ERK and p38MAPK combine to improve survival in patients with BRAF mutant colorectal cancer

Running Title: MAPK pathway in colorectal cancer patients

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ABSTRACT

Background: In colorectal cancer (CRC), BRAF mutations influence tumour progression. In mismatch repair deficient (dMMR) tumours, BRAF mutations are associated with a good prognosis whereas in MMR competent tumours they are detrimental. The differential expression of the downstream MAPK pathway members, which are constitutively activated in BRAF mutant patients, may account for these differences.

Methods: Phosphorylation of ERK, p38MAPK and JNK was assessed by immunohistochemistry utilising CRC tissue microarrays. A discovery cohort (n=187) and a validation cohort (n=801) were analysed for associations with BRAF mutations, clinicopathological characteristics and cancer-specific survival (CSS).

Results: In 801 CRC patients, nuclear ERK phosphorylation (HR 0.65 95% CI 0.48-0.88, p=0.004) and the combined nuclear pERK/p-p38 score (HR 0.61 95% CI 0.45-0.82, p=0.001) independently associated with CSS and were further associated with increased BRAF mutations (p=0.003 and p=0.002). When stratified for BRAF status, only MMR competent patients harbouring the mutation and a strong combined nuclear pERK/p-p38 score (HR 0.49 95% CI 0.27-0.89, p=0.016) demonstrated improved CSS. This improvement in CSS was specific to stage III CRC (HR 0.25 95% CI 0.10-0.64, p=0.002).

Conclusions: MMR competent stage III tumours harbouring BRAF mutations have an improved prognosis when strong nuclear phosphorylation of both ERK and p38MAPK is present.

Keyword: ERK, p38MAPK, BRAF mutations, colorectal cancer, and prognosis.

INTRODUCTION

Colorectal cancer (CRC) is the second most common cause of cancer death in Europe[1]. Although survival has improved, this is predominantly a result of better surgical technique and adjuvant/neo-adjuvant therapies. Despite this, 5- year survival remains poor at 60% across all stages of disease[2]. The present TNM-based staging of CRC is suboptimal, given the heterogeneity in survival among patients across the same stage of disease. There is an obvious clinical need to identify characteristics pertaining to both the tumour and the host, which may not only guide prognosis but also novel adjuvant therapies.

BRAF V600E mutations are currently being investigated as a predictive biomarker for selecting patients for EGFR inhibitor treatment[3]. BRAF mutations exhibit different associations with prognosis dependent on the subset of CRC patients. In sporadic CRC, patients with mismatch repair (MMR) deficient (dMMR) tumours harbouring BRAF mutations have an improved survival. Conversely, in MMR competent (cMMR) patients, BRAF mutations convey a poor prognosis and their prognosis declines further in BRAF mutant metastatic disease[4]. Furthermore, dMMR/BRAF mutant patients have a 5-year survival rate of 65% compared to 46% for cMMR/BRAF mutant patients. The reason for this difference in survival for BRAF mutant patients is still unclear, but may lie in the expression of downstream targets such as extracellular regulated kinase (ERK).

ERK is part of the mitogen-activated protein kinase (MAPK) family along with two other members, p38MAPK and c-Jun-regulated kinase (JNK). All MAPKs are serine-threonine kinase activated by dual phosphorylation. The effects of MAPKs on patient survival in various cancers are varied[5-8], with some studies showing a survival advantage and some a detrimental survival effect. Most studies look at the three members in isolation, overlooking potential cross talk between the pathways, which may explain the different associations with

survival. Therefore, the aim of the present study was to assess the effect of phosphorylation of ERK, p38MAPK and JNK alone and in combination on cancer-specific survival in a discovery and validation cohort of CRC patients. The study also assessed associations between the MAPK pathway, BRAF mutations, MMR status, and clinicopathological factors.

METHODS

Patients

Discovery cohort patients (n=272) were identified from retrospectively retrieved routine CRC resections performed within Glasgow Royal Infirmary between 1997 and 2007. This cohort was extended to the validation cohort (n=1030) with the addition of retrospectively identified CRC resections performed in the Western Infirmary and Stobhill Hospital, Glasgow in the same time period. Patients who had undergone a potentially curative resection for stage I-III CRC and were included within previously constructed tissue microarrays (TMAs) were studied. Resections were considered curative based on pre-operative computed tomography and intra-operative findings. Patients who had died within 30 days of surgery were excluded. Ethical approval was obtained from the West of Scotland Research Ethics Committee.

