown an increased expression of this protein in colorectal tumor samples compared to normal tissue, suggesting that PLCγ could be activated in cancer cells, independently of the receptor[80,81]. Therefore, we hypothesize that PLCγ1 expression could be associated to increased resistance of colorectal cancer cells to Cetuximab. To investigate the association between PLCγ1 expression and sensitivity of colon cancer cell lines to Cetuximab, we examined the basal level of PLCγ1 protein expression in our cell line panel. As shown in Figure 7A, higher expression of PLCγ1 protein was seen in colorectal cancer cell lines with increased resistance to Cetuximab. We also access the expression of activated PKC, one downstream effector of PLCγ1 signaling, however, no correlation between p-PKC levels and Cetuximab response was found (Figure 7A).

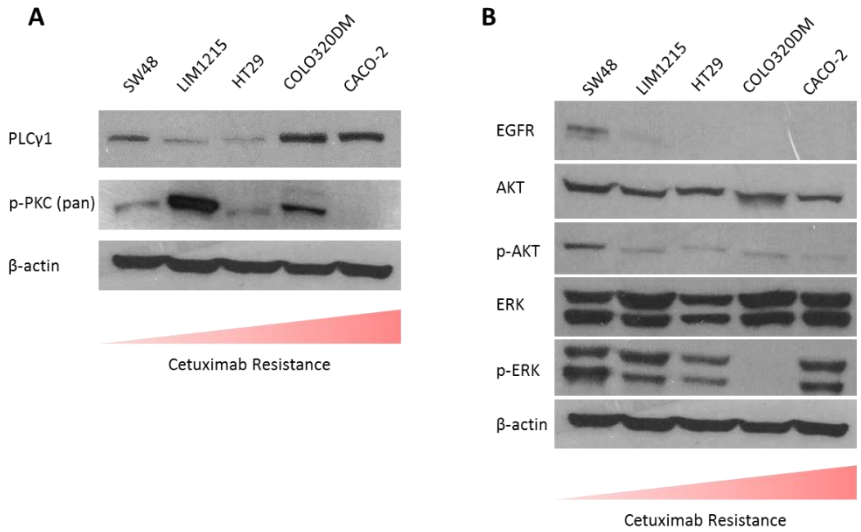

Figure 7: Basal expression of PLCγ1, p-PKC, EGFR, AKT, p-AKT, ERK and p-ERK in colorectal cancer cell line panel. (**A**) PLCγ pathway. (**B**) EGFR downstream pathways: ERK and AKT.

We went further to investigate whether EGFR or its known downstream effector pathways AKT and ERK were also altered in colorectal cancer cell lines resistant to Cetx (Figure 7B). It is worth to note that activation of MAPK and PI3K-AKT pathways has already been associated with poor response to anti-EGFR therapy[21,100]. Nevertheless, in our panel of CRC cell lines, EGFR protein expression was only detected in SW48 cell line, which is the most sensitive cell line to Cetuximab (Figure 7B). Furthermore, activated ERK and AKT also do not seem to correlate with Cetuximab sensitivity in our panel of cells.

4.1.3. PLCγ1 Knockdown Increases Sensitivity to Cetuximab Treatment in CACO-2 Cell Line

CACO-2 was the most resistant cell line to Cetuximab, in our panel of CRC cells, and was the line with higher PLCγ1 protein expression (Figures 6 and 7). Therefore, we started by knocking-down PLCγ1 expression in CACO-2 cell line by using shRNA lentiviral particles in order to establish stable cells expressing PLCγ1 shRNA. In this way, we obtained CACO-2$^{PLCγ1\ KD}$ and control CACO-2^{Control} cells (scrambled shRNA) after antibiotic selection. Figure 8 indicates that PLCγ1 shRNA vector effectively downregulated the expression of PLCγ1 in comparison with cells transduced with control shRNA (Figure 8A). Results were further confirmed by RT-qPCR showing a reduction of approximately 40% on PLCγ1 mRNA level (Figure 8B).

