CANCER GENOMICS & PROTEOMICS *21*: 533-548 (2024)
doi:10.21873/cgp.20470

PIK3CA Mutated Colorectal Cancers Without KRAS, NRAS and BRAF Mutations Possess Common and Potentially Targetable Mutations in Epigenetic Modifiers and DNA Damage Response Genes

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Abstract. *Background/Aim: Despite therapeutic advancements, metastatic colorectal cancer is usually fatal, necessitating novel approaches based on the molecular pathogenesis to improve outcomes. Some colorectal cancers have no mutations in the extended RAS panel (KRAS, NRAS, BRAF) genes and represent a special subset, which deserves particular therapeutic considerations. Materials and Methods: The genomic landscape of colorectal cancers from publicly available genomic series was interrogated, using the cBioportal platform. Colorectal cancer cohorts with cancers devoid of KRAS/NRAS or BRAF mutations were evaluated for the presence of mutations in the catalytic sub-unit alpha of kinase PI3K, encoded by the gene PIK3CA. Results: PIK3CA mutations in the absence of KRAS/NRAS/BRAF mutations were observed in 3.7% to 7.6% of colorectal cancers in the different series examined. Patients with all four genes in wildtype configuration (quadruple wild type) represented 32.2% to 39.9% of cases in the different series examined. Compared with quadruple wild type cancers, triple (KRAS/NRAS/BRAF) wild type/PIK3CA mutated cancers had a higher prevalence of high TMB cases and additional mutations in colorectal cancer associated genes except for mutations in TP53. Mutations in genes encoding for epigenetic modifiers and the DNA damage response (DDR) were also more frequent in triple wild type/PIK3CA mutated cancers. The prognosis of the two groups was comparable. Conclusion: Colorectal cancers with PIK3CA mutations in the absence of KRAS/NRAS/BRAF mutations have frequently mutations in epigenetic modifiers and DDR response genes, which may provide opportunities for targeting. These mutations are present in a smaller subset of quadruple wild type cancers.*

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Key Words: PI3K, genomic studies, targeted therapy, quadruple wild type, extended RAS panel.

Progress in the elucidation of the molecular pathogenesis of colorectal cancer has had a positive impact on therapeutics for the disease (1). Several targeted therapies are currently available for the treatment of molecularly defined groups of colorectal cancers, which have improved the outcomes of metastatic disease. Targetable alterations are observed only in a minority of colon cancers and therefore the treatment of most patients relies on cytotoxic chemotherapy. Colorectal cancer patients with targeted treatment options include those with microsatellite unstable tumors (Microsatellite Instability high, MSI-high) who are candidates for checkpoint inhibitor immunotherapy (2). In addition, patients with *BRAF* mutations are candidates for BRAF kinase inhibitors in combination with anti-EGFR monoclonal antibodies, patients with *KRAS* G12C mutations are candidates for KRAS inhibitors in combination with anti-EGFR monoclonal antibodies, and those with HER2 amplifications are candidates for HER2 targeted therapies (3-5). Additional cancers with mutations of the KRAS/BRAF cascade, including those with mutations in *KRAS* other than G12C, may also acquire additional therapeutic options in the future, as inhibitors targeting other *KRA*S mutations are in development (6). For example, inhibitors of *KRAS* G12D mutations, which are more prevalent in colorectal cancer than *KRAS* G12C mutations, have been discovered and are in pre-clinical development (7, 8). In contrast, cancers with no extended RAS panel mutations (mutations in *KRAS*, *NRAS* or *BRAF*) will not have these options of treatment and will potentially benefit from drugs targeting alternative molecular alterations.

Phosphoinositide 3-kinase (PI3K) is a lipid kinase that phosphorylates membrane lipid phosphatidyl-inositol 4,5-biphosphate to phosphatidyl-inositol 3,4,5-triphosphate, creating binding sites for target enzymes, including phosphoinositide dependent kinase 1 (PDK1) and the mechanistic target of rapamycin complex 2 (mTORC2) (9). Localization of these proteins in the cell membrane results in the activation of downstream cascades (9). Kinase AKT is a major effector of PI3K but other effectors with a role in cancer include kinase SGK, Ras family GTPase RAC1 and Rho family GTPase CDC42 (10, 11). PI3K consists of a catalytic and a regulatory sub-unit, with each having several isoforms (9). The alpha isoform of the catalytic sub-unit, p110α, encoded by gene *PIK3CA*, is mutated in a significant percentage of colorectal cancers and these mutations contribute to colorectal cancer propagation, through activation of effectors leading to cell proliferation and inhibition of apoptosis (12). In addition, two other pathways, the KRAS/BRAF/MAPK pathway and the WNT/APC/β-catenin pathway, that are also involved in colorectal cancer pathogenesis, co-operate with the PI3K pathway signaling to promote colorectal cancer (13).

The present investigation takes advantage of published genomic data to determine the genomic landscape and prognosis of *PIK3CA* mutated colorectal cancers without mutations in the extended RAS panel genes (triple wild type/*PIK3CA* mutated) and compares them with colorectal cancers that are wild type for all four genes (quadruple wild type). The overarching aim is to characterize common alterations, which could become therapeutic targets.

Materials and Methods

Genomic series. Four colorectal or colon cancer genomic series with publicly available data were interrogated for determining the prevalence of single nucleotide variants of interest and, when available, copy number alterations. These included the colorectal cancer study of The Cancer Genome Atlas (TCGA), the colorectal cancer study from the Dana Farber Cancer Institute (DFCI), a colorectal cancer cohort from the Memorial Sloan Kettering Cancer Center (MSKCC, MSK cohort) and a colon cancer genomic cohort published from the Sidra- LUMC AC-ICAM international collaboration (Sidra) (14-17). Different genomic platforms and pipelines were used in the four series for identification of molecular alterations (18). The studies of TCGA have used a standardized mutation calling that was derived by seven algorithms of mutation calling (MuTect, MuSE, VarScan2, Radia, Pindel, Somatic Sniper and Indelocator) used by various member institutions (18). The MuTect algorithm was used for somatic mutation calling in the DFCI and the Sidra studies (15, 17). The MSK study used the MutSigCV and the MuSiC algorithms for mutation calling (16, 19, 20). The groups of interest in the four cohorts consisted of cancers without mutations of the extended RAS gene panel (*KRAS*/*NRAS* and *BRAF*), with or without mutations in *PIK3CA* gene.

Data extraction and analysis from online databases. Analyses of the alterations in the four source genomic studies were performed in the cBioCancer Genomics Portal (cBioportal, http://www.cbioportal.org), a cancer genomics site maintained by MSKCC and other academic institutions (21, 22). The four genomic cohorts, which are included in cBioportal, were analyzed at the level of individual samples and of individual genes contained in the source studies. Molecular alterations available for analysis in the four colorectal series are point mutations in all four studies, copy number alterations in three studies (TCGA, MSK and Sidra) and structural gene alterations, such as fusions, in two studies (TCGA, MSK).

An aneuploidy score (AS) is provided in TCGA as a measure of chromosomal instability and was calculated as the sum of the number of chromosome arms that possessed either copy number gains or copy number losses in each sample. Chromosome arms were considered copy number altered, gained or lost, if there was a somatic copy number alteration, gain or loss, in more than 80% of their length, as measured by the ABSOLUTE algorithm from Affymetrix 6.0 SNP arrays (23). Chromosomal arms with somatic copy number alterations in 20% to 80% of the arm length were considered indeterminate and therefore not added in the denominator of the AS calculation. Chromosomal arms with somatic copy number alterations in less than 20% of the arm length were considered not altered. Another metric of chromosomal instability provided in TCGA is the Fraction Genome Altered (FGA) which is defined as the sum of the length of segments with log2 greater than 0.2 divided by the total length of all segments measured in the sample within source segment files

Statistical analysis. Statistical comparisons of categorical data were carried out with the Fisher exact test or the χ^2 test and comparisons of continuous parameters were performed with the Student *t* test. The log-rank test was used to compare Kaplan-Meier survival curves. All statistical comparisons were considered significant if $p<0.05$.