Clinicopathological characteristics

Tumours were staged using the fifth edition of the AJCC/UICC-TNM staging system[9]. Tumour differentiation was graded in accordance with Royal College of Pathologists[10]. The presence of venous invasion was assessed using Elastica staining. Differentiation, margin involvement, peritoneal involvement, and necrosis were taken from pathology reports issued following resection. Data on Ki67 was already available for both cohorts using a threshold of 50%. MMR status was assessed as previously described[11]. Patients were followed up for at least five years and date and cause of death were crosschecked with electronic case records. Cancer-specific survival (CSS) was measured from date of surgery until date of death from CRC.

Assessment of inflammatory responses

Stromal infiltration was assessed using tumour stroma percentage (TSP) as previously described[12]. The local inflammatory cell infiltrate was assessed using the Klintrup-Makinen (KM) grade[13]. The Glasgow microenvironment score (GMS) was calculated as previously described[14]. Tumor-infiltrating lymphocyte (TIL) counts were established from pathology reports issued following resection. For systemic inflammation, serum C-reactive protein (CRP) and albumin were recorded prospectively and measured within 30 days prior to surgery. The pre-operative systemic inflammatory response was defined using the modified Glasgow prognostic score (mGPS)[15].

Immunohistochemistry

BRAF V600E, phosphorylated ERK1/2 (pERK), phosphorylated p38MAPK (p-p38) and phosphorylated JNK (pJNK) were assessed via immunohistochemistry (Figure S1) in the discovery (n=272) and validation TMAs (n=758). Antibody validation was performed with a single band on a western blot and EGF or UV stimulated cell pellet +/- inhibitors. For BRAF V600E antibody was also validated using BRAF WT and BRAF V600E mouse colon tissue (Figure S2).

Sections were dewaxed in histoclear then rehydrated using graded alcohols. Antigen retrieval for pERK was performed in citrate buffer at 96ºC for 20 minutes or for BRAF V600E, p-p38, and pJNK using Tris EDTA buffer pH8 under pressure for 5 minutes. Endogenous peroxidase activity was blocked using 3% hydrogen peroxidase. 10% Casein (BRAF V600E), 1.5% horse serum (pERK) or 5% horse serum (p-p38/pJNK) was applied as a blocking solution. TMA sections were incubated overnight at 4ºC with primary BRAF V600E (1:200, clone VE1, Spring Biosciences #E1929), pJNK (Thr183/Tyr185, 1:50, cell signalling #4668) or p-p38 (Thr180/Tyr182, 1:100, cell signalling #4511) antibody. Primary pERK (Thr202/Tyr204, 1:200, cell signalling #9101) antibody was incubated for 6 hours at room temperature.

Envision (Dako) was used as a secondary antibody before DAB substrate was added for colour development. Slides were counterstained with haematoxylin and blued with Scott's tap water before being dehydrated through a series of graded alcohols and histoclear. Cover slips were applied using distrene, plasticizer, xylene (DPX).

Scoring

Stained TMA sections were scanned using a Hamamatsu NanoZoomer (Welwyn Garden City, Hertfordshire, UK) at x20 magnification and visualized on Slidepath Digital Image Hub (Leica Biosystems, Milton Keynes, UK). Assessment of BRAF V600E, ERK1/2, JNK and p38MAPK phosphorylation was performed by a single examiner blinded to clinical data at x20 magnification (total magnification x400) using the weighted histoscore. The weighted histoscore was calculated as follows: 0x% not stained + 1x% weakly stained + 2x% moderately stained + 3x% strongly stained. This gave a range of scores from 0 to 300 with cytoplasmic and nuclear staining scored separately. 10% of tumours were co-scored by a co-investigator and the interclass correlation coefficient calculated to be <0.7 for all proteins.

Statistical analysis

Histograms were assessed for each protein and BRAF V600E and pERK histograms determined that negative and positive expression was the appropriate threshold. Receiver operator characteristic (ROC) curves were employed to identify the optimal threshold for low/high expression of p-p38 and pJNK in the discovery cohort and validated using the validation cohort, the following thresholds were identified for each protein: 40 for nuclear p-p38, 10 for cytoplasmic p-p38, 70 for nuclear pJNK, and 145 for cytoplasmic pJNK.

SPSS (version 22) was used for statistical analysis. By using a two-sided α=0.05 analysis and assuming a hazard ratio (HR) of 0.65 and a both high prevalence of 40% for the combined

nuclear score, a sample size of >178 patients gave >90% power to detect a survival difference between the both low or one high and both high groups. Pearson's $\chi 2$ test assessed associations between MAPK members, BRAF mutations, and clinicopathological features. Kaplan-Meier curves and log-rank analysis compared CSS. HRs and confidence intervals (CI) were calculated from univariate cox regression survival analysis. Multivariate cox regression survival analysis using a backward conditional elimination model and a significance threshold of $p<0.05$ was performed to identify independent prognostic biomarkers. The study is reported in line with the REMARK guidelines[16] and significance was set as $p<0.05$.

