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Association of FGF2, CD39, ANGPT1, and MMP9 Genetic Variants with Clinical Outcomes in Metastatic Colorectal Cancer Patients Treated with Bevacizumab and Chemotherapy

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Abstract

Genetic variations in angiogenesis-related genes lead to marked differences among patients in how well they respond to drugs that block new blood vessel formation. In a study of 57 individuals with metastatic colorectal cancer treated with bevacizumab plus chemotherapy, researchers tested 20 single nucleotide polymorphisms (SNPs) in 13 genes from the angiogenesis pathway to find markers that could forecast tumor shrinkage, progression-free survival (PFS), and overall survival (OS). DNA was obtained from archived formalin-fixed paraffin-embedded tumor tissue and genotyped with the iPLEX® Assay. Patients carrying the variant allele of CD39 rs11188513 were significantly more likely to achieve a good tumor response (p = 0.024). Those with two copies of the common (wild-type) allele at FGF2 rs1960669 had a median PFS of 10.95 months, whereas patients with at least one rare A allele had a median PFS of only 5.44 months (hazard ratio 3.30, 95% CI 1.52–7.14, p = 0.001). Having two common alleles at either MMP9 rs2236416 or MMP9 rs2274755 was linked to longer PFS (median 9.48 months for both) compared with 6.00 and 6.62 months, respectively, in patients carrying at least one rare allele (p = 0.022 and p = 0.043). Median overall survival reached 30.92 months in patients homozygous for the common allele of ANGPT1 rs2445365 versus 22.07 months in those with at least one rare A allele (p = 0.034).

These polymorphisms in angiogenesis pathway genes appear useful for predicting both treatment response and survival in metastatic colorectal cancer patients receiving bevacizumab-based therapy.

Keywords: Colorectal cancer, Bevacizumab, Polymorphisms, Angiogenesis

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Introduction

Colorectal cancer (CRC) is among the most commonly diagnosed cancers worldwide, ranking fourth in incidence and fifth in cancer-related mortality, with 1.85 million new cases and 861,663 deaths reported in 2018 [1]. The lethality of CRC is largely driven by metastasis, which is responsible for approximately 90% of deaths in colon cancer patients [2]. The development and progression of metastatic CRC (mCRC) are influenced by a combination of lifestyle, dietary habits, and socioeconomic factors, emphasizing the need for strategies aligned with predictive, preventive, and personalized medicine (PPPM) [1]. Increasing evidence suggests that genetic variation between individuals can significantly influence susceptibility to mCRC [3], and single nucleotide polymorphisms (SNPs) are emerging as valuable

biomarkers for predicting both disease risk and therapeutic outcomes [4, 5].

Bevacizumab (BVZ), a humanized monoclonal antibody targeting vascular endothelial growth factor (VEGF), is frequently combined with chemotherapy (CT) to treat mCRC [6]. By blocking VEGF signaling, BVZ disrupts the formation of new blood vessels and induces regression of existing tumor vasculature, thereby restricting tumor growth. VEGF and its receptor (VEGFR) are central mediators of angiogenesis, which is a hallmark of advanced cancers. Multiple studies have reported that combining CT with BVZ leads to improved progression-free survival (PFS), overall survival (OS), and response rates (RR) compared to chemotherapy alone [7–12]. Nonetheless, clinical responses to BVZ-based regimens vary widely, likely due to mechanisms of resistance such as activation of alternative proangiogenic pathways, recruitment of proangiogenic or inflammatory cells into the tumor microenvironment, and heterogeneity among genetically unstable tumor cells [13, 14].

Previous research has also highlighted associations between SNPs in angiogenesis-related genes and clinical outcomes in mCRC patients treated with BVZ. Specific gene variants have been linked to both disease susceptibility and treatment response [15–20]. Despite growing recognition of the importance of VEGF-dependent and independent angiogenic genes in mCRC pathogenesis and prognosis, our understanding of how these genetic differences affect BVZ efficacy and resistance remains limited [21].

In this study, we investigated whether genetic polymorphisms in key regulators of angiogenesis—including growth factors, cytokine signaling molecules, angiopoietins, and components of the adenosine pathway—can serve as predictive markers for BVZ-based chemotherapy in mCRC. We analyzed SNPs in 13 VEGF-dependent and independent angiogenic genes (ANGPT1, IGF1, A2BR, CCL5, CD39, ANGPT2, EDN1, MKNK1, FGF2, NT5E, MMP9, TOP1, and VEGF-A) and assessed their association with tumor response OS, PFS, and RR in patients receiving BVZ in combination with chemotherapy.

Results and Discussion

The study included 57 patients diagnosed with metastatic colorectal cancer (mCRC). The majority of primary tumors were located in the proximal (left-sided) colon, accounting for 70% of cases (40/57), while the distal (right-sided) colon was involved in 30% (17/57). Most patients (55/57, 96%) presented with advanced-stage disease (stage III or IV), and KRAS mutations were detected in 76% (42/55) at diagnosis. Patients had a mean of 2 metastatic sites, ranging from 1 to 5. The average age at diagnosis was 61.8 years, with a range of 27 to 81 years. The cohort was predominantly male, comprising 72% of patients (41/57) **(Table 1)**.

Table 1. Patient characteristics (n = 57).

