Original Article

# Prognostic evaluation of *KRAS* and *BRAF* mutations in relation to Mismatch Repair protein status: preliminary survival analysis of patients with Colorectal Cancer

## Valutazione prognostica delle mutazioni di *KRAS* e *BRAF* in relazione allo stato delle proteine del *Mismatch Repair*: analisi preliminare di sopravvivenza dei pazienti con adenocarcinoma colorettale

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**Key words:** Colorectal Cancer, survival analysis, MAPK pathway, Mismatch Repair.

## ABSTRACT

**Aims:** prognostic impact of mutations in *BRAF* and *KRAS* genes in patients with colorectal cancer was evaluated to detect variations in survival among patients with these mutations, in relation to different morphologic features identified at diagnosis and the expression of mismatch repair proteins. The final purpose of the project will be to establish models to predict variations in survival in different subsets of patients with colorectal cancer.
**Materials and Methods:** partial survival analyses were conducted on 32 subjects in relation to three different variables: mutation in *BRAF* or *KRAS* genes, expression of mismatch repair system and morphological features of the tumor.
**Results:** preliminary results of this study provide evidence that survival in different subsets of patients with colorectal cancer can be influenced by several factors, which include, in addition to immunohistochemical and molecular investigations, the assessment of different morphological features.
**Conclusions:** Integrating different characteristics may reveal which factors have a more significant impact on survival in these subgroups. More patients will be needed to design different survival models and to check these preliminary results more effectively.

**Obiettivi:** l'impatto prognostico delle mutazioni nei geni *BRAF* e *KRAS* nei pazienti con tumore del colon-retto è stato valutato per individuare le variazioni di sopravvivenza tra i pazienti con queste mutazioni, in relazione alle diverse caratteristiche morfologiche identificate alla diagnosi ed all'espressione delle proteine di Mismatch Repair. Lo scopo finale del progetto sarà quello di stabilire modelli per prevedere le variazioni di sopravvivenza in diversi sottoinsiemi di pazienti con tumore del colon-retto.
**Materiali e Metodi:** sono state condotte analisi di sopravvivenza parziale su 32 soggetti in relazione a tre diverse variabili: mutazione nei geni *BRAF* o *KRAS*, espressione delle proteine dei Mismatch Repair e caratteristiche morfologiche del tumore.
**Risultati:** i risultati preliminari di questo studio dimostrano che la sopravvivenza in diverse sottopopolazioni di pazienti con tumore del colon-retto può essere influenzata da diversi fattori, che includono, oltre alle indagini immunoistochimiche e molecolari, la valutazione di diverse caratteristiche morfologiche.
**Conclusioni:** l'integrazione di diverse caratteristiche può rivelare quali fattori abbiano un impatto più significativo sulla sopravvivenza in questi sottogruppi. Sarà necessario un numero maggiore di pazienti per progettare diversi modelli di sopravvivenza e verificare in maniera più efficace questi risultati preliminari.



## Introduction

Colorectal Cancer (CRC) is a major health problem in Western countries, representing the third most diagnosed malignancy in men, and the second in women,[1] and with more than 2 million people worldwide affected each year.[2] Despite recent advances in new therapies, overall 5-year survival for this malignancy stands at about 50%, highlighting the need to develop early diagnostic, prognostic, and predictive biomarkers that can be used in routine clinical practice to reduce the morbidity and mortality associated with this disease.[3]

Most colorectal carcinomas (70-80%) are sporadic and originate from precancerous lesions termed polyps. In contrast, a minority (20-30%) of colorectal carcinomas develop because of a genetic predisposition:[4] the most common inherited form of colorectal cancer is Lynch syndrome. An additional group, constituting 1-2% of

all cases, evolves because of chronic inflammatory bowel disease, such as Crohn's disease and ulcerative rectocolitis.[5]

The molecular events underlying the development of colorectal adenocarcinoma are highly variable and include not only genetic but also epigenetic abnormalities. In fact, progression from adenoma to carcinoma is manifested by three main phenotypes: Chromosomal Instability (CIN), which characterizes most colorectal cancers, Microsatellite Instability (MSI) leading to instability of the DNA mismatch repair system,[6] and the phenotype caused by epigenetic DNA modifications due to hypermethylation of CpG islands (CIMP)[7].

MSI is due to loss of DNA Mismatch Repair (MMR) activity and accounts for about 12-15% of all colorectal carcinomas. Of these, 2-3% of MSI, CRCs are associated with Lynch syndrome, while the rest represent the sporadic or acquired form of the disease.

