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Analysis of *KRAS*, *NRAS*, *BRAF*, *PIK3CA* and *TP53* mutations in a large prospective series of locally advanced rectal cancer patients

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Abbreviations:

CE-SSCA: capillary electrophoresis-single strand conformational analysis. CI: confidence intervals. EGFR: epidermal growth factor receptor. EMVI: extramural venous invasion. HR: hazard ratio. LARC: locally advanced rectal cancer. MMR: mismatch repair. MMS: microsatellite stable. MRI: magnetic resonance imaging. MSI: microsatellite instability. NGS: next-generation sequencing. PCR: polymerase chain reaction. pCR: pathological complete response. PFS: progression-free survival. pTRG: pathological tumour regression grade. OS: overall survival.

Novelty and Impact

This article reports the incidence, heterogeneity and clinical significance of *KRAS*, *NRAS*, *BRAF*, *PIK3CA* and *TP53* mutations in a prospective series of 210 non-metastatic rectal cancer patients. Main findings include an association between *TP53* mutations and poor pathological regression grade after neoadjuvant treatment, and worse survival outcome among patients with tumours harbouring concomitant *TP53* and *RAS* mutations. Upon confirmation, these results may be used for patient stratification in future clinical trials.

Abstract

Little information is available on the clinical significance of cancer-related genes such as KRAS, NRAS, BRAF, PIK3CA and TP53 in non-metastatic rectal cancer. We investigated mutations of these genes in a large prospective series of locally advanced rectal cancer (LARC) patients who were recruited into two phase II trials. Mutational analyses were performed with diagnostically validated methods including polymerase chain reaction, capillary electrophoresis-single strand conformational analysis, Sanger sequencing and next-generation sequencing. Associations between single or multiple gene mutations and clinico-pathological characteristics and treatment outcomes were explored. 210/269 (78%) patients were assessable. Mutations of *KRAS*, *NRAS*, *BRAF*, *PIK3CA* and *TP53* occurred in 43%, 9%, 4%, 9% and 60% of patients, respectively. Concordance between paired biopsy and resection specimens was 82% for *KRAS*, 95% for *NRAS*, 99% for *BRAF*, 96% for *PIK3CA* and 63% for *TP53*. *TP53* mutations were associated with extramural venous invasion on baseline MRI (78% vs 65%, p=0.04), good pathological tumour regression (36% vs 23%, p=0.05) and a trend towards a better 5-year progression-free survival (74% vs 60%, HR 1.59, p=0.06). Patients with tumours harbouring mutation of *TP53* and either *KRAS* or *NRAS* (32%) had a worse 5-year progression-free survival than those with *TP53/KRAS/NRAS* wild-type tumours (54% vs 72%, HR 1.75, p=0.02). In univariate analysis *BRAF* mutation predicted poor 5-year overall survival only among patients treated without cetuximab (20% vs 73%, HR 3.29, p=0.03). This is one of the largest biomarker studies in a prospective, largely homogeneous, LARC population. Our findings are hypothesis-generating and require validation in independent series.

Introduction

In early stage colon cancer and metastatic colorectal cancer molecular tests are routinely performed to capture useful information on tumour biology and guide treatment decisions. Testing for microsatellite instability (MSI)/mismatch repair (MMR) deficiency allows for identification of good prognosis stage II colon cancer patients who do not require adjuvant chemotherapy while mutational analysis of *KRAS*, *NRAS* and *BRAF* provides a tool to predict resistance to anti-EGFR monoclonal antibodies and prognosis in stage IV colorectal tumours.

In contrast, the management of non-metastatic rectal cancer still lacks biomarkers that could refine prognostication and treatment response prediction as currently provided by conventional clinical, pathological and imaging factors (1). While important advances have been made in the definition of risk categories and implementation of risk-stratified treatment approaches (2-9), much still needs to be done to capture the underlying inter-individual tumour heterogeneity and to identify molecular determinants of treatment responsiveness or resistance. As a result, therapeutic algorithms for non-metastatic rectal cancer remain suboptimal and different outcomes are generally seen among patients who share similar clinico-pathological risk features and are elected to receive the same treatment.