Patient and Treatment Characteristics	Number (%)
Tumor response to bevacizumab	
– Responders	30 (52.6%)
– Non-responders	27 (47.4%)
Gender	
– Female	16 (28%)
– Male	41 (72%)
Age at treatment (years)	
– Younger than 55	13 (22.8%)
– 55 to 65	20 (35.1%)
– Older than 65	24 (42.1%)
Number of metastatic sites (liver, lung, peritoneum, etc.)	
– 1 site	16 (27.7%)
– 2 sites	22 (38.6%)
– More than 2 sites	19 (33.3%)
Location of primary tumor	
– Right-sided colon	17 (30%)
– Left-sided colon / rectum	40 (70%)
Tumor stage at initial diagnosis	
– Stage I	0

– Stage II	2 (3.5%)
– Stage III	16 (28.1%)
– Stage IV	39 (68.4%)
KRAS mutation status	
– Mutated	42 (76%)
– Wild-type (normal)	13 (24%)
Chemotherapy regimen combined with bevacizumab	
– FOLFOX	27 (47%)
– FOLFIRI	21 (37%)
– Other (capecitabine, 5-FU, irinotecan alone, etc.)	9 (16%)
Line of bevacizumab-containing therapy	
– 1st line	33 (58.9%)
– 2nd line	15 (26.7%)
– 3rd line	6 (10.7%)
– 4th line	1 (1.8%)
– 5th line	1 (1.8%)
Primary tumor surgically removed	
– Yes	20 (35%)
– No	37 (65%)

Only 35% of patients underwent surgical resection as their initial treatment. All participants received chemotherapy combined with bevacizumab (CT + BVZ), with this regimen serving as the first-line therapy in 58.9% of cases (33/57). Among the first-line chemotherapy protocols, FOLFOX was the most commonly administered (47%, 27/57) **(Table 1)**. Most patients (79%) did not require dose adjustments of BVZ. Treatment discontinuation due to either lack of response or significant toxicity occurred in only 11% of patients, as determined by the treating physician. Regarding adverse events, 13% of patients (7/57) experienced grade III toxicity, while 87% experienced milder grade I–II toxicity (50/57). The most frequently observed side effects included hypertension (34%, 19/57), intestinal perforation (11%, 6/57), and bleeding events (32%, 18/57), which were primarily epistaxis, followed by rectal fistulas and hemorrhoids.

The median follow-up period was 28.5 months. At the end of the study, 61% of patients had died. The median progression-free survival (PFS) was 11.09 months, and the median overall survival (OS) was 34.79 months. Tumor response was observed in 30 patients (52.6%), whereas 27 patients (47.4%) experienced disease progression **(Table 1)**.

Gene variants

The accuracy of genotyping was validated through direct DNA sequencing, yielding a concordance rate of 99%. Across the cohort, genotyping was successfully performed in 97.98% of samples for each SNP examined, with the exception of MMPA-rs17577 and CDKAL1-rs7453577, which were excluded from further analysis. The distribution of the 20 SNPs across the 13 genes is presented in **Table 2**.

Several SNPs exhibited a high prevalence of the wild-type homozygous genotype, including ANGPT2 rs10102851 (92.98%), FGF2 rs1960669 (82.14%), VEGF-A rs3025039 (75.44%), TOP1 rs34282819 (77.19%), MMP9 rs2236416 (75.44%), EDN1 rs5370 (60.71%), MMP9 rs2274755 (76.78%), ANGPT1 rs2445365 (57.89%), CCL5 rs2280789 (82.46%), and MKNK1 rs8602 (53.70%) **(Table 2)**. In contrast, a large proportion of patients carried at least one variant allele for certain genes, including NT5E rs2229523 (94.64%), ANGPT2 rs2515462 (92.98%), VEGFA rs833061 (82.46%), CD39 rs11188513 (89.47%), and IGF1 rs6220 (92.86%), and ANGPT2 rs1375668 (89.29%),. Notably, approximately 50% of patients were heterozygous for VEGFA polymorphisms rs833061, rs833068, and rs833069, as well as for TOP1 rs6072249 and A2BR rs2015353. **Table 2** also classifies the analyzed genes according to their associated biological processes based on Gene Ontology. This classification confirms that all SNPs investigated are located in genes with a direct or indirect role in angiogenesis, which is the principal biological pathway targeted by bevacizumab (BVZ).

Table 2. Distribution of SNP genotypes and gene classification according to biological process (Gene Ontology). Reference allele frequencies are provided from the 1000 Genomes database [22] (W = wild-type allele, V = variant allele).