MSI results in the accumulation of insertion and/or deletion mutations within microsatellite DNA regions. As a result, mismatches that occur during DNA replication cannot be corrected and mutations accumulate in short repetitive stretches called microsatellites.[8] These DNA sequences, distributed throughout the genome due to their characteristic repetitive structure, are particularly inclined to replication errors. Deficit in the repair system leads to increased mutational rate: the result is an increased endogenous immune response, prerogative to increased sensitivity to immunotherapy.[9]

Mutations in *KRAS* and *BRAF* genes are statistically significantly associated with both Disease-Free Survival (DFS) and Overall Survival (OS) in stage II and III tumors.[10] However, the prognostic value of these mutations within subsets with Microsatellite Stability (MSS) or instability remains controversial.[11]

*KRAS* mutations are found in 30-50% of patients with CRC. Ninety percent of these occur in codons 12 and 13 within the second exon (G12/13 changed to valine),[12] causing constitutive activation of the Mitogen-Activated Protein Kinase (MAPK) cascade. There are conflicting results regarding the association between *KRAS* mutations and prognosis of CRC.[13] Stratification according to primary tumor location and according to the state of instability (MSI) in stages I-III suggests that the negative prognostic effect of *KRAS* mutations is found for tumors with left-sided localization and in the absence of instability (MSS).[14] In metastatic CRC, patients with *KRAS* mutations are currently not candidates for anti-EGFR therapy.[15]

*BRAF* mutations have also been well studied for their prognostic and predictive roles in CRC. *BRAF* is a serine/threonine RAF family isoform downstream of *KRAS* in the MAPK/ERK[16] pathway and is mutated in about 10% of CRC patients, with primary mutations at residue 600 resulting in valine to glutamate substitution (V600E) in most CRCs with *BRAF* mutation.[17] *BRAF V600E* mutations often occur in patients with MSI and elevated CIMP. *BRAF V600E* is associated with a shorter OS across tumor stages, with a negative prognostic impact that is most evident in tumors with MSS sited on the left side.[18] Previous studies have suggested that *BRAF* and *KRAS* mutations are independent (mutually exclusive) of each other and do not occur simultaneously. However, this scientific conception has recently been updated as cases of colorectal cancer with concomitant mutation have been reported.[19] The role of *KRAS* and *BRAF* mutations in the survival of patients with these cancers remains controversial. Some studies confirm the role of these genes as prognostic biomarkers, while others are inconclusive.

To this end, in this pilot study we aimed to perform a prognostic evaluation on overall and disease-free survival in patients with colorectal adenocarcinoma presenting mutation in the *KRAS* and/or *BRAF* gene in relation to mismatch repair protein expression, recruited to Oncology Unit of ASL AL. There are already studies in literature that have evaluated survival by linking the presence of mutations with the stability or instability of microsatellites. The innovative character of the current study lies in the intention to identify possible differences in the survival of stable and unstable patients, based on individual delete proteins, to design different survival models that are based not only on molecular characteristics and thus on the mutations investigated, but also considering the morphologic features that characterize the tumor. For this reason, further analyses, in addition to the preliminary ones reported in this article, will be conducted at the conclusion of the study to characterize patients regarding: i) mutations in *KRAS* and *BRAF* genes and correlation of mismatch repair protein expression status in immunohistochemistry; ii) survival analysis in relation to single delete proteins, where there is no expression of these proteins; iii) survival analysis by relating previous data to morphological features of the tumor, such as: site of location, grade of differentiation, histotype, tumor budding, vascular invasion, perineural invasion, and presence of metastasis; iv) finally, survival will be assessed considering clinical data, such as therapies and radiological reassessments.

We investigated for this pilot study all the cohort of colorectal adenocarcinomas diagnosed in Surgical pathology of ASL AL in which the mutations under study were present, in a period between January 2018 and December 2022.

A sample size of about 40 patients eligible for the inclusion criteria in the study was estimated.

Subjects eligible for the study were enrolled upon signing of informed consent during the follow-up visits conducted at the oncology Unit of Alessandria Local Health Authority.

Subsequent to enrollment, data referring to demographic variables, tumor morphology at diagnosis and on type of treatment performed were collected through access to the health care provider's information systems.

## Materials and Methods

Subjects enrolled for this study had a diagnosis of colorectal cancer performed at the Surgical Pathology at Local Health Authority of Alessandria in a period from January 2018 to December 2022, with *KRAS* and/or *BRAF* mutation gene.

Variables collected for the purpose of analysis were input into electronic data collection forms (eCRFs) making use of REDCap platform.

### Ethical approval

This study is a retrospective, monocenter, non-industrial observational study in accordance with M.D. 30/11/2021 (No-Profit trial). It was sponsored by Research and Innovation Department (DAIRI), "SS. Antonio and Biagio and Cesare Arrigo University Hospital of Alessandria".

The study, identified with the acronym "MAPK-colon", was approved by Local Ethics Committee, and complied with the Declaration of Helsinki and Good Clinical practice guidelines. Participants provided written informed consent.

### *Statistical analysis*

Statistical analysis of the partial data was conducted by SS. Clinical Epidemiology and Biostatistics from Research and Innovation Department of SS. Antonio and Biagio and Cesare Arrigo University Hospital of Alessandria and Local Health Authority of Alessandria.

