ESMO Translational Research Fellowship

(September 2015 – September 2016)

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FINAL REPORT

Host Institute: **German Trias i Pujol Health Sciences Institute and Hospital**
Mentor: **Rafael Rosell, MD. Director Cancer Biology and Precision Medicine Program**
Project title: **Therapeutic opportunities for squamous cell carcinoma of the lung: the Hedgehog pathway and beyond**

Home Institute: **Ospedale Santa Maria delle Croci, Oncology Department, Ravenna, Italy**

Introduction

Lung cancer is a major cause of cancer death, with a 5-year survival rate of 16%. Different histologies, genetic and epigenetic changes, and sites of origin define non-small cell lung cancer (NSCLC) subtypes accounting for ~80% of all lung cancers. Lung squamous cell carcinoma (LSCC) represents 30% of NSCLC diagnoses and is characterized by poor treatment response and prognosis [1,2] . Discovery of genomic alterations in kinase genes has changed the clinical history of patients with lung adenocarcinoma, but only a few therapeutically tractable genome alterations have been found in LSCC [3,4]. This disparity in lung adenocarcinoma has expanded interest in understanding alterations that drive LSCC and identifying novel therapeutic targets.

Rationale and Aim

Existence of stem-like cells in several cancer types responsible for tumour initiation, maintenance, relapse and metastases has been demonstrated by several studies [5,6]. These cells, resistant to common therapeutics, have capability to activate pathways such as Wnt, Hedgehog (HH) and Notch by aberrant expression of different genes [7].Primary LSCC tumours co-ordinately overexpress protein kinase C iota (PKCi), SOX2 and hedgehog acyltransferase (HHAT) and require PKCi-SOX2 –HHAT signalling to maintain a stem cell phenotype [8].

In other tumors (such as gastrointestinal tumors, prostate cancer, haematological malignancies and gliomas) this pathway is ligand-dependent for growth, survival or both [9].Patched homolog 1 (PTCH1) and GLI1 are HH target genes and their expression serve as a marker of HH pathway activation; furthermore NSCLC cells overexpressing GLI1 can overcome the growth-inhibitory effects of HH antagonist such as cyclopamine [10]. This can occur through crosstalk between the Hedgehog and EGFR signaling pathways and cooperation between Hedgehog and GLI—EGFR synergistically induce the expression of SOX2 [11]. Simultaneous stimulation of the HH—GLI pathway and EGFR signaling synergistically activates the pathways' cooperation response genes, thereby promoting malignant transformation. Recent genome-wide transcriptional profiling showed that atypical PKCι/λ (aPKCι/λ) and smoothened (SMO) control the expression of similar genes in tumor cells, as aPKCι/λ functions downstream of SMO to phosphorylate and activate GLI1, resulting in maximum transcription activation [12]. At the same time, PKCi is a critical lung

cancer gene that activates RAC1-PAC-MEK1/2-ERK1/2 signaling pathway required for transformed growth and for BIM down-regulation [11,12]. Furthermore, SMO-independent stimulation of GLI can occur through interactions with the MAPK and the PI3K/AKT pathway. Inhibition of HH pathways may be therapeutically beneficial in LSCC, but it is necessary first to discover drug-receptive targets within them.

The fibroblast growth factor receptor (FGFR1), amplified in about 20% of cases, is the most frequently altered tyrosine kinase family in LSCC [13]. Correlation between FGFR1 activation and tumor growth in lung cancer cell lines harboring FGFR1 amplification and in mice engrafted with FGFR1-amplified cells has been demonstrated. It signals downstream to four different pathways: mitogen-activated protein kinase (MAPK) extracellular signal–regulated kinase 1/2 (ERK1/2); phosphatidylinositol 3' -kinase-AKT (PI3K/AKT); signal transducer and activator of transcription (STAT); and phospholipase C [14]. Discoidin Domain Receptor 2 (DDR2) is a tyrosine kinase that binds collagen as its endogenous ligands and once activated, interacts with Src and Shc [14]. Mutations may alter kinase activity, ligand binding or DDR2 localization [15]. About 4% of LSCC harbor DDR2 mutations, cell lines exhibiting mutations are selectively killed by RNA interference or dasatinib [4]. Literature review indicates no published data related to the possibility that these alterations could be connected to the HH pathway.

Experimental design

Molecular studies on FFPE samples and cell lines

RNA was isolated from the cells through proprietary procedure (EU patent EP1945764-B1). Samples were briefly lysed in a trischloride, EDTA, sodium dodecyl sulphate (SDS) and proteinase K containing buffer. RNA was then extracted with phenol-chloroform-isoamyl alcohol, followed by precipitation with isopropanol in the presence of glycogen and sodium acetate. RNA was re-suspended in water and treated with DNAse to avoid contamination. cDNA was synthesized using M-MLV retrotranscriptase enzyme. Template cDNA was added to Taqman Universal Master Mix in a 12.5 µl reaction with specific primers and probe for each gene. The primer and probe sets were designed using Primer Express 3.0 Software according to their Ref Seq. Quantification of gene expression was performed using ABI Prism 7900HT Sequence Detection System. Quantification of gene expression was calculated according to the comparative Ct method. Final results were determined as follows: $2^{-(\Delta Ct\ sample-\Delta Ct\ calibrator)}$, where ΔCt values of the calibrator and sample are determined by subtracting the Ct value of the target gene from the value of the endogenous gene (β-actin). Commercial RNA controls were used as calibrators. In all quantitative experiments, samples were considered not evaluable when standard deviation of Ct values is >0.30 in two independent analyses. Thus, the number of evaluable samples were various among the several genes analysed.

