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Variations in genes involved in immune response checkpoints and association with outcomes in patients with resected colorectal liver metastases

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Abstract

In patients with colorectal liver metastases (CLM), liver resection offers the possibility of cure and long-term survival. The liver is a highly immunogenic organ harboring ~80% of the body's tissue macrophages. Emerging data demonstrate a critical role of the immune response for cancer treatment. We investigated variations within genes involved in immune response checkpoints and their association with outcomes in patients with CLM who underwent neoadjuvant chemotherapy including bevacizumab and liver resection. Single-nucleotide polymorphisms (SNPs) in nine genes (*CCL2, CCR2, LAG3, NT5E, PDCD1, CD274, IDO1, CTLA4* and *CD24*) were analyzed in genomic DNA from 149 patients with resected bevacizumab-pretreated CLM by direct Sanger DNA sequencing, and correlated with response, recurrence-free survival (RFS), overall survival (OS), probability of cure and recurrence patterns. *IDO1* (indoleamine 2, 3-dioxygenase) rs3739319 G>A and *CD24* rs8734 G>A showed a significant difference in 3-year OS rates. In addition, *IDO1* rs3739319 G>A was significantly associated with extrahepatic recurrence. Recursive partitioning analyses revealed that *IDO1* rs3739319 G>A was the dominant SNP predicting RFS and OS. Our data suggest that variants within genes involved in immune response checkpoints are associated with outcomes in patients with resected CLM and might lead to improved treatment strategies modulating anti-tumor immune response by targeting novel immune checkpoints.

INTRODUCTION

Approximately 50% of the patients with colorectal cancer present with metastases either at the time of diagnosis or at a later time.[1,2] If metastases are limited to the liver, patients can be cured or survival can be prolonged in a multidisciplinary treatment approach.[3] In patients with resectable colorectal liver metastases (CLM), perioperative chemotherapy has demonstrated to prolong progression-free survival, and in patients with unresectable CLM, chemotherapy can reduce tumor burden to allow secondary liver resection.[4,5] The addition of the monoclonal antibody bevacizumab to chemotherapy, which inhibits angiogenesis by targeting the vascular endothelial growth factor (VEGF), has been approved in the metastatic setting and is being used successfully in patients with liver-limited disease.[6,7]

The aim of this multidisciplinary treatment approach is cure, which can be achieved in ~ 25% of the patients.[4] However, predictive and prognostic biomarkers are needed to select patients benefiting from treatment. The liver is a highly immunogenic organ harboring ~80% of the body's tissue macrophages. In addition, T cells and other immune cells infiltrate the microenvironment of CLM, representing the tumor's immunogenic nature.[8] Thereby, investigating the immune system is a promising approach to identify biomarkers in patients with CLM. Previously, it has been demonstrated that these cells facilitate the anti-tumoral immune response and thereby influence clinical outcome in the treatment of CRC.[9] Emerging data show that VEGF and other factors involved in angiogenesis are having a significant role in modulation of the immune system, suggesting a potential role of immune response on the efficacy of anti-VEGF targeted therapy.[10]

The regulation of the immune response upon activation by antigens is facilitated via immune checkpoints. They represent an entity of cellular immune regulators and are considered attractive targets for drug development.[11] Therefore, these immune checkpoints may be predictive or prognostic biomarkers in the treatment of metastatic CRC. However, the influence of these checkpoints on clinical outcome in patients is still unclear and specifically, in patients with CLM undergoing perioperative bevacizumab-based chemotherapy has not yet been studied. Our goal was to test the clinical significance of single-nucleotide polymorphisms (SNPs) in genes coding for immune checkpoints on response and clinical outcome to bevacizumab-based perioperative chemotherapy. We investigated 10 SNPs in a panel of nine genes involved in immune response checkpoints (Figure 1). This comprised genes that are involved in inhibition of cytotoxic T cells and activation of inhibitory T-regulatory cells (Tregs). These mechanisms of action are facilitated via adenosine induced through adenosine monophosphate dephosphorylation by NT5E, via cell–cell interaction by CD274, PDCD1, LAG3 and CTLA4, via tryptophan degeneration by IDO1 (indoleamine 2, 3-dioxygenase), via the CCL2/CCR2 axis and via homeostatic proliferation by CD24.

MATERIALS AND METHODS

In this study, 149