Data collected for the analysis were processed anonymously and aggregated. A descriptive analysis of the data was performed by representing the continuous variable referring to age as median and Interquartile Range (IQR), following its distribution. The categorical variables were represented within the frequency table (Table 1, Table 2) as absolute frequency and percentage frequency. Statistical analyses were conducted using the Log Rank statistical test and a 5% significance level was selected. Patient survival analyses were conducted using the Kaplan-Meier method. All analyses were performed with R software, version 4.2.2.

## Results

Overall survival was evaluated in relation to the variables collected for morphological features, mutations, and expression of mismatch repair proteins.

Preliminary analyses were performed on 32 subjects enrolled in the study. To date, 17 men and 15 women have been enrolled. Age at diagnosis shows a median of 68.5 years, IQR (60.5-73.0).

For every recruited patient, the presence of mutations in *KRAS* gene and/or the punctiform mutation in *BRAF V600E* gene was assessed. In the cases analyzed, 19 mutations in *KRAS* gene (codons 12, 13, 61 and 146) were found while 13 *BRAF V600E* mutation positivity cases were detected.

The expression of mismatch repair proteins, detected by immunohistochemical investigation, was also investigated for each patient. Preliminary data on the cohort enrolled until now identifies 5 cases of MSI, almost all of them with deletion of LMH1/PMS2 dimeric complex.

As required by the protocol, data were concern morphological variables, identified at post-surgery diagnosis, were finally collected. From the analyzed cohort, 25 of 32 cases were evaluated. Of these, the frequencies referring to the individual morphological features of interest were collected (Table 2), which are absent in 7 individuals who underwent diagnostic biopsy only.

Survival curves were subsequently evaluated by holding the individual morphological features previously reported and relating them to mutation pattern and mismatch repair protein expression status. Variables were then merged into a single curve to evaluate the impact on survival by considering both mutations, mismatch repair expression and morphological characteristics under investigation, to assess whether the integration of these elements would have a different impact on the survival of patients with colorectal cancer.

Survival analysis of morphological features tumor budding, in comparison to mismatch repair expression and mutation in *KRAS* and *BRAF* genes, did not reveal statistically significant results ($p>0.05$).

Survival curves related to other morphological variables, such as histological grade, vascular invasion, histotype, metastasis and site of location, showed statistically significant results when these morphological characteristics are associated with mismatch repair expression (Figure 1) and when both variables are integrated with *KRAS* and *BRAF* mutations (Figure 2).

Analysis of these morphological features in association with

**Table 1.** Frequency of mutations and expression of Mismatch Repair.

| Characteristic | N=32 |
|---|---|
| Sex | |
| Male | 17 (53.1%) |
| Female | 15 (46.9%) |
| Age (years) | 68.5 (60.5-73.0) |
| Gene mutation | |
| *BRAF* | 13 (40.6%) |
| *KRAS* | 19 (59.4%) |
| Tumor located in one or more colon site | |
| Single-site | 22 (68.8%) |
| Multi-site | 10 (31.3%) |
| Mismatch Repair System | |
| MSS | 27 (84.4%) |
| MSI | 5 (15.6%) |

MSS, microsatellite stability; MSI, microsatellite instability.

**Table 2.** Morphological features of colorectal cancer at diagnosis.

| Morphological characteristics | Resection performed (N=25) | Resection not performed (N=7) |
|---|---|---|
| Histotype | | |
| NOS | 13 (52.0%) | 0 (NA%) |
| Mucinous | 10 (40.0%) | 0 (NA%) |
| Neuroendocrine | 1 (4.0%) | 0 (NA%) |
| Another subtype | 1 (4.0%) | 0 (NA%) |
| Missing | 0 | 7 |
| Grade | | |
| G1 | 1 (4.0%) | 0 (NA%) |
| G2 | 11 (44.0%) | 0 (NA%) |
| G3 | 13 (52.0%) | 0 (NA%) |
| Missing | 0 | 7 |
| Tumor budding | | |
| High grade | 20 (80.0%) | 0 (NA%) |
| Low grade | 3 (12.0%) | 0 (NA%) |
| Not evaluable | 2 (8.0%) | 0 (NA%) |
| Missing | 0 | 7 |
| Vascular invasion | | |
| Present | 13 (52.0%) | 0 (NA%) |
| Absent | 8 (32.0%) | 0 (NA%) |
| Not evaluable | 4 (16.0%) | 0 (NA%) |
| Missing | 0 | 7 |
| Perineural invasion | | |
| Present | 12 (48.0%) | 0 (NA%) |
| Absent | 5 (20.0%) | 0 (NA%) |
| Not evaluable | 8 (32.0%) | 0 (NA%) |
| Missing | 0 | 7 |
| Metastasis | | |
| Presents | 20 (80.0%) | 0 (NA%) |
| Absents | 5 (20.0%) | 0 (NA%) |
| Missing | 0 | 7 |
| Tumor location | | |
| Single site | 16 (64.0%) | 6 (85.7%) |
| Multi-site | 9 (36.0%) | 1 (14.3%) |

NA, not available; NOS, not otherwise specified.