Cellular studies

Squamous lung cancer cell lines (EBC1, H1703, SK-MES-1, HCC366, H2286) were maintained in RPMI medium supplemented with 10% fetal bovine serum (FBS), 50 µg/mL penicillin-streptomycin and 2 mM L-Glutamine. Cells were grown in a humidified atmosphere with 5% CO_2 at 37ºC. Cell viability was assessed by the Thiazolyl Blue Tetrazolium Bromide (MTT) (Sigma) assay. Cells from each cell line were seeded at 2000 to 6000 per well in 96-well plates and allowed to attach for 24 h. The concentration of drug required for 50% growth inhibition (IC50) after a 72h treatment was assessed. After treatment, cells were incubated with medium containing MTT (0.75 mg/mL in medium) for 1 h at 37ºC. Culture medium with MTT was removed and formazan crystals reabsorbed in 100 µL DMSO (Sigma). Cell viability was determined by measuring the absorbance at 590 nm, using a microplate reader (BioWhittaker). The interaction between drugs was determined by calculating the combination index according to method of isobologram-combination index (Chou-Talalay method). IC values of IC<0.7, 0.7<IC<0.85, 0.85<IC<1, 1 and >1 indicate strong synergism, moderate synergism, slight synergism, additive interaction and antagonism, respectively

Results, Conclusions and Future Perspectives

Considering more recent publications and according with my Mentor, Dr. Rafael Rosell, we decided to explore the potential impact of other signalling pathways that may be relevant in the evolution of squamous cell lung cancer:

- **PAK1:** Ong et al. found that PAK1 amplification is high in non-basal tumours and copy-number gain is well-correlated with PAK1 mRNA expression [16].Expression of PAK1 protein was also analysed on tissue microarrays of 27 SCLC and 97 NSCLC (30 adenocarcinomas and 67 squamous cell carcinomas) and 130 head and neck squamous cell carcinomas. 64% squamous NSCLC samples were positive for PAK1 expression, and 52% of all cases showed staining of moderate (2+) or strong (3+) intensity in the malignant cells. 61% of head and neck squamous cell carcinomas showed staining of moderate (2+) or strong (3+) intensity in the malignant cells [16]. Interestingly, EGF is found to stimulate PAK1 activity and PAK1 expression or activity is related to high EGFR in head and neck cancer cells [17]. The contribution of PAK1 to tumour cell growth and survival was further examined in a panel of lung cancer cell lines. In the EBC-1, NCI-H520, KNS-62, SK-Mes-1, and NCI-H441 squamous NSCLC cell lines that highly express PAK1, PAK1 blockade encompassed pronounced cytostatic effects in squamous NSCLC cells [16]. Furthermore, combined PAK1 knockdown with antagonists of inhibitor of apoptosis proteins (IAP) epidermal growth factor receptor (EGFR), MEK1/2, and Src family kinases displayed dramatically enhanced efficacy. None of these agents showed a profound single-agent effect on the growth and survival of EBC-1 cells in the absence of PAK1 knockdown. Therefore interfering with PAK1 signalling could have therapeutic efficacy in squamous lung cancer and PAK1 inhibition can greatly augment the efficacy of several classes of well-characterized molecularly targeted therapeutics [16].

- **EGFR**: Aberrant expression of EGFR is a common characteristic of NSCLC, especially in LSCC.

One approach is to inhibit the activation of EGFR with monoclonal antibodies (mAbs) that bind the extracellular domain of EGFR to block natural ligand binding. However, the majority of patients acquire resistance. Impaired EGFR internalization and degradation lead to increased EGFR surface level expression, increased EGFR kinase activity and dependence on EGFR induced signalling pathways [18].

- **AXL**: Cetuximab resistance has been related to AXL over-expression. EGFR directly regulates expression of AXL mRNA through MAPK signalling and the transcription factor c-Jun in cetuximab resistant cells, creating a positive feedback loop that maintains EGFR activation by AXL. In HNSCC patient-derived xenografts, AXL was over-expressed in tumour resistance to cetuximab.

- **Rac1 and Rac1b**: in our experiments Rac1 and specifically rac1b is over expressed in NSCLC especially in squamous NSCLC. Protein kinase Cι (PKCι) promotes NSCLC by binding to Par6α and activating a Rac1-Pak-Mek1,2-Erk1,2 signalling cascade [19].

- **SYK total, short and long**: SYK over-expression causes paclitaxel resistance [20]. This finding is rather intriguing since SYK could be involved in the TLR4 pathway. Secondly, up to now the co-activation of receptor tyrosine kinases (RTK) has been under appreciated and could affect the response to targeted therapies. For example, RTK activation patterns in lung carcinoma cell lines A549 and H1299 show phosphorylation of EGFR as well as AXL, MET and also EPHA2 in the A549 [21]. Therefore, crosstalk between different signalling pathways can be followed by a single EGFR blockade, including AXL, NOTCH and SMO signalling pathways (See Figure 3 in Rosell, Bivona, Karachaliou, Lancet, 2013) [11].

- **YAP:** YAP1 has been recognized as a marker of resistance to cetuximab in colorectal cancer patients (18) and also the activation of YAP1 leads to EGFR over-expression [22].