Results

In the colorectal cancer cohort of TCGA, the prevalence of *PIK3CA* mutations was 27.5% (147 of 534 patients profiled). Thirty-nine patients (7.3% of the profiled cohort and 26.5% of patients with *PIK3CA* mutations) had *PIK3CA* mutations without concomitant *KRAS*/*NRAS*/*BRAF* mutations (triple wild type/*PIK3CA* mutated group). The prevalence of *PIK3CA* hotspot mutations at codons E542, E545, Q546 and H1047 did not differ between cases with and without concomitant mutations in *KRAS*/*NRAS*/*BRAF* (Figure 1). The group of colorectal cancer patients with no *KRAS*/*NRAS*/*BRAF* or *PIK3CA* mutations (quadruple wild type) included 191 patients or 32.2% of the entire TCGA colorectal cancer cohort.

The group of quadruple wild type colorectal cancers did not differ from the triple wild type/*PIK3CA* mutated group in their mean age, sex prevalence, or distribution of cancer stages (Table I). Quadruple wild type cancers were more commonly located in the rectum (32.8% of cases) than triple wild type/*PIK3CA* mutated cancers (13.2%, Fisher's exact test $p=0.01$, Table I). Quadruple wild type cancers were also more often of the CIN phenotype (χ^2 test $p=0.005$) and had higher AS and FGA scores (although the latter did not reach statistical significance) as well as lower rates of elevated TMB (higher than 10 mutations/Mb, Fisher's exact test $p=0.01$) (Table I).

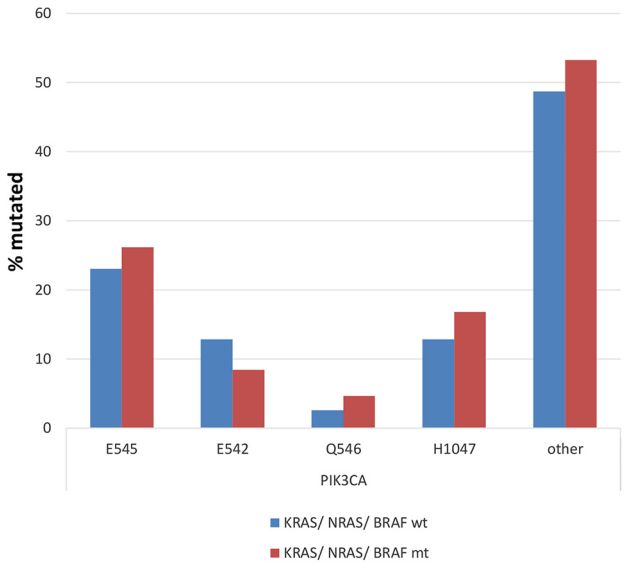

Figure 1. *Prevalence of PIK3CA hotspot mutations at codons E542, E545, Q546 and H1047 in colorectal cancers with and without concomitant mutations in KRAS/NRAS/BRAF. Differences in the prevalence of the hotspots' mutations were not statistically significant between the two groups. Data are from The Cancer Genome Atlas (TCGA). wt: Wildtype; mt: mutated.*

Compared with quadruple wild type colorectal cancer patients, patients with triple wild type/*PIK3CA* mutated cancers had no significantly different prevalence in other common colorectal cancer associated gene mutations, except for *ATM* and *AMER1*, which were significantly more prevalent in the latter group (Fisher's exact test p=0.05 and 0.003, respectively; Figure 2A). Higher mutation rates in many other of these commonly mutated genes were observed in the *PIK3CA* mutated group, which did not reach statistical significance, except for *ATM* and *AMER1* (Figure 2A). A notable exception was in *TP53* mutations, which were more prevalent in the group with quadruple wild type colorectal cancers (66.5% *versus* 51.3%, Fisher's exact test p=0.09; Figure 2A). Despite higher *TP53* mutation rates in quadruple wild type cancers, the expression of p53 target genes *BBC3* (encoding for apoptosis inducer PUMA), *PMAIP1* (encoding for apoptosis inducer NOXA), *FAS*, *TNFRSF10B*, *PIDD1* and *CDKN1A* (encoding for the cyclin dependent kinase inhibitor p21) were not significantly different between the two groups (Figure 3). Other less frequently mutated genes in colorectal cancers, such as the epigenetic modifiers *KMT2A*, *KMT2D*, *KMT2B* and *ARID1A*, genes encoding for polymerases epsilon (*POLE*) and theta (*POLQ*), *BRCA2*, and β-catenin had a higher prevalence in triple wild type/*PIK3CA* mutated cases (Fisher's exact test p<0.05 for all comparisons, Figure 4A). In addition, the genes of the PI3K kinase own pathway, *PIK3R1* and *MTOR*, had a higher prevalence in triple wild type/*PIK3CA* mutated cases (Fisher's exact test p<0.05 for both comparisons, Figure 4A).

Regarding copy number alterations, the most prevalent colorectal cancer amplification at locus 20q11 showed similar prevalence in patients with quadruple wild type colorectal cancers and with triple wild type/*PIK3CA* mutated cancers (12.8% *versus* 14.6%, Fisher's exact test p=0.49). The OS of patients with triple wild type/*PIK3CA* mutated cancers was not significant different from the OS of patients with quadruple wild type colorectal cancers (log rank p=0.3, Figure 5A). In addition, the OS of patients with mutations in *KRAS* or *NRAS* or *BRAF* was not different in the sub-groups with or without mutations in *PIK3CA* (log rank p=0.2, Figure 5B).

The DFCI cohort with a total of 619 patients included 304 patients (49.1%) without *KRAS*/*NRAS*/*BRAF* mutations. *PIK3CA* mutations were present in 47 of these patients (7.6% of the entire cohort and 15.5% of patients without *KRAS*/*NRAS*/*BRAF* mutations). Similarly to TCGA, the groups of colorectal cancer patients in the DFCI cohort without mutations in *KRAS*/*NRAS*/*BRAF* with or without *PIK3CA* mutations did not differ in their mean age, sex prevalence or cancer stages (Table II). The prevalence of high tumor grade and CpG island methylator phenotype (CIMP) were also not different in the two groups. The group with *PIK3CA* mutations had higher prevalence of MSI tumors (22% *versus* 8% in tumors of the quadruple wild type group, Fisher's exact test p=0.02) and a higher prevalence of cases with TMB above 10 mutations/Mb (29.8% *versus* 8.6% in the quadruple wild type group, Fisher's exact test p=0.0002).

Regarding additional mutations, similar to the TCGA cohort, triple wild type/*PIK3CA* mutated colorectal cancers in the DFCI cohort had a higher prevalence of *AMER1* mutations (17%) than quadruple wild type cancers (3.5%, Fisher's exact test p=0.001, Figure 2B). In contrast to TCGA, mutations in *FAT4* were also significantly more prevalent in the triple wild type/*PIK3CA* mutated group (23.4% *versus* 11.7% in the quadruple wild type group, Fisher's exact test p=0.03). *ATM* mutations displayed no statistically significant difference in prevalence between the two groups (Fisher's exact test p=0.69, Figure 2B). *TP53* mutations were more prevalent in the quadruple wild type group (59.9% *versus* 44.7%, Fisher's exact test p=0.05, Figure 2B). Epigenetic modifiers *KMT2A* and *KMT2D*, polymerases *POLE* and *POLQ*, *CTNNB1* and *BRCA2* mutations were more prevalent in the triple wild type/*PIK3CA* mutated group (Figure 4B).

