Report for Pathological Society Small Grant Award

Title: The role of Focal Adhesion Kinase (FAK) in response to anti-EGFR therapy in KRAS wild-type colorectal cancers

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Background:
KRAS mutation status in colorectal cancer (CRC) is a negative predictive marker for response to anti-EGFR therapy. However, response rates in wild-type KRAS CRC patients are still low, indicating that other factors, next to KRAS mutation status, influence the response to EGFR inhibitors. We recently showed that wild-type KRAS CRCs present heterogeneity at the DNA copy number level and that copy number gain of FAK, mapping at 8q24.3, leading to its overexpression is likely to affect the response to anti-EGFR therapy (unpublished results).
Focal adhesion kinase (FAK), also known as Protein tyrosine kinase 2 (PTK2), is a cytoplasmic tyrosine kinase identified as a key mediator of signalling by integrins.
FAK is activated by integrins through disruption of an auto-inhibitory intra-molecular interaction between its kinase domain and the amino terminal FERM domain. The activated FAK forms a binary complex with Src family kinases, which phosphorylate additional sites on FAK leading to its full activation. The complex can phosphorylate other substrates and trigger multiple signalling intracellular pathways to regulate various cellular functions. The RAS/RAF/MAPK signalling pathway is one of the pathways that can be triggered by FAK. We hypothesized that wild-type KRAS CRC cells, where FAK is amplified and overexpressed, would be resistant to treatment with EGFR inhibitors, as the downstream RAS/RAF/MAPK pathway would be activated by FAK.

Original Aims (copied from original application):
To functionally test the role of FAK in CRC cell lines with wild-type KRAS.
The plan of investigation included:
1. To select two KRAS (and BRAF) wild-type CRC cell lines, one of which with amplification and overexpression of FAK (FAK+) and the other without amplification and overexpression of FAK (FAK-).
2. To treat the two selected colorectal cancer cell lines (FAK+ and FAK-), both KRAS (and BRAF) wild-type, with Cetuximab (an anti-EGFR antibody) and evaluate the response to the treatment.
3. To inhibit FAK expression in FAK+ cells by shRNA treatment and evaluate if cells become sensitive to the Cetuximab treatment.
4. To analyse the phosphorylation status of FAK and other proteins in the downstream pathway in FAK+ cells and shRNA-treated FAK+ cells by phosphoproteomics to elucidate the signalling pathway of FAK.

Results:
Results obtained in Activity 1:
In this part we aimed to select two CRC cell lines, both wild-type for genes in the EGFR pathway, in which one would have amplification and overexpression of FAK (FAK+) and the other one would have neither amplification nor overexpression of FAK (FAK-). To reach this aim we performed mutation analysis, by high resolution melting (HRM) analysis, for KRAS, BRAF, EGFR and PIK3CA on 13 CRC cell lines available in our lab. From this analysis, three cell lines, namely Caco2, Colo320 and KM12, showed to be wild-type for all the four genes analysed (Table 1).
Next, we evaluated the copy number status of FAK in these cell lines, by looking at the available array CGH profiles (run on Agilent 180k or 244k oligonucleotide platform).

Moreover, expression values of FAK in each cell line were determined by qRT-PCR. Caco2 showed neither amplification nor overexpression of FAK. On the other hand, Colo320 and KM12 both show amplification of FAK, although only Colo320 shows clear overexpression (Figure1). We decided to proceed with the Colo320 (FAK+) and Caco2 (FAK-).

Results obtained in Activity 2:
In this part of the work we aimed to evaluate the sensitivity of the selected cell lines to EGFR inhibitors. As inhibitor we first used cetuximab, an antibody directed against EGFR. However, in vitro analysis with this antibody did not work, as extreme high concentrations of cetuximab are necessary to inhibit the KRAS pathway, which is toxic for the cells (results not shown). We decided to treat the cells with either gefitinib or erlotinib (small molecule kinase inhibitors targeting EGFR). As depicted in Figure 2, the cell line Colo320 with FAK amplification did not show less sensitivity to both drugs as hypothesized when compared to the cell line without amplification (IC50 = 5-10 µM in Colo320 compared to approximately 20 µM in Caco2).