Retrospective analyses of clinical trials suggest that *KRAS* mutation (especially for left-sided and rectal tumours) and *BRAF* mutation (at least in microsatellite stable [MMS] or MMR proficient tumours) predict poor prognosis of colon cancer patients (10-13) while *PIK3CA* and *TP53* mutation are associated with increased risk of local recurrence and resistance to radiotherapy, respectively (14, 15). Nevertheless, data are scant overall and more studies are needed. Furthermore, there is very limited information regarding the prognostic/predictive

value of these genetic alterations when simultaneously detected in the same tumour. Therefore, we investigated baseline clinical characteristics, treatment outcome and survival of a large prospective series of LARC patients according to the mutational status of five genes including *KRAS*, *NRAS*, *BRAF*, *PIK3CA* and *TP53*.

Material and Methods

Patient population

PAN-EX was a pooled analysis of two phase II trials sponsored by The Royal Marsden NHS Foundation Trust (16). EXPERT was a single-centre, single-arm, phase II trial (2001-2005) while EXPERT-C was an international, multicentre, randomised phase II trial (2005-2008) (17, 18). Main eligibility criteria for both studies included non-metastatic rectal cancer with at least one of the following high-risk features on high-resolution pelvic MRI at baseline: tumour <1mm of the mesorectal fascia, extramural invasion >5mm (T3c/d), T4 stage, T3 tumour at/below levator muscles. Additional high-risk imaging features for eligibility included N2 stage (EXPERT) and extramural venous invasion (EMVI) (EXPERT-C) (17, 18)

Treatment

All patients were treated with 4 cycles of neoadjuvant CAPOX chemotherapy followed by capecitabine-based chemo-radiotherapy (54 Gy in EXPERT and 50.4 Gy in EXPERT-C). Surgery was performed according to the principles of total mesorectal excision 4-6 weeks after completion of chemo-radiotherapy. Four cycles of adjuvant chemotherapy (capecitabine in EXPERT and CAPOX in EXPERT-C) were also administered. Patients in the EXPERT-C study were randomised in a 1:1 ratio to receive cetuximab in combination with neoadjuvant chemotherapy, chemoradiotherapy and adjuvant chemotherapy. Follow-up was carried out for 5 years after surgery (17, 18).

Mutation analyses

All mutation analyses were performed in a central laboratory (Centre for Molecular Pathology, The Royal Marsden Hospital NHS Foundation Trust) on DNA extracted from formalin-fixed, paraffin-embedded tissue sections from diagnostic biopsy and/or post-treatment primary resection samples. Given that the PAN-EX study analysed all mutational data that were obtained over time from samples of patients included in the EXPERT and EXPERT-C trials, different analytic techniques were used. In the EXPERT-C study, analysis of *KRAS* (exon 2 and 3) and *BRAF* (exon 15) was performed prospectively using the INFINITI platform (AutoGenomics, Vista, CA), as per the manufacturer's instructions. Mutations in *PIK3CA* exon 9 and 20 and *NRAS* exon 3 were screened for by capillary electrophoresis-single strand conformational analysis (CE-SSCA) followed by bi-directional Sanger sequencing. Mutations in exon 4 of *KRAS* and exons 2 and 4 of *NRAS* were screened for by bi-directional Sanger sequencing. All mutations detected were confirmed on an independent PCR and sequencing analysis (17-19). *TP53* mutational analysis (exon 4-9) was performed by CE-SSCA and bi-directional Sanger sequencing analysis performed on an independent PCR (20). In the EXPERT study, mutations in *KRAS* (exon 2-4), *NRAS* (exon 2-4), *BRAF* (exon 15), *PIK3CA* (exon 9 and 20) and *TP53* (exon 4-11) were retrospectively screened for using a next-generation sequencing (NGS) panel which was developed in house (Centre for Molecular Pathology, The Royal Marsden Hospital NHS Foundation Trust) and subsequently validated for routine clinical application. All the analyses were performed by investigators who were blinded to the clinical data.