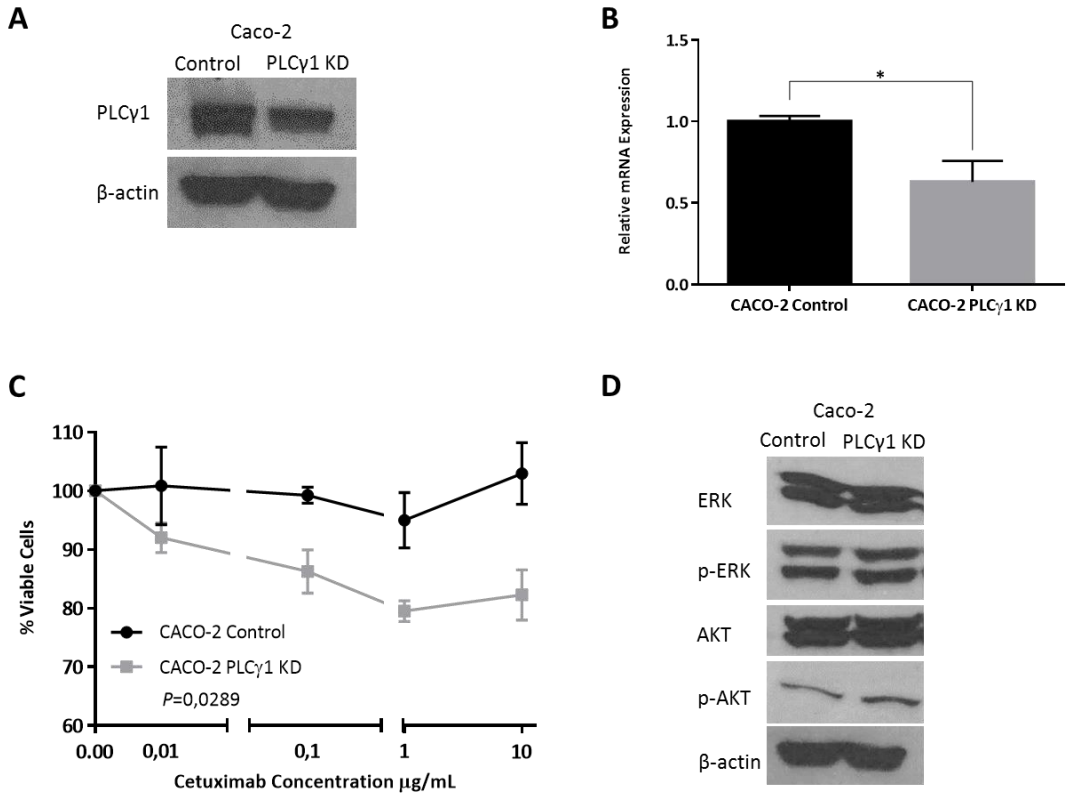

Figure 8: PLCγ1 knockdown in human colorectal adenocarcinoma CACO-2 cell line. (**A**) PLCγ1 protein expression was evaluated by Western Blot. (**B**) Relative mRNA expression was quantified by RT-qPCR and normalized to GAPDH gene. Experiments were performed in triplicate and data is presented as the mean ± SEM, *p<0.05. (**C**) Differential sensitivity to 72-hours Cetuximab treatment. The AlamarBlue™ assay was used to determine the growth response of CACO-2$^{PLCγ1\ KD}$ and CACO-2^{Control} cell lines to Cetuximab (0,01 – 10 µg/mL). Data are present as means ± SEM; p value was calculated using paired t-test. Four independent experiments were performed, n=4. (**D**) Effect of PLCγ1 knockdown in MAPK and PI3K/AKT pathways was accessed by Western Blot.

To determine whether PLCγ1 is involved in the resistance to anti-EGFR targeted therapy, we accessed the effects of PLCγ1 reduction in CACO-2 cell line. Cells were exposed to different Cetx concentrations (0,01; 0,1; 1; 10 µg/mL) for 72h. As shown in Figure 8C, cells expressing lower levels of PLCγ1 exhibited a statistically significant increase in Cetuximab sensitivity (p=0,0289).

Then, we examined if knocking-down PLCγ1 leads to alterations in other signaling pathways downstream of EGFR. Therefore, we analyzed by Western Blotting the activation of ERK and AKT pathways. Although previous reports have associated ERK and AKT pathways to PLCγ1 regulation[31,100], in this cell line we did not observe inhibition of AKT or ERK pathways in CACO-2$^{PLCγ1\ KD}$ cells, suggesting that Cetuximab sensitization induced by knocking-down PLCγ1 does not signal through ERK or AKT pathways.

4.1.4. PLCγ1 Knockdown Increases Sensitivity to Cetuximab Treatment in HT-29 Cell Line

In our panel of CRC cell lines, HT-29 are the only cells with alterations in *BRAF* and *PIK3CA* genes. *PIK3CA* P449T mutation, present in this cell line, is not described as oncogenic or related to therapeutic resistance[101]. However, *BRAF* V600E mutation leads to constitutively activation of MAPK pathway which has already been associated with a poor prognosis and poor response to EGFR antibody therapy[32,100]. In this way, we decided to investigate if, even in the presence of *BRAF* V600E activating mutation, PLCγ1 knockdown can sensitive cells to the treatment with Cetuximab. HT-29$^{PLCγ1\ KD}$ and HT-29^{Control} cells were obtained by transducing cells with target-specific and control shRNA particles, respectively, as described before. Figure 9 shows that PLCγ1 shRNA virus effectively inhibited the expression of PLCγ1 protein in comparison with cells transduced with control shRNA. Western blotting and RT-qPCR show a reduction of about 30% on protein and mRNA levels, respectively (Figure 9A and B).