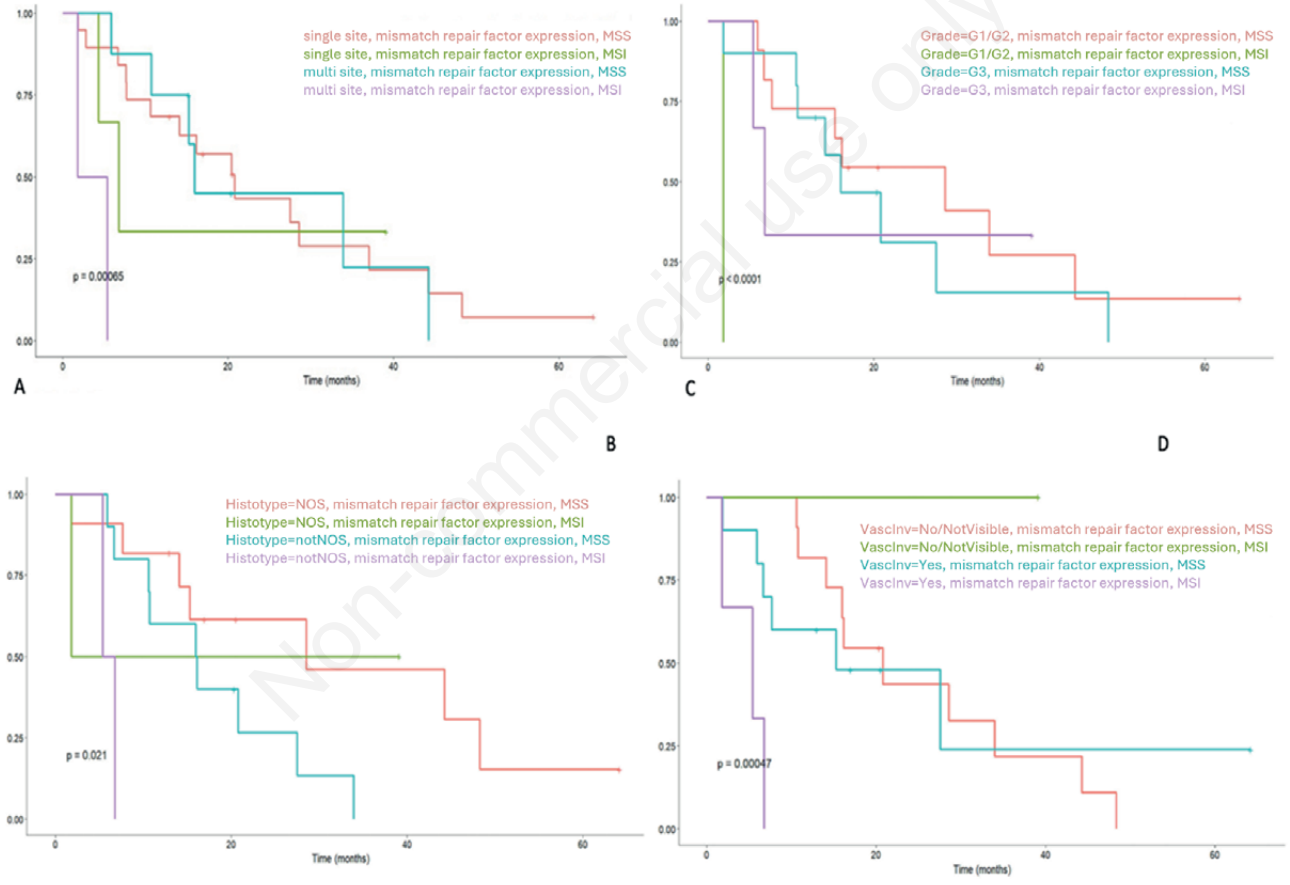
only *BRAF* and *KRAS* mutation, on the other hand, did not provide statistically significant results (p>0.05). Regarding morphological variables relating to perineural invasion, statistically significant results were found in all three survival curves (Figure 3).

## Discussion

As mentioned previously, colorectal carcinogenesis follows three main mechanisms: Chromosomal Instability (CIN), epigenetic DNA modifications (CIMPs) and instability of the DNA mismatch repair system.[6]

With regard to MSI, when this repair system is intact and efficient, MMR proteins identify and correct DNA mismatches caused by DNA polymerase during replication, working in pairs and forming MutLα (MLH1 and PMS2) and MutSα (MSH2 and MSH6) complexes, respectively. MutSα recognizes the single wrong base pair, creates a sliding clamp around the DNA and binds MutLα complex. This system interacts with many enzymes, including DNA polymerase, to excision the single mismatch and resynthesize DNA strand.[20]

These MSI, which occurs in some colorectal carcinomas, can occur as a result of two different events: an inherited germline mutation in one allele followed by somatic inactivation of wild-type allele (in case of Lynch syndrome) or somatic inactivation of both alleles.[21]




NOS, not otherwise specified.