Comparison of NGS against other sequencing techniques

Given the use of different sequencing techniques between EXPERT and EXPERT-C, 45 samples from 37 EXPERT-C patients with available tumour tissue were also analysed with the same NGS panel which was used for EXPERT patients. Concordance rates were as follows: 89% (40/45) for *KRAS* (4 new mutations detected while 1 mutation missed), 98% (44/45) for *NRAS* (1 mutation missed), 100% (45/45) for *BRAF*, 98% (44/45) for *PIK3CA* (1 new mutation detected) and 91% (41/45) for *TP53* (3 new mutations detected while 1 mutation missed; a second mutation was missed in 2 mutant samples, one each by CE-SSCA and NGS).

Outcome measures and statistical analysis

Only patients who had tumour samples assessable for genetic analyses were included in this study. Pathological tumour regression grade (pTRG) was assessed (prospectively in EXPERT-C and retrospectively in EXPERT) by local independent pathologists according to Dworak et al (21). For the purpose of this analysis, good tumour regression corresponded to pTRG 3 or 4 while pTRG 0-2 indicated poor tumour regression. Pathological complete response (pCR) was defined as the absence of viable tumour cells in the tumour bed and resected lymph nodes. Progression-free survival (PFS) and overall survival (OS) were calculated from trial start date to date of recurrence and death, respectively. Patients alive and without evidence of tumour progression were censored at last follow-up. Patients who died without tumour progression were censored at the time of death.

All pooled analyses were stratified by treatment arm and trial. For the PFS and OS endpoints the Kaplan-Meier method was used and median survival along with 95% confidence intervals (CI) were estimated according to mutational status. Cox proportional hazards regression models were fitted to produce the estimated hazard ratios (HR) and survival probabilities.

Univariate analyses were performed to examine the crude relationship between marker and PFS/OS. In view of the exploratory nature of the study, a multivariable model was fitted initially with standard clinico-pathological variables while *KRAS*, *NRAS*, *BRAF*, *PIK3CA*, and *TP53* were added in a forward selection procedure. Prognostic variables were retained in the model if they demonstrated significance at the ≤10% level (i.e., p≤0.10). Logistic regression was used for the dichotomised endpoints (i.e., pTRG 3-4 vs pTRG 0-2 and pCR vs non-pCR).

Data are available upon request.

Regulatory approval

EXPERT and EXPERT-C were approved by the relevant National Regulatory Agencies and Research Ethics Committee. All patients provided written informed consent. The PAN-EX study was approved by the Committee for Clinical Research at The Royal Marsden NHS Foundation Trust.

Results

Two-hundred and sixty-nine patients were included in the PAN-EX study (105 from EXPERT and 164 from EXPERT-C) (Supplementary Table 1). Of these, 210 (78% [58% and 91% of the EXPERT and EXPERT-C patient population, respectively]) were assessable for ≥1 biomarker while 59 (22%) were not assessable due to lack of tumour tissue (i.e., archived material not retrievable and/or pCR on resection specimen) or poor quality of the available samples (i.e., low DNA concentration). The majority of assessable patients had only one sample available for analysis (either baseline biopsy or resection sample) while a variable number (ranging from 57 to 71 depending on the biomarker) could be successfully analysed in paired (baseline biopsy and resection) samples (Figure 1).

Analysis of single mutations

Mutation rates in biopsy and resection samples are shown in Table 1 (full list of mutations including type and frequency available in Supplementary Table 2). Overall 43%, 9%, 4%, 9% and 60% of patients had *KRAS*, *NRAS*, *BRAF*, *PIK3CA* and *TP53* mutant tumours, respectively. Two *TP53* mutations co-occurred in 7 cases. The concordance rates between paired specimens was 82% for *KRAS*, 95% for *NRAS*, 99% for *BRAF*, 96% for *PIK3CA* and 63% for *TP53* (Table 2). No statistically significant associations were observed between mutations and baseline prognostic factors with the only exception of *TP53* mutations which were associated with MRI-detected EMVI (78% in *TP53* mutant tumours vs 65% in *TP53* wild-type tumours, p=0.04) (Supplementary Tables 3-7).