RESULTS

For the discovery cohort, a total of 187 patients were studied, and they underwent a potentially curative resection for stage I-III CRC and had a valid score for pERK, p-p38 and pJNK. The patient characteristics for the discovery cohort are shown in Table S1. In brief, 64% were 65 or older and 53% were male. 5% had stage I disease, 49% had stage II disease, and 46% had stage III disease. 38% had right-sided colon cancer, 30% had left-sided colon cancer and 32% had rectal cancer. MMR deficiency was identified in 10% and 20% had the BRAF V600E mutation. 31% received adjuvant chemotherapy. The median follow-up for patients was 11.6yrs (range 7.3-16.0yrs) with 65 cancer deaths and 45 non-cancer deaths.

The association between MAPK phosphorylation and CSS within the cytoplasm or nucleus was investigated in Table 1. p-p38 and pJNK were not associated with CSS at either cellular location. However, phosphorylation of ERK significantly improved CSS within the nucleus (HR 0.61 95% CI 0.38-1.00, p=0.048). To assess if analysing the MAPK family in combination would provide any additional power, combined prognostic scores were established as follows: combined pERK/p-p38 score, combined pERK/pJNK score and combined p-p38/pJNK score. For all combined scores, patients were split into two groups; patients with weak activation of both proteins or strong activation of one protein was termed both weak or one strong, and patients with strong activation of both proteins termed both strong. Only the combined nuclear pERK/p-p38 score provided some additional power to improve CSS (HR 0.56 95% CI 0.33-0.95, p=0.030, Table 1). Next, to assess if this increased power is relevant, this combined pERK/p-p38 score and pERK alone were taken forward into the validation cohort.

To validate theses findings the cohort was extended to an 801 CRC patient validation cohort. CRC patients with a valid score for pERK and p-p38 were included. The characteristics of

this cohort are shown in Table S1. In Brief, 68% were 65 or older and 53% were male. 14% had stage I disease, 48% had stage II disease, and 38% had stage III disease. 41% had right-sided colon cancer, 34% had left-sided colon cancer and 24% had rectal cancer. MMR deficiency was identified in 16% and 21% had the BRAF V600E mutation. The median follow-up for patients was 12.0yrs (range 7.3-16.0yrs) with 235 cancer deaths and 258 non-cancer deaths. As this data was similar to that of the discovery cohort it was deemed appropriate to validate the findings.

The validation cohort was assessed for associations with CSS (Table 2); p-p38 did not associate with CSS at either cellular location. However, pERK was associated with improved CSS within both the nucleus (HR 0.76 95% CI 0.59-0.99, p=0.037) and cytoplasm (HR 0.77 95% CI 0.60-0.99, p=0.047). When the combined pERK/p-p38 score was assessed, associations with improved CSS were strengthened for both nuclear (HR 0.69 95% CI 0.53-0.90, p=0.005) and cytoplasmic localisation (HR 0.68 95% CI 0.51-0.92, p=0.010).

pERK and the combined pERK/p-p38 score were then assessed for associations with clinicopathological factors as shown in Table 3. Patients with phosphorylation of nuclear ERK were more likely to have a BRAF V600E mutation (p=0.003), be MMR competent (p=0.010), and have lower CRP levels (p=0.030). Similarly, patients with phosphorylation of cytoplasmic ERK were more likely to have a BRAF V600E mutation (p=0.009) and be MMR competent (p=0.032). For the combined nuclear pERK/p-p38 score, patients with a both strong score associated with increased BRAF V600E mutations (p=0.002). Whereas a both strong score for the combined cytoplasmic pERK/p-p38 score associated with older age (p=0.039). No associations with other clinicopathological characteristics or local inflammation were seen for any MAPK members.

