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Article

Unraveling the Role of Molecular Profiling in Predicting Treatment Response in Stage III Colorectal Cancer Patients: Insights from the IDEA International Study

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Simple Summary: The treatment and prognosis of colorectal cancer (CRC) patients vary depending on their disease stage at diagnosis. Understanding the processes of tumorigenesis and disease development can reveal new therapeutic targets and guide patient management. On the basis of this, we investigated the molecular profiles of patients with stage III CRC enrolled in the international IDEA study. We also correlated the molecular profile with Toll-like and vitamin D receptor polymorphisms, clinicopathological and epidemiological characteristics, and patient outcomes. This study suggests that the molecular characterization of tumor cells may contribute to the understanding of the biological disease course. Mutations can serve as promising prognostic biomarkers leading to better treatment options. If the results will be confirmed in larger patient cohorts, then this is expected to guide clinical decision-making, personalized and improved care, and reduce treatment toxicity and patient and health system costs.

Abstract: Background: This study aimed to investigate the molecular profiles of stage III CRC patients from the international IDEA study. It also sought to correlate these profiles with Toll-like and vitamin D receptor polymorphisms, clinicopathological and epidemiological characteristics, and patient outcomes. **Methods:** Whole Exome Sequencing and PCR-RFLP on surgical specimens and blood samples, respectively, were performed to identify molecular profiling and the presence of Toll-like and vitamin D polymorphisms. Bioinformatic analysis revealed mutational status. **Results:** Among the enrolled patients, 63.7% were male, 66.7% had left-sided tumors, and 55.7% received CAPOX as adjuvant chemotherapy. Whole exome sequencing identified 59 mutated genes in 11 different signaling pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG) CRC panel. On average, patients had 8 mutated genes (range, 2-21 genes). Mutations in ARAF and MAPK10 emerged as independent prognostic factors for reduced DFS ($p=0.027$ and $p<0.001$, respectively), while RAC3 and RHOA genes emerged as independent prognostic factors for reduced OS ($p=0.029$ and $p=0.006$, respectively). Right-sided tumors were also identified as independent prognostic factors for reduced DFS ($p=0.019$) and OS ($p=0.043$). Additionally, patients with tumors in the transverse colon had mutations in genes related to apoptosis, PIK3-Akt, Wnt, and MAPK signaling pathways. **Conclusions:** Molecular characterization of tumor cells can enhance our understanding of the disease course. Mutations may serve as promising prognostic biomarkers, offering improved treatment options. Confirming these findings

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with require larger patient cohorts and international collaborations to establish correlations between molecular profiling, clinicopathological and epidemiological characteristics and clinical outcomes.

Keywords: colorectal cancer; stage III; molecular profiling; IDEA study; whole exome sequencing; bioinformatics

1. Introduction

Colorectal cancer (CRC) is one of the most common malignancies and the second most common cause of death from cancer[1]. In 2020, 1.9 million new cases of CRC and approximately 935,000 deaths were reported[2]. By 2030, the global burden of CRC is predicted to be 60%, with more than 2.2 million new cases and 1.1 million deaths. By 2035, the total number of deaths from rectal and colon cancer is estimated to increase by 60% and 71.5%, respectively[2]. Depending on the stage of the disease at the time of diagnosis, both the treatment and prognosis differ. Patients with stage III CRC have an overall 5-year survival rate of 60%. Adjuvant chemotherapy aims to increase this rate and extend both the overall and disease-free survival[3]. Since 2004, folinic acid, fluorouracil, and oxaliplatin (FOLFOX) or capecitabine and oxaliplatin) for six months has been the standard treatment regimen[3,4]. However, oxaliplatin can lead to adverse effects, particularly peripheral sensory neuropathy[5].

Considering the increased incidence of the disease, toxicity of the treatment, cost, and efforts to reduce the duration of treatment for all the aforementioned reasons[5], the international IDEA study was designed to evaluate the hypothesis of non-inferiority of the 3-month vs. 6-month adjuvant chemotherapy with FOLFOX or CAPOX[6].

Although strong, AJCC/UICC-TNM staging (American Joint Committee on Cancer/Union Internationale Contre le Cancer—extent of primary tumor, regional lymph node involvement, presence of distant metastases) often fails to provide complete prognostic information because the outcome varies even among patients of the same stage[7]. Therefore, there is an urgent need to identify new tools that can contribute to CRC prognosis. The aim of the current study was to investigate the molecular profile of surgical specimens from stage III CRC patients enrolled in the international IDEA study, for whom paraffin-embedded cancer tissue was available. Following genetic profiling, correlations were performed with clinicopathological characteristics, as well as with patient outcomes. Additionally, the patients were tested for vitamin D receptor (*VDR*) and toll-like receptor (*TLR*) gene polymorphisms in peripheral blood, as our previous studies have demonstrated the role of such polymorphisms in tumor development and progression[8–10].

2. Patients and methods

2.1. Patient enrollment

The Hellenic Oncology Research Group (HORG) enrolled 708 patients with CRC in the international IDEA study (ClinicalTrials.gov Identifier: NCT01308086). Of these, 237 stage III patients with formalin-fixed paraffin-embedded (FFPE) tissues were included in the current study (Supplementary Table S1). All patients were aged > 18 years and received adjuvant chemotherapy with FOLFOX or CAPOX. None of the enrolled subjects had any other documented malignancies.

2.2. Formalin-Fixed Paraffin-embedded (FFPE) tissues

All surgical materials were evaluated by a specialized pathologist at the Department of Pathology of the University General Hospital of Heraklion, Crete, and the most representative and enriched tumor areas were selected for dissection. Healthy tissue was used as the control tissue. The specifications of the FFPE sections used for DNA extraction were as follows: tissue surface area, 25 mm^2, section thickness, 10 μm; c. At least 10 sections with >50% cancer cells, d. Cancer cells were collected from areas rich in cancerous tissue and avoiding healthy tissue, adipose tissue, necrotic

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areas, and lymphocytes that decrease the content of cancer DNA. To facilitate the collection of appropriate cells from the 10 sections, an additional section was stained with hematoxylin-eosin to locate the cancerous areas.