In the entire study population, patient outcomes did not differ according to the mutational status of *KRAS*, *NRAS*, *BRAF*, or *PIK3CA*. Numeric differences were found between *TP53* wild-type and *TP53* mutant patients in terms of pCR (17% vs 9%, p=0.08), good tumour regression (36% vs 23%, p=0.05) and 5-year PFS (74% vs 60%, HR 1.59 [95% CI: 0.98-2.58], p=0.06) but not for 5-year OS (77% vs 72%, HR 1.20 [95% CI: 0.72-2.00], p=0.48). (Table 3). The association between *TP53* mutation and PFS remained unaltered (HR 1.65 [95% CI: 0.99-2.75], p=0.06) after multivariate analysis.

When the analyses were restricted to the group of patients who did not receive cetuximab no statistically significant associations were detected between single gene mutations and patient outcome with the only exception of *BRAF* and *TP53* mutations. The former was associated with a worse 5-year OS (20% vs 73%, HR 3.29 [95% CI: 1.16-9.28], p=0.03) and a trend towards a worse 5-year PFS (20% vs 66%, HR 2.54 [95% CI: 0.91-7.10], p=0.08). The latter

predicted poor tumour regression (84% vs 61%, p=0.01). After multivariate analysis, the association between *BRAF* mutation and OS did not reach statistical significance (HR 2.54 [95% CI: 0.91-7.13], p=0.08).

Analysis of mutation combinations

KRAS or *NRAS* mutations were detected in 105/203 patients (52%), *KRAS*, *NRAS* or *BRAF* mutations in 111/202 (55%), *KRAS*, *NRAS*, *BRAF* or *PIK3CA* mutations in 114/202 (56%), *KRAS*, *NRAS*, *BRAF*, *PIK3CA* or *TP53* mutations in 170/205 (83%). No associations between any of these mutation combinations and either prognostic factors or treatment outcomes were found to be significant at 5% level association. There were 63 out of 199 patients (32%) with tumours harbouring mutations of *TP53* and either *KRAS* or *NRAS*. These had an older age (median age 64.2 vs 60.4 years, p=0.02) and different stage distribution at diagnosis (i.e., stage II/III tumours 30% vs 70%, p=0.01) than patients with *TP53*/*KRAS*/*NRAS* wild-type tumours. While no association was observed between *TP53* and *KRAS*/*NRAS* mutations and early outcome efficacy measures (i.e., pCR and tumour regression), patients with *TP53* and *KRAS*/*NRAS* mutant tumours had a worse 5-year PFS than those with *TP53*/*KRAS*/*NRAS* wild-type tumours (54% vs 72%, HR 1.75 [95% CI: 1.10-2.78], p=0.02) (Figure 2). This association remained significant after adjusting for prognostic factors in multivariate analyses (HR 1.74 [1.07-2.85], p=0.03). In the group of patients who did not receive cetuximab, none of the mutation combinations was statistically significantly associated with either short- or long-term outcome measures.

Discussion

In this study we analysed the clinical significance of mutations of five genes, including *KRAS*, *NRAS*, *BRAF*, *PIK3CA* and *TP53*, in a large prospective series of LARC patients. The

results of our analysis suggest an association between *TP53* mutations and MRI-detected EMVI at baseline and poor tumour regression after neoadjuvant treatment. Furthermore, we found that patients with tumours harbouring concomitant *TP53* and *RAS* mutations had a worse PFS than those with wild-type tumours for either of these genes. Finally, in the group of patients who were treated with chemotherapy and chemoradiotherapy but without cetuximab, *BRAF* mutations were associated with a worse OS in univariate analysis.

The genes which were selected for this analysis are all known to be biologically and clinically relevant as they play a key role in the processes of colorectal cancer carcinogenesis and tumour progression and in the mechanisms of treatment resistance (22, 23). Nevertheless, the bulk of evidence underlying this information consists of studies that were conducted in metastatic colorectal cancer patients while limited data exist for rectal cancer especially in the non-metastatic setting. Therefore, investigation in a largely homogeneous, prospective cohort of LARC patients is warranted. In this regard, PAN-EX (a pooled analysis of two academic phase II rectal cancer trials of similar design, EXPERT and EXPERT-C) provides a unique, valuable platform for exploratory biomarker analyses (16).