**Figure 1.** Survival analysis for different morphological features in correlation with Mismatch Repair protein expression. A) Survival curves for morphological variable "site of location". The curve shows a reduced survival for the subgroup with multi-site colon-rectal location and Microsatellite Instability (<10 months) compared to the unstable subgroup with single-site tumor location (40 months). B) Survival curves for "histotype" variable. The analysis shows a reduction in survival in the subgroup of patients with instability with non-NOS histotype (not otherwise specified). Survival for the unstable subgroup with NOS tumor histotype is higher than for patients with a non-NOS histotype but Microsatellite Stability (40 months *vs* 35 months). C) Survival curves for morphological variable 'histological grade'. There is a clear reduction in survival for the subgroup with Microsatellite Instability related to a histological grade lower than G3, which is also explained by the small number of patients in this subgroup. Survival for the unstable subgroup with histological grade G3 is around 40 months, compared with patients with the same grade but no instability (about 50 months). D) Survival curves for morphological variable 'vascular invasion'. The curve for subjects with vascular invasion in association with Microsatellite Instability is lower than the subset with instability but no vascular invasion (10 months *vs* 40 months). The curve for stable subjects presenting vascular invasion shows a significantly higher survival curve (>60 months).

MSI can be detected by Polymerase Chain Reaction (PCR), while in immunohistochemistry it is possible to identify the loss of one or more mismatch proteins (MLH1, PMS2, MSH2, MSH6). Mutations in the *KRAS* and *BRAF* genes are statistically significantly associated with both Disease-Free Survival (DFS) and Overall Survival (OS) in stage II and III tumours.[10] However, the prognostic value of these mutations within subgroups with MSS or instability remains controversial.[11]

Molecular characterization of colorectal cancer plays a key role in the choice of therapeutic strategy, particularly in the setting of metastatic colorectal cancer. In these patients, mutational status of *KRAS* and *NRAS* genes is assessed before starting treatment. Indeed, in case of somatic mutations in these genes, there is a mechanism of resistance to anti-EGFR[22] monoclonal antibodies.

*KRAS* mutations are encountered in 30-50% of CRC patients and 90% of these occur in codons 12 and 13 within the second exon (G12/13 changed to valine).[12] Results regarding the associa-