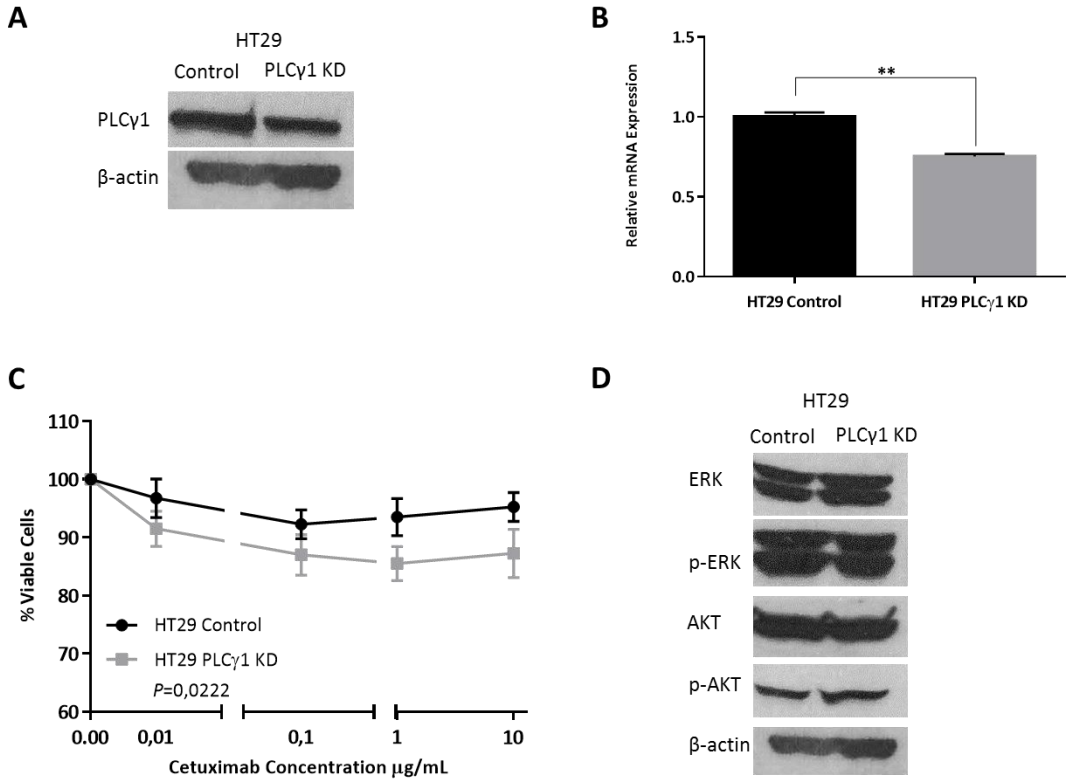

Figure 9: PLCγ1 knockdown in human colorectal adenocarcinoma HT-29 cell line. (**A**) PLCγ1 protein expression was evaluated by Western Blot. **B**) Relative mRNA expression was quantified by RT-qPCR and normalized to GAPDH gene. Experiments were performed in triplicate and data is presented as the mean ± SEM, $^{**}p<0.01$. (**C**) Differential sensitivity to 72-hours Cetuximab treatment. The AlamarBlue™ assay was used to determine the growth response of HT-29$^{PLCγ1\ KD}$ and HT29^{Control} cell lines to Cetuximab (0,01 – 10 µg/mL). Data are present as means ± SEM; *p* value was calculated using paired t-test. Four independent experiments are represented, n=4. (**D**) Effect of PLCγ1 knockdown in MAPK and PI3K/AKT pathways accessed by Western Blot.

Next, we investigated if downregulation of PLCγ1 in HT-29 cells influence the resistance to Cetuximab. Cells were exposed to Cetuximab (0,01; 0,1; 1; 10 µg/mL) for 72h, as previously. As shown in Figure 9C, cells expressing lower levels of PLCγ1 exhibited lower resistance to Cetuximab treatment (*p*=0,0222). It is interesting to note that sensitization of HT-29$^{PLCγ1\ KD}$, which harbor *BRAF* V600E mutation, although significant, was quite modest when compared to sensitization induced by knocking-down PLCγ1 in CACO-2 cell line. Although knocking-down PLCγ1 in this cell line was also less effective than in CACO-2 line, care should be taken when inhibiting PLCγ1 as a way to sensitize cells to Cetx, since other pathways downstream of EGFR can prevent the full potential of inhibiting PLCγ1.

Finally, we examined if knocking-down PLCγ1 leads to alterations in other known signaling pathways downstream of EGFR. As seen previously, no association between PLCγ1 knockdown and inhibition of EGFR downstream pathways was found (Figure 9D).

4.1.5. PLCγ1 Overexpression Increases Resistance to Cetuximab Treatment in SW48 Cell Line

SW48 cell line was the most sensitive to Cetuximab treatment in our panel of CRC cells (Figure 6). Therefore, we decided to overexpress full length wild-type PLCγ1 and a lipase constitutively active mutant (PLCγ1 ΔSA) in this cell line. The ΔSA mutant encodes an in-frame deletion of γSA PLCγ1 region known to own auto-inhibitory functions (deletion of amino acids 488-933 of *PLCG1*), therefore, ΔSA is expected to have increased catalytic activity. In order to confirm the activity of these variants, we started by performing a lipase catalytic assay in COS-7 cells overexpressing both constructs (Figure 10A). Results show that under non-stimulated conditions ΔSA mutant has increased lipase activity when compared to PLCγ1 wild-type. Furthermore, both constructs seem to have similar activities when stimulated with EGF for 1h (Figure 10A). Overall, this experiment shows that both constructs are well expressed and functional in cells.

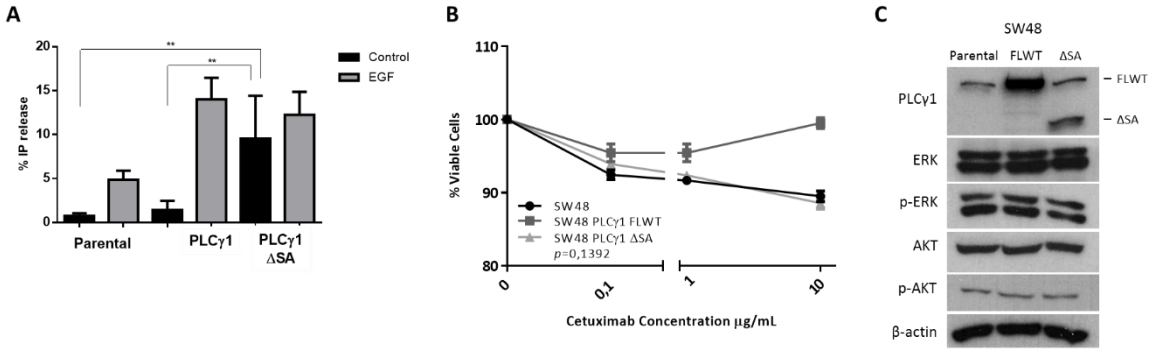

Figure 10: PLCγ1 overexpression in human colorectal adenocarcinoma SW48 cell line. (**A**) PLCγ1 activity assay. Results are representative of two independent experiments, n=2. Data are present as mean ± SEM; *p* value was calculated using unpaired t-test, ***p*<0.01. (**B**) Differential sensitivity to 72-hours Cetuximab treatment. The AlamarBlue™ assay was used to determine the growth response of SW48, SW48 PLCγ1 FLWT and SW48 ΔSA cell lines to Cetuximab (0,1 – 10 µg/mL). Data are present as means ± SEM; *p* value was calculated using paired t-test for comparison of SW48 and SW48^{FLWT}. Two independent experiments are represented, n=2. (**C**) Effect of PLCγ1 overexpression in MAPK and PI3K/AKT pathways accessed by Western Blot.