TP53 mutations occur in the late phase of the step-wise, colorectal carcinogenesis process and are particularly common among non-hypermutated tumours (22, 24). In line with the role of the TP53 pathway in the mechanisms of DNA repair following genotoxic stress, studies have demonstrated an association between *TP53* mutations and resistance to radiotherapy (15). This association has been confirmed by the results of our study which are also in line with previous data showing a higher incidence of vascular invasion in *TP53* mutant colorectal cancers (25). Interestingly, the improved pathological regression observed in our study among patients with *TP53* wild-type tumours translated into numerically higher but not

statistically significant survival outcomes. While this inconsistency may be secondary to the limited sample size and the relatively low number of survival events, it is also possible that the long-term prognostic effect of *TP53* could have been diluted by the inclusion of patients who were treated without cetuximab and in fact accounted for the majority of the study population. Indeed, in a previous exploratory biomarker analysis of the EXPERT-C trial, we showed that *TP53* was an independent predictive factor for PFS and OS only in the group of cetuximab-treated patients, possibly due to a selective therapeutic effect of EGFR inhibition on micrometastatic foci of *TP53* wild-type tumours (20, 26). Therefore, beyond the confirmation of reduced pathological regression of *TP53* mutant tumours after neoadjuvant therapy, the findings of our analysis appear to provide further indirect support to the design of prospective trials investigating *TP53* as predictive biomarker for cetuximab in LARC.

Mutations of the *KRAS* gene occur before *TP53* mutations in the early stages of the adenoma-carcinoma sequence (27). While testing for these genetic aberrations (alongside *NRAS* mutations) is a routine procedure to select metastatic colorectal cancer patients for treatment with anti-EGFR monoclonal antibodies, the significance of *KRAS/NRAS* mutations in the setting of non-metastatic rectal cancer is unknown. Data from previous studies are inconsistent. *KRAS* mutations were associated with a reduced rate of pCR or worse long-term outcome in some series (28-32) but not in others (33-38). Of note, in a study of 229 rectal cancer patients who had received neoadjuvant chemotherapy either before or after chemoradiotherapy, Chow et al showed that patients who had *KRAS/TP53* double mutant tumours achieved a lower rate of pCR compared to patients with non-double mutant tumours (10% vs 31%, p=0.001), this association being likely driven by the negative prognostic effect of *KRAS* mutations in the same series (29). In our study, we did not find any difference in outcome between patients with *KRAS* (or *KRAS*/*NRAS*) wild-type and *KRAS* (or *KRAS*/*NRAS*)

mutant tumours. On the other hand, in line with the study by Chow et al, we observed a poorer PFS among patients with tumours harbouring concomitant *RAS* and *TP53* mutations. While, this finding may actually be biased by the reduced response to treatment and poor outcome of *TP53* mutant tumours in our series, further investigation of the prognostic role of concurrent *RAS* and *TP53* mutations in future studies may be warranted. In contrast, none of the other mutation combinations which were tested in our study appeared to have any impact on treatment outcome or patient prognosis.

As expected for rectal cancers, mutations of the *BRAF* gene were detected in a small proportion of our patients (4%). Bearing in mind that the rarity of this alteration precludes any meaningful analysis, we found an association between *BRAF* mutations and poor OS which was significant in univariate analysis only and limited to the group of patients treated without cetuximab. The absence of differences between patients with *BRAF* mutant and *BRAF* wild-type tumours in terms of pathological tumour regression or PFS suggests that this association may be secondary to the poor prognosis conferred by *BRAF* mutation after tumour recurrence as previously reported (39). Larger series are certainly needed to clarify the prognostic and predictive value of *BRAF* mutation in this disease setting. Of note, recent studies have shown that non-V600 *BRAF* mutations account for 22% of all *BRAF* mutations. These occur more frequently in rectal cancers and are associated with more favourable clinico-pathological features and better outcomes than canonical V600 *BRAF* mutations (40, 41). In our series, approximately one third of *BRAF* mutations were non-V600 but the small numbers did not allow us to explore any association with clinical data.