Functional Category	Gene & Variant (dbSNP)	Variant Type	Genotype Distribution in 57 Patients	Wild-type Allele (W) Frequency	Variant Allele (V) Frequency	1000 Genomes European Population Allele Frequency	Most Common Substitution
Adenosine signaling pathway	ADORA2B (A2BR) rs2015353	Coding sequence variant	TT: 8 (15.1%) TC: 26 (49.1%) CC: 19 (35.8%)	T = 0.39	C = 0.61	T = 0.46 C = 0.54	T → C
	ENTPD1 (CD39) rs11188513	Intronic variant	CC: 6 (10.5%) CT: 24 (42.1%) TT: 27 (47.4%)	C = 0.31	T = 0.69	C = 0.36 T = 0.64	C → T
	NT5E (CD73) rs2229523	Missense variant	AA: 3 (5.4%) AG: 26 (46.4%) GG: 27 (48.2%)	A = 0.29	G = 0.71	A = 0.29 G = 0.71	A → G
Direct regulators of angiogenesis	ANGPT1 rs2445365	Intronic variant	GG: 33 (57.9%) GA: 20 (35.1%) AA: 4 (7.0%)	G = 0.75	A = 0.25	G = 0.75 A = 0.25	G → A, C
	ANGPT2 rs10102851	Intronic variant	AA: 53 (93.0%) AG: 4 (7.0%) GG: 0	A = 0.97	G = 0.03	A = 0.97 G = 0.03	A → G
	ANGPT2 rs1375668	Intronic variant	GG: 6 (10.7%) GA: 18 (32.1%) AA: 32 (57.1%)	G = 0.27	A = 0.73	G = 0.34 A = 0.66	G → A, C
	ANGPT2 rs2515462	Intronic variant	AA: 4 (7.0%) AG: 25 (43.9%) GG: 28 (49.1%)	A = 0.29	G = 0.71	A = 0.32 G = 0.68	A → C, G, T
	VEGFA rs833061	Upstream regulatory variant	CC: 10 (17.5%) CT: 28 (49.1%) TT: 19 (33.3%)	C = 0.42	T = 0.58	C = 0.50 T = 0.50	C → G, T
	VEGFA rs833068	Intronic variant	GG: 17 (29.8%) GA: 28 (49.1%) AA: 12 (21.1%)	G = 0.54	A = 0.46	G = 0.69 A = 0.31	G → A
	VEGFA rs833069	Intronic variant	TT: 16 (29.1%) TC: 27 (49.1%) CC: 12 (21.8%)	T = 0.54	C = 0.46	T = 0.69 C = 0.31	T → C, G
	VEGFA rs3025039	3′ UTR variant	CC: 43 (75.4%) CT: 13 (22.8%) TT: 1 (1.8%)	C = 0.88	T = 0.12	C = 0.88 T = 0.12	C → T
	FGF2 rs1960669	Intronic variant	CC: 46 (82.1%) CA: 9 (16.1%) AA: 1 (1.8%)	C = 0.90	A = 0.10	C = 0.84 A = 0.16	C → A
	MMP9 rs2236416	Intronic variant	AA: 43 (75.4%) AG: 12 (21.1%) GG: 2 (3.5%)	A = 0.86	G = 0.14	A = 0.83 G = 0.17	A → G
	MMP9 rs2274755	Intronic variant	GG: 43 (76.8%) GT: 12 (21.4%) TT: 1 (1.8%)	G = 0.87	T = 0.13	G = 0.83 T = 0.17	G → T
Cytokine and chemokine signaling	EDN1 rs5370	Missense variant	GG: 34 (60.7%) GT: 20 (35.7%) TT: 2 (3.6%)	G = 0.79	T = 0.21	G = 0.78 T = 0.22	G → T
	CCL5 rs2280789	Intronic variant	AA: 47 (82.5%) AG: 7 (12.3%) GG: 3 (5.3%)	A = 0.89	G = 0.11	A = 0.89 G = 0.11	A → G, C, T
DNA topology regulation	TOP1 rs34282819	5′ regulatory variant	CC: 44 (77.2%) CA: 13 (22.8%) AA: 0	C = 0.89	A = 0.11	C = 0.92 A = 0.08	C → A
	TOP1 rs6072249	Upstream regulatory variant	AA: 18 (31.6%) AG: 29 (50.9%) GG: 10 (17.5%)	A = 0.57	G = 0.43	A = 0.55 G = 0.45	A → G
Intracellular signaling	MKNK1 rs8602	Non-coding	CC: 29 (53.7%) CA: 22 (40.7%) AA: 3 (5.6%)	C = 0.74	A = 0.26	C = 0.72 A = 0.28	C → A

Growth factor signaling	IGF1 rs6220	transcript variant 3′ UTR variant	GG: 4 (7.1%) GA: 18 (32.1%) AA: 34 (60.7%)	G = 0.23	A = 0.77	G = 0.27 A = 0.73	G → A

All examined allele frequencies were consistent with Hardy-Weinberg equilibrium. In univariate analysis of SNP distribution among patients, a significant association was observed only for the variant allele of CD39 rs11188513. Specifically, carriers of the homozygous variant genotype (VV/TT) had higher odds of a particular outcome compared with wild-type homozygotes or heterozygotes (CT/CC) (OR 3; 95% CI: 1.015–8.864; p = 0.047). No other SNPs or patient characteristics included in the analysis **(Table 1)** demonstrated statistically significant differences within the cohort.

To assess tumor genetic heterogeneity, the number of mutated genes was calculated according to tumor stage **(Table 3)**. Patients with stage III and IV disease exhibited the highest accumulation of mutated genes. Notably, the variant ANGPT1 rs2445365 was detected in 19 out of 39 patients with stage IV disease.

Table 3. Distribution of genetic heterogeneity in mCRC tumors according to tumor stage. The table presents the number of mutated genes observed per patient at each stage of disease.