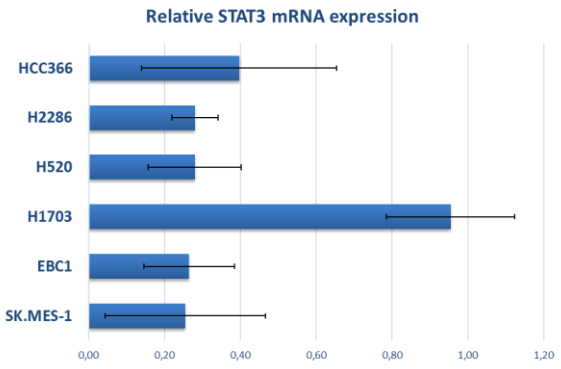
- **GLI2:** The strong and unique expression pattern of GLI2 prompted the query whether it is necessary for LSCC tumour progression [23].

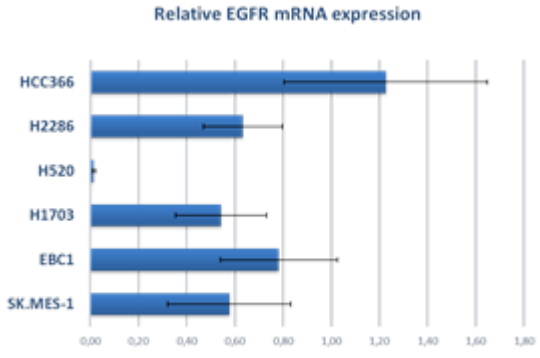
Here the list of primers designed for the project. For Rac1 and Rac1b after several attempts we decided to use an assay.

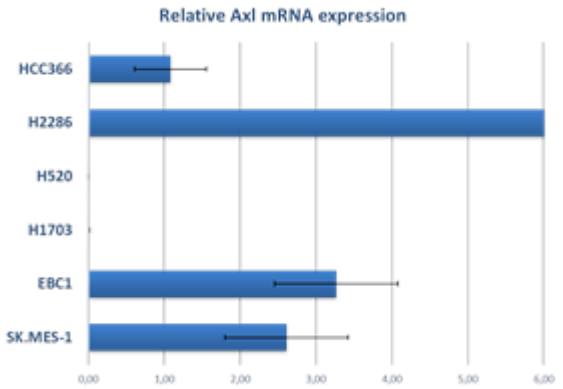
PRIMERS						
Gene	Exons	Intron	Sequence Lenght	Forward	Complementary Reverse	Probe
PKCi	5 and 6	2.420 pb	68pb	5'CTTTCCAAGCCAAGCGTTTC 3'	5'AAGTCCCCATATTCGGTCTGTG3'	5'ACAGGCGTGCTCACT 3'
BIM	2 and 3	25940 pb	75 pb	5'TCCTTGCCAGGCCTTCAA 3'	5'GGGCGCATATCTGCAGGTT 3'	5'AATGGCTTCCATGAGGCA 3'
FGFR1	5 and 6	1675 pb	77pb	5'CCACACTGCGCTGGTTGA 3'	5'GGCTACAAGGTCCGTTATGCC 3'	5'AACCTGACCACAGAATT 3'
HHAT	10 and 11	35426 pb	65pb	5'GTGGAGACTCCCTGCATCCA 3'	5'AATCGACGGCGAGCTTGT 3'	5'ACAGTCTGGCCCGATAC 3'
DISP1	2 and 3	39747 pb	69 pb	5'CATTTTCAGCATCAGCCTGTG 3'	5'TGGCAACTTGAAAGGTCTGGA 3'	5'CATAGCCAACATAAGACC 3'
SOX2	1 exon	no intron	60pb	5' ATGTCCTACTCGCAGCAGGG 3'	5' GACCACCGAACCCATGGAG 3'	5' FAM CTGGCATGGCTCTT 3'
GLI1	3 and 4	581 pb	70 pb	5'GGCCCCTCCCCAGTCAG 3'	5'CTTCTCCCCGGAGTGCAGT 3'	5'ACAGAAGGCCCACTCT 3'
Rac1	Using an assay					
Rac1b						
Axl	4 and 5	8910 pb	65pb	5'CAGCGCAGCCTGCATGT 3'	5'GCGTTATGGGCTTCGCAG 3'	5'CAGGGCTGAACAAGAC 3'
YAP	1 and 2	48061pb	72 pb	5'TTGGGAGATGGCAAAGACATC3'	5'TCGATCAGACAACAACATGGC 3'	5' TCAGAGATACTTCTTAAATCACA3'
SYK short	6 and 8	2033 pb and 7004 pb	60 pb	5'ATCCTGCGTCCTCCCCTG 3'	5'TGGCTCATACGGATTGAATGAC 3'	5' CAAGGGAACCGGCAAG 3'
SYK long	6 and 7	2033 pb	79 pb	5'AACTTCCAGGTTCCCATCCTG 3'	5' GGCTTTGGGAAGGAGTATGATTT 3'	5'GACTTGGTCAGCGGGT 3'
SYK total	3 and 4	16611 pb	69 pb	5'TGTCCTGATAGGATCAAAGACAAATG 3'	5'GCGTAGGAGCCGTTGTTGTC 3'	5'AAGTTCCTGATCCGAGCCA 3'
PAK1	2 and 3	12336 pb	67 pb	5' GGACCGATTTTACCGATCCA 3'	5'TGGCCGCTCTTTCTCTTTCTT 3'	5' TTACCTGGAGATAAAACA 3'
EGFR	1 and 2		60 pb	5' GAGTCGGGCTCTGGAGGAA 3'	5'AACTGCGTGAGCTTGTTACTCG 3'	5'GAAAGTTTGCCAAGGCA 3'
GLI2	2 and 3	129892 pb	80 pb	5' CCTCTCCTTTGGTGGTGG 3'	5' GAATGGTGGCAAGAGATGCTG 3'	5' TGCCCAAGGAGTGC 3'

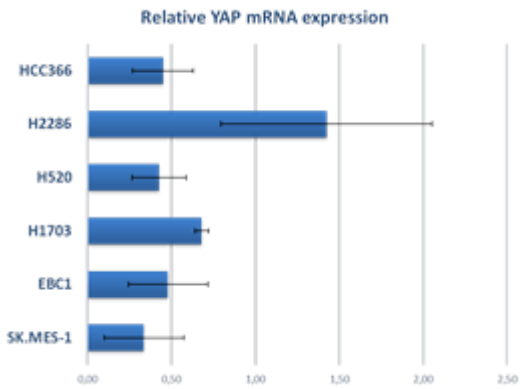
All the primers were validated on validated on paraffined samples to be sure they can detect the expression of the genes on tumour samples from patients.