Spatial and temporal intra-tumour molecular heterogeneity is a landmark of many malignancies including colorectal cancer (42, 43). Studies addressing this phenomenon in

non-metastatic rectal cancer are limited and results not always consistent (44-48). By analysing a relatively large number of paired tumour tissues from pre-treatment biopsies and post-treatment resection samples we found a high concordance (≥95%) for *NRAS*, *BRAF* and *PIK3CA* while the concordance for *KRAS* and *TP53* was lower at 82% and 63%, respectively. Notably, the vast majority of discordant cases in our series were due to the detection at baseline of mutant clones which were not subsequently detectable on the resection specimens, this likely reflecting an artefact secondary to the reduced tumour cellularity after neoadjuvant treatment. As previously shown, the rate of discordance can be significantly reduced by analysing post-treatment resection samples with more sensitive detection techniques than those used for diagnostic purposes on the pre-treatment biopsy (47). On the other hand, sampling errors may account for the few remaining "false negative" (i.e., wild-type) biopsy cases and highlight the potential value of multiple sampling at baseline as well as further investigation and validation of circulating tumour DNA mutational analyses (49).

The results of our analysis should be interpreted with extreme caution due to a number of limitations. The PAN-EX study was meant to analyse all mutational data that were obtained over time from samples of patients included in two prospective trials, this inevitably resulting in the use of several analytic platforms. Nevertheless, in view of the high concordance rates between NGS and other sequencing techniques (ranging from 89% for *KRAS* to 100% for *BRAF*) as observed in a small sample of the study population, it is unlikely that this heterogeneity could have significantly affected the final results. While all "false negative" cases by NGS were secondary to the poor quality of the re-tested samples, the "false negative cases" by other sequencing techniques were due to either technical issues or mutations which were below the detection level. Other study limitations include the retrospective analysis, the relatively high proportion of non-assessable patients (especially for the EXPERT study), the

small number of genes analysed, the limited number of exons tested for each gene, and some treatment heterogeneity between the two study populations. Furthermore, in light of the multiple testing some of the statistically significant associations between mutated genes and clinico-pathological characteristics or treatment outcomes could be random effects. It should be noted that the PAN-EX study was originally designed as an exploratory analysis and no formal a-priori sample size calculation was made. Despite the relatively large population, this study does not have sufficient power to detect meaningful effects and the lack of sufficient events/observations is confirmed by the very wide confidence intervals even in the presence of results which ultimately met the criteria for statistical significance. Larger studies of independent series are needed to support our findings which remain hypothesis-generating. Nevertheless, useful insights can be obtained from this analysis, which is one of the largest of its kind, including a better understanding of the potential clinical utility of testing LARC patients for genetic variants which are commonly evaluated in routine oncology practice.

There is no doubt that refinement of currently adopted risk-stratified treatment strategy for non-metastatic rectal cancer is needed and unlikely to happen without the identification and validation of clinically actionable molecular alterations. Studies providing a comprehensive and integrated molecular characterisation of rectal tumours and exploring clinical correlations in relation to prognosis and response to treatment are highly desirable and likely to shape the future treatment landscape of this disease.