A third colorectal cancer cohort examined, the MSK cohort, included 1516 patients. Eighty-two patients (5.4%) had *PIK3CA* mutations in the absence of *KRAS*/*NRAS*/*BRAF* mutations (triple wild type/*PIK3CA* mutated group) and 574 patients (37.9%) had all four genes in the wild type

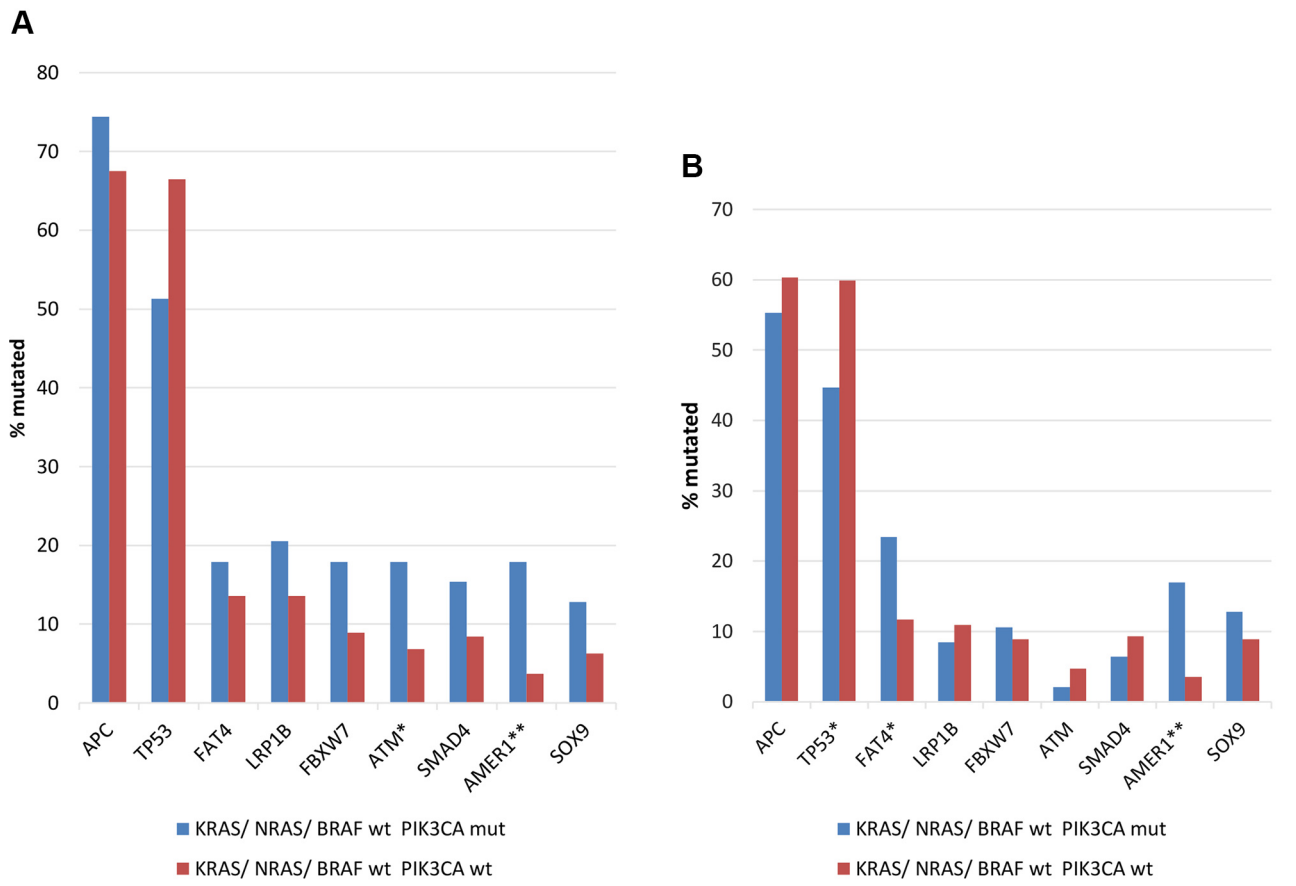

Figure 2. *Prevalence of colorectal cancer associated gene mutations in KRAS/NRAS/BRAF wild type, PIK3CA mutated (triple wild type/PIK3CA mutated) and quadruple wild type colorectal cancers from (A) The Cancer Genome Atlas (TCGA) and (B) the Dana Farber Cancer Institute (DFCI) cohort. In TCGA, mutations in AMER1 and ATM were significantly more prevalent in triple wild type/PIK3CA mutated cancers (Fisher's exact test p=0.003 and 0.05, respectively). In DFCI, mutations in AMER1 and FAT4 were significantly more prevalent in triple wild type/PIK3CA mutated cancers (Fisher's exact test p=0.001 and 0.03, respectively). TP53 mutations were more prevalent in the quadruple wild type group (Fisher's exact test p=0.05). *p<0.05 and **p<0.01. wt: Wild type; mt: mutated.*

configuration (quadruple wild type group). The two groups did not differ significantly in age, sex, prevalence of obesity or prevalence of metastatic disease (Table III). The triple wild type/*PIK3CA* mutated cancers group had a higher prevalence of right colon cancers and a higher prevalence of the MSI/POLE molecular subtype and of cases with high (above 10 mutations/Mb) TMB compared with the quadruple wild type group. In contrast, the triple wild type/*PIK3CA* mutated group had a lower prevalence of poorly differentiated cancers and of cases with a high FGA (Table III).

In the MSK cohort, mutations in *FBXW7*, *ATM* and *AMER1* were more frequent in the triple wild type/*PIK3CA* mutated group compared with the quadruple wild type group (Fisher's exact test p=0.03, 0.03 and 0.01, respectively; Figure 6A). In contrast, *TP53* were more common in the latter group (87.1% *versus* 69.5%, Fisher's exact test p=0.0001; Figure 6A). Similarly to the other cohorts, several epigenetic modifiers (*KMT2A*, *KMT2D*, *ARID1A*) displayed significantly more frequent mutations in triple wild type/*PIK3CA* mutated cancers (Figure 7A). In addition, other colorectal cancer associated genes, including *MTOR*, *CTTNB1*, *POLE* and *BRCA2*, with intermediate prevalence of mutations were more frequently mutated in the same group of triple wild type/*PIK3CA* mutated cancers (Figure 7A). Similar to TCGA, the OS of patients with triple wild type/*PIK3CA* mutated cancers in the MSK cohort was not significantly different from the OS of patients with quadruple wild type cancers (log rank p=0.5, Figure 8).

The Sidra-LUMC AC-ICAM cohort included a total of 348 patients among who 160 patients (46%) had quadruple wild type tumors and just 13 patients (3.7%) had triple wild type/*PIK3CA* mutated cancers. The groups were not different regarding their mean age, sex and stage. High TMB (above 10 mutations/Mb) was more frequent in *PIK3CA* mutant

Table I. *Characteristics of colorectal cancers in the entire TCGA cohort and in the subsets without KRAS/NRAS/BRAF mutations, with or without PIK3CA mutations. Percentages are shown in parentheses.*

Characteristic	Entire (n=594) (%)	KRAS/NRAS/BRAF wt		*p*-Value
		PIK3CA mt (n=39)	PIK3CA wt (n=191)	
Age				
Mean (±SD)	66.1±13.4	65.1±12	63.6±13.1	0.5
>65 years old	332 (56.1)	19 (50)	92 (48.2)	0.86
≤65 years old	260 (43.9)	19 (50)	99 (51.8)	
NA	2	1		
Sex				
Male	312 (52.7)	22 (57.9)	102 (53.4)	0.72
Female	280 (47.3)	16 (42.1)	89 (46.6)	
NA	2	1		
Stage				
I	104 (17.9)	6 (16.3)	34 (18.1)	0.3
II	220 937.9)	17 (45.9)	63 (33.5)	
III	170 (29.3)	12 (32.4)	62 (33)	
IV	86 (14.8)	2 (5.4)	29 (15.4)	
NA	14	2	3	
Primary location				
Colon	436 (74.1)	33 (86.8)	127 (67.2)	0.01
Rectal	152 (25.9)	5 (13.2)	62 (32.8)	
NA	6	1	2	
Subtype				
CIN	328 (71.4)	23 (76.7)	132 (91)	0.005
MSI	63 (13.7)	1 (3.3)	8 (5.5)	
GS	58 (12.7)	3 (10)	5 (3.5)	
POLE	10 (2.2)	3 (10)	0	
NA	135	9	46	
AS				
≤4	108 (18.4)	7 (17.9)	13 (6.9)	0.05
>4	478 (81.6)	32 (82.1)	176 (93.1)	
NA	8		2	
FGA				
≤0.08	118 (20.2)	6 (16.3)	14 (7.5)	0.11
>0.08	465 (79.8)	31 (83.7)	172 (92.5)	
NA	11	2	5	
TMB				
≤10 mutations/Mb	451 (84.5)	33 (84.6)	183 (95.8)	0.01
>10 mutations/Mb	83 (15.5)	6 (15.4)	8 (4.2)	
NA	60			

Wt: Wildtype; mt: mutated; CIN: chromosomal instability; MSI: microsatellite instability; GS: genomically stable; AS: aneuploidy score; FGA: fragment genome altered, TMB: tumor mutation burden; NA: not available.

patients (23.1% *versus* 9.7% in patients with quadruple wild type cancers), although not statistically significant, possibly due to the lower numbers of the *PIK3CA* mutated group (Fisher's exact test p=0.16, Table IV). The trends in the prevalence of mutations in several colorectal cancer associated genes in the two groups were similar to the other cohorts, although they did not reach statistical significance (Figure 6B and Figure 7B). Amplifications of the most commonly amplified locus of colorectal cancer at 20q11.21 were equally distributed in the two groups with or without *PIK3CA* mutations.