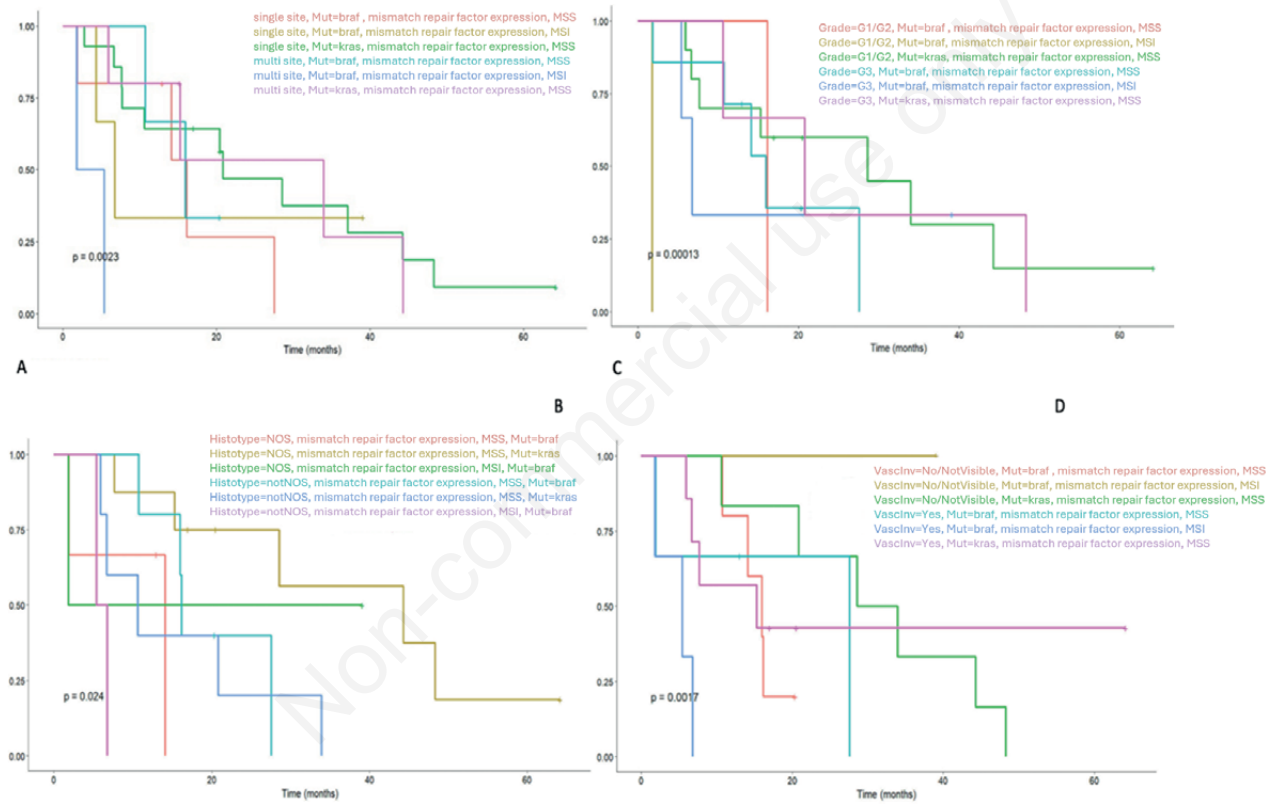



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**Figure 2.** Survival analysis for different morphological features in correlation with Mismatch Repair protein expression and mutation in BRAF and KRAS gene. A) Survival curves for morphological variable 'site of location'. Patients with BRAF mutation related to instability and multi-site tumor location have a significantly reduced survival (<10 months) compared to BRAF mutated unstable subgroup with single-site tumor location (40 months). Compared to the last group, BRAF mutated patients with single-site location but no Microsatellite Instability show a reduced survival curve (40 months *vs* 25 months). For stable mutated KRAS subgroups with both single- and multi-site location, survival is clearly increased. B) Survival curves for morphological variable 'histotype'. The unstable mutated BRAF subgroup with non-NOS histotype has a reduced survival curve compared to the unstable BRAF group with NOS histotype (10 months *vs* 40 months). The subgroup with BRAF mutation, NOS histotype but no Microsatellite Instability also showed a reduced survival compared to the unstable mutated BRAF counterpart with a more aggressive non-NOS histotype (15 months *vs* 30 months). C) Survival curves for morphological variable "histological grade". The unstable BRAF mutated subgroup with a grade below G3 has a reduced survival (also justified by the small number of subjects) compared to the counterpart with no instability (<20 months). Among the stable G3 grade subgroups, the sub-cohort of BRAF mutated patients has a lower survival compared to KRAS mutated counterpart (30 months *vs*. 50 months). D) Survival curves for morphological variable 'vascular invasion'. There is a reduction in survival for the unstable mutated BRAF subgroup with evidence of vascular invasion compared to the group with Microsatellite Stability (10 months *vs* 30 months). The graphic also shows a reduction in survival for BRAF mutated subjects without vascular invasion but Microsatellite Stability, compared to the unstable counterpart (20 months *vs* 40 months).

tion between *KRAS* mutations and CRC prognosis remain conflicting.[13] Regarding stratification according to primary tumor location and instability status (MSI) in stages I-III there is a greater negative prognostic effect of KRAS mutations when the tumor is located on the left side and there is no instability (MSS).[14]

Another molecular evaluation concerns the analysis of the mutational status of *BRAF*, a protein belonging to serine/threonine kinase family. In normal cells, *BRAF* activation occurs because of many complex processes involving protein and ligand binding, conformational changes, and numerous regulatory phosphorylation events.[23] *BRAF* mutation in codon 600 leads to a substitution of a valine for a glutamic amino acid, which results in a constitutive activation of *BRAF* protein and the subsequent elements of the MAPK cascade.[24] *BRAF* mutations are considered oncogenic driver mutations as they occur early in carcinogenesis.[25]

*BRAF V600E* mutation can be found in 10% of all patients with colorectal cancer and, in metastatic disease, the mutation is associated with other features such as location in the right colon, presence of metastases to the peritoneum and in distant lymph nodes.[26] *BRAF* mutation is considered a negative prognostic factor in patients with metastatic colorectal cancer.[27] Furthermore,