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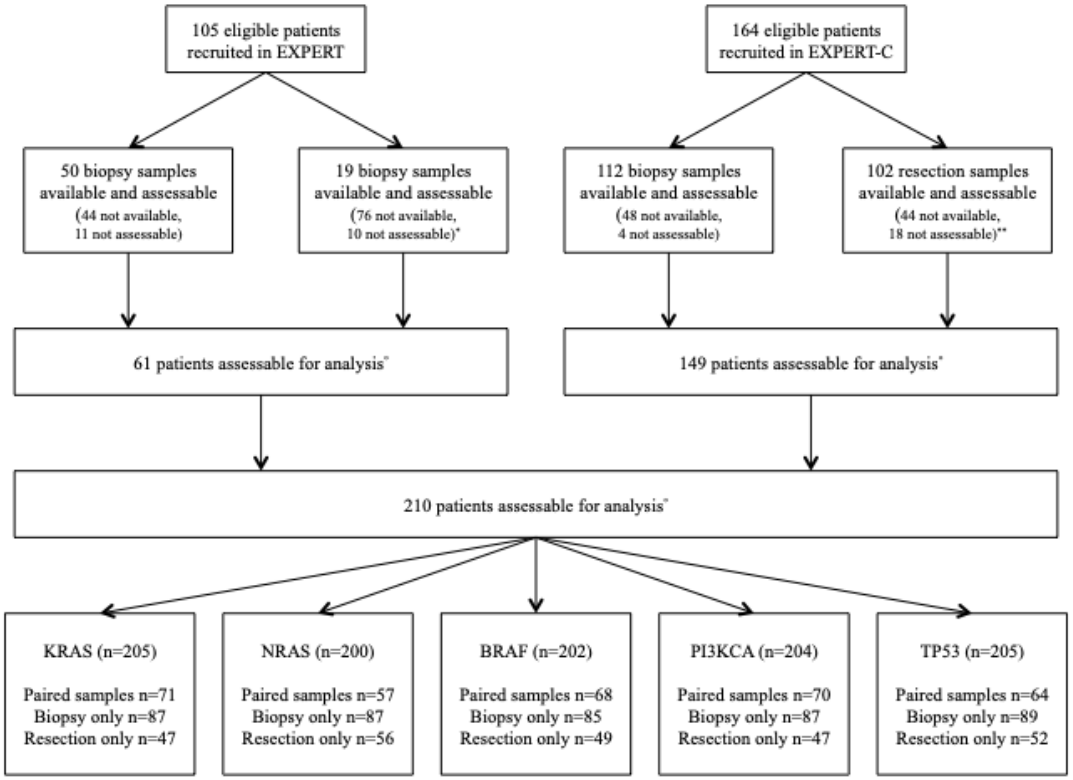
Figure legends

Figure 1. Study flow diagram

Figure 2. Progression-free survival by *TP53* and *KRAS/NRAS* status



* As a result of pathological complete response in 21 cases
** As a result of pathological complete response in 22 cases
^ Assessable for at least one of the biomarkers

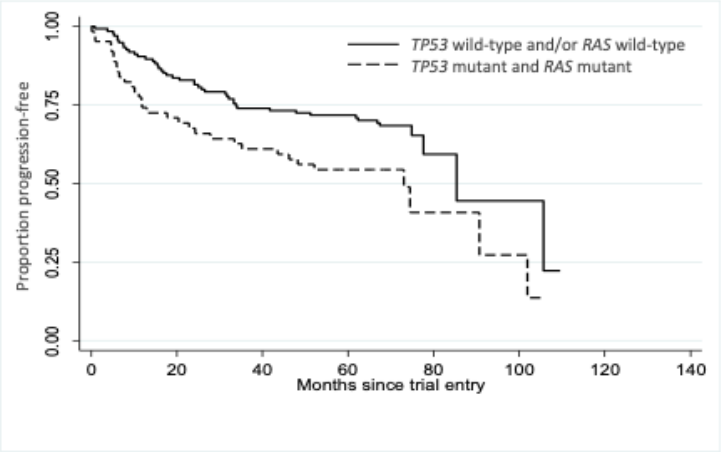


Table 1. Mutation rates in biopsy and resection samples, and overall number of patients with mutant tumours

Gene	Biopsy samples harbouring mutations	Resection samples harbouring mutations	Overall number of patients with mutant tumours
KRAS	72/158 (46%)	35/118 (30%)	89/205 (43%)
NRAS	13/144 (9%)	6/113 (5%)	17/200 (9%)
BRAF	7/153 (5%)	2/117 (2%)	7/202 (4%)
PI3KCA	17/157 (11%)	4/117 (3%)	18/204 (9%)
TP53	98/153 (64%)	46/116 (40%)	123/205 (60%)

Table 2. Mutation concordance between biopsy and resection in patients with paired specimens