Tumor Stage	<7 Mutated Genes	7 Mutated Genes	8 Mutated Genes	9 Mutated Genes	10 Mutated Genes	11 Mutated Genes
II	–	1 (50%)	1 (50%)	–	–	–
III	–	4 (25%)	5 (31%)	5 (31%)	2 (13%)	–
IV	6 (15.5%)	11 (28%)	6 (15.5%)	11 (28%)	3 (8%)	2 (5%)

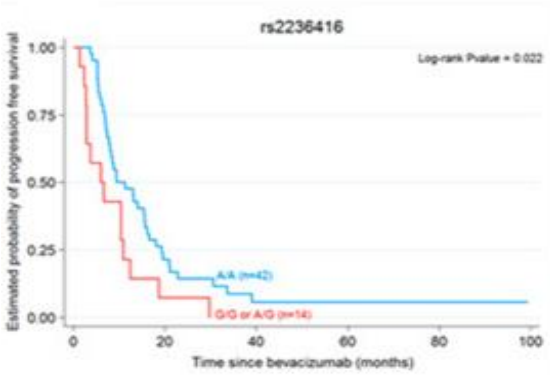
Gene variants and tumour response

To investigate whether specific SNPs influenced the effectiveness of CT combined with bevacizumab (CT+BVZ), tumor response after six months of therapy was analyzed in relation to patients' genotypes. Patients were divided into responders (R) and non-responders (nR), and the frequency of each polymorphism was compared between these groups. Two analytical approaches were applied: first, comparing patients with at least one variant allele (VV or W/V) to those carrying only wild-type alleles (WW); second, comparing patients homozygous for the variant allele (VV) to those with either a single variant or only wild-type alleles (W/V or WW).

Among all SNPs evaluated, only CD39 rs11188513 showed a significant correlation with treatment response. Individuals carrying this variant allele were more frequently observed in the responder group (62.07%), whereas non-responders were predominantly heterozygous or homozygous for the wild-type C allele (67.86%) (p = 0.024). No other polymorphisms demonstrated a statistically meaningful difference in distribution between responders and non-responders (data not shown).

Gene variants, progression-free survival and overall survival

In univariate analyses, certain wild-type alleles were found to have a significant impact on progression-free survival (PFS). Specifically, patients carrying the CC genotype of FGF2 rs1960669 exhibited a median PFS of 10.95 months, whereas individuals with at least one variant-A allele had a substantially shorter median PFS of 5.44 months (HR 3.30; 95% CI: 1.52–7.14; p = 0.001). Similarly, for MMP9, patients homozygous for the wild-type alleles of rs2236416 and rs2274755 demonstrated longer median PFS (9.48 months) compared to 6 months and 6.62 months, respectively, in those harboring one or more variant alleles (p = 0.022 and p = 0.043). No additional SNPs included in the analysis were significantly associated with PFS **(Table 4 and Figure 1)**.



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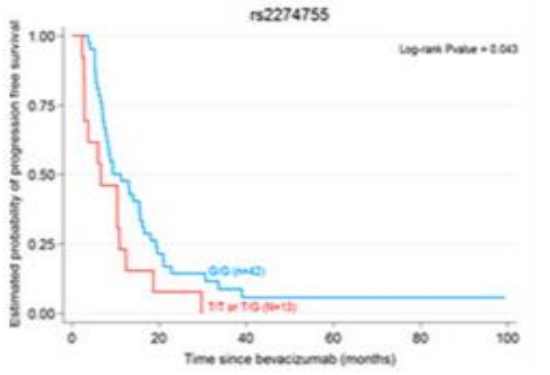

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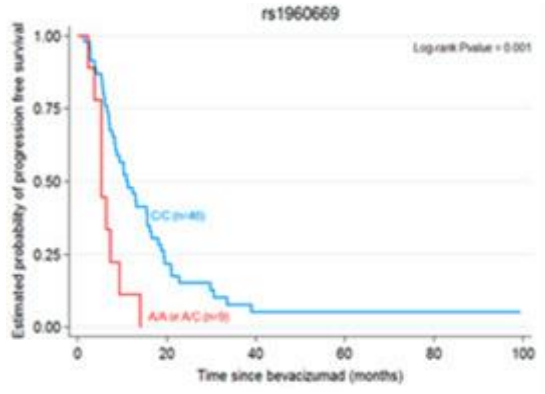

c)

Figure 1. Genotype-specific progression-free survival (PFS) in metastatic colorectal cancer patients treated with bevacizumab: (A) Comparison of MMP9 rs2236416: patients with A/A genotype versus those carrying one or two G alleles (A/G or G/G) showing median PFS of 9.48 versus 6 months (p = 0.022). (B) Comparison of MMP9 rs2274755: G/G homozygotes versus G/T or T/T carriers with median PFS of 9.48 versus 6.82 months (p = 0.043). (C) Comparison of FGF2 rs1960669: C/C homozygotes versus patients with at least one A allele (C/A or A/A) showing median PFS of 10.95 versus 5.44 months (p = 0.001).

Table 4. Summary of clinical outcomes according to SNP genotype: median overall survival (OS) and progression-free survival (PFS) for each polymorphism analyzed.