Next, we evaluate the effects of PLCγ1 overexpression in Cetuximab sensitivity. After transfection, SW48 cells were exposed to three different Cetuximab concentrations (0,1; 1; 10 µg/mL) for 72h, as before. As shown in Figure 10B, cells expressing high levels of wild-type PLCγ1 exhibited an increase in resistance to Cetuximab when compared to mutant ΔSA which exhibit a sensitivity similar to parental non-transfected cells. This result is in agreement with previous knockdown results suggesting that increased expression of PLCγ1 is involved in the resistance

mechanism to EGFR-targeted therapy. Surprisingly, however, ΔSA constitutively active mutant does not seem to induce such resistance in SW48 cells (Figure 10B), possibly indicating a lipase independent mechanism of action.

Finally, we examined the effects of PLCγ1 overexpression in MAPK and PI3K-AKT pathways. Therefore, Western Blot analysis of ERK, AKT protein expression and its activated forms show no involvement of any of these pathways in the resistance mechanism to Cetuximab treatment (Figure 10C).

4.1.6. Upregulation of PLCγ1 is Associated with Acquired Resistance to Cetuximab

Clinical data indicate that even the best responders to anti-EGFR target therapies are transient, and that patients eventually acquired resistance to this therapies[29]. In order to address a possible involvement of PLCγ1 in the acquired resistance to Cetuximab, we exposed three independent SW48 cultures to a fixed concentration of Cetuximab during five months. SW48 cell line was chosen because it is the most sensitive cell line in our panel of CRC cells (Figure 6). The growth profile of SW48 treated cells (SW48 Cetx 1, 2 and 3) was further evaluated towards Cetuximab sensibility. Preliminary results show that exposed cell cultures have an increase in Cetuximab resistance when compared with parental control, but with different resistant profiles (Figure 11A).

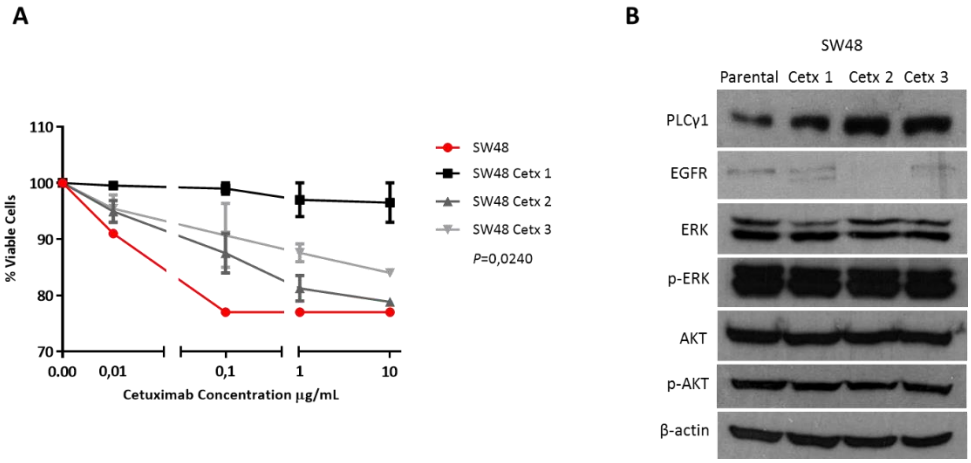

Figure 11: Characterization of Cetx-resistant culture lines. (**A**) Growth profile of SW48 parental cell line and Cetx-resistant cells to 72-hours Cetuximab treatment. The AlamarBlue™ assay was used to determine the growth response of SW48 Cetx-resistant and SW48 parental cells to Cetuximab (0,01 – 10 µg/mL). Data are present as means ± SEM; p value was calculated using repeated measures One-Way ANOVA. Two independent experiments are represented, n=2. (**B**) Effects of prolonged expression to Cetx in PLCγ1, EGFR, MAPK and PI3K/AKT pathways. Protein expression was accessed by Western Blot analysis

Cetuximab resistant cultures were further characterized and compared with the parental cell lines for EGFR, PLCγ1, AKT and ERK protein expression (Figure 11B). Despite the fact that no linear correlation between resistant lines and PLCγ1 levels can be seen, this analysis reveals an increase in PLCγ1 expression in the three cultures exposed to Cetx, when compared to non-exposed cells (Figure 11B). On the other hand, activation of ERK or AKT pathways in Cetx resistant cultures is clearly nonexistent. Even though, these preliminary results lack further confirmation, they seem to suggest an overactivation of PLCγ1 pathway in adaptive Cetx-resistant cells.