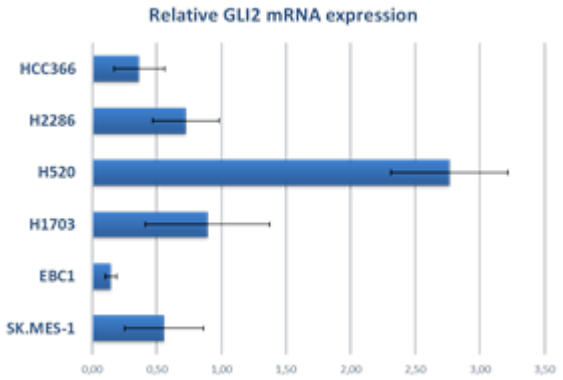
Following the basal expression of several genes involved in my project. The experiments were conducted three times in different samples of mRNA collected from "fresh" cell lines:

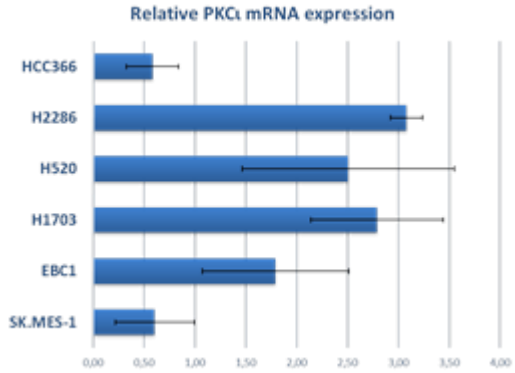


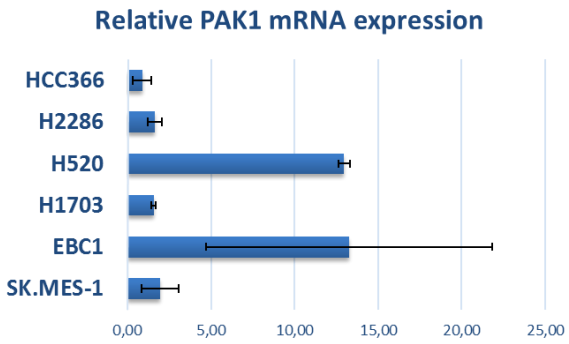


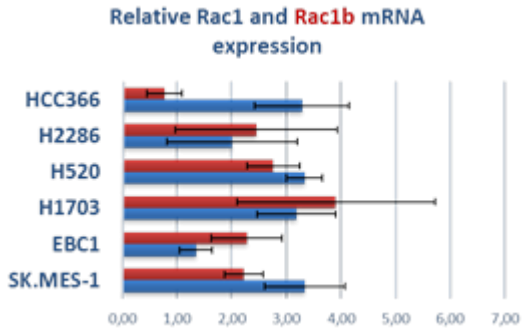

Relative Axl mRNA expression
HCC366
H2286
H520
H1703
EBC1
SK.MES-1
0,00
1,00
2,00
3,00
4,00
5,00
6,00

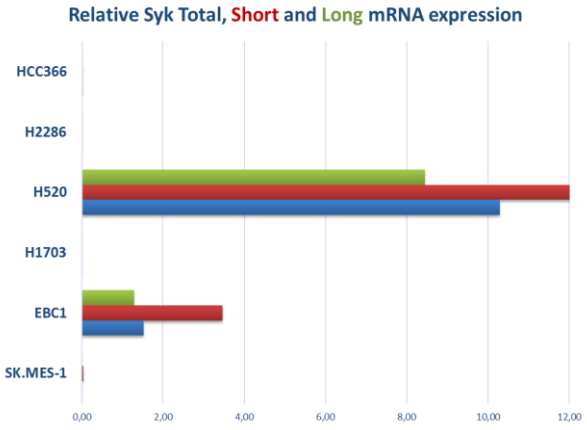

Relative YAP mRNA expression
HCC366
H2286
H520
H1703
EBC1
SK.MES-1
0,00
0,50
1,00
1,50
2,00
2,50


Relative GLI2 mRNA expression
HCC366
H2286
H520
H1703
EBC1
SK.MES-1
0,00
0,50
1,00
1,50
2,00
2,50
3,00
3,50


Relative PKCι mRNA expression
HCC366
H2286
H520
H1703
EBC1
SK.MES-1
0,00
0,50
1,00
1,50
2,00
2,50
3,00
3,50
4,00


Relative PAK1 mRNA expression
HCC366
H2286
H520
H1703
EBC1
SK.MES-1
0,00
5,00
10,00
15,00
20,00
25,00


Relative Rac1 and Rac1b mRNA expression
HCC366
H2286
H520
H1703
EBC1
SK.MES-1
0,00
1,00
2,00
3,00
4,00
5,00
6,00
7,00