As the combined nuclear pERK/p-p38 score associated with increased BRAF mutations as well as pERK alone, firstly associations between nuclear p-p38 and BRAF status were assessed as they are normally within divergent MAPK pathways. High nuclear p-p38 did associate with increase BRAF mutations (p=0.039, Table S2) suggesting they do interact. Next, patients were stratified into BRAF wildtype or BRAF mutant and effects on patients CSS assessed (Figure 1). For phosphorylation of nuclear ERK, CSS was only improved in patients with BRAF mutations (HR 0.51 95% CI 0.29-0.90, p=0.018, Figure 1A). This effect on CSS was slightly potentiated for the combined nuclear pERK/p-p38 score (HR 0.50 95% CI 0.29-0.88, p=0.014, Figure 2B). To further assess BRAF mutant patients, the combined nuclear pERK/p-p38 score was assessed for effects pertaining to MMR status (Figure 2). In MMR competent patients, a both strong combined nuclear pERK/p-p38 score improved CSS (HR 0.49 95% CI 0.27-0.89, p=0.016). Whereas in MMR deficient patients, a both strong combined pERK/p-p38 score did not significantly improve patient survival (HR 0.45 95% CI 0.08-2.16, p=0.285).

To further investigate the utility of the combined nuclear pERK/p-p38 score within BRAF mutant patients, patients were further stratified for TNM-stage. For MMR competent patients (Figure S3), only BRAF mutant patients with stage III CRC had improved survival with a both strong score (HR 0.25 95% CI 0.10-0.64, p=0.002, Figure S3C). No survival advantage was seen for stage I (HR 1.84 95% CI 0.19-17.94, p=0.593, Figure S3A) or stage II patients (HR 0.97 95% CI 0.36-2.63, p=0.952, Figure S3B). No effect of stage was seen in MMR deficient patients.

pERK and the combine pERK/p-p38 score were then taken forwards into multivariate analysis with common clinicopathological factors (Table 4). Under multivariate analysis for all patients (n=606), TNM-stage (p<0.001), venous invasion (p=0.006), margin involvement

(p=0.032), peritoneal involvement (p<0.001), TSP (P=0.030), KM grade (p=0.030), TILs (p=0.002), mGPS (p<0.001), nuclear pERK (p=0.004) and the combined nuclear pERK/p-p38 score (p=0.001) were independent prognostic factors for CSS. When stratified for MMR competent BRAF mutant patients (n=136), venous invasion (p=0.003), margin involvement (p=0.009), mGPS (p<0.001) and nuclear pERK (p=0.042) remained independently prognostic. When further stratified for stage III patients (p=53), margin involvement (p=0.029), mGPS (p=0.001) and nuclear pERK (P<0.001) remained independent for CSS.

DISCUSSION

The results of the present study suggest that patients with CRC need strong nuclear phosphorylation of both ERK and p38MAPK for a good prognosis. The data shows that this survival improvement is enhanced in MMR competent patients with stage III CRC harbouring BRAF mutations. However, patients with strong activation of only one protein have a poorer survival outcome suggesting that these patients may benefit from a ERK or p38MAPK activation.

ERK has long been associated with malignant transformation in various cancers including CRC, with upstream KRAS/BRAF harbouring driver mutations[6,8,17]. Therefore, it is interesting that this improvement in patient survival is specific to BRAF mutant tumours, with strong activation of ERK. Previous studies have mainly associated phosphorylation of ERK with reduced survival in patients with CRC[18,19], however they assessed ERK in isolation, and therefore differences in the activation of p38MAPK between cohorts may account for the difference in survival effects compared to the present study. p38MAPK is thought to suppress cell proliferation in normal cells but can promote proliferation in certain cancer cells, and this has been linked to activation levels[20]. This observation is similar to ERK, which has also been shown to adapt its proliferative effects depending on activation levels, with strong activation of ERK causing cell cycle arrest and decreased proliferation[5]. Furthermore, p38MAPK has also been shown to suppress ERK activity, which may be important in BRAF mutant tumours where ERK is hyper-activated[21]. In the current study, patients with BRAF mutant tumours have a 3-fold increase in nuclear ERK phosphorylation compared to BRAF WT tumours (data not shown), supporting the hypothesis that ERK is hyper-activated in BRAF mutant patients. Although p38MAPKs is thought to be a poor prognostic factor in CRC that promotes cancer cell survival[22], most research to date has been

restricted to cell lines and mouse models[23,24]. However, the present tissue data suggests that high levels of phosphorylated p38MAPK promote proliferation potentially by inhibiting ERK activation. Therefore, in patients with high phosphorylation of both nuclear p38MAPK and ERK, p38MAPK may dampen the anti-proliferative effects of ERK to maintain cell proliferation. This is in line with previous literature that suggests proliferation measured by Ki67 is a good prognostic factor in patients with CRC[25,26]. However, if only ERK phosphorylation is high, then the hyper-activation in BRAF mutant tumours will be uncontrolled and start to suppress the cell cycle leading to decreased proliferation and reduced patient survival.