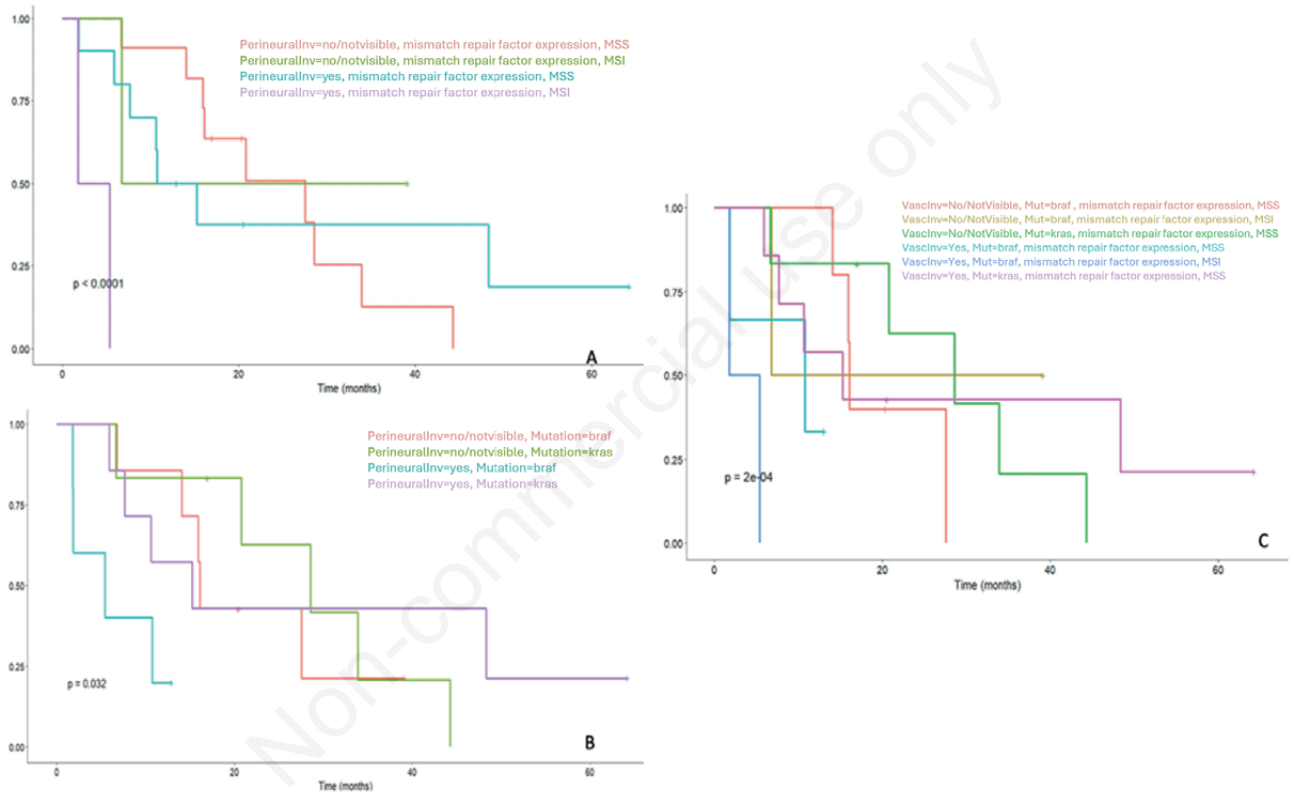




**Figure 3.** Survival analysis related to perineural invasion. A) Survival curves for morphological variable "perineural invasion" related to the expression of Mismatch Repair proteins. There is a survival reduction for subjects with instability and perineural invasion (also justified by the reduced number of subjects belonging to the subgroup) compared to the subgroup with the same characteristics except for the absence of perineural invasion (<10 months *vs* 40 months). In the presence of Microsatellite Instability, vascular invasion related to this reduces survival compared to stable subjects. For mutated KRAS subgroups, there is a reduction in the curve for subjects without perineural invasion compared to those presenting this morphological feature (45 months *vs* >60 months). B) Survival curves for morphological variable "perineural invasion" related to KRAS and BRAF mutation genes. The survival curve for BRAF mutated subgroup with perineural invasion appears reduced compared to the group with the same mutation but absence of perineural invasion (15 *vs* 40 months). In the presence of KRAS mutation, sub-cohort without perineural invasion show reduced survival compared to their perineural invasion counterparts (45 months *vs* >60 months). In mutated BRAF patients, the presence of vascular invasion would appear to have a more significant impact on survival than mutated KRAS subgroup. C) Survival curves for morphological variable "perineural invasion" related to BRAF and KRAS mutation genes and Mismatch Repair status expression. The graph shows a reduction in survival for unstable mutated BRAF patients characterized by morphological features of interest (considering the reduced number of subjects), compared to non-perineural invasion counterparty (<10 months *vs* 40 months). For BRAF patients mutated without Microsatellite Instability, the presence of perineural invasion shows a reduced survival compared to subgroup with the same characteristics except for the absence of the investigated morphological variable (15 months *vs* 30 months). Regarding KRAS subgroups not unstable, there is a reversal of survival trend, as the curve appears more reduced for individuals that do not have perineural invasion than the subgroup characterized by this morphological variable (30 months *vs* >60 months).

in case of recurrence after resection of the primary tumor, if *BRAF* mutation is present, it is associated with reduced post-recurrence survival.

*BRAF V600E* mutations frequently occur in patients with MSI and increased CIMP. *BRAF V600E* is associated with a shorter OS at various tumor stages, with a negative prognostic impact that is most pronounced in tumors with left-sided and MSS.[18]

The role of *KRAS* and *BRAF* mutations in the survival of patients with these cancers remains controversial. Some studies confirm the role of these genes as prognostic biomarkers, while others are inconclusive.