4.2. Involvement of PLCγ1 in the Resistance Mechanism to Cetuximab in a Clinical Setting

4.2.1. Elevated PLCγ1 Expression is Associated with Resistance to Cetuximab Treatment

To access the predictive value of PLCγ1 in the treatment of metastatic colorectal cancer with Cetuximab, we analyzed the expression of PLCγ1 in 25 FFPE primary CCR tumors by IHC. Staining slides were evaluated by a medical pathologist and intensities were scored as: (0) negative, (1) weak, (2) moderated and (3) strong staining, in both normal mucosa and tumor cells. Representative images of IHC staining of PLCγ1 are shown in Figure 12. An homogenously weak expression was observed in the cytoplasm of non-neoplasic cells. In comparison to the normal tissue, tumor cells showed increased expression of PLCγ1, as previously reported[80,81]. In neoplastic cells, staining was predominantly cytoplasmatic, but could also be found in the nucleus. PLCγ1 expression was also present in different elements of neoplasic stroma.

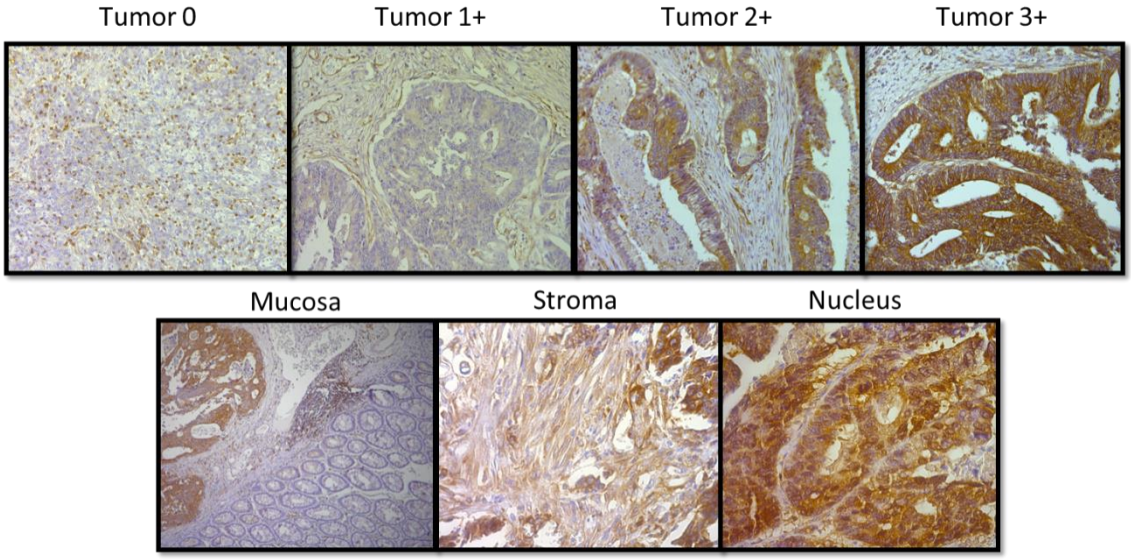

Figure 12: Immunohistochemical analysis of PLCγ1 in human CRC samples. Intensity of PLCγ1 staining in tumor cells range from 0 (absence of staining) to 3 (maximal intensity) (magnification, 200x). Staining was also found in the normal mucosa (magnification, 200x), in neoplasic stroma (magnification, 200x) and in the nucleus of some neoplasic cells (magnification, 400x).

For the analysis, Histoscore was evaluated uniquely based on the cytoplasmic staining of neoplasic cells. Samples were scored according to the percentage of cells with different intensity staining (final score ranges from 0 to 300) and dichotomized in low or high PLCγ1 expression based

in the average value of the final score, as described in Materials and Methods. Ten of twenty-five (40%) samples had low PLCγ1 expression and fifteen (60%) high PLCγ1 expression. In this cohort, PLCγ1 staining did not correlate with clinicopathological characteristics such as age, gender and TMN at diagnosis, as shown in Table 3. The treatment characteristics, such as number of cycles of Cetx and backbone chemotherapy, are equally balanced between both groups of patients. All samples used in this study are from patients with *KRAS* and *NRAS* wild-type tumors but may harbor *BRAF* V600E mutations (n=3) (Table 3). All patients in this cohort had disease progression under Cetuximab treatment and eventually died.

Table 3: Association between PLCγ1 expression and clinicopathological characteristics of patients.

Characteristics	PLCγ1 (%)		*p*
	Low	High	
No. of Patients	10 (40.0)	15 (60.0)	
Age at diagnosis (years)			0,6950#
<50	1 (10.0)	1 (6.7)	
50-65	5 (50.0)	7 (46.7)	
>65	4 (40.0)	7 (46.7)	
Sex			0,3781*
Female	4 (40.0)	3 (20.0)	
Male	6 (60.0)	12 (80.0)	
Lesion site			0,3577*
Right colon	3 (30.0)	2 (13.3)	
Left colon	7 (70.0)	13 (86.7)	
TMN stage			0,4422*
< IV	5 (50.0)	5 (33.3)	
IV	5 (50.0)	10 (66.7)	
Metastasis			0,6882*
Single	6 (60.0)	7 (46.7)	
Multiple	4 (40.0)	8 (53.3)	
Cycles of Cetx			0,2486#
≤10	3 (30.0)	8 (53.3)	
11-20	4 (40.0)	6 (40.0)	
>20	3 (30.0)	1 (6.7)	
Backbone chemotherapy			0,5824#
Oxaloplatin-based	2 (20.0)	1 (6.7)	
Irinotecan-based	7 (70.0)	13 (86.7)	
Monotherapy	1 (10.0)	1 (6.7)	
BRAF			1,0000*
WT	9 (90.0)	13 (86.7)	
V600E	1 (10.0)	2 (13.3)	

All values are presented as the number of patients followed by percentages in parentheses. Statistical analysis for categorical variables were performed using *Fisher's exact test or #Chi-square test. Abbreviations: PLCγ1, Phospholipase C gamma 1; WT, wild-type; TNM system – evaluation of tumor progression: T-Primary Tumor, N-Regional lymph nodes, M-Distant metastasis.