This was observed when assessing the combined nuclear ERK/p38 score in BRAF mutant patients. In BRAF mutant patients with strong activation of both ERK and p38MAPK, survival is significantly improved. It is interesting to note that in these patients of the 115 patients with strong activation of ERK, 95 patients also had strong activation of p38. This suggests that dual activation is common in BRAF mutant tumours and accounts for why only a slight increase in power is seen between nuclear pERK and the combined pERK/p-p38 score (figure 1). This dual activation protects the patient against the hyper-activation of ERK allowing the tumor to continue to proliferate, which has been previously shown to convey a good prognosis to patients with CRC[25]. However, when only one MAPK is highly active or both are weak, survival is decreased, which suggests that for a protective influence to ensue, high activation levels of both members are required. When only one member is activated, both proliferation and survival rates are lowered to a similar level to that observed for patients that have low activation of both. This suggests that proliferation needs to be driven by both members for improved patient prognosis. This effect is not seen in BRAF WT patients, suggesting that only when ERK is hyper-activated can it affect the cell cycle inhibiting proliferation, which leads to a worse prognosis for these patients. In BRAF WT

patients the levels of ERK activation never reach the threshold to affect the cell cycle, so the reduction in proliferation and survival is never produced.

As BRAF mutations are commonly associated with MMR deficient patients[4], we next stratified BRAF mutant patient by MMR status. In patients with MMR competent CRC, similar results were seen with a both strong nuclear ERK/p38 score conveying a survival advantage to the patient, suggesting that this score would be a useful prognostic marker in these patients. To confirm the utility of this prognostic marker across all disease stages of CRC, we next stratified MMR competent BRAF mutant patients by stage, and found that this survival advantage with the nuclear ERK/p38 score was potentiated in patients with stage III CRC. Patients with a both weak/one strong score had significantly poorer survival rates. In contrast, the survival difference previously observed was lost in stage I/II patients. These finding suggest that BRAF mutational analysis should be extended beyond it's current clinical application in metastatic disease to the adjuvant setting to further aid clinicians with patient prognosis.

In conclusion, this is the first study to show a combined survival advantage of ERK and p38MAPK in MMR competent BRAF mutant patients with stage III CRC; confirmation in an independent cohort is needed. One limitation of this study is that it does not cover metastatic disease and therefore further analysis of this combine score in BRAF mutant metastatic patients is also warranted. Overall, these results suggest that patients within the adjuvant setting with MMR competent Stage III CRC should not only be routinely tested for BRAF mutations but should also be further tested for phosphorylation of ERK and p38MAPK to fully stratify prognosis. Furthermore, strong expression of only one of these proteins (ERK or p38MAPK) could be used as a predictive biomarker for clinical trials in BRAF mutant MMR competent CRC patients, establishing the benefit of treatment with an agonist to the other

member (ERK for p38MAPK agonist and p38MAPK for ERK agonist). This approach would assess if this combined MAPK score truly has a prognostic benefit and would help move towards a precision medicine approach for patients with CRC.

ADDITIONAL INFORMATION

Ethics approval - Ethical approval was obtained from the West of Scotland Research Ethics Committee for use of surplus tissue (16WS0207) and was performed in accordance with the Declaration of Helsinki.

Availability of Data and Material – All clinical information is held within a database available from the NHS GGC Safehaven and tissue microarrays are available from the NHS Research Scotland GGC Biorepository.

Conflict of Interest – The authors declare no conflicts of interest.

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Author Contributions – AKR designed the experiments, performed experiments, analyzed the data, and wrote the manuscript; ESH performed experiments and reviewed the manuscript, SC performed experiments and reviewed the manuscript, AGMTP performed experiments and analyzed the data, DCM helped developed the concept and revised the manuscript; PGH helped developed the concept and reviewed the manuscript; and JE developed the concept, designed the experiments and revised the manuscript.

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TITLES AND LEGENDS TO FIGURES

Figure 1. Combined nuclear pERK/p-p38 score stratifies CSS in BRAF mutant CRC patients (n=782). Kaplan Meier curves showing associations between (A) pERK or (B) the combined nuclear pERK/p-p38 score and CSS in BRAF WT and BRAF mutant patients with CRC.

Figure 2. Combined nuclear pERK/p-p38 score differentially stratifies BRAF mutant CRC patient survival in MMR competent and MMR deficient patients (n=165). Kaplan Meier curves showing association between the combined nuclear pERK/p-p38 score and CSS in BRAF mutant patients with (A) MMR competent or (B) MMR deficient CRC.