Discussion

PI3K kinase possesses a key position in the center of signal transduction cascades with colorectal cancer promoting functions, including proliferation, inhibition of apoptosis, stemness characteristics and metastasis (24). A main downstream target of PI3K is kinase AKT, which then regulates the activity of several effectors through phosphorylation (9). Examples include activation of the ubiquitin ligase MDM2, which is a negative regulator of tumor suppressor p53, phosphorylation and inhibition of pro-

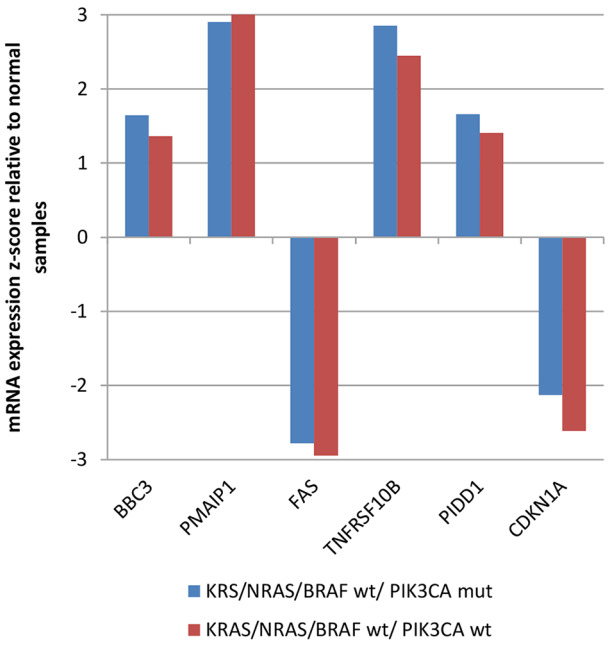

Figure 3. *mRNA expression (z-score related to normal samples) of p53 target genes in KRAS/NRAS/BRAF wild type, PIK3CA mutated (triple wild type/PIK3CA mutated) and quadruple wild type colorectal cancers from TCGA. wt: Wild type; mt: mutated.*

apoptotic protein BAD and activation of the mTORC1 complex. Moreover, AKT phosphorylates and inhibits kinase GSK3, which is a negative regulator of the WNT/β-catenin pathway, leading to an indirect activation of the pathway that may act synergistically with *APC* mutations. The PI3K/AKT pathway is also intertwined with the KRAS/BRAF/MAPK pathway and its inhibition contributes to decreased activity of MEK kinases (25). A third pathway frequently deregulated in colorectal cancer, the TGF-β/SMAD cascade, may also co-operate with the activation of PI3K/AKT to promote colorectal tumorigenesis (26). Molecular alterations occurring in all these pathways destabilize normal regulatory circuits and create the novel neoplasia-associated cellular networks that promote cancer hallmarks.

Mutations in the gene encoding for the alpha catalytic subunit of kinase PI3K, *PIK3CA*, are present in 20% to 25% of colorectal cancers (14-16). *PIK3CA* mutations are most often associated with mutations of the upstream RAS family members KRAS and less often NRAS, as well as mutations of their target kinase BRAF. A smaller number of colorectal cancers harbor *PIK3CA* mutations without concomitant *KRAS*/*NRAS*/*BRAF* mutations. These cases represent a distinct subgroup, which together with the group devoid of mutations in all four genes (*KRAS*/*NRAS*/*BRAF* and *PIK3CA*) do not benefit from new combination treatments targeting KRAS or BRAF. The landscapes of these two groups of colorectal cancers were explored in detail and compared in the current report. *PIK3CA* mutations without *KRAS*/*NRAS*/*BRAF* mutations (triple wild type/*PIK3CA* mutated cancers) were observed in 3.7% to 7.6% of colorectal cancers in the series examined and 32.2% to 39.9% of patients had tumors with wild type *KRAS*, *NRAS*, *BRAF* and *PIK3CA* (quadruple wild type). Triple wild type cancers with PIK3CA mutations had co-mutations of several epigenetic modifiers and DDR genes in a significant minority of cases. These mutations were observed with lower frequency in quadruple wild type colorectal cancers.

The association of *KRAS* mutations and lack of benefit from therapy with monoclonal antibodies blocking the EGFR receptor in colorectal cancer was discovered shortly after these therapies were introduced for the treatment of metastatic colorectal cancer (27, 28). Response rates with chemotherapy/cetuximab combinations were higher in metastatic *KRAS* wild type colorectal cancers than KRAS mutated cancers (29). Later, it became evident that mutations in *NRAS* and *BRAF* as well as mutations in *KRAS* outside the classic exon 2 mutations at codons 12 and 13 were also associated with lack of benefit from the addition of anti-EGFR antibodies to the chemotherapy backbone in metastatic colorectal cancer (30, 31). The median PFS in patients with KRAS exon 2 wild type tumors receiving 5-Fluorouracil/folinic acid/irinotecan (FOLFIRI) plus cetuximab was 11.1 months for tumors with no *KRAS* mutations in other exons or *NRAS* mutations *versus* 8.9 months in tumors with presence of non-exon 2 *KRAS* or *NRAS* mutations (32). Patients with localized colorectal cancer and exon 3 and 4 *KRAS* mutations or *NRAS* mutations have a better prognosis than patients with *KRAS* exon 2 mutations, suggesting a prognostic value of the specific location of these mutations, in addition to the predictive value for anti-EGFR based therapy response (33). Regarding *PIK3CA* mutations, a meta-analysis showed worse outcomes with anti-EGFR based therapies in patients with *PIK3CA* mutated colorectal cancers compared with patients with wild type *PIK3CA* colorectal cancers (34). This meta-analysis also suggested a differential effect of specific mutations, with resistance being associated mostly with exon 20 *PIK3CA* mutations. In a study that compared responses to chemotherapy and anti-EGFR monoclonal antibody therapy, patients with exon 2 *KRAS* wild type tumors had an overall response rate of 34.8%, while when all RAS/*BRAF* and *PIK3CA* mutations were considered, quadruple wild type patients had an overall response rate of 41.5% (35). These data confirm that patients with quadruple wild type tumors are the optimal candidates for anti-EGFR based combinations with chemotherapy.