Survival analysis showed a statistically significant association of higher PLCγ1 expression with lower progression-free survival (*p*=0,0460; HR 0,4239 95%CI 0,1824-0,9849) and a trend towards a lower overall survival (*p*=0,0839; HR 0,4755 95%CI 0,2046-1,105) (Figure 13). Median PFS was 9,7 months in patients with low PLCγ1 expression compared with 6,4 months in patients with high PLCγ1 expression. Median of OS was 19,7 months in low PLCγ1 expression group compared with 12,1 months in high PLCγ1 levels group. Taken together, these results indicate that PLCγ1 can be a predictor of poor response to anti-EGFR target therapy.

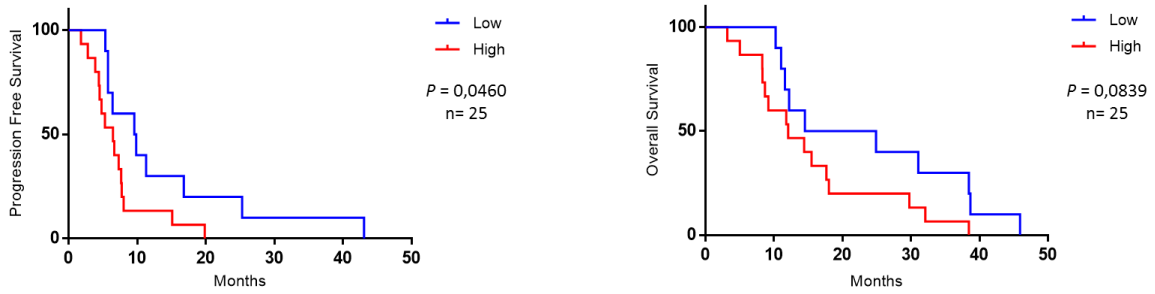

Figure 13: Kaplan-Meier estimates of progression-free survival (PFS) and overall survival (OS) according to PLCγ1 expression in primary CRC samples (n=25). *p* value was calculated using log-rank test.

5. Discussion

EGFR is a relevant player in colorectal cancer, being deregulated in about 60 to 80% of cases[102,103]. The development and approval of new therapies, including the monoclonal antibodies that specifically target EGFR, have increased the median survival of patients with metastatic colorectal cancer[24,104]. However, the efficacy of these therapies is restricted to a small percentage of patients, pointing out the extreme importance of new biomarkers capable of accurately select patients in this context.

In this study, we proposed to investigate the possible contribution of PLCγ1 to the resistance mechanism to EGFR-target therapies, namely Cetuximab, by using an *in vitro* approach and analysis of patient samples. PLCγ1 belongs to a family of phospholipase C that are activated by direct binding and phosphorylation by EGFR[40]. PLCγ1 activity is involved in the regulation of multiple oncogenic processes, such as growth-factor induced mitogenesis[66], cell migration[70], tumor development and progression of different cancers[72,86].

Here we show that basal levels of PLCγ1 are higher in cells intrinsically resistant to Cetuximab, when compared with more sensitive ones (Figures 6 and 7). Correlation between PLCγ1 protein levels and therapy resistance, namely Cetuximab and other RTK inhibitors, was never reported before. However, different colon cancer cell lines have already been screened for Cetuximab sensitivity, showing that alterations in MAPK and PI3K/AKT pathways could predict Cetx response[100]. Nevertheless, in our panel of *KRAS*, *NRAS*, *PIK3CA* and *PTEN* wild-type CRC cells, analysis of ERK and AKT signaling pathway does not seem to correlate with Cetuximab sensitivity (Figure 7). Interestingly, PKCs activation (PLCγ1 downstream effectors) were also not associated with Cetx sensitivity nor with PLCγ1 expression (Figure 7). It is, however, worthy of note that PKC isozymes are activated by DAG and calcium release and, therefore, could be regulated by multiple families of PLCs in these cell lines[105]. Nevertheless, the fact that p-PKC levels are not correlated with Cetuximab sensitivity, suggests that this class of proteins is not involved in the possible mechanism of resistance to Cetuximab induced by PLCγ1.

Furthermore, Cetuximab was initially approved for use in mCRC patients with EGFR overexpression[35], however, early studies found no evidence between Cetx response and EGFR expression[36]. In our cell line panel, EGFR protein expression was only observed in SW48 cell line, which is the most sensitive to Cetuximab (Figure 7). We could not detect EGFR expression in any of

the other cell lines studied, therefore, indicating that EGFR protein expression is unlikely to be responsible for differences in Cetx sensitivity.

Based on the Cetuximab sensitivity assay, we decide to knockdown PLCγ1 expression in CACO-2 and HT-29 (*BRAF* V600E mutant) cell lines and evaluate its consequences in Cetx response. Our findings indicate that reduction of PLCγ1 expression leads to increased Cetuximab sensitivity in both cell lines, independently of its *BRAF* status (Figures 8 and 9). Different studies have found that patients with *BRAF* alterations had worse clinical outcome when receiving anti-EGFR target therapies[31]. Indeed, V600E mutation is the most common *BRAF* genetic alteration in CRC and leads to activation of MAPK pathway[32]. However, there are no consistent evidences that *BRAF* V600E could be used as a predictive biomarker in clinical practice[33]. Indeed, in our panel of cell lines, HT-29 shows intermediate sensitivity to Cetx although harboring *BRAF* mutation. Additionally, PLCγ1 knockdown was able to sensitize this cell line to Cetx treatment, showing an important role of PLCγ1 in Cetuximab resistance even in the presence of constitutively active MAPK signaling.