In a recent study, in which prognostic impact in relation to mutations, MMR status and clinicopathological features was assessed, a decreased OS in *KRAS* mutated patients compared to wild-type phenotype was observed in presence of advanced (stage IV) cancer, while MMR deletion status appeared to be associated with a higher OS than MSS.[28]

A meta-analysis of studies conducted on stage II-III colorectal tumors reported a statistically significant reduction of OS in *KRAS* mutated tumors when they adjusted for MSI, while the effect of *BRAF* was significant on OS both in the presence and absence of adjustment for MSI.[29]

Several evidence have also supported the hypothesis that MSI can be considered as a predictor of response to immunotherapy, in particular the response to immunological checkpoint inhibitors in metastatic tumours.[30]

Preliminary survival analyses conducted on patients enrolled so to date have reported statistically significant values for all morphological variables identified, except for tumor budding. This is characterized by the presence of single cells or small groups of cells (less than 5 elements) resulting from the transition of loose and de-differentiated glandular structures at the tumor progression front.[31]

The classification of tumor budding into low-grade or high-grade reflects the dedifferentiation of cells in which high-grade budding correlates with the development of regional and distant metastases with a worse prognosis.[32] It would therefore be logical to assume that patients with high-grade budding have a poorer prognosis than subjects with low-grade budding. The survival curve obtained is based on a small number of subjects; for this reason, there is a need to re-evaluate the analysis after completion of the study and with a larger sample size.

Regarding all other morphological variables investigated, survival analyses revealed some differences in survival curve between the morphological character suggestive of greater aggressiveness, compared to the absence of that character or a reduction in the grade for that morphological characteristic. In this survival analysis also considering mismatch repair protein expression status, a reduction in survival was observed in most of the cases for patients presenting instability (MSI) and morphological features of greater tumor aggressiveness, such as vascular and perineural invasion.

Survival analysis in which *KRAS* and *BRAF* mutation was also considered confirmed reduced survival curves for *BRAF* and MSI mutation in most cases. In a few cases, however, the impact of the mutation on survival would appear to be independent of morphological character, as in case of histological grade.

As these are preliminary results and considering that MSI occurs in a minority of colorectal cancers, the reduced number of individuals enrolled may partially influence the results of these survival analyses.

In these partial analyses, the presence of a possible difference in survival of unstable patients, in relation to individual delete proteins, molecular mutation and morphological features, was not investigated, as the number of patients with MSI is currently not sufficient to be evaluated.

This analysis, as well as the preliminary ones, will be re-evaluated at the end of the study.

## Conclusions

Because of the wide molecular heterogeneity of colorectal cancer, therapeutic strategies for the treatment of this disease are manifold. They differ according to the mutational status of oncogenes such as *BRAF* and *KRAS*, the presence of metastases and the tumor stage.

Adjuvant chemotherapy is useful in improving overall survival. The first-line chemotherapeutic agent used in the treatment of colorectal cancer is 5-fluorouracil, which can be combined in chemotherapy regimens with oxaliplatin (FOLFOX) or irinotecan (FOLFIRI).[33]

More recently, targeted therapies have been introduced, including monoclonal antibodies against the Epidermal Growth Factor Receptor (EGFR), such as cetuximab, and Endothelial Growth Factor (VEGF), such as bevacizumab, which inhibit tumor cell proliferation and angiogenesis, respectively.[34]

Finally, immunotherapy, with immune checkpoint inhibitors, has revolutionized cancer treatment, as although response rates are not only high, patients who respond to such therapy have a durable response.[35]

As despite recent advances in new therapies overall 5-year survival for this neoplasm remains limited, there is still a need to develop early diagnostic, prognostic and predictive biomarkers that can be used in routine clinical practice to reduce morbidity and mortality associated with this illness.

For this reason, the response to different therapies, and consequently survival in different subgroups of patients, may be influenced by many factors, which include, in addition to immunohistochemical and molecular investigations, the assessment of different morphological features, some of which are already known in literature as characteristics associated with a poor prognosis. Preliminary results of this study provide evidence that survival in different subsets of patients with colorectal cancer can be influenced by several factors.

From survival curves examined, it appears that for several morphological variables, particularly perineural invasion which occurs in some colorectal cancers, when *BRAF* gene mutation is present, this morphological character, if present, increases the unfavorable prognosis in terms of survival for these patients. The same is evident for subjects with MSI. This trend does not seem to be detectable in case of *KRAS* mutation, in which there is an upside-down of the survival trend for some morphological characteristics, which generally seems to be greater for patients presenting the morphological feature than for those who do not present it.

Integrating different characteristics may reveal which factors have a more significant impact on survival in these subgroups. More patients will be needed to design different survival models and to check these preliminary results more effectively.

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