In addition to potentially being resistant to anti-EGFR based therapies, triple wild type tumors with *PIK3CA* mutations have fewer targeting options as they would not be candidates for treatments with BRAF or specific KRAS inhibitor combinations with anti-EGFR monoclonal antibodies. In contrast to BRAF and KRAS G12C inhibitors, no inhibitors of PI3K in *PIK3CA*

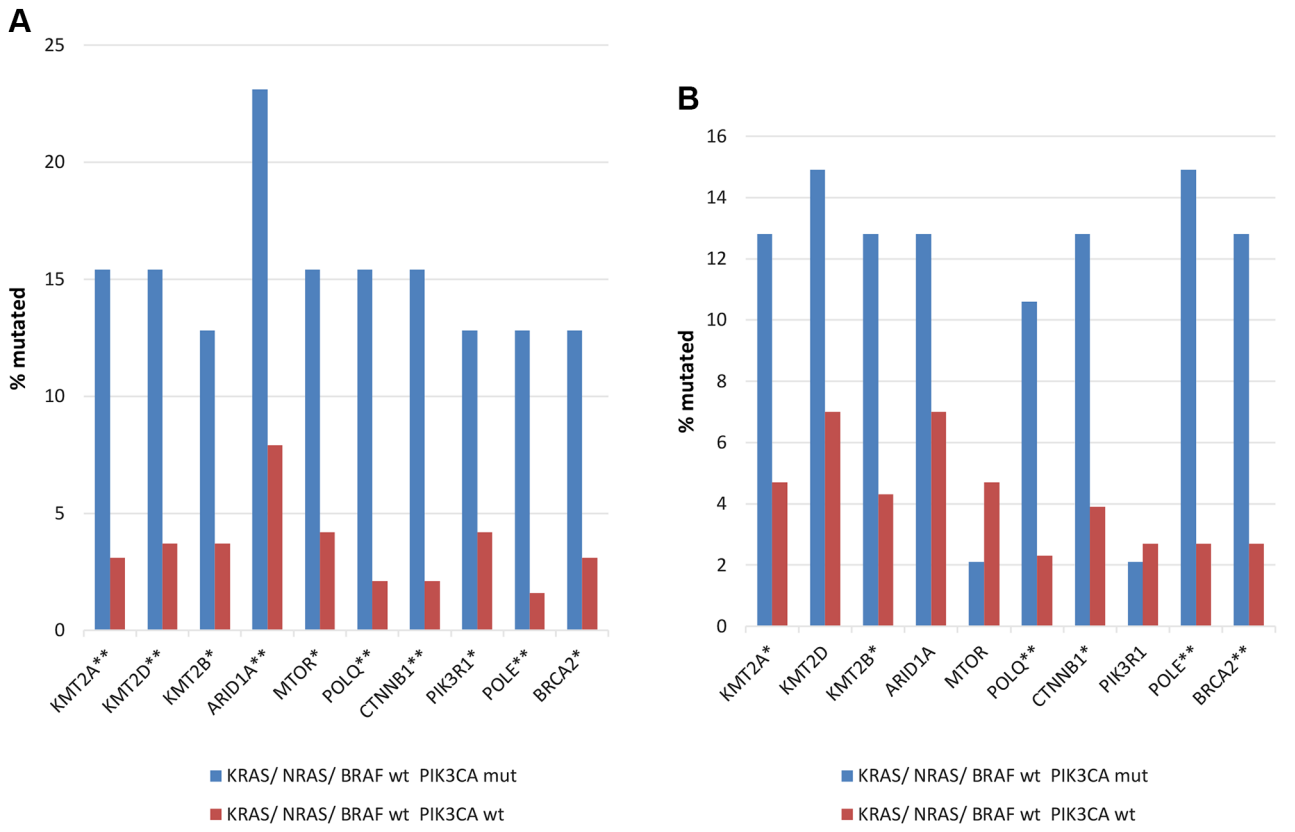

Figure 4. *Mutations in epigenetic modifiers KMT2A, KMT2D, KMT2B and ARID1A, mutations in genes encoding for polymerases epsilon (POLE) and theta (POLQ), in BRCA2, CTNNB1, PIK3R1 and MTOR in KRAS/NRAS/BRAF wild type, PIK3CA mutated (triple wild type/PIK3CA mutated) and in quadruple wild type colorectal cancers from (A) The Cancer Genome Atlas (TCGA) and (B) the Dana Farber Cancer Institute (DFCI) cohort. In TCGA, all genes had a higher prevalence in triple wild type/PIK3CA mutated cases (Fisher's exact test p<0.05 for all comparisons). In DFCI, epigenetic modifiers KMT2A and KMT2D, polymerases POLE and POLQ, CTNNB1 and BRCA2 mutations were more prevalent in the triple wild type/PIK3CA mutated group. The prevalence of PIK3R1 and MTOR mutations were not significantly different between the two groups. *p<0.05 and **p<0.01. wt: Wild type; mt: mutated.*

mutant colorectal cancers have been approved yet. Several inhibitors have been in early clinical trials showing low to moderate efficacy at best (36, 37). An arm with the alpha subunit specific PI3K inhibitor taselisib from the multi-arm NCI-MATCH basket trial showed no responses and a median PFS of 3.1 months (36). Median OS was 7.2 months. The trial included 61 evaluable patients with metastatic *PIK3CA* mutated cancers, among whom were seven colorectal cancer patients. Six colorectal cancer patients had stable disease and one patient had progressive disease as best response. These results together with the results of a randomized phase 3 trial in breast cancer patients, which showed minimal clinical benefit, led to the discontinuation of further development of taselisib (37). In another example, the alpha selective inhibitor alpelisib has been examined in early phase trials that included colorectal cancer patients (38). The first phase I study of alpelisib in cancers with *PIK3CA* mutations included an expansion cohort of 35 colorectal cancer patients (39). The overall response rate in these patients was 5.7%, (2 of 35 patients, both responses were partial). The disease control rate was 34.3% and the clinical benefit rate was 8.6%. Three fourths of colorectal cancer patients in this trial had concomitant mutations in *KRAS* (39). In another phase I trial that studied alpelisib in combination with capecitabine in metastatic colorectal and breast cancers, six patients with colorectal cancer were included (NCT04753203) (40). Three of the six patients had *PIK3CA* mutations and 4 had *KRAS* mutations. No responses were observed in colorectal cancer patients, who all had progressive disease as their best response. Therefore, current results suggest that PI3K inhibitors as monotherapy or as treatment in non-selected populations of colorectal cancer patients are minimally effective. A more selective development of these drugs in colorectal cancer in the smaller subset of patients with triple wild type *PIK3CA* mutant colorectal cancers, as defined in the current report would be a more rational way forward. Combinations with other targeted drugs in this group of patients