Gene (SNP)	Genotype	Median PFS (months, 95% CI)	HR (95% CI)	p-value	Median OS (months, 95% CI)	HR (95% CI)	p-value
A2BR rs2015353	TT	6.20 (2.89–16.56)	0.57 (0.26–1.24)	–	12.52 (4.89–…)	0.70 (0.31–1.60)	–
	TC/CC	10.39 (7.05–15.64)	–	0.149	28.39 (21.48–34.89)	–	0.397
CD39 rs11188513	CC	9.41 (2.43–…)	1.71 (0.65–4.46)	–	43.84 (12.52–…)	2.32 (0.71–7.54)	–
	CT/TT	8.75 (7.05–13.18)	–	0.272	23.51 (19.38–30.92)	–	0.150
NT5E rs229523	AA	9.48 (6.62–…)	0.8 (0.25–5.59)	–	27.54 (11.84–…)	0.59 (0.18–1.95)	–
	AG/GG	9.41 (7.05–13.18)	–	0.708	23.87 (20.72–34.89)	–	0.386
ANGPT1 rs2445365	GG	9.48 (6.62–14.03)	1.20 (0.69–2.06)	–	30.92 (21.48–37.87)	1.91 (1.04–3.50)	0.034
	GA/AA	8.75 (5.74–15.70)	–	0.519	22.07 (12.52–29.28)	–	–
ANGPT2 rs10102851	AA	8.75 (6.89–13.05)	0.97 (0.30–3.15)	–	–	–	–
	AG/GG	14.03 (12.43–…)	–	0.953	–	–	–
ANGPT2 rs1375668	GG	10.43 (5.44–…)	0.66 (0.27–1.58)	–	21.48 (12.20–…)	0.72 (0.24–2.08)	–
	GA/AA	9.41 (7.21–14.03)	–	0.343	23.87 (20–34.89)	–	0.544
ANGPT2 rs2515462	AA	10.39 (5.74–…)	0.74 (0.26–2.09)	–	21.48 (12.20–…)	0.82 (0.19–3.51)	–
	AG/GG	8.75 (7.05–13.18)	–	0.568	27.54 (20–32.89)	–	0.787
VEGFA rs833061	CC	5.61 (2.79–15.64)	0.71 (0.36–1.43)	–	21.48 (4.89–32.89)	0.77 (0.37–1.61)	–
	CT/TT	9.48 (7.21–13.18)	–	0.337	23.87 (18.85–35.02)	–	0.481
VEGFA rs833068	GG	9.48 (5.44–15.64)	1.00 (0.56–1.80)	–	22.20 (14.75–36.52)	0.93 (0.48–1.78)	–
	GA/AA	9.41 (6.62–13.18)	–	0.999	23.87 (18.85–34.89)	–	0.821
VEGFA rs833069	TT	8.52 (5.44–15.48)	0.98 (0.54–1.79)	–	28.39 (14.75–36.52)	1.03 (0.53–2.02)	–
	TC/CC	9.41 (6.62–13.18)	–	0.946	23.87 (18.62–34.89)	–	0.926
VEGFA rs3025039	CC	9.41 (6.89–13.18)	1.10 (0.59–2.03)	–	23.87 (19.38–32.89)	0.84 (0.40–1.75)	–
	CT/TT	8.52 (5.74–16.56)	–	0.769	27.54 (11.74–…)	–	0.634

FGF2 rs1960669	CC	10.95 (7.84–15.70)	3.30 (1.52–7.14)	0.001	27.54 (20–34.89)	1.45 (0.69–3.05)	0.324
	CA/AA	5.44 (2.43–9.48)	–	–	22.20 (9.97–32.89)	–	–
MMP9 rs2236416	AA	9.48 (7.84–15.64)	2.04 (1.09–3.80)	0.022	23.51 (19.38–29.74)	0.91 (0.46–1.81)	–
	AG/GG	6.00 (2.79–10.95)	–	–	32.74 (12.20–43.84)	–	–
MMP9 rs2274755	GG	9.48 (7.84–15.64)	1.91 (1.01–3.63)	0.043	23.51 (19.38–29.74)	0.90 (0.44–1.84)	–
	GT/TT	6.62 (2.89–10.95)	–	–	35.02 (12.20–43.84)	–	–
EDN1 rs5370	GG	10.43 (7.21–16.26)	1.29 (0.74–2.26)	–	23.51 (18.85–34.89)	1.02 (0.55–1.88)	–
	GT/TT	7.84 (4.20–14.03)	–	0.367	27.54 (11.84–35.02)	–	0.957
CCL5 rs2280789	AA	9.48 (7.21–15.48)	1.44 (0.70–2.98)	–	27.54 (21.48–32.89)	1.17 (0.49–2.79)	–
	AG/GG	7.44 (2.89–12.43)	–	0.323	16.03 (7.31–…)	–	0.723
TOP1 rs34282819	CC	8.52 (6.89–12.43)	0.80 (0.42–1.54)	–	23.87 (20.72–32.72)	0.93 (0.46–1.91)	–
	CA/AA	16.26 (4.20–21.08)	–	0.501	27.54 (9.97–44.49)	–	0.853
TOP1 rs6072249	AA	11.34 (5.44–18.10)	1.05 (0.58–1.91)	–	23.51 (12.10–35.02)	0.87 (0.45–1.65)	–
	AG/GG	8.75 (7.05–13.05)	–	0.862	23.87 (19.38–34.89)	–	0.662
MKNK1 rs8602	CC	8.75 (6.89–13.18)	1.04 (0.59–1.83)	–	23.51 (16.03–36.52)	1.18 (0.63–2.20)	–
	CA/AA	11.34 (7.21–16.56)	–	0.904	28.39 (21.48–34.89)	–	0.614
IGF1 rs6220	GG	13.05 (12.43–…)	1.09 (0.38–3.07)	–	21.48 (4.89–32.89)	1.70 (0.41–7.07)	–
	GA/AA	8.52 (6.62–11.34)	–	0.874	23.87 (18.85–35.02)	–	0.457

Analysis of overall survival (OS) revealed that patients harboring the wild-type ANGPT1 rs2445365 genotype had a notably longer median survival of 30.92 months, compared with 22.07 months for those carrying at least one variant-A allele (p = 0.034) **(Table 4 and Figure 2)**. No other SNPs examined in this study showed a significant impact on OS **(Table 4 and Figure 2)**.