Results obtained in Activity 3:
Although we did not observe more resistance to the drugs in Colo320 cell line (FAK+) compared to caco2 (FAK-), we proceeded to knockdown the FAK gene, by shRNA targeting FAK, in Colo320 (amplification and overexpression). If FAK plays a role in response to anti-EGFR therapy, then knockdown (KD) of the gene would have an effect in sensitivity, when compared to the WT cells. We did not observe a change in sensitivity when comparing Colo320 KD versus WT (Figure 3).

Results obtained in Activity 4:
Because of the negative results obtained in the previous tasks, we did not proceed with phosphoproteomics analysis to evaluate the phosphorylation status of FAK and other proteins in the downstream pathway of FAK.

Conclusions:
No clear role for FAK amplification and overexpression in the resistance to anti-EGFR therapy could be demonstrated.

How Closely Have the Original Aims been Met:
Task 1: Selection of the cell lines, based on their mutation, copy number and expression status was successfully achieved.
Task 2: Treatment of the cell lines with two different EGFR inhibitors was successful.
Task 3: Knockdown of FAK in the cell line with amplification and overexpression of FAK, followed by drug treatment, was successful.
Task 4: Not done, as results obtained in the previous tasks were not confirming the hypothesis that FAK amplification and overexpression would play a role in resistance to anti-EGFR therapy.

Table 1. HRM mutation analysis results for KRAS, BRAF, EGFR and PIK3CA in 13 CRC cell lines.

	KRAS	BRAF	EGFR	PIK3CA
CaCO2	WT	WT	WT	WT
Colo205	WT	V600E	WT	WT
Colo320	WT	WT	WT	WT
HCT116	codon 13: GGC=>GAC	WT	WT	codon 1047: CAT=>CGT
HCT15	codon 13: GGC=>GAC	WT	WT	codon 545: GAG=>AAG
HT29	WT	V600E	WT	WT
LS513	codon 12:GGT=>GAT	WT	codon 836: CGC=>CG	WT
RKO	WT	V600E	WT	codon 1047: CAT=>CGT
SW480	codon 12:GGT=>GTT	WT	WT	WT
SW48	WT	WT	codon 719: GGC=>GA	WT
LS174T	codon 12:GGT=>GAT	WT	WT	codon 1047: CAT=>CGT
SW1398	WT	V600E	WT	WT
KM12	WT	WT	WT	WT

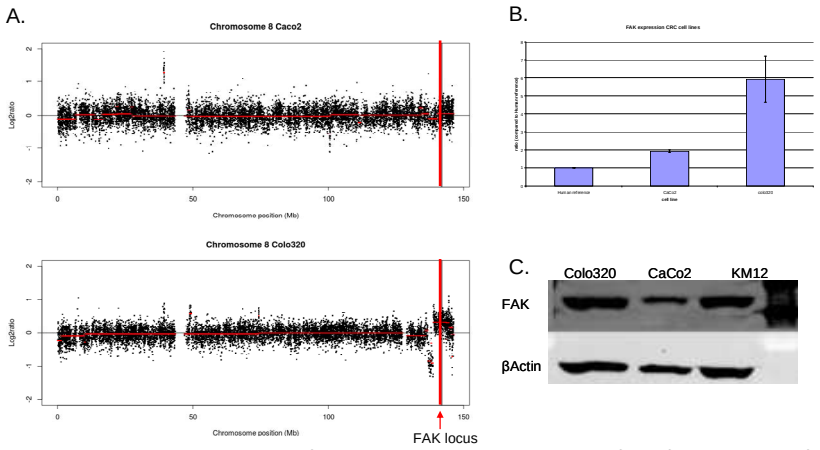

Figure 1. A: Chromosome 8 copy number profile (Agilent 244k oligonucleotide platform, for array CGH) of Caco2 and Colo320 colorectal cancer cell lines. Vertical red line depicts the location of FAK gene at 142 Mb. Copy number gain of this locus is observed in Colo320 but not in Caco2. B: mRNA expression levels of FAK, determined by RT-PCR in Colo320 and Caco2 in relation to the human reference (Strategene). C: Protein expression levels of FAK, determined by Western blotting in Colo320 and Caco1. β-actin used as loading control.