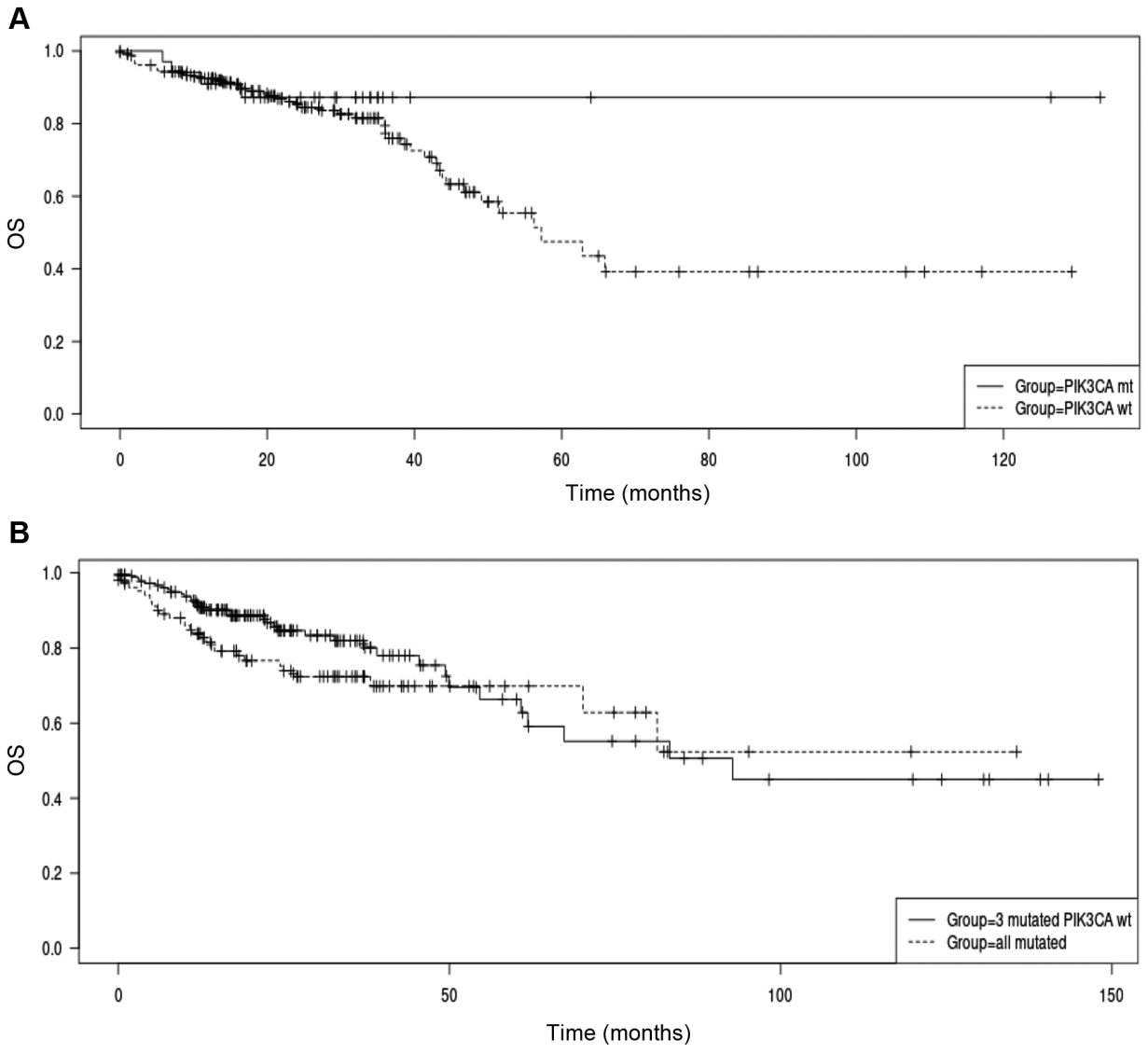

Figure 5. *(A) Overall survival (OS) of patients with triple wild type/PIK3CA mutated cancers (PIK3CA mt, solid line) and of patients with quadruple wild type colorectal cancers (PIK3CA wt, interrupted line, log rank p=0.3). (B) Overall survival (OS) of patients with mutations in KRAS or NRAS or BRAF without PIK3CA mutations (3 mutated PIK3CA wt, solid line) or with PIK3CA mutations (all mutated, interrupted line). Log rank p=0.2. Data are from TCGA. wt: Wild type; mt: mutated.*

will probably be needed to prevent emergence of drug resistance. A rational combination with EGFR targeting monoclonal antibodies, modeled after the respective combinations with activity in cancers with *BRAF* V600E mutations or *KRAS* G12C mutations could be envisioned (3, 4).

A new avenue for targeting of *PIK3CA* mutations arises with the discovery of next generation allosteric inhibitors that inhibit the tumor mutated/activated alleles present in tumor cells but not the normal PI3K present in normal tissues (41, 42). These inhibitors are devoid of the on-target adverse effects, which are a significant clinical hurdle in the therapy with orthosteric inhibitors.

Targeted treatment opportunities for quadruple wild type colorectal cancers, beyond chemotherapy combined with anti-EGFR monoclonal antibodies, may be guided by alterations that are present in this subset of colorectal cancers. Among the common colorectal cancer alterations, *TP53* mutations stand out as being the only frequent alterations that are consistently more prevalent in quadruple wild type cancers than in triple wild type cancers with *PIK3CA* mutations. *TP53* mutations occur in 57% to 87% of quadruple wild type colorectal cancers in the different series evaluated. A high pressure for acquiring mutations in this essential tumor suppressor may relate to the fact that

Table II. *Characteristics of colorectal cancers in the entire DFCI cohort and in the subsets without KRAS/NRAS/BRAF mutations, with or without PIK3CA mutations. Percentages are shown in parentheses.*

Characteristic	Entire cohort (n=619) (%)	KRAS/NRAS/BRAF wt		*p*-Value
		PIK3CA mt (n=47) (%)	PIK3CA wt (n=257) (%)	
Age				
Mean (±SD)	70.7±8.6	71.1±10.8	70±8.8	0.44
>65 yo	160 (25.9)	37 (78.7)	178 (69.3)	0.22
≤65 yo	457 (74.1)	10 (21.3)	79 (30.7)	
NA	2			
Sex				
Male	239 (38.6)	20 (42.6)	114 (44.4)	0.87
Female	380 (61.4)	27 (57.4)	143 (55.6)	
Stage				
I	152 (27)	10 (25)	73 (31.6)	0.77 (Stage I-III *versus* IV)
II	187 (33.2)	18 (45)	59 (25.6)	
III	159 (28.2)	9 (22.5)	74 (32)	
IV	65 (11.6)	3 (7.5)	25 (10.8)	
NA	56	7	26	
Grade				
Well-moderately	501 (91.4)	41 (93.2)	222 (93.7)	1
Poorly	47 (8.6)	3 (6.8)	15 (6.3)	
NA	69	3	20	
Primary location				
Cecum	114 (18.4)	7 (14,9)	34 (13.2)	0.01
Ascending to transverse	201 (32.5)	17 (36.2)	48 (18.7)	
Splenic flexure to sigmoid	166 (26.9)	16 (34)	89 (34.6)	
Rectal	137 (22.2)	7 (14.9)	86 (33.5)	
NA	1			
CIMP				
High	95 (19)	3 (7.7)	12 (5.6)	0.7
Negative/low	405 (81)	36 (92.3)	204 (94.4)	
NA	119	8	41	
MSI status				
MSI high	91 (17.2)	9 (22)	18 (8)	0.02
MSS	438 (82.8)	32 (78)	207 (92)	
NA	90	6	32	
TMB				
≤10 mutations/Mb	491 (79.3)	33 (70.2)	235 (91.4)	0.0002
>10 mutations/Mb	128 (20.7)	14 (29.8)	22 (8.6)	

Wt: Wildtype; mt: mutated; CIMP: CpG island methylator phenotype; MSI: microsatellite instability; TMB: tumor mutation burden; NA: not available.

quadruple wild type cancers do not possess mutations in PIK3CA which interfere with the function of wild type p53, through MDM2 activation (43). Although most mutations in *TP53* are pathogenic, they are not currently targetable. Attempts to target the mutated p53, which displays increased stability due to inability of ubiquitin ligase MDM2 to ubiquitinate it for proteasome degradation, have been met with limited success (44, 45). Despite years of intensive research, the relative relevance of mutated p53 gain of function cancer promoting properties as compared to loss of normal p53 function is not entirely resolved (45). Several inhibitors have advanced to early clinical development, but no inhibitor has been approved for clinical use. Alternative approaches for indirect targeting of p53 mutations are also being studied. One such effort involved targeting with a bispecific single chain antibody that binds concomitantly with the HLA-A2 allele presenting a peptide from the R175H mutant p53 on tumor cells and the T cell receptor (TCR) on cytotoxic T cells (46). The bispecific construct was effective in T cell activation for lysis of tumor cells bearing the mutation in *in vitro* and *in vivo* mouse models.