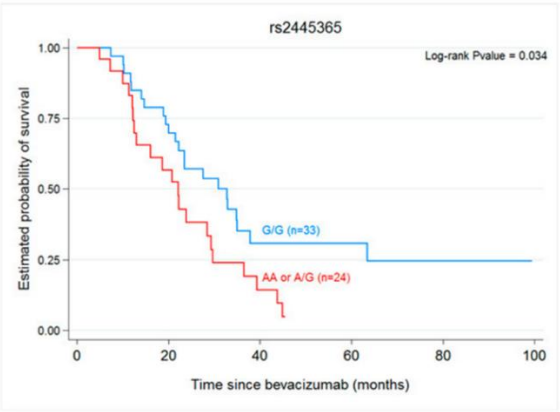

Figure 2. OS in response to bevacizumab treatment in patients with G/A/AA vs. ANGPT1 rs2445365 G/G (22.07 vs. 30.92 months, p = 0.034).

When combined with chemotherapy, the widely used anti-VEGF monoclonal antibody bevacizumab (BVZ) demonstrates variable efficacy depending on the cancer type and even among patients with the same cancer [6, 23–25]. In metastatic colorectal cancer (mCRC), multiple studies have reported that treatment with chemotherapy plus BVZ (CT+BVZ) leads to higher response rates, improved progression-free survival (PFS), and increased overall survival (OS) compared to chemotherapy alone [7–9, 11, 12]. Similarly, in breast cancer, BVZ has been associated with significantly improved pathological complete response (pCR) rates [24]. Nevertheless, clinical evidence also indicates that both the effectiveness of BVZ as an anti-angiogenic agent and the development of resistance vary considerably among mCRC patients [21]. This variability has driven the search for predictive biomarkers to assess treatment efficacy and forecast outcomes.

Over the past decades, certain polymorphisms in genes related to VEGF-dependent and independent pathways have been proposed as potential determinants of improved clinical outcomes in mCRC patients receiving first-line CT+BVZ [24, 26]. Studies in other cancers, such as breast cancer, have also suggested that genetic variants located in regulatory regions, beyond coding sequences, may be particularly relevant [24]. Since angiogenic mechanisms are implicated in resistance to BVZ, we analyzed a panel of single nucleotide polymorphisms (SNPs) associated with tumor angiogenesis to identify predictors of individual treatment response. We selected 20 SNPs from 13 genes, focusing on regulatory region variants (intron, 3'UTR, 5'UTR) capable of altering gene expression, as

well as non-synonymous coding SNPs affecting amino acid sequences. The allele frequencies of these 20 SNPs in our mCRC cohort were generally comparable to those reported in healthy European populations [22].

A notable observation was the association between the CD39 rs11188513 SNP and tumor response to BVZ treatment. CD39 is a key enzyme in the adenosine pathway, and extracellular adenosine has well-documented immunosuppressive and pro-angiogenic effects within the tumor microenvironment. Adenosine is produced by CD39 and CD73, primarily expressed on cancer cells, B cells, or regulatory T cells (Tregs), influencing both cancer cell proliferation through A2BR receptors and immune cell activity via A2AR and A2BR [27–29]. In our study, patients homozygous for the variant allele of CD39 rs11188513 showed favorable responses, defined as the absence of tumor progression after six months of BVZ therapy, although this did not translate into improved PFS or OS. These findings align with those of Tokunaga *et al*., who reported that the presence of any wild-type allele (C) corresponded to poorer PFS and OS compared to homozygous variant patients [30]. Their study, involving 451 mCRC patients divided into control (FOLFIRI + cetuximab), analysis (FOLFIRI + BVZ), and validation (FOLFIRI + BVZ) groups, identified CD39 rs11188513 as a strong predictor of BVZ treatment outcomes. Located in the 3'-UTR of CD39, this SNP is a potential binding site for miR-155, which may regulate CD39 function [31, 32]. Tokunaga *et al*. also suggested that it could act as a tag SNP affecting other functional polymorphisms within CD39 [30]. While the mechanistic impact of this variant remains unclear, CD39 is increasingly recognized as a potential checkpoint target due to its role in mitigating adenosine-mediated immunosuppression and influencing tumor progression [33]. Recent studies have highlighted the prognostic and predictive potential of this SNP in the context of BVZ therapy [30], warranting further research.

The underlying reasons for inter-patient variability in BVZ efficacy in mCRC remain incompletely understood. In our cohort, MMP9 polymorphisms rs2236416 and rs2274755 were associated with PFS after CT+BVZ treatment. MMP9 functions as a type IV collagenase and is critical for tumor growth, invasion, metastasis, and angiogenesis [34, 35]. Prior research has proposed MMP9 as a biomarker in various cancers [36–38], and genetic variants may influence susceptibility to cancer. Despite the numerous reported MMP9 polymorphisms, their clinical significance remains largely unclear. Based on findings by Makhoul *et al*., we assessed the impact of rs2236416 and rs2274755 on CT+BVZ response in mCRC. Their work in breast cancer showed that tumors with these MMP9 variants were more likely to achieve pCR following neoadjuvant BVZ therapy [24]. In our study, patients homozygous for the wild-type alleles of rs2236416 and rs2274755 had a median PFS of 9.48 months, whereas carriers of at least one variant allele had reduced PFS of 6 and 6.62 months, respectively ($p = 0.022$, $p = 0.043$). The rs2274755 SNP is located at the third base of the fourth intron and may influence RNA splicing, although its functional significance remains to be determined. The rs2236416 SNP, also intronic, has been linked to Henoch-Schönlein purpura nephritis (HSPN), though no functional data are currently available [39]. These MMP9 variants may modulate gene expression in mCRC, potentially diminishing the response to BVZ.