Moreover, we also overexpressed PLCγ1 in the most sensitive cell line, SW48, and cells became more resistant to Cetuximab treatment (Figure 10). This result reinforces the idea that differences in PLCγ1 levels could predict Cetx response. Previous studies have already shown that PLCγ1 is upregulated in tumor cells, including in CRC tumors, when compared to normal tissue[80,81], having a tumor promoting role and being involved in tumorigenesis[85] and tumor progression[84]. Our work further reveals an enormous potential of this protein as a predictive biomarker of response to anti-EGFR therapy, namely Cetuximab. Of particular importance, analysis of the TCGA data, through cBioPortal, shows that *PLCG1* is upregulated or amplified in approximately 40% of colorectal cancers, furthermore, being mutually exclusive to *KRAS* activating mutations (p=0.032) (Figures S1 and S2, Supplementary Information)[106–108]. This suggests that patients that are prescribed with Cetuximab therapy (harboring *KRAS* wild-type), are very likely to have increased expression of PLCγ1 and therefore being also resistant to these treatments.

Remarkably, when we overexpressed a lipase constitutively active PLCγ1 mutant (ΔSA), cells exhibit sensibility to Cetuximab similar to control cells (Figure 10). This result, in concordance with the lack of association between p-PKC and Cetx resistance (mentioned above), indicate that PLCγ1 could be involved in Cetx resistance by a mechanism independent of its lipase activity. Indeed, several studies support that PLCγ1 catalytic activity is not required for its proliferative mediated signals[69]. Fibroblasts lacking catalytic active PLCγ1 display normal proliferative responses to diverse growth factors[109]. EGF-induced mitogenesis of squamous cell carcinoma requires PLCγ1 but not its

catalytic activity[68]. In the same line of evidence, diverse reports show that PLCγ1 SH3 domain (absent in our ΔSA mutant) can promote cell growth and, therefore, have proliferative activity[68]. PLCγ1 SH3 domain also interacts with multiple proteins, including AKT[54], RAC1[70], dynamin-1[110] and PIKE[55] and this interactions regulate diverse cell processes, such as cell growth and migration.

In this study remains to be clarified the downstream effectors of PLCγ1 that are involved in therapy resistance. At present, we suspect that the mechanism involved in PLCγ1 mediated resistance to Cetuximab goes through direct activation of mammalian target of rapamycin (mTOR) and downstream S6-kinase. Markova and colleagues[111] found that PLCγ1 siRNA led to diminished activation of mTOR and S6 pathway, with consequent inhibition of cell proliferation. In other report, immunoprecipitation of mTOR from lysates of VEGF-treated HUVEC cells revealed a band of phosphorylated PLCγ1, suggesting that PLCγ1 and mTOR do exist in the same complex, allowing PLCγ1 mediated responses independently of its catalytic activity. Nevertheless, more experiments are needed in order to confirm this hypothesis.

Most mCRC cancer patients do not respond to EGFR target therapy[24,36]. Yet, the majority of patients who do achieve good tumor responses will eventually develop an acquired resistance to this therapies[24]. Therefore, another objective of this study was to investigate the possible involvement of PLCγ1 in the development of adaptive resistance to Cetuximab treatment. SW48 cell line was exposed to a fixed Cetuximab concentration for five months and the growth profile of resistant cells and PLCγ1 protein levels were further evaluated. Cell viability assays of resistant cells shows different growth profiles (Figure 11). This can be explained by the clonal diversity within the tumor cells and the selective pressure exerted by Cetuximab[112]. Furthermore, Cetx is a cytostatic and not a cytotoxic agent, that may permit viability of many cells in a senescent and less proliferative phenotype[22]. Nevertheless, treated cells show a different proliferative profile in comparison with parental control. Immunoblotting analysis of cell lysates obtained from resistant lines revealed an increase of PLCγ1 expression, especially in Cetx 2 and Cetx 3 culture lines (Figure 11). Thus, this result suggests an overactivation of the PLCγ1 signaling by action of Cetuximab selection. Activation of PLCγ1 was already accessed in cell lines resistant to PI3Kα inhibition[113]. Further work is still needed to confirm these preliminary results. Acquisition of more evident and consistent differences in PLCγ1 expression between parental and resistant lines might probably be achieved by increasing the Cetx exposure period.

Increased expression of PLCγ1 has been reported in tumors samples of different cancers, including CRC, when compared with normal tissue[81,82]. Overexpression of PLCγ1 was also associated

with increased risk for distant metastasis and faster tumor progression[87,91]. However, differences in PLCγ1 expression were never correlated with therapy response. Therefore, we decided to use patient samples to access the predictive value of PLCγ1 in the treatment of metastatic colorectal cancer. Immunohistochemical analysis of 25 primary CRC tumors shows a significant association of higher PLCγ1 expression with lower progression-free survival of patients under Cetx treatment and a trend towards a lower overall survival. Our analysis showed a predominant cytoplasmatic localization of PLCγ1 staining, however, PLCγ1 could also be found in the cell nucleus of some neoplasic cells. PLCγ1 nuclear localization has been described in highly proliferative cells[114,115]. PLCγ1 also translocate for the nucleus where activates PIKE and promotes growth factor-induced cell proliferation[55]. Nevertheless, we could not found a correlation between nuclear staining and PFS or OS. PLCγ1 staining was also present in different elements of neoplasic stroma, namely in endothelium, and also in lymphocytes. PLCγ1 signaling is known to be very important in angiogenesis downstream of VEGF signaling during arterial development[109]. Furthermore, PLCγ1 deficient mice have absence of erythrogenesis and vasculogenesis[63]. However, we could not found a correlation between staining of neoplsic stroma and PFS or OS.