Table III. *Characteristics of colorectal cancers in the entire MSK cohort and in the subsets without KRAS/NRAS/BRAF mutations, with or without PIK3CA mutations. Percentages are shown in parentheses.*

Characteristic	Entire (n=1516) (%)	KRAS/NRAS/BRAF wt		*p*-Value
		PIK3CA mt (n=82)	PIK3CA wt (n=574)	
Age				
Mean (±SD)	50.8±12.9	50.2±10.9	48.7±11.9	0.28
>65 yo	226 (14.9)	10 (12.2)	59 (10.3)	0.56
≤65 yo	1287 (85.1)	72 (87.8)	515 (89.7)	
NA	3			
Sex				
Male	801 (54.8)	49 (63.6)	328 (59.1)	0.46
Female	660 (45.2)	28 (36.4)	227 (40.1)	
NA	55	5	19	
Stage				
I	53 (3.6)	4 (5.2)	26 (4.7)	0.32 (Stage I-III *versus* IV)
II	163 (11.2)	17 (22.1)	49 (8.8)	
III	378 (25.9)	16 (20.8)	157 (28.4)	
IV	865 (59.3)	40 (51.9)	322 (58.1)	
NA	57	5	20	
Grade				
Well-moderately	1124 (78.7)	68 (89.5)	427 (78.1)	0.02
Moder-Poorly	304 (21.3)	8 (10.5)	120 (21.9)	
NA	88	6	27	
Primary location				
Right	374 (25.8)	16 (21.3)	60 (10.9)	0.009
Left	664 (45.8)	45 (60)	321 (58.2)	0.03 (colon *versus* rectal)
Rectal	411 (28.4)	14 (18.7)	171 (30.9)	
NA	67	7	22	
BMI category				
Underweight	28 (1.9)	2 (2.6)	11 (2)	0.55
Normal weight	532 (36.7)	28 (36.4)	200 (36.9)	
Overweight	481 (33.2)	29 (37.7)	168 (31)	
Obese	408 (28.2)	18 (23.3)	163 (30.1)	
NA	67	5	32	
Molecular subtype				
MSI high	98 (6.7)	12 (15.6)	18 (3.2)	<0.0001 (MSI + POLE *versus* MSS)
MSS	1357 (92.4)	62 (80.5)	539 (96.8)	
POLE	13 (0.9)	3 (3.9)		
NA	48	5	17	
FGA				
≤0.08	506 (33.4)	32 (39)	141 (24.7)	0.007
>0.08	1007 (66.6)	50 (61)	430 (75.3)	
NA	3		3	
TMB				
≤10 mutations/Mb	1246 (88.7)	55 (67.1)	519 (92.3)	<0.0001
>10 mutations/Mb	158 (11.3)	27 (32.9)	43 (7.7)	
NA	112		12	

Wt: Wildtype; mt: mutated; BMI: body mass index; MSI: microsatellite instability; FGA: fragment genome altered; TMB: tumor mutation burden; NA: not available.

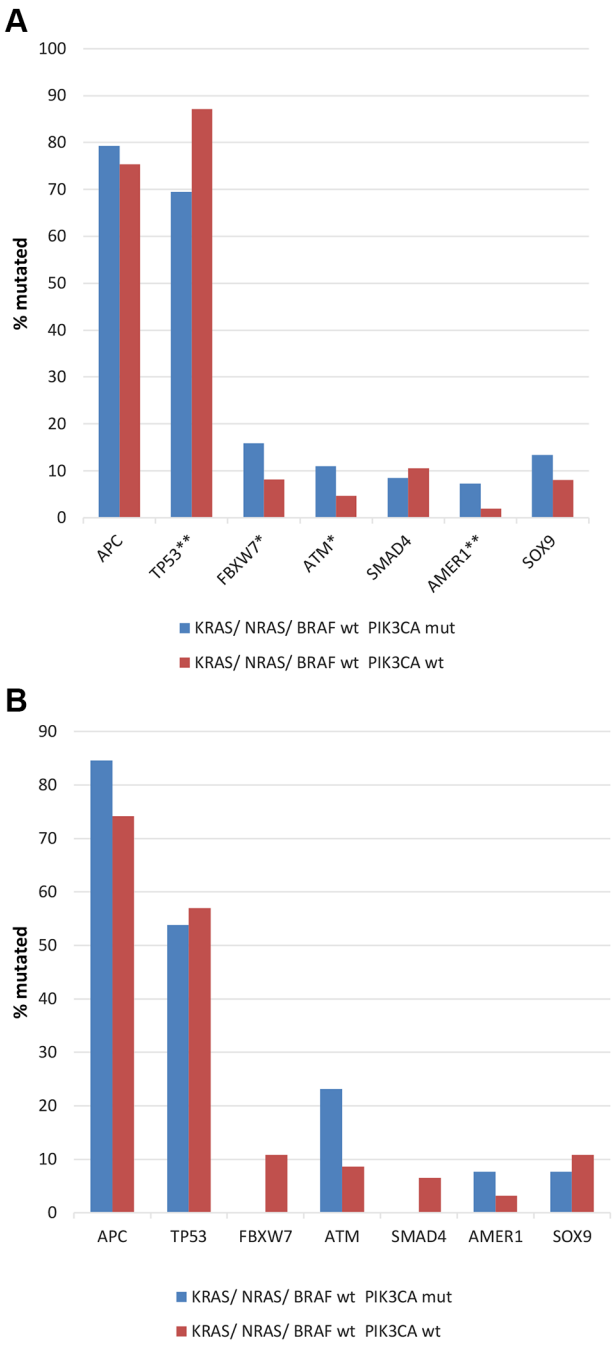

Figure 6. *Prevalence of colorectal cancer associated gene mutations in KRAS/NRAS/BRAF wild type, PIK3CA mutated (triple wild type/PIK3CA mutated) and quadruple wild type colorectal cancers from (A) the Memorial Sloan Kettering Cancer Center (MSK) cohort and (B) the Sidra-LUMC AC-ICAM cohort. In MSK, mutations in FBXW7, ATM and AMER1 were more frequent in the triple wild type/PIK3CA mutated group compared with the quadruple wild type group (Fisher's exact test p=0.03, 0.03 and 0.01, respectively). In contrast, TP53 were more common in the quadruple wild type group (Fisher's exact test p=0.0001). In the Sidra-LUMC AC-ICAM cohort, although several trends were similar, differences did not reach statistical significance. *p<0.05 and **p<0.01. wt: Wild type; mt: mutated.*

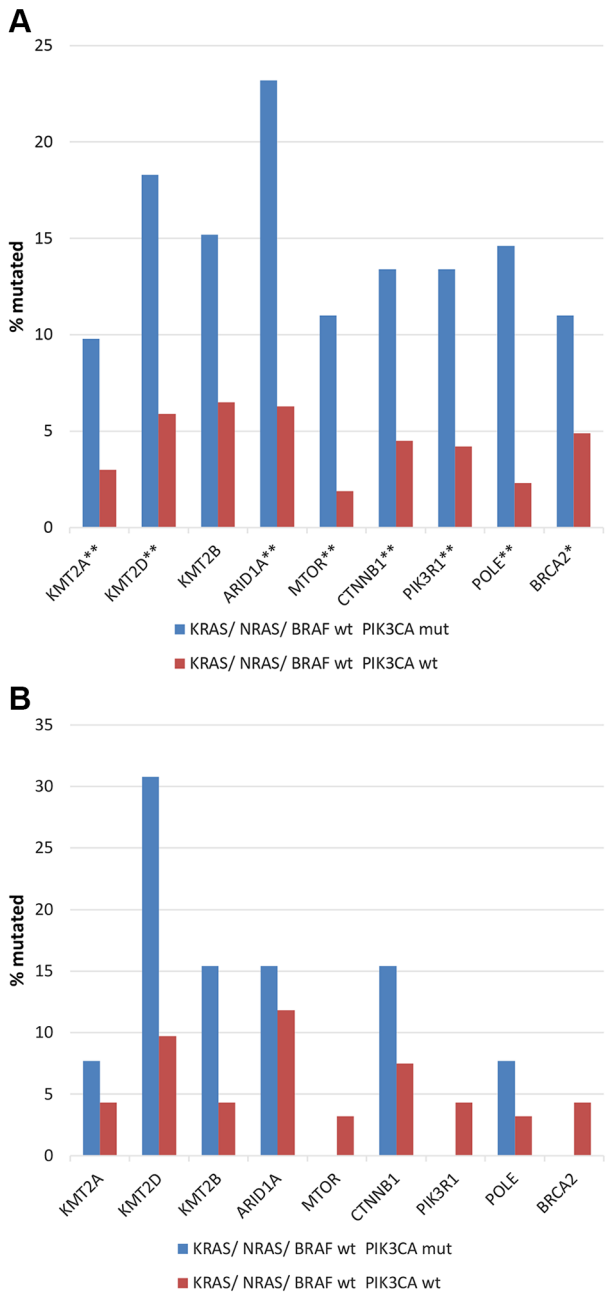

Figure 7. *Mutations in epigenetic modifiers KMT2A, KMT2D, KMT2B and ARID1A, genes encoding for polymerases epsilon (POLE) and theta (POLQ), BRCA2, CTNNB1, PIK3R1 and MTOR in KRAS/NRAS/BRAF wild type, PIK3CA mutated (triple wild type/PIK3CA mutated) and quadruple wild type colorectal cancers in (A) the Memorial Sloan Kettering Cancer Center (MSK) cohort and (B) the Sidra-LUMC AC-ICAM cohort. In MSK, epigenetic modifiers (KMT2A, KMT2D, ARID1A) displayed significantly more frequent mutations in triple wild type/PIK3CA mutated cancers than in quadruple wild type colorectal cancers (Fisher's exact test p=0.007, 0.0004 and 0.0001, respectively). MTOR, CTTNB1, POLE and BRCA2 were also more frequently mutated in the group of triple wild type/PIK3CA mutated cancers. In the Sidra-LUMC AC-ICAM cohort, although several trends were similar, differences did not reach statistical significance. *p<0.05 and **p<0.01. wt: Wild type; mt: mutated.*