Additionally, we observed a significant univariate association between FGF2 rs1960669, an intronic tag SNP, and PFS. FGF2 is a potent angiogenic factor implicated in tumor progression, metastasis, and response to therapy [40, 41]. Elevated serum FGF2 levels correlate with disease burden and mortality in metastatic cancers [42]. Makhoul *et al*. also reported a relationship between FGF2 rs1960669 and pCR in breast cancer patients treated with neoadjuvant BVZ [24]. Future research should explore the role of this SNP in mCRC prognosis and survival. If BVZ efficacy can be predicted by genotype, it may inform personalized anti-angiogenic treatment strategies. The FGF2 variant may influence the expression of specific isoforms, which could differentially affect tumor progression under BVZ therapy.

VEGF-independent angiogenesis is also relevant in colorectal cancer. ANGPT1 is a key mediator of this pathway. In our study, carriers of the wild-type ANGPT1 rs2445365 allele exhibited improved OS, while other SNPs showed no effect. This supports the established efficacy of VEGF-targeting agents such as BVZ [23]. Resistance to anti-angiogenic therapy may result from intrinsic tumor heterogeneity, redundant angiogenic signaling, or recruitment of hematopoietic and inflammatory cells [14]. Genetic variations in ANGPT1 may alter its expression and function. SNPs in ANGPT genes have been linked to autoimmune diseases, portopulmonary hypertension, and colorectal cancer risk and outcomes [43–45]. Makhoul *et al*. reported that ANGPT1 rs2445365 correlates with pCR in breast cancer patients treated with BVZ [24]. In our mCRC cohort, the wild-type allele of rs2445365 may improve OS. Although the phenotypic impact remains uncertain, the ANGPT-TIE signaling pathway plays a critical role in VEGF-independent angiogenesis, vascular homeostasis, and links angiogenesis to inflammation [46]. ANGPT1 upregulation is associated with malignancy in several cancers [46], suggesting rs2445365 may influence protein function and metastasis. Some studies also suggest ANGPT1 can inhibit pathologic vascular expansion, indicating tumor-suppressor functions in certain contexts [47–50]. These findings imply that ANGPT1's role in prognosis may be cancer-specific and influenced by other angiogenesis-related genes.

This study has limitations, including a relatively small sample size and heterogeneous tumor and treatment characteristics. Nevertheless, the clinical relevance of these SNPs underscores the need for larger, more uniform studies. Validating these findings could enable genotyping to identify patients with likely favorable responses, while sparing others from potential side effects. Personalized approaches using BVZ could be cost-effective, easily implemented in clinical practice, and improve outcomes in mCRC treatment.

Materials and Methods

Design and patients

This retrospective study enrolled 57 individuals with confirmed metastatic colorectal cancer treated at the Oncology Unit of Fuenlabrada University Hospital (Madrid, Spain) from 2009 through 2019. All received bevacizumab together with various chemotherapy backbones (including FOLFOX, FOLFIRI, capecitabine, 5-fluorouracil monotherapy, or irinotecan-based schemes). A medical oncologist (Dr. D. Malón) gathered the clinical information by reviewing patient charts. Records also documented whether the primary tumor was located in the right or left colon/rectum and whether the analyzed tissue came from surgical resection or needle biopsy.

The research was performed at Fuenlabrada University Hospital in accordance with the Declaration of Helsinki and ICH Good Clinical Practice standards. It received approval from the local Ethics Committee (code APR 15/38, August 2015) and from the European University Research Committee (code CIPI/18/106, April 2018). Every patient signed an informed consent form before inclusion.

Patients underwent clinical evaluation and staging CT scans every three weeks while on treatment. Objective response was assessed using RECIST criteria. Individuals showing complete or partial response after six months of bevacizumab were classified as responders (R group), whereas those with stable disease or progression at that time point were considered non-responders (nR group). The decision to continue bevacizumab beyond progression was left to the treating physician.

Study endpoints were progression-free survival (PFS) and overall survival (OS). PFS was defined as the interval from treatment initiation to radiologic progression or death from any cause, and OS as the interval from treatment start to death from any cause. For patients without progression or who remained alive, survival times were censored at the date of their last contact.

Candidate polymorphisms

Genes and polymorphisms involved in both VEGF-dependent and VEGF-independent angiogenic pathways were selected based on existing literature and publicly available databases. The selection criteria included: (a) documented evidence supporting the gene's role in angiogenesis signaling pathways; (b) polymorphisms previously reported as predictive biomarkers of response to bevacizumab (BVZ) therapy in cancer, particularly colorectal cancer (CRC); and (c) a minor allele frequency greater than 5% in Caucasian populations.

A total of 20 SNPs across 13 genes were examined: ANGPT1 (rs2445365), A2BR (rs2015353), IGF1 (rs6220), ANGPT2 (rs10102851, CCL5 (rs2280789), rs1375668, rs2515462), CD39 (rs11188513), EDN1 (rs5370), MKNK1 (rs8602), FGF2 (rs1960669), MKNK1 (rs8602), TOP1 (rs34282819, rs6072249), and VEGF-A (rs3025039, MMP9 (rs2236416, rs2274755), NT5E (rs2229523), rs833061, rs833068, rs833069). An overview of these genes classified according to Gene Ontology–Biological Processes is presented in **Table 2**. This categorization confirmed that all analyzed SNPs are located in genes that play a direct or indirect role in angiogenesis.