Overall, our results reveal a potential new biomarker, easily detected by IHQ, which is a technique widely used in clinical practice, able to reliably identify people more likely to respond to Cetuximab therapy. Ultimately, our work also unravels the relevance of this PLC as a possible target of therapy, given that inhibiting PLCγ1 can have major consequences sensitizing tumor cells to Cetuximab therapy. Unfortunately, there are no specific PLCγ1 inhibitors available. The only commercially available inhibitor that has been routinely used as a general PLC inhibitor, named U73122, was recently identified as an inhibitor of calcium channels (downstream effector of PLCs) and not directly affecting PLCs activity[116]. In this context, future studies involving the development and test of new specific inhibitors for PLCγ1 are of great importance, forecasting important consequences for the health of patients.

6. Conclusion and Future Perspectives

Over the past decade, health care has been evolving from the traditional medicine towards the recognition that patients have distinctive inherent traits which cause variations in response to therapy. Nowadays, the great challenge of personalized medicine is precisely to be able to reliably identify biomarkers allowing accurate selection of patients to different therapies.

During the recent years, the treatment of metastatic colorectal cancer has made great advances thanks to the development of novel target therapies such as anti-EGFR medicines. Nevertheless, multiple drug resistance is common, and a large percentage of patients still do not benefit from these innovative treatments.

PLCγ1 enzyme is involved in tumorigenic signals downstream of receptor tyrosine kinases such as EGFR and VEGFR. This was the rationale behind our study, aiming at identifying the relevance of PLCγ1 for the resistance mechanism to anti-EGFR target therapies.

Overall, our results indicate, for the first time, a correlation between PLCγ1 protein expression levels and resistance to Cetuximab. To confirm the predictive value of PLCγ1 in Cetuximab sensitivity, we aim to generate a colorectal cancer cell line with inducible expression of PLCγ1 which will be xenografted at the back of nude mice. Responses of animals to Cetuximab treatment will be evaluated after expression and repression of PLCγ1 protein. Evidently, further studies are needed in order to consolidate our results, nevertheless, our findings are expected to have an enormous impact in the cancer field.

Besides the fact that our data has identified a new potential predictive biomarker of response to Cetuximab in the treatment of metastatic colorectal cancer, PLCγ1 could possibly be used as a biomarker of response in a more general way. Anti-EGFR targeted therapies (Cetuximab and Panitumumab) are not only used in metastatic colorectal cancer, but also, in the treatment of other metastatic malignancies, specifically HNSCC. In this context, being PLCγ1 constitutively expressed in several organs, it is natural to imagine that it can also be involved in resistance to EGFR-targeted therapies in other cancer diseases. Presumably, the same can also be true for other RTK-targeted therapies, especially anti-VEGFR, given that PLCγ1 is widely involved in angiogenic process[62,63]. Bevacizumab, a VEGFR-specific antibody, is approved as a first-line treatment for metastatic colorectal cancer and other metastatic diseases such as NSCLC. Therefore, it would be very interesting to study PLCγ1 contribution to Bevacizumab resistance both in metastatic colorectal cancer and other relevant cancers.

Additionally, of its important role as a biomarker of response, this scientific study has also unraveled PLCγ1 as a potential target of therapy, given that inhibiting PLCγ1 could have important consequences sensitizing tumor cells to EGFR-targeted therapy. Unfortunately, there is no available inhibitor specific to this PLC isoenzyme whereby we could not test this fact. In the near future, we also plan the possible development of a specific molecular inhibitor, in collaboration with the computational medical organic chemistry group (ORCHIDS) at Universidade do Porto. Specific inhibitor for PLCγ1 would, very likely, improve efficacy of the standard anti-EGFR therapy, being an outstanding tool, both in scientific research, and in the clinical practice, having certainly a great impact in the treatment of CRC metastatic disease.

Finally, we truly believe that this scientific research study may have important implication for the future wellbeing of patients, not only, allowing a better selection of patients more likely to respond to Cetuximab, avoiding unnecessary toxicity, but also, revealing novel therapeutic options.

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Supplementary Information

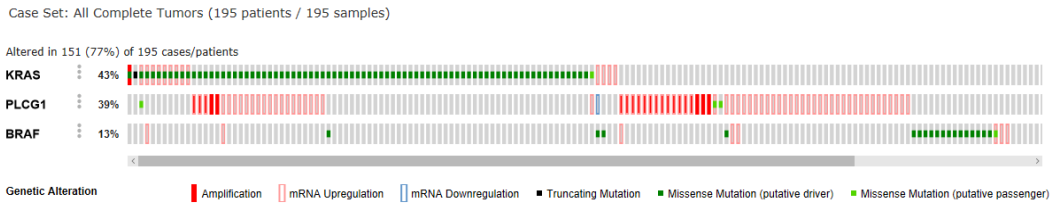

Figure S1: *KRAS*, *PLCG*1 and *BR*AF alteration in human CRC tumors. Analysis of TGGA data from cBioPortal.

Gene A	Gene B	p-Value	Log Odds Ratio	Association	
KRAS	BRAF	**0.006**	-1.305	Tendency towards mutual exclusivity	Significant
KRAS	PLCG1	**0.032**	-0.603	Tendency towards mutual exclusivity	Significant
PLCG1	BRAF	0.055	-0.857	Tendency towards mutual exclusivity	

Figure S2: Analysis of mutually exclusive alterations in *KRAS, PLCG1* and *BRAF* in human CRC tumors. Analysis of TGGA data from cBioPortal.