2.3. TLR and VDR genotyping in blood samples

A total of 5 ml of peripheral blood in EDTA was collected from each patient, and DNA was extracted using the QIAamp DNA Blood Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The DNA concentration was determined using a NanoDrop ND-1000 v3.3 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

To determine the genetic variants of *TLRs* and *VDRs*, Polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) were used to determine the genetic variants of TLRs and VDRs. For *TLR2* 196-to-174 Ins/Del genetic variants, PCR was used, while *TLR4* (Asp299Gly and Thr399Ile) and *TLR9* (T1237C and T1486C) genetic variants were determined using PCR-RFLP. The materials and conditions for each gene target have been previously described[8,9]. Similarly, for genotyping of VDR genetic variants at the *Taq*I, *Apa*I, *Fok*I, and *Bsm*I positions, PCR-RFLP was used. The reagents and PCR conditions have been previously described, in detail[8–10]. The patients were classified as wild-type, heterozygous, or homozygous for each single nucleotide polymorphism, based on the absence or presence of the restriction site in both alleles.

2.4. Whole Exome Sequencing (WES)

The Illumina DNA Prep with Enrichment kit (Illumina, San Diego, CA 92122) was used for library preparation and enrichment, and sequencing of both tumor and normal tissues was conducted using the NovaSeq 6000 system (Illumina). For each sample, at least 250 ng of high-quality DNA was quantified using a Qubit Fluorometer (Thermo Fisher Scientific, Paisley, UK), was used. Following the WES, two files (. fastq) containing sequencing data were extracted from each tissue (tumor and normal) using forward and reverse reads.

2.5. Bioinformatic analysis

After obtaining the raw data from the WES analysis, a bioinformatics pipeline was used to process the data and generate interpretations. This included alignment to the human genome, variant calling, variant filtering, and annotation of the variants. The processed data were analyzed to identify somatic variants and evaluate their functional significance, particularly those associated with CRC (Figure 1). Initially, the raw sequences were aligned to the human genome (version hg19/GRCh37)[11], and variant calling was conducted using the genome analysis toolkit (GATK) for SNPs and insertion/deletions (INDELs). This generates two variant call format files (VCF) for each patient, one for the tumor and one for the normal tissue, respectively[12]. The somatic variants were isolated by subtracting the germline variants from the tumor, and a custom gene panel composed of 62 genes associated with 11 signaling pathways was utilized based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database on CRC-correlated genes[13] (Tables 1 and 2). Subsequently, ANNOVAR software was employed to annotate the SNPs and INDELs, providing functional information that can determine the biological significance of each variant and identify CRC-associated variants[14].

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Figure 1

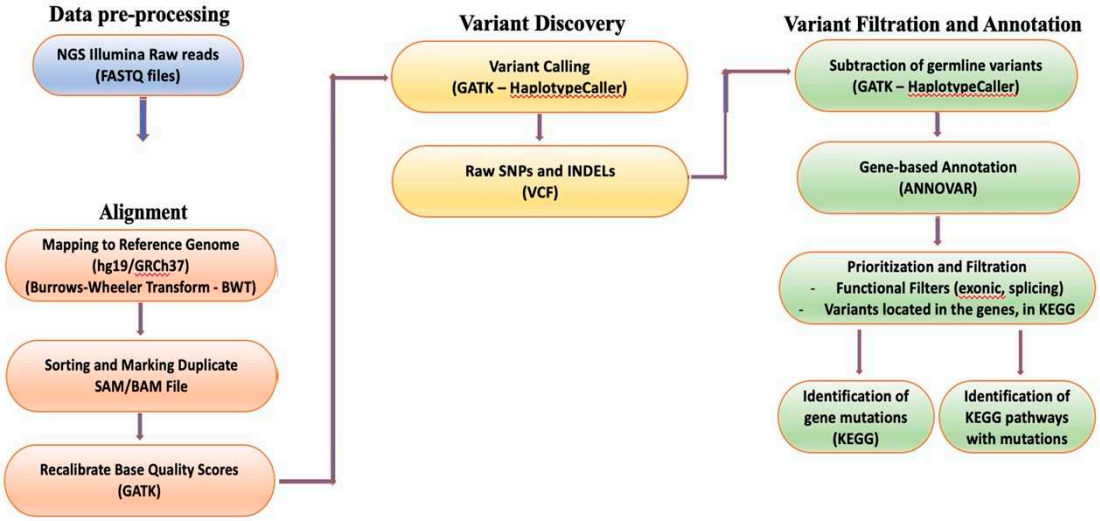

Figure 1. Overview of the next generation sequencing (NGS) analysis pipeline.

Table 1. Gene panel for colorectal cancer (CRC) based on Kyoto Encyclopedia of Genes and Genomes (KEGG).

Gene	Chromosome	Location based on GRCh37.p13 assembly		Source [https://www.ensembl.org/index.html]
		Start	End	
AKT1	14	105235686	105262085	Ensembl:ENSG00000142208
AKT2	19	40736224	40791252	Ensembl:ENSG00000105221
AKT3	1	243651535	244014381	Ensembl:ENSG00000117020
APC	5	112043195	112181936	Ensembl:ENSG00000134982
APC2	19	1450120	1473243	Ensembl:ENSG00000115266
APPL1	3	57261757	57307499	Ensembl:ENSG00000157500
ARAF	X	47420604	47431307	Ensembl:ENSG00000078061
AXIN1	16	337440	402723	Ensembl:ENSG00000103126
AXIN2	17	63524681	63557766	Ensembl:ENSG00000168646
BAD	11	64037300	64052176	Ensembl:ENSG00000002330
BAX	19	49458132	49465055	Ensembl:ENSG00000087088
BCL2	18	60790579	60987002	Ensembl:ENSG00000171791
BIRC5	17	76210334	76221716	Ensembl:ENSG00000089685
BRAF	7	140413128	140624729	Ensembl:ENSG00000157764
CASP3	4	185548850	185570601	Ensembl:ENSG00000164305
CASP9	1	15817896	15851285	Ensembl:ENSG00000132906
CCND1	11	69455924	69469242	Ensembl:ENSG00000110092
CTNNB1	3	41240996	41281934	Ensembl:ENSG00000168036
CYCS	7	25158275	25164879	Ensembl:ENSG00000172115
DCC	18	49866567	51062269	Ensembl:ENSG00000187323
FOS	14	75745531	75748933	Ensembl:ENSG00000170345
GSK3B	3	119540168	119813294	Ensembl:ENSG00000082701
JUN	1	59246463	59249719	Ensembl:ENSG00000177606
KRAS	12	25358180	25403863	Ensembl:ENSG00000133703
LEF1	4	108968704	109090088	Ensembl:ENSG00000138795
MAP2K1	15	66679250	66783882	Ensembl:ENSG00000169032
MAPK1	22	22113946	22221970	Ensembl:ENSG00000100030
MAPK10	4	86931558	87374348	Ensembl:ENSG00000109339
MAPK3	16	30125426	30134541	Ensembl:ENSG00000102882