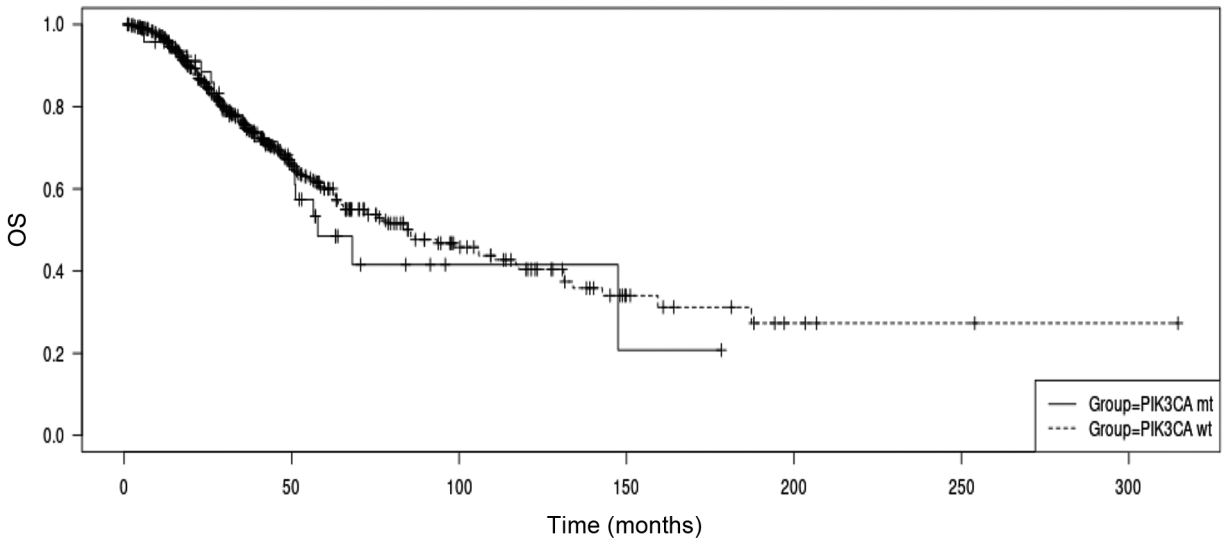

Figure 8. *Overall survival (OS) of patients with triple wild type/PIK3CA mutated cancers (PIK3CA mt, solid line) and of patients with quadruple wild type colorectal cancers from the MSK cohort (PIK3CA wt, interrupted line, Log Rank p=0.5). wt: Wild type; mt: mutated.*

Other common alterations, which have comparable prevalence in quadruple wild type colorectal cancers and in triple wild type cancers with *PIK3CA* mutations, are *APC* mutations. These occur in 60% to 75% of quadruple wild type colorectal cancers in the different series examined. The activation of the WNT/APC/β-catenin pathway that results from *APC* mutations is critical in colorectal cancer pathogenesis (24). Mutations in APC lead to increased stabilization of β-catenin, which enters the nucleus and acts a transcription co-factor, contributing to the increased expression of several tumor promoting genes (47). Similar to p53 mutations, the WNT/β-catenin signaling is at present not targeted pharmacologically with clinically approved drugs. Drugs in development, that include tankyrase inhibitors and porcupine inhibitors could be candidates for combining with PI3K inhibitors in selected patients with the targeted molecular alterations (48).

Mutations in DDR associated genes such as BRCA2, observed in a sizeable minority of triple wild type/*PIK3CA* mutated cancers could be targeted with PARP inhibitors. However, monotherapy with this class of drugs has only shown minimal activity in colorectal cancers with homologous recombination defects and therefore combination approaches taking into consideration additional molecular alterations such as PIK3CA mutations will be required (49).

The most frequent copy number alteration in colorectal cancer, amplification at chromosome locus 20q11.21 is more prevalent in quadruple wild type and triple wild type/ *PIK3CA* mutated cancers (12.8% and 14.6%, respectively) than in colorectal cancers with extended RAS mutations where the 20q11.21 amplification prevalence is 7% to 9% (50). Several candidate oncogenes have been identified in the amplified locus and could serve as therapeutic targets in these cancers.

Conclusion

In conclusion, colorectal cancers without extended RAS mutations, with or without *PIK3CA* mutations represent subsets of the disease with targetable alterations. Besides the established treatment with chemotherapy combinations with anti-EGFR monoclonal antibodies, other rational combinations selected according to the presence of targetable alterations will be required and will also be facilitated by the discovery of more effective targeted drugs (51). A personalized approach based on these principles, replacing the traditional one-size-fit-all approach for drug therapies will increase the probability of successful clinical development.

Conflicts of Interest

None to be disclosed.

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Table IV. *Characteristics of colorectal cancers in the entire Sidra-LUMC AC-ICAM cohort and in the subsets without KRAS/NRAS/BRAF mutations, with or without PIK3CA mutations. Percentages are shown in parentheses.*

Characteristic	Entire (n=348) (%)	KRAS/NRAS/BRAF wt		*p*-Value
		PIK3CA mt (n=13)	PIK3CA wt (n=160)	
Age				
Mean (±SD)	68.2±11.5	69.8±10	67.2±12.1	0.45
>65 years old	233 (67)	10 (76.9)	101 (63.1)	0.38
≤65 years old	115 (33)	3 (23.1)	59 (36.9)	
Sex				
Male	182 (52.3)	7 (53.8)	91 (56.9)	1
Female	166 (47.7)	6 (46.2)	69 (43.1)	
Stage				
I	55 (15.8)	7 (53.8)	15 (9.4)	
II	122 (35.1)	3 (23.1)	58 (36.3)	0.48 (Stage I-III *versus* IV)
III	110 (31.6)	2 (15.4)	57 (35.5)	
IV	61 (17.5)	1 (7.7)	30 (18.8)	
Primary location				
Cecum-ascending colon-hepatic flexure	158 (45.4)	5 (38.5)	53 (33.1)	0.76
Transverse colon-splenic flexure-descending colon-sigmoid	190 (54,6)	8 (61.5)	107 (66.9)	
Consensus Mol Subtype				
CMS1	43 (12.4)	0	8 (5)	0.85
CMS2	76 (21.8)	4 (30.8)	53 (33.1)	
CMS3	66 (19)	0	18 (11.3)	
CMS4	82 (23.6)	4 (30.8)	46 (28.7)	
Mixed	81 (23.3)	5 (38.5)	35 (21.9)	
FGA				
≤0.08	88 (31.3)	3 (23.1)	12 (12.9)	0.39
>0.08	193 (68.7)	10 (76.9)	81 (87.1)	
NA	67		67	
TMB				
≤10 mutations/ Mb	208 (74)	10 (76.9)	84 (90.3)	0.16
>10 mutations/ Mb	73 (26)	3 (23.1)	9 (9.7)	
NA	67		67	

Wt: Wildtype; mt: mutated; CMS: consensus molecular subtype; FGA: fragment genome altered; TMB: tumor mutation burden; NA: not available.

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Received May 11, 2024
Revised June 15, 2024
Accepted June 17, 2024