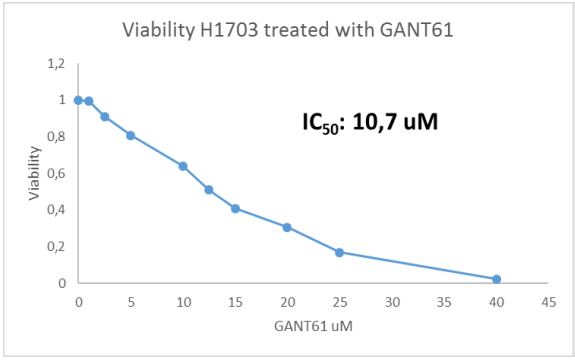

Relative Syk Total, Short and Long mRNA expression
HCC366
H2286
H520
H1703
EBC1
SK.MES-1
0,00
2,00
4,00
6,00
8,00
10,00
12,00

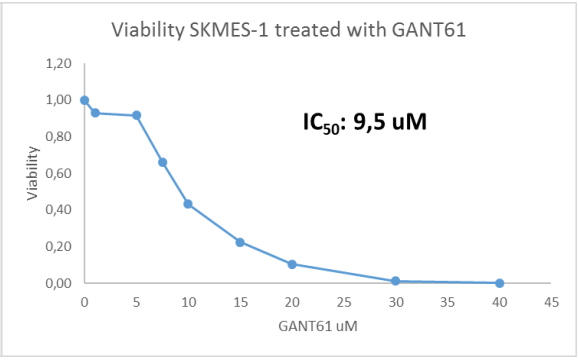
Squamous NSCLC cell lines classification according to their mRNA profile (our data):

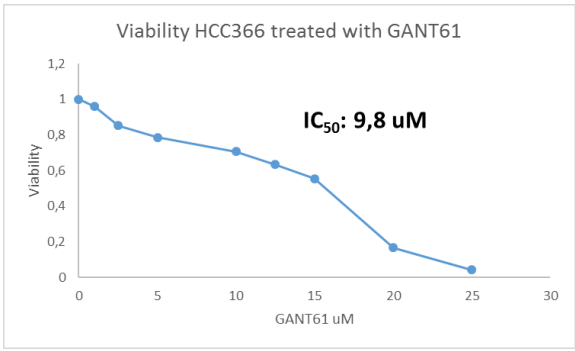
Squamous NSCLC cell lines	Signaling pathways*
SK MES-1, HCC366	**EGFR**
H1703	**STAT3/YAP1/Src**
H520	**Sonic Hedgehog** (GLI1/2, HHAT, DISP1, PRKCI)
H520, H2286	**PAK1/PRKCI/Rac1**
H2286, EBC1	**Other RTKs** (AXL, Gas6, Shpk1)

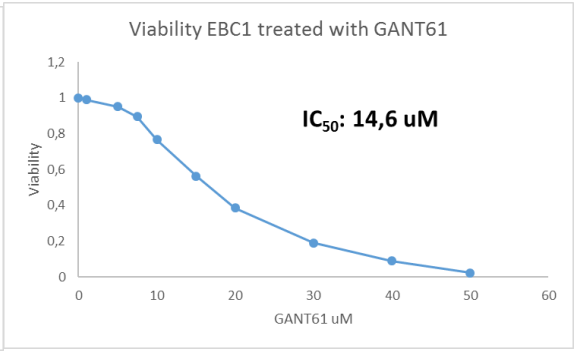
** qRT-PCR was used for the mRNA expression of EGFR, STAT3, YAP1, Src, GLI1, GLI2, HHAT, DISP1, PRKCI, PAK1, Rac1, Rac1b, AXL, Gas6 and Shpk1. The cell lines were classified as high or low expressors for each gene according to the median relative expression*

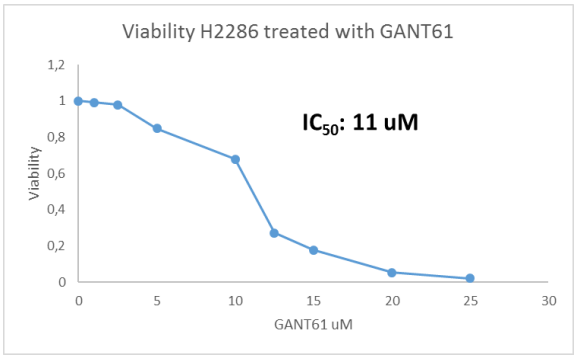
GANT61 and Mebendazole are well known inhibitor of the Sonic Hedgehog pathways, in particular they the inhibit the activity of GLI1 and GLI2 [24,25]. Here the results of the viability of some cell lines treated with the two drugs:

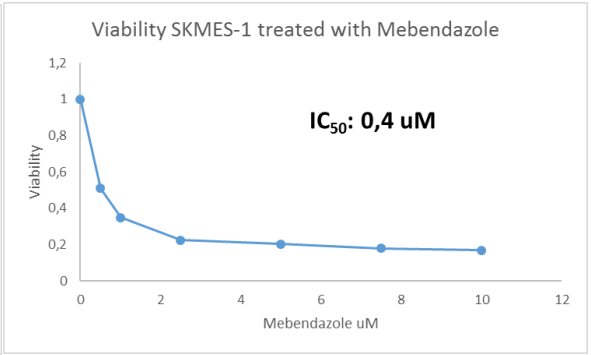










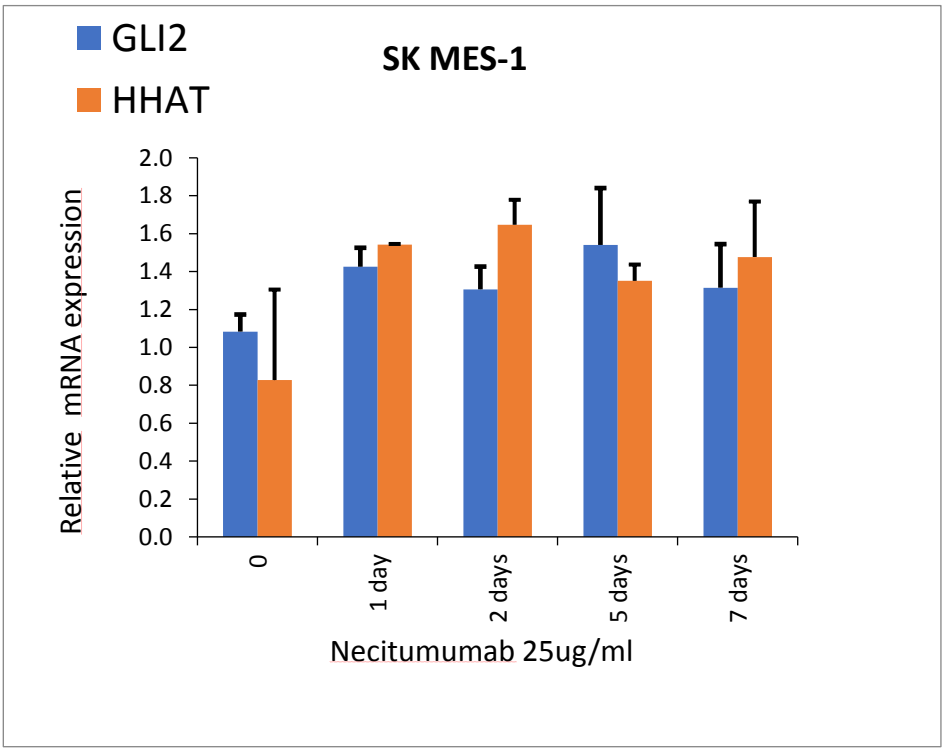


The H520 cell line, previously reported to be sensitive to GANT61 with a IC_{50} 5 uM/L [23], in our hands resulted to be resistant to that drug with a IC_{50} 18,8 uM/L (data not shown). We repeated the experiment 3 times with 3 different cell cultures with overlapping results.

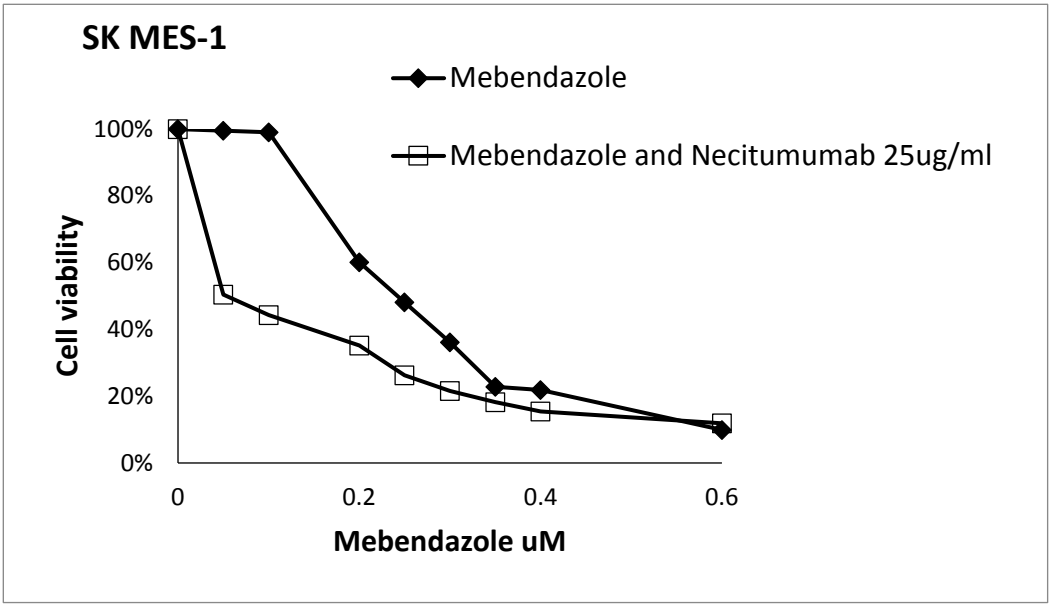
Aberrant expression of EGFR is a common characteristic of NSCLC, especially in LSCC. One approach is to inhibit the activation of EGFR with monoclonal antibodies (mAbs) that bind the extracellular domain of