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MAPK8	10	49514720	49647403	Ensembl:ENSG00000107643
MAPK9	5	179660143	179719083	Ensembl:ENSG00000050748
MLH1	3	3703500	37092337	Ensembl:ENSG00000076242
MSH2	2	47630206	47710367	Ensembl:ENSG00000095002
MSH3	5	79950471	80172634	Ensembl:ENSG00000113318
MSH6	2	48010284	48034092	Ensembl:ENSG00000116062
MYC	8	128747680	128755197	Ensembl:ENSG00000136997
PIK3CA	3	178866145	178957881	Ensembl:ENSG00000121879
PIK3CB	3	138371540	138553770	Ensembl:ENSG00000051382
PIK3CD	1	9711789	9789172	Ensembl:ENSG00000171608
PIK3CG	7	106505727	106549425	Ensembl:ENSG00000105851
PIK3R1	5	67511584	67597649	Ensembl:ENSG00000145675
PIK3R2	19	18263973	18281342	Ensembl:ENSG00000105647
PIK3R3	1	46505812	46640573	Ensembl:ENSG00000117461
PIK3R5	17	8782233	8869024	Ensembl:ENSG00000141506
RAC1	7	6414158	6443598	Ensembl:ENSG00000136238
RAC2	22	37621310	37640309	Ensembl:ENSG00000128340
RAC3	17	79989554	79992080	Ensembl:ENSG00000169750
RAF1	3	12625100	12705616	Ensembl:ENSG00000132155
RALGDS	9	135973109	136024597	Ensembl:ENSG00000160271
RHOA	3	49396578	49449409	Ensembl:ENSG00000067560
SMAD2	18	45335328	45457243	Ensembl:ENSG00000175387
SMAD3	15	67357940	67487507	Ensembl:ENSG00000166949
SMAD4	18	48556583	48611412	Ensembl:ENSG00000141646
TCF7	5	133450372	133483901	Ensembl:ENSG00000081059
TCF7L1	2	85360515	85537510	Ensembl:ENSG00000152284
TCF7L2	10	114710006	114927437	Ensembl:ENSG00000148737
TGFB1	19	41836228	41859827	Ensembl:ENSG00000105329
TGFB2	1	218518678	218617961	Ensembl:ENSG00000092969
TGFB3	14	76424440	76449354	Ensembl:ENSG00000119699
TGFBR1	9	101867395	101916474	Ensembl:ENSG00000106799
TGFBR2	3	30647994	30735634	Ensembl:ENSG00000163513
TP53	17	7571739	7590808	Ensembl:ENSG00000141510

Table 2. Signaling pathways and the associated genes for colorectal cancer (CRC) based on Kyoto Encyclopedia of Genes and Genomes (KEGG).

Pathway	KEGG CRC genes	References [https://www.genome.jp]
Cell cycle	*CCND1, GSK3B, MYC, SMAD2, SMAD3, SMAD4, TGFB1, TGFB2, TGFB3, TP53*	https://www.genome.jp/pathway/hsa04110
p53 signaling pathway	*BAX, BCL2, CASP3, CASP9, CCND1, CYCS, TP53*	https://www.genome.jp/pathway/hsa04115
Apoptosis	*AKT1, AKT2, AKT3, BAD, BAX, BCL2, BIRC5, CASP3, CASP9, CYCS, FOS, JUN, KRAS, MAP2K1, MAPK1, MAPK10, MAPK3, MAPK8, MAPK9, PIK3CA, PIK3CB, PIK3CD, PIK3R1, PIK3R2, PIK3R3, RAF1, TP53, DCC, APPL1*	https://www.genome.jp/pathway/hsa04210
mTOR signaling pathway	*BRAF, GSK3B, KRAS, MAP2K1, MAPK1, PIK3CA, PIK3CB, PIK3CD, PIK3R1, PIK3R2, PIK3R3, RAF1, RHOA*	https://www.genome.jp/pathway/hsa04150

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PI3K-Akt signaling pathway	*AKT1, AKT2, AKT3, BAD, BCL2, CASP9, GSK3B, KRAS, MAP2K1, MAPK1, MAPK3, MYC, PIK3CA, PIK3CB, PIK3CD, PIK3CG, PIK3R1, PIK3R2, PIK3R3, PIK3R5, RAC1, RAF1, TP53*	https://www.genome.jp/pathway/hsa04151
Wnt signaling pathway	*APC, APC2, AXIN1, AXIN2, CCND1,CTNNB1, GSK3B, JUN, LEF1, MAPK10, MAPK8, MAPK9, MYC, RAC1, RAC2, RAC3, RHOA, SMAD3, SMAD4, TCF7, TCF7L1, TCF7L2, TP53*	https://www.genome.jp/pathway/hsa04310
TGF-beta signaling pathway	*MAPK1, MAPK3, MYC, RHOA, SMAD2, SMAD3, SMAD4, TGFB1, TGFB2, TGFB3, TGFBR1, TGFBR2,*	https://www.genome.jp/pathway/hsa04350
MAPK signaling pathway	*AKT1, AKT2, AKT3, ARAF, BRAF, CASP3, FOS, JUN, KRAS, LEF1, MAP2K1, MAPK1, MAPK10, MAPK3, MAPK8, MAPK9, MYC, RAC1, RAC2, RAC3, RAF1, TGFB1, TGFB2, TGFB3, TGFBR1, TGFBR2, TP53*	https://www.genome.jp/pathway/hsa04010
ErbB signaling pathway	*AKT1, AKT2, AKT3, ARAF, BAD, BRAF, GSK3B, JUN, KRAS, MAP2K1, MAPK1, MAPK10, MAPK3, MAPK8, MAPK9, MYC, PIK3CA, PIK3CB, PIK3CD, PIK3R1, PIK3R2, PIK3R3, RAF*	https://www.genome.jp/pathway/hsa04012
MSI pathway	*APC, AXIN1, AXIN2, BAD, BAX, BCL2, GSK3B, MLH1, MSH2, MSH3, MSH6, TGFBR2*	https://www.genome.jp/dbget-bin/www_bget?path:map05210
Ras signaling pathway	*AKT1, AKT2, AKT3, BAD, KRAS, MAP2K1, MAPK1, MAPK10, MAPK3, MAPK8, MAPK9, PIK3CA, PIK3CB, PIK3CD, PIK3R1, PIK3R2, PIK3R3, RAC1, RAC2, RAC3, RAF1, RALGDS, RHOA*	https://www.genome.jp/pathway/hsa04014