Tumour DNA extraction and genotyping

Tissue samples obtained through either open surgical biopsy (incisional or excisional) or core-needle biopsy were handled using routine pathology laboratory procedures, which included immediate fixation in 10% neutral buffered formalin and subsequent embedding in paraffin. Once the pathological diagnosis had been confirmed by conventional histology and/or immunohistochemistry, the leftover formalin-fixed paraffin-embedded (FFPE) blocks were repurposed for genomic studies. In cases where multiple blocks existed for the same patient, the block containing the greatest proportion of tumor cells was selected; no further macro- or microdissection was carried out to enrich tumor content. For DNA isolation, 3–5 sections of 5 μm thickness were cut from each chosen block on a Leica EM UC7 ultramicrotome (Wetzlar, Germany); the very first sections were routinely discarded to avoid contamination or degradation artifacts. DNA was isolated with the Maxwell® 16 FFPE Plus LEV DNA Purification Kit (Promega, Madison, WI, USA) exactly as recommended by the manufacturer, and the purified DNA was finally dissolved in 50 μL of nuclease-free water. Yield and purity were measured on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Only specimens providing a DNA concentration ≥50 ng/μL and an A260/A280 ratio between 1.8 and 2.0 (indicating acceptable purity) were accepted for downstream genotyping. Samples meeting these criteria were aliquoted into 96-well plates and kept at −20 °C until use.

Twenty selected single-nucleotide polymorphisms (SNPs) were genotyped at the Spanish National Genotyping Centre (CeGen-PRB2-ISCIII, www.cegen.org) on the

MassARRAY system using iPLEX® Gold chemistry (Agena Bioscience, San Diego, CA, USA), following the supplier's standard protocols. All assays were designed against the GRCh38 human genome assembly with Agena Bioscience Assay Designer software version 4.0, and the 20 SNPs were divided into two multiplex reactions.

Each PCR was performed in a 5 µL volume containing 20 ng of genomic DNA, 1× PCR buffer, 2 mM $MgCl_2$, 500 µM of each dNTP, and 1 unit of hot-start polymerase (reagents from Agena Bioscience). Final primer concentrations were 100 nM each. Amplification conditions were: 94 °C for 2 min, then 45 cycles of 94 °C/30 s → 56 °C/30 s → 72 °C/1 min, ending with 72 °C for 1 min. Excess dNTPs were removed by adding 0.6 units of shrimp alkaline phosphatase, incubating at 37 °C for 40 min, and inactivating the enzyme at 85 °C for 5 min.

The single-base extension reaction (iPLEX Gold) was carried out in 9 µL containing 0.222× iPLEX Buffer Plus, 0.5× termination mix, and 0.5× iPLEX enzyme, with extension primers at final concentrations of 0.73–1.46 µM. Cycling parameters were: 94 °C for 30 s, followed by 40 outer cycles that each incorporated five inner cycles (94 °C/5 s → 52 °C/5 s → 80 °C/5 s), and a final extension at 72 °C for 3 min.

Products were purified with Clean Resin (Agena Bioscience), transferred to a 384-well SpectroCHIP II using an RS1000 Nanodispenser, and analyzed on a MassARRAY Analyzer 4 (MA4) mass spectrometer. Spectra were visually inspected and called with Typer 4.0.26 software. Every 384-well plate included no-template controls and three Coriell reference DNAs (NA10861, NA11994, NA11995). Two samples and two SNPs (MMP9-rs17577 and CDKAL1-rs7453577) failed reproducibility checks and were removed, as was the intergenic variant rs444903. Quality-control replicates showed 100% concordance and complete call rates.

Genotype and allele counts for the retained SNPs (covering 13 genes in total; **(Table 2)**) were determined by direct enumeration and compared with published frequencies in European-ancestry individuals from the 1000 Genomes Project [22]. All loci conformed well to Hardy–Weinberg expectations.

Statistical analysis

Continuous variables are presented either as medians with interquartile ranges (IQR) or as means ± standard deviations (SD), depending on their distribution, which was assessed using the Shapiro–Wilk test for normality. Categorical variables are expressed as absolute numbers and percentages.

The relationships between genetic polymorphisms and baseline clinical characteristics **(Table 1)**, as well as tumor response, were analyzed using contingency tables and Fisher's exact test. Hazard ratios (HRs) and 95% confidence intervals (CIs) were calculated using Cox proportional hazards models. For each polymorphism and patient subgroup, HRs for overall survival (OS) and progression-free survival (PFS), along with 95% CIs, were estimated.

Survival differences according to genotype were evaluated using Kaplan–Meier curves and the log-rank test. Comparisons included patients carrying at least one variant allele (VV or VW) versus those with two wild-type alleles (WW), as well as individuals with two variant alleles (VV) versus those with one or no variant allele (VW or WW) for each SNP. Kaplan–Meier plots were generated, and all statistical analyses were performed using Stata (STATA IC, version 14, StataCorp LLC, Texas, USA). A p-value of ≤ 0.05 was considered statistically significant.

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Conflict of interest: None.

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Ethics statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of University Hospital of Fuenlabrada (protocol code APR 15/38, August 2015) and Research Committee of the European University (identification code: CIPI/18/106, April 2018).

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