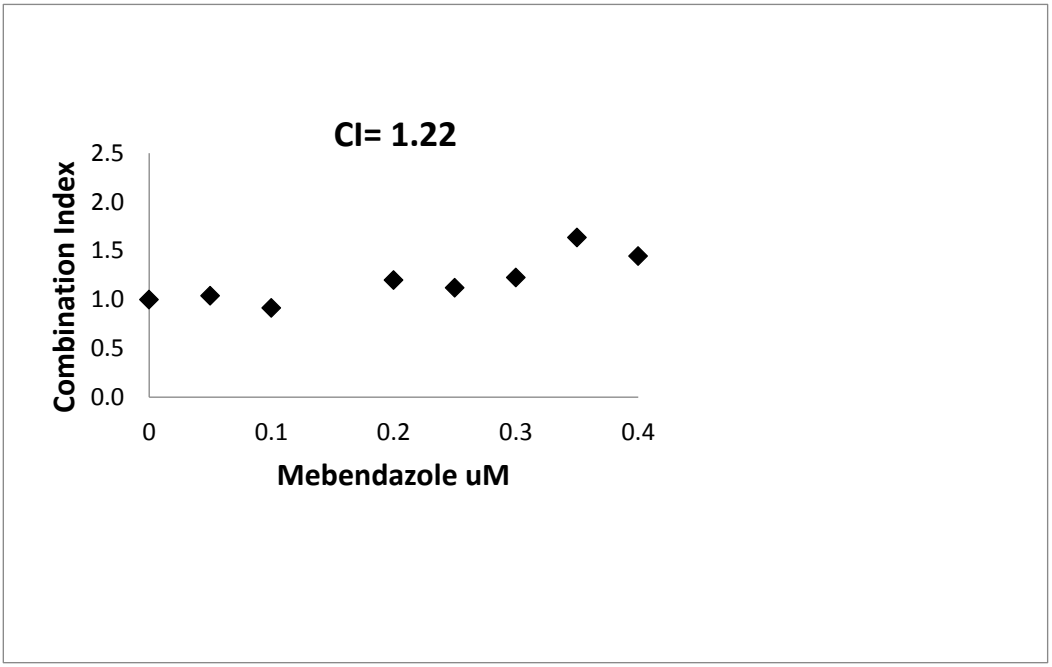
EGFR to block natural ligand binding. Necitumumab, a second generation, recombinant human EGFR mAb has demonstrated better survival in a phase III study (Squamous NSCLC treatment with the Inhibitor of EGF Receptor (SQUIRE) in LSCC, treated with gemcitabine/cisplatin/necitumumab versus gemcitabine/cisplatin (median survival 11.5 vs 9.9 months; HR= 0.84, p=0.01) [26]. However, the majority of patients acquire resistance. A model of Hedgehog EGFR cooperation has been shown in basal cell carcinoma and pancreatic cancer cells [27]. Not surprisingly, hedgehog signalling alters reliance on EGF receptor signalling and mediates resistance to anti-EGFR therapy in HNSCC [28]. A subgroup of LSCC over-expresses hedgehog GLI signalling components, specifically GLI2. GANT61 blocks GLI1 and GLI2 [29]. GANT61 has demonstrated activity in xenograft models of GLI positive cell lines, such as the NCI-H520 (a cell line available in our lab), and NCI-H226, however it has no activity in NCI-H2170 and does not express GLI [23]. According to the above we decided to test a multiple therapeutic approach on LSCC cell lines using Necitumumab plus a HH inhibitors to avoid possible mechanisms of resistance and maybe obtaining a synergistic effect.

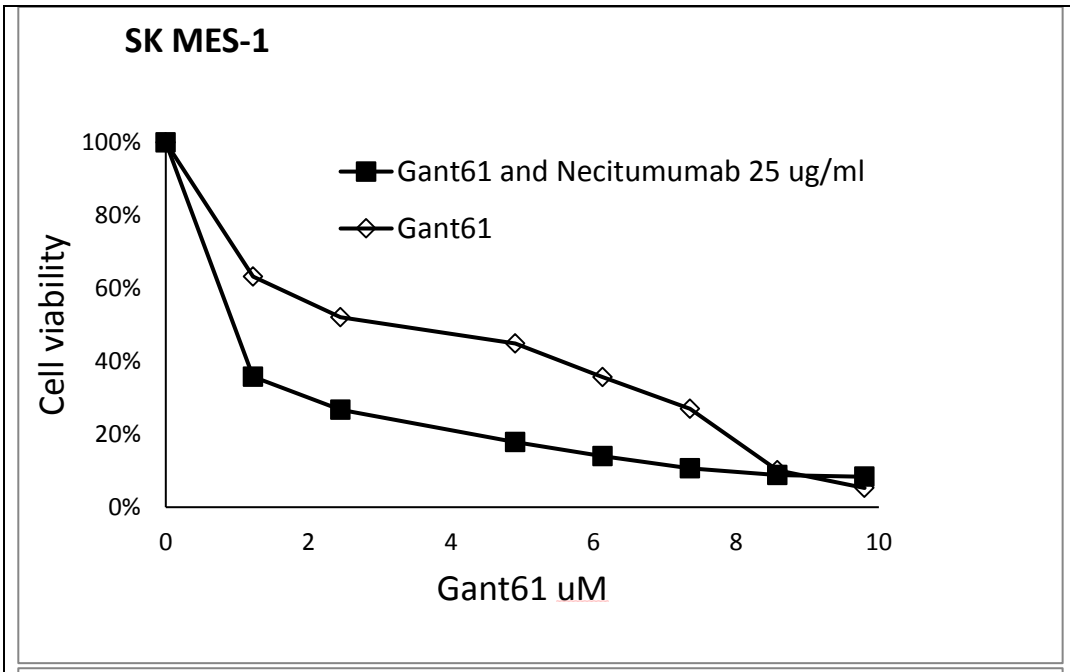
Here the relative mRNA expression of GLI2 and HHAT after exposure to necitumumab in the SK MES-1 cell line:

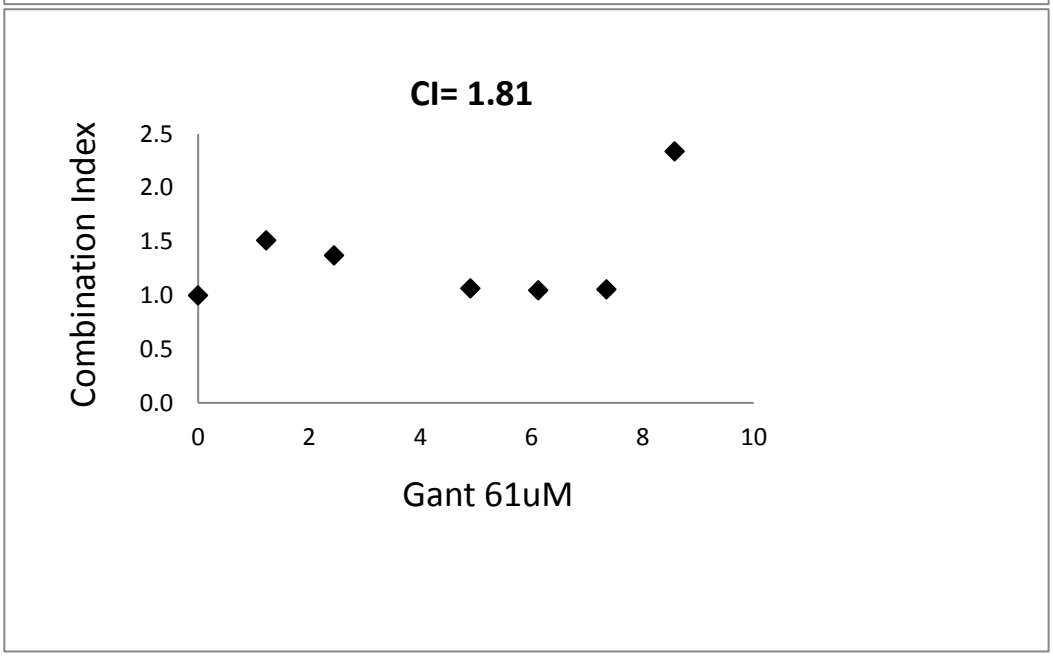


Considering the results above I tested the combination necitumumab + GANT61 and Mebendazole in the SM MES-1 cell line:









The next step should have been profiling samples from patient with advanced squamous lung cell carcinoma. Unfortunately, almost 50 samples collected, stored in paraffin, were between 4 and 6 years old and the mRNA extraction resulted in poor quality material to carry on the pre-planned analysis. We are still working to collect new samples with a full clinical history profile.

Conclusion:

The activation of the HH pathways was found in about 36% of LSCC samples: in particular in a population with a heaviest smoking history [23]. Despite the attempt to abrogate the HH signal pathway the results let think that the Sonic Hedgehog pathways is not a driving mutation in the development of squamous cell lung carcinoma. Furthermore, we tested the possibility that the sonic hedgehog pathways could be implicated as mechanism of resistance to the anti EGFR therapy in squamous cell carcinoma of the lung. The results demonstrated no synergism with the abrogation of both signals. LSCC is not a unique entity, nor single EGFR nor HH inhibition will not be adequate for LSCC and new biomarker-driven synthetic lethal approaches should tailor the treatment of this disease.

Preclinical studies provide insights to therapy mechanisms of resistance that are not feasible with clinical studies, providing a perspective for new approaches to more and better evidence based treatments [30].

***List of Publications and Presentations Resulting from the Translational Research Project* "Therapeutic opportunities for squamous cell carcinoma of the lung: the Hedgehog pathway and beyond"**

Poster Presentation at ELCC 2016:

Differential expression profile of lung squamous cell carcinoma (LSCC) cell lines as a mean to predict drug interaction effects Verlicchi, A. et al. Journal of Thoracic Oncology, Volume 11, Issue 4, S85 - S86

STAT3 and Src-YAP1 Inhibition Results in Greater Necitumumab Sensitivity in Lung Squamous Cell Carcinoma Verlicchi, Alberto et al. Journal of Thoracic Oncology, Volume 12, Issue 1 , S114

Poster Presentation at WCLC 2016

STAT3 and Src-YAP1 Inhibition Results in Greater Necitumumab Sensitivity in Lung Squamous Cell Carcinoma Verlicchi, Alberto et al. Journal of Thoracic Oncology, Volume 12, Issue 1, S1143

Oral Presentation at "First cancer cell signalling pathways meeting" (Institut Germans Trias i Pujol (IGTP), Conference Meeting Room, Badalona, Barcelona, Spain - June 15th 2016): An in depth look at trends in poly-targeted lung cancer therapy": Squamous cell carcinoma of the lung: Combinatorial targeted therapies based on anti-EGFR monoclonal antibodies. · Aberrations on the EGFR pathway in squamous cell carcinoma cell lines Co-targeting YAP/STAT3, PAK1, AXL, and Hedgehog · In vitro findings in squamous cell carcinoma cell lines

Molecular Bases for Combinatorial Treatment Strategies in Patients with KRAS Mutant Lung Adenocarcinoma and Squamous Cell Lung Carcinoma. Lazzari, C., Verlicchi, A., Gkountakos, A. et al. Pulm Ther (2016) 2: 1. https://doi.org/10.1007/s41030-016-0013-3

List of Publications and Presentations resulting from other projects during the fellowship period (if applicable)

Poster Presentation as co-author at ELCC 2016

Molecular bases for combinatorial treatment strategies in KRAS mutant (KRASm) lung adenocarcinoma (LAC) Lazzari, C. et al. Journal of Thoracic Oncology, Volume 11, Issue 4, S82

Poster Presentation as co-author at ESMO 2016

Biomarker driven combinations for synthetic lethal approaches in KRAS mutant (KRASm) lung adenocarcinoma (LAC). Lazzari C et al. Annals of Oncology, Volume 27, Issue suppl_6, 1 October 2016, 1526P

Publications:

Co-activation of STAT3 and YES-Associated Protein 1 (YAP1) Pathway in EGFR-Mutant NSCLC. Chaib I et al. J Natl Cancer Inst. 2017 Sep 1;109(9).

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