To identify clinically significant variants and the signaling pathways involved, a filtering strategy was employed based on the functional position of the variants. Variants located in exons and splice sites are isolated as they are more likely to cause diseases[15]. In addition, the variants located in exons were further filtered based on their functional consequences, excluding variants causing synonymous mutations. This step was considered synonymous mutations that do not affect the amino acid sequence of proteins and are therefore less likely to be clinically relevant. All analyses were run in the Anaconda Powershell Prompt (Anaconda3, Inc.) on Ubuntu 20.04.3 LTS.

2.6. Statistical analysis

After characterizing the patients' molecular profiles, the clinical, pathological, and epidemiological characteristics were examined to determine their association with patient outcomes. Disease-free (DFS) and overall survival (OS) were calculated from the day of tumor excision until the first documented recurrence or death, respectively. Recurrence was defined as the presence of metastatic disease, local recurrence, or a second primary tumor. The possible associations between baseline characteristics, recurrence, and individual or concurrent mutations were compared using the 2-sided Fisher exact test for categorical variables. The association between risk factors and time-to-event endpoints was evaluated using the log-rank test, and the Kaplan-Meier method was used to generate DFS and OS curves. Univariate and multivariate Cox regression analyses were conducted

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to evaluate the correlation between the potential prognostic factors and DFS or OS. Statistical significance was defined as $p \leq 0.05$, and the statistical tool used was SPSS v. 26.

3. Results

3.1. Patients

The current investigation enrolled 237 patients with stage III CRC, and their characteristics are displayed in Table 3 and Supplementary Table S1. Among these patients, 151 (63.7%) were male, 159 (67.1%) were <70 years old (median:64 years, range:18-84), 158 (66.7%) had tumor localization in the left colon, and 132 (55.7%) patients underwent CAPOX as adjuvant chemotherapy. Of the entire patient population, 116 (48.9%) were administered a 3-month treatment regimen, while 121 (51.1%) were administered a 6-month treatment regimen. Moreover, 84 patients were analyzed for *VDR* and *TLR* gene polymorphisms. Regarding *VDRs*, 10 (11.9%), 7 (8.2%), 5 (6.0%), and 17 (20.2%) patients presented *Taq*I, *Apa*I, *Fok*I, and *Bsm*I homozygous phenotypes, respectively. Moreover, regarding *TLRs*, 48 (57.1%), 38 (46.4%), 38 (46.4%), 37 (44.0%), and 37 (44.0%) patients presented *TLR2* 196-to-174, *TLR4*—Asp299Gly, *TLR4*—Thr399Ile), *TLR9*—T1237C and *TLR9*—T1486C homozygous phenotype, respectively.

Table 3. Patients characteristics.

Characteristics	Number of patients (N=237)	%
Median age (range)	64 (18–84)	
<70	159	67.1
≥70	78	32.9
Gender		
Males	151	63.7
Females	86	36.3
Tumor location		
Cecum	38	16.0
Ascending	42	17.7
Transverse	22	9.3
Descending	24	10.1
Sigmoid	111	46.8
Sidedness		
Left	158	66.7
Right	79	33.3
Performance status		
0-1	236	99.6
>2	1	.4
Regimen		
Folfox	105	44.3
Capox	132	55.7
Treatment duration		
3 months	116	48.9
6 months	121	51.1

3.2. Annotated variants for each position

The KEGG CRC panel detected 85,871 uniquely annotated variants. Of these, 602 were detected in exonic positions, including 25 splice variants. The remaining variants were non-coding, with 81,555 intronic variants being the most common, followed by 2,184 UTR variants, 788 downstream variants, and 742 upstream variants.

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3.3. Identification of mutated genes

Mutated genes within the KEGG CRC gene panel for each patient were identified. On average, the patients exhibited eight mutated genes (range, 2–21 genes). The frequencies of mutations in each gene, specifically in exons and splicing sites, are presented in Table 4 and Figure 2.

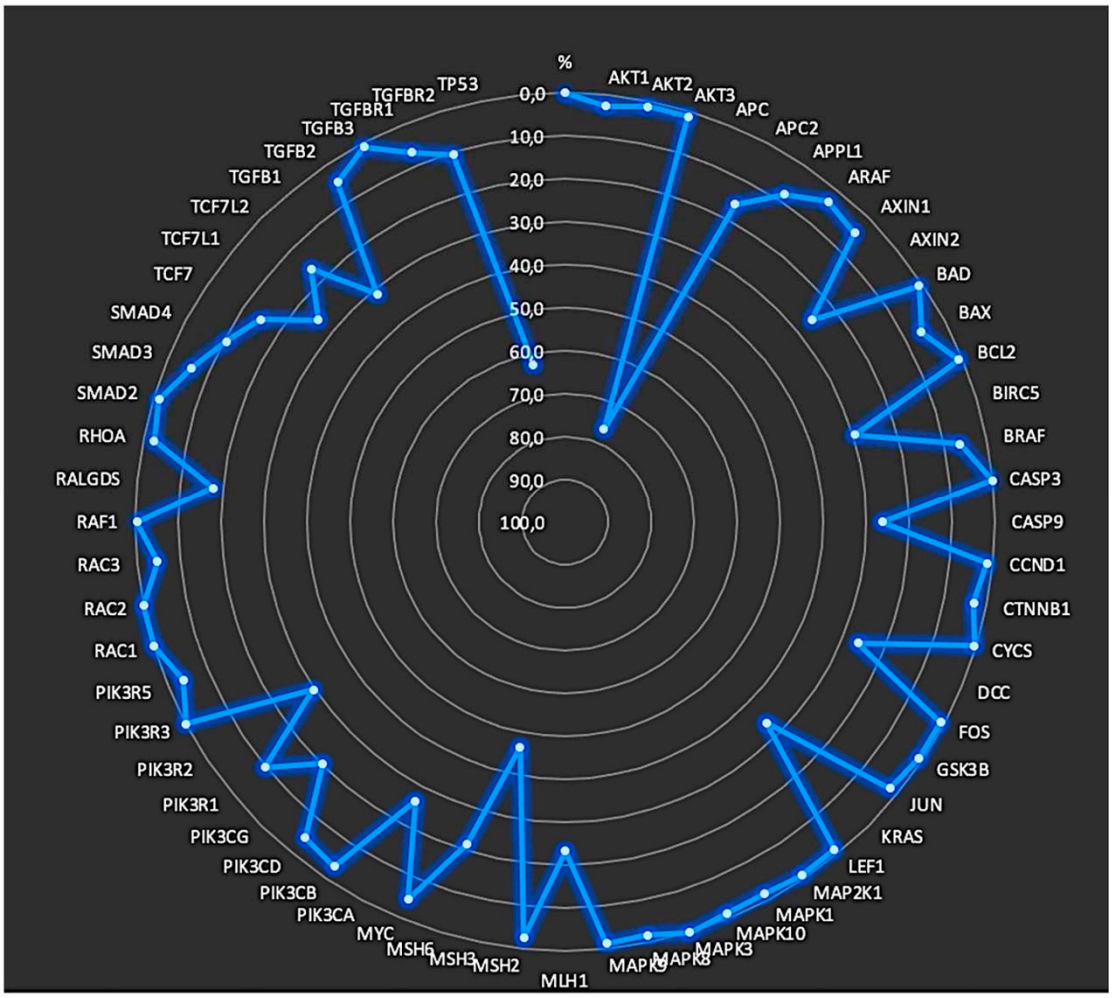

Figure 2. Frequency of mutations in each gene located in exons and splicing sites.

Table 4. Frequency of mutated patients in each gene.

Gene	Mutant patients
AKT1	6
AKT2	3
AKT3	3
APC	181
APC2	38
APPL1	19
ARAF	8
AXIN1	11
AXIN2	61
BAD	2
BAX	14
BCL2	2
BIRC5	70
BRAF	15

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CASP3	0
CASP9	62
CCND1	3
CTNNB1	7
CYCS	1
DCC	62
FOS	2
GSK3B	2
JUN	5
KRAS	80
LEF1	3
MAP2K1	2
MAPK1	4
MAPK10	3
MAPK3	0
MAPK8	4
MAPK9	3
MLH1	55
MSH2	6
MSH3	110
MSH6	51
MYC	11
PIK3CA	62
PIK3CB	8
PIK3CD	11
PIK3CG	48
PIK3R1	23
PIK3R2	70
PIK3R3	0
PIK3R5	9
RAC1	0
RAC2	0
RAC3	11
RAF1	1
RALGDS	42
RHOA	6
SMAD2	3
SMAD3	14
SMAD4	25
TCF7	35
TCF7L1	61
TCF7L2	39
TGFB1	74
TGFB2	11
TGFB3	2
TGFBR1	16
TGFBR2	25
TP53	148

From this analysis, it was observed that certain groups of patients had a higher mutation frequency in specific genes (Table 5). In brief, males had a significantly higher frequency of mutations in the *JUN* and *MAPK3* genes than females (p=0.05, p=0.05, respectively). In terms of age groups,

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patients below 70 years of age had a higher frequency of mutations in *TGFBR1* than those ≥70 years of age (p=0.012). Similarly, patients between 51-70 years old had more frequent mutations in *BAD* (p<0.001), *RAC* (p=0.016), *AKT, AKT2, AKT3, APC, APPL1, AXIN1, AXIN2, BIRC5, DCC, GSK3B, KRAS, MAPK1, MAPK8, MAPK9, MAPK10, MLH1, MSH6, PIK3CA, PIK3R1, PIK3R2, PIK3R5, RAF1, RALGDS, SMAD2, SMAD3, TCF7L2, TGFB1* and *TGFB2* genes (p=0.037). Moreover, mutation rates in ARAF, MAPK10, CASP, TCF7, and TGFB3 genes were significantly higher in patients relapsed after adjuvant treatment (p=0.027, p=0.044, p=0.003, and p=0.037, respectively).

Table 5. Frequency of mutations according to patients' characteristics.

Characteristics	Gene	No % (p value)
Gender		
Male vs Female	*JUN*	43.4% vs 25.0% (0.05)
	MAPK3	57.5% vs 31.1% (0.05)
Sideness		
Left vs Right	*MLH1*	80% vs 20% (0.007)
	MSH6	84.3% vs 15.7% (0.001)
	TCF7L1	77% vs 33% (0.019)
Location		
Colon vs Sigmoid	*DCC*	90.3% vs 9.7% (0.04)
	KRAS	88.8% vs 11.2% (0.048)
	TGFBR2	96% vs 4% (0.043)
Age		
<70 vs ≥70	*TGFBR1*	57.5% vs 33% (0.012)
51-70 vs ≥70 vs <50	*BAD*	56.1% vs 30.7% vs 12.7% (<0.001)
51-70 vs ≥70 vs <50	*RAC*	56.1% vs 30.7% vs 12.7% (0.016)
51-70 vs ≥70 vs <50	*AKT, AKT2, AKT3, APC, APPL1, AXIN1, AXIN2, BIRC5, DCC, GSK3B, KRAS, MAPK1, MAPK8, MAPK9, MAPK10, MLH1, MSH6, PIK3CA, PIK3R1, PIK3R2, PIK3R3, PIK3R5, RAF1, RALGD5, SMAD2, SMAD3, TCF7L2, TGFB1, TGFB2*	56.1% vs 30.7% vs 12.7% (0.037)
Relapse post Adj Chemotherapy		
Yes vs No	*ARAF, MAPK10*	71.7% vs 20.5% (0.027)
	CASP3	75.5% vs 22.6% (0.044)
	TCF7	75% vs 21.2% (0.003)
	TGFB3	74.5% vs 22.2% (0.037)

Subsequently, it was demonstrated that patients with tumors located in the transverse colon, homozygous for mutated *VDR* alleles *(TaqI, ApaI, FokI, BsmI)* and homozygous for mutated *TLR9* alleles (T1237C and T1486C), had mutations in genes that are mainly involved in the apoptosis, *PIK3-AKT*, *Wnt* and *MAPK* signaling pathways (Table 6).

Table 6. Correlation of mutated signaling pathways and patients characteristics.

	Cell cycle	Apoptosis	PI3K-Akt	Wnt	MAPK	ErbB	MSI	RAS
Transverse Colon		X	X	X	X			
TaqI Homozygous	X	X	X	X		X	X	X
ApaI Homozygous		X	X	X	X			
FokI Homozygous		X	X	X				
BsmI Homozygous		X	X	X				

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TLR9 -T1237C Homozygous	X	X	X
TLR9–T1486C Homozygous	X	X	X

3.4. Clinical outcome based on molecular profile and patients' characteristics

Regarding disease-free survival (DFS), it was demonstrated that patients with *ARAF* mutations had a significantly shorter DFS (74 months, 95% CI:29.3 – 154.9 months) compared to those with wild-type ARAF mutations (133 months, 95% CI:101 – 138 months, p=0.017) (Figure 3A). Similarly, patients with *MAPK10* mutations exhibit a significantly shorter DFS compared to those wild-type (12.5 months, 95% CI:0.0 – 29 months vs 108 months, 95% CI:101 – 114 months; p<0.001) (Figure 3B).

Figure 3

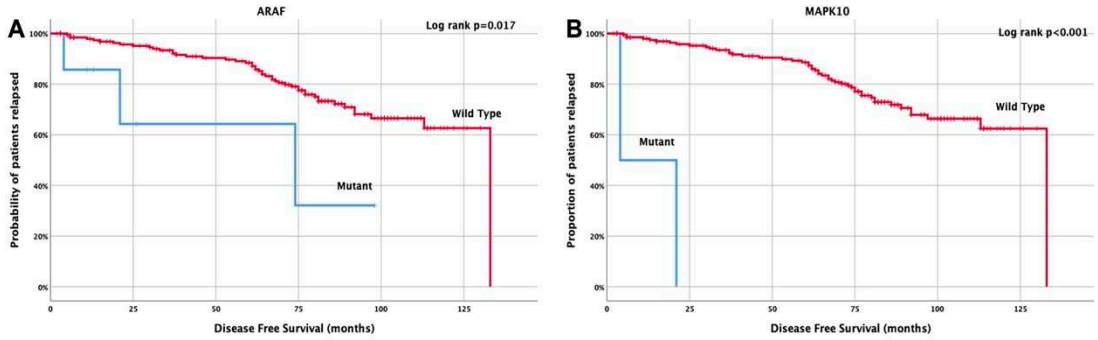

Figure 3. Kaplan Meier curve for disease-free survival (DFS) according to (A) *ARAF* and (B) *MAPK10* mutations.

Similarly, it was demonstrated that patients with right-sided tumors experienced a significantly shorter overall survival (OS) compared to patients with left-sided tumors (92.1 months, 95% CI:82.5 – 122 months vs 111.3 months, 95% CI:104 – 135 months; p=0.011) (Figure 4A). Moreover, relapsed patients demonstrated a significantly shorter OS compared to those non-relapsed (81.1 months, 95% CI:70.4 – 118 months vs 104.3 months, 95% CI:93.9 – 130 months; p=0.008) (Figure 4B). Moreover, patients with *AKT1, APC2, ARAF, BAD, MAPK10, RAC3, RHOA, TGFB2* and *TGFB3* mutations exhibited a significantly shorter OS compared to wild-type patients (68.9 vs 107.9 months, p=0.03; 89.7 vs 109.4 months, p=0.001; 43.3 vs 109.4 months, p<0.001; 34 vs 107.7 months, p=0.011; 19.3 vs 108.6, p>0.001; 55.6 vs 109, p<0.001; 45.2 vs 108.5 months, p=0.004; 69.5 vs 109.7 months, p=0.032 και 30.5 vs 108.1 months p<0.001, respectively) (Figures 4C-K). Finally, patients with *MSH6* gene mutations had a significantly longer OS compared to those wild-type (120.7 months, 95% CI:111.3 – 130.1 months vs 100.9 months, 95% CI:93.7 – 108.1 108.1 months; p=0.008) (Figure 4L).

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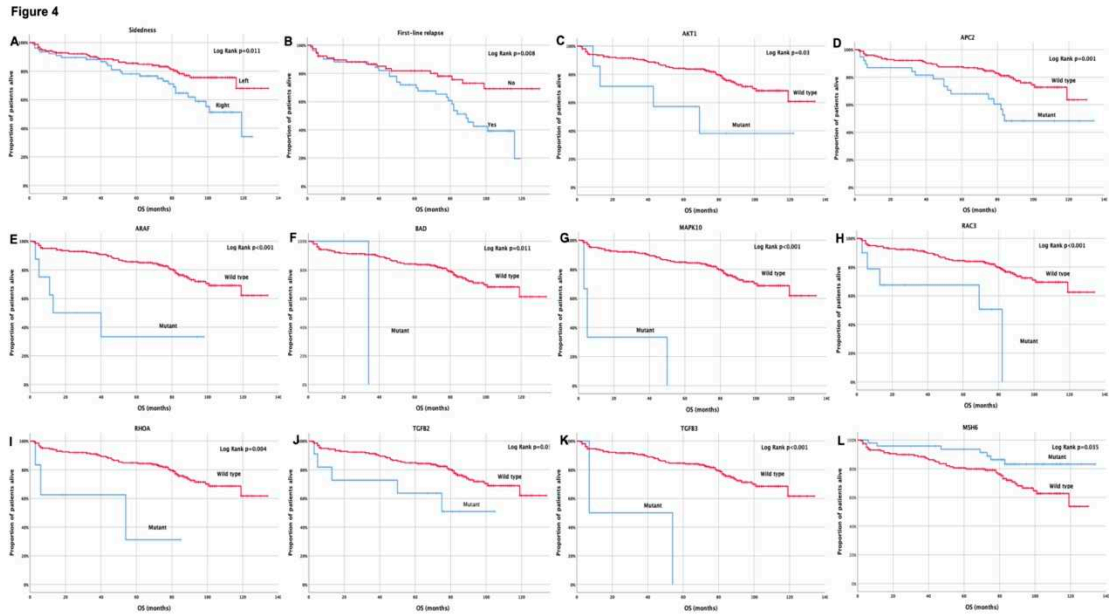

Figure 4. Kaplan Meier curve for overall survival (OS) according to (A) tumor sidedness, (B) relapse status, (C–L) *AKT1, APC2, ARAF, BAD, MAPK10, RAC3, RHOA, TGFB2, TGFB3* and *MSH6* mutations.

3.5. Univariate and multivariate Cox-regression analysis

Univariate analysis revealed that tumor localization in the right colon, and *ARAF* and *MAPK10* mutations were associated with reduced DFS (Table 7). Multivariate analysis confirmed that tumor localization and *ARAF* and *MAPK10* mutations were independent predictive factors of reduced DFS (HR=2.1; 95% CI:1.1-4.0; p=0.019; HR=3.9 ; 95% CI:1.2-13.1; p=0.027; HR=49; 95% CI:9.8-244.1; p<0.001 (Table 7).

Table 7. Univariate and multivariate Cox regression analysis.

	Univariate				Multivariate			
	DFS		OS		DFS		OS	
Feature	**HR (95%CI)**	***p*-value**	**HR (95%CI)**	***p*-value**	**HR (95%CI)**	***p*-value**	**HR (95%CI)**	***p*-value**
Sidedness (Right vs Left)	1.9 (1.2–3.2)	0.012	2.1 (1.0–4.0)	0.043	2.1 (1.1-4.0)	0.019	2.2 (1.0-4.5)	0.043
***AKT1* (Mutant vs Wild type)**			2.9 (1.1-8.2)	0.039				
***APC2* (Mutant vs Wild type)**			2.5 (1.4-4.5)	0.002				
***ARAF* (Mutant vs Wild type)**	3.8 (1.2-12.1)	0.027	5.0 (2.0-12.7)	0.001	3.9 (1.2-13.1)	0.027		
***BAD* (Mutant vs Wild type)**			8.6 (1.1-64.4)	0.036				
***MAPK10* (Mutant vs Wild type)**	43.0 (9.1-203.7)	<0.001	15.1 (4.6-50.3)	<0.001	49.0 (9.8-244.1)	<0.001		
MSH6 (Wild type vs Mutant)			2.3 (1.0-5.1)	0.041				
***RAC3* (Mutant vs Wild type)**			4.7 (1.8-11.9)	0.001			3.5 (1.1-10.7)	0.029
***RHOA* (Mutant vs Wild type)**			4.8 (1.5-15.6)	0.009			9.5 (1.9-47.7)	0.006
***TGFB2* (Mutant vs Wild type)**			2.6 (1.1-6.7)	0.040				
***TGFB3* (Mutant vs Wild type)**			9.0 (2.1-37.8)	0.003				

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Similarly, right-sided tumors and *AKT1, APC2, ARAF, BAD, MAPK10, RAC3, RHOA, TGFB2,* and *TGFB3* were associated with an increased risk of shorter OS. In contrast, *MSH6* mutations were demonstrated to be a good prognostic factor, as they were associated with a reduced risk for shorter OS. Multivariate analysis revealed that right-sided tumors and *RAC3* and *RHOA* gene mutations emerged as independent predictors of reduced OS (HR=2.2; 95% CI:1.0-4.5; p=0.043; HR=3.5; 95% CI:1.1-10.7; p=0.029 and HR=9.5; 95% CI:1.9-47.7; p=0.006 (Table 7).

4. Discussion

Despite the potential benefits of presymptomatic screening and available treatments, CRC continues to be a significant public health concern[16]. Understanding the processes involved in CRC development and progression can help to identify new targets for treatment. Structural and functional changes in the DNA can offer vital insights into patient management[17,18]. As the normal colonic epithelium transforms into cancerous tissue, various mutations occur, leading to adenoma formation[19–26]. Extensive cancer cell proliferation through the *RAS-RAF-MEK-ERK* signaling pathway drives carcinogenesis, tumor invasion, and metastasis[27]. Moreover, the immune responses to cancer cells differ among patients with mutations[28–34].

The objective of this study was to analyze genetic changes in surgical samples from patients with stage III CRC. The study included 237 patients, and the WES and KEGG gene panel for CRC revealed 59 mutated genes belonging to 11 distinct signaling pathways. Of these, mutations in *APC2, BRAF, MAPK10, MLH1, MSH6, RHOA, TGFβ,* and *TGFβ2* have been linked to a significant impact on patient survival. *APC, TP53, KRAS,* and *MSH3* were the most commonly observed mutations in this study.

APC encodes an anti-tumor protein that competes with the Wnt signaling pathway and is involved in cell migration, adhesion, and apoptosis. *APC* mutations are responsible for familial adenomatous polyposis (FAP), an autosomal dominant precancerous disease that typically leads to malignancy. *APC* mutations are commonly observed in CRC cases[35]. Similarly, *APC2* mutations, which are directly associated with *APC*'s tumor-suppressive function[36], have been linked to worse prognosis in CRC patients[37,38]. This study confirms that *APC2* mutations in patients with stage III CRC are associated with lower overall survival but do not represent an independent prognostic factor. *TP53* encodes an anti-tumor protein that regulates the expression of target genes, leading to cell cycle arrest, apoptosis, senescence, DNA repair, or metabolic changes. Similar to *APC*, *TP53* are frequently observed in CRC cases[35]. Furthermore, mutations in the *APC2* gene, which is directly linked to *APC*'s tumor-suppressive function[36], are also associated with worse prognosis in CRC patients[37,39]. This study confirms that while *APC2* mutations in stage III CRC patients are linked to lower overall survival, they do not represent an independent prognostic factor. Various human cancers, including approximately 60% of CRC, are associated with mutations in the *TP53* gene[40,41]. Prior studies have shown that mutations in *TP53* resulting in the loss of its transcriptional activity, can lead to uncontrolled cellular proliferation in multiple organs, including the colon[42]. Similarly, *KRAS* mutations are the primary indicators of gastrointestinal cancers and are found in approximately 40% of patients with CRC (stage II-IV)[43]. They serve as negative prognostic factors for carcinogenesis and anti-*EGFR* therapy[44] because intracellular signal interruption leads to uncontrolled cellular proliferation and cancer. *MSH3* mutations have been mainly linked to endometrial cancer, but there are reports of its relationship with inflammatory processes, such as ulcerative colitis and Crohn's disease, which considerably increase the likelihood of CRC development[45,46]. *MSH3*-associated CRC seems to follow the classic *APC* pathway, as patients with adenomas and CRC carrying *APC* mutations showed *MSH3* deficiency[47], as confirmed in this study. In addition to the common mutations detected in the patient group, mutations in *AKT1*, *ARAF*, *BAD*, *MAPK10*, *RAC3*, *RHOA*, *TGFB2*, and *TGFB3* were associated with worse prognosis in this study. Furthermore, mutations in *ARAF* and *MAPK10* were identified as independent prognostic factors for DFS, whereas mutations in *RAC3* and *RHOA* were identified as independent prognostic factors for decreased OS.

This study sheds light on the association between mutations in genes involved in signaling pathways such as PI3K-Akt, MAPK, apoptosis, and CRC. To the best of our knowledge, this is the

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first report of its kind in the literature. The reactivation of embryonic self-renewal pathways, such as Hedgehog, Notch, and *TGFβ/Stat3*, is characteristic of most tumors, including CRC. The *Wnt* pathway is also essential in most CRC. Targeting embryonic pathways directly is likely to be more effective against stem and differentiated cancer cells [48–50]. Tumors that are addicted to increased regulated activity of the embryonic pathway, in combination with high tumor heterogeneity, may be more vulnerable to such therapies [51–53]. Patients with *VDR* polymorphisms had concurrent mutations in genes involved in cell cycle, apoptosis, *PI3K-Akt*, *WNT*, *MAPK*, *ErbB*, MSI, and *RAS*. Similarly, *TLR9* polymorphisms were associated with mutations in genes involved in apoptotic signaling pathways, *PI3K-Akt*, and *Wnt*. Previous studies from our group have demonstrated that higher detection of *TLR* and *VDR* polymorphisms in CRC patients, especially advanced-stage patients, highlights the role of these polymorphisms in carcinogenesis, disease progression, and ultimately, patient survival[9,10,54]. Regarding DFS, tumors in the sigmoid or right colon and mutations in the *ARAF* and/or *MAPK10* genes were associated with shorter DFS, a fact that has been confirmed in previous studies[55–57]. To our knowledge, this is the first study to highlight the role of *ARAF* and *MAPK10* mutations as independent prognostic factors for decreased DFS.

The observation of statistically lower OS in patients with right colon tumors and gene mutations has been confirmed in the literature. Borakati *et al*. conducted A retrospective study and found that tumors in the right colon were independent prognostic factors for reduced OS after hepatic metastasectomy, regardless of the higher rates of liver metastases and larger metastases in the left colon[58]. Patients with mutations in genes, such as *AKT1*, *APC2*, *ARAF*, *BAD*, *MAPK10*, *RAC3*, *RHOA*, *TGFB2*, and *TGFB3* had significantly reduced OS, as reported in other studies that also linked *APC2*, *RHOA*, and *TGFB* mutations to worse prognosis[37,38,59]. Conversely, studies have shown that mutations in *MSH6* are associated with a lower risk of developing CRC, and patients with such mutations have a milder clinical presentation[60,61]. In the present study, patients with *MSH6* mutations had a significantly longer OS, confirming *MSH6* mutations are good prognostic factors. Concurrent mutations (co-mutations) are a significant factor that have been minimally investigated in CRC. Studies in patients with non-small cell lung cancer have shown distinct biological behavior and prognosis in *KRAS/LKB1*, *KRAS/TP53*, or *KRAS/p16* mutated tumors[62]. Additionally, our group has previously reported the importance of evaluating the loss of *LKB1* through immunohistochemistry in early stage CRC, particularly in $BRAF^{V600E}$ mutated tumors[63]. In the present study, several concurrent mutations were detected in patients, but no correlation was found with clinical/pathological characteristics or patient prognosis.

5. Conclusions

In conclusion, molecular characterization of cancer cells can enhance our understanding of the biological progression of this disease[64,65]. The findings of this study suggest that mutations are promising prognostic biomarkers. As personalized medicine has become the primary mode of therapy, knowledge of the precise mutation status of patients with CRC can lead to better therapeutic choices. However, further research is necessary with a larger patient cohort and international collaborations to confirm the correlation between patients' molecular profiles, clinicopathological and epidemiological characteristics, and outcomes. Such research is expected to contribute to more precise clinical decision-making, personalized and improved care, and reduced toxicity of treatment, costs to patients, and burden on health systems.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: Raw patient data.

Author Contributions: Conceptualization, I.M. and J.S.; methodology, I.M., E.P., K.V., M.S. and M.T.; software, I.M., E.P., P.T. and I.I.; validation, I.M., P.T. and M.T.; formal analysis, I.M.; data curation, I.M, D.M., J.T., N.G., M.T. and J.S..; writing—original draft preparation, I.M.; writing—review and editing, I.M., P.T., I.L. and J.S.; supervision, I.M., P.T. and J.S.; funding acquisition, I.M. and J.S. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was approved by the Ethics Committee/Institutional Review Board of the University Hospital of Heraklion (Number 7302/19-8-2009). All procedures were performed in accordance with the ethical standards of the institutional and/or national research committee and the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. All patients signed a written informed consent form for participation.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files. Supplementary Table S1: Raw patient data

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Conflicts of Interest: The authors declare no conflicts of interest.

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