		KRAS **Resection**			***p*** **value**
		Wild type	Mutant	Total	
KRAS ***Biopsy***	Wild type	40 (93%)	3 (7%)	43 (60.6%)	0.05
	Mutant	10 (35.7%)	18 (64.3%)	28 (39.4%)	
	Total	50 (70.4%)	21 (29.6%)	71 (100%)	
		NRAS **Resection**			
		Wild type	Mutant	Total	
NRAS ***Biopsy***	Wild type	52 (100%)	0	52 (91.2%)	0.25
	Mutant	3 (60%)	2 (40%)	5 (8.8%)	
	Total	55 (96.5%)	2 (3.5%)	57 (100%)	
		BRAF **Resection**			
		Wild type	Mutant	Total	
BRAF ***Biopsy***	Wild type	65 (100%)	0	65 (95.6%)	1.0
	Mutant	1 (33.3%)	2 (66.7%)	3 (4.4%)	
	Total	66 (97.1%)	2 (2.9%)	68 (100%)	
		PI3KCA **Resection**			
		Wild type	Mutant	Total	
PI3KCA ***Biopsy***	Wild type	64 (100%)	0	64 (91.4%)	0.25
	Mutant	3 (50%)	3 (50%)	6 (8.6%)	
	Total	67 (95.7%)	3 (4.3%)	70 (100%)	
		TP53 **Resection**			
		Wild type	Mutant	Total	
TP53 ***Biopsy***	Wild type	19 (79.2%)	5 (20.8%)	24 (37.5%)	0.004
	Mutant	19 (47.5%)	21 (52.5%)	40 (62.5%)	
	Total	38 (59.4%)	26 (40.6%)	64 (100%)	

*Mc Nemar's test

Table 3. Patient outcomes by single gene mutational status

Gene	pTRG*	pCR	5-year PFS	5-year OS
KRAS Wild type *vs* Mutant	29% *vs* 32% p=0.77	11% *vs* 16% p=0.34	70% *vs* 61% HR 1.25 (95% CI: 0.80-1.94), p=0.33	76% *vs* 70% HR 1.30 (95% CI: 0.81-2.10), p=0.27
NRAS Wild type *vs* Mutant	31% *vs* 8% p=0.11	13% *vs* 6% p=0.70	67% *vs* 59% HR 1.40 (95% CI: 0.69-2.86), p=0.35	74% *vs* 70% HR 1.17 (95% CI: 0.54-2.50), p=0.69
BRAF Wild type *vs* Mutant	29% *vs* 33% p=1.00	12% *vs* 29% p=0.22	67% *vs* 43% HR 1.60 (95% CI: 0.58-4.39), p=0.36	74% *vs* 43% HR 2.23 (95% CI: 0.81-6.15), p=0.12
PI3KCA Wild type *vs* Mutant	29% *vs* 41% p=0.30	12% *vs* 22% p=0.27	67% *vs* 67% HR 0.74 (95% CI: 0.32-1.71), p=0.48	74% *vs* 67% HR 0.99 (95% CI: 0.43-2.29), p=0.98
TP53 Wild type *vs* Mutant	36% *vs* 23% p=0.05	17% *vs* 9% p=0.08	74% *vs* 60% HR 1.59 (95% CI: 0.98-2.58), p=0.06	77% *vs* 72% HR 1.20 (95% CI: 0.72-2.00), p=0.48

* Includes TRG 3 and 4 according to Dworak et al.

Abbreviations: *CI: confidence intervals; HR: hazard ratio; pCR: pathological complete response; PFS: progression-free survival; pTRG: pathological tumour regression; OS: overall survival.*

Novelty & Impact Statement:

Mutational analysis of cancer-related genes can yield critical insight into therapeutic response and disease prognosis in patients with advanced metastatic colorectal cancer (CRC). The ability of mutational analysis to predict disease progression in nonmetastatic CRC, however, remains uncertain. Here, investigation of the significance of mutations in cancer-related genes in nonmetastatic CRC patients reveals an association specifically between *TP53* mutation and poor tumor regression following neoadjuvant treatment. Survival was especially poor in patients with concomitant mutations in *TP53* and *RAS*. The findings are relevant to the future generation of risk-stratified treatment approaches for nonmetastatic CRC.

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