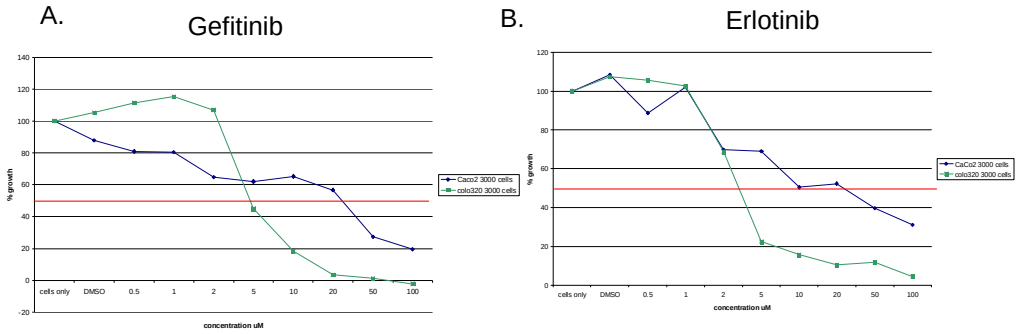

Figure 2. Growth curves of Colo320 and Caco2 under the different concentrations of Gefitinib (A) and Erlotinib (B). The half maximal inhibitory concentration (IC50; horizontal red line) of Gefitinib is 5 uM for Colo320 (blue line) and >20 uM for Caco2 (green line). The IC50 of Erlotinib is <5 uM for Colo320 (blue line) and 10 uM for Caco2 (green line).

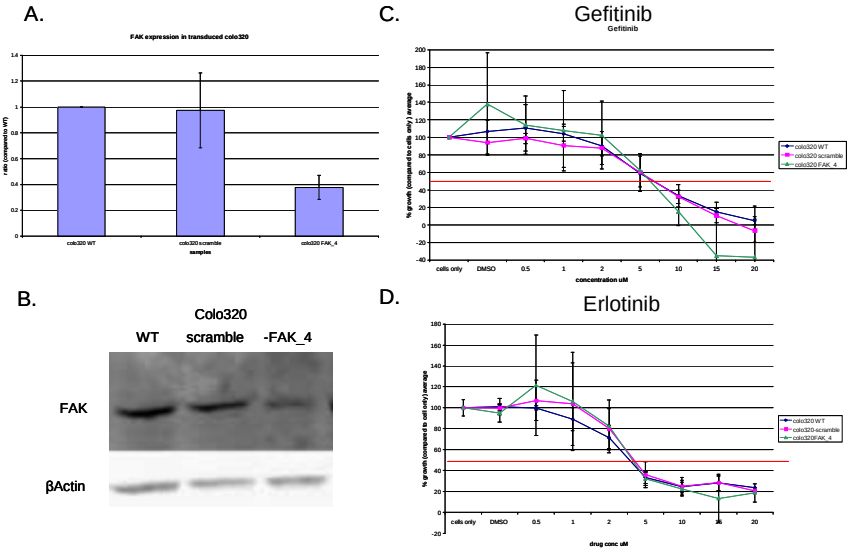

Figure 3. Knockdown by shRNA of FAK expression in Colo320 cell line. A: mRNA expression levels of FAK, determined by RT-PCR. Knockdown efficiency of 60% relative to the WT cells. B: Protein expression levels of FAK, determined by WB. A clear reduction of FAK expression is observed in the Knckdown cell line. C and D: Growth curves of Colo320-WT (blue line), Colo320-scramble control (pink line) and Colo320-shFAK (green line) under different concentrations of Gefitinib (C) and Erlotinib (D). No differences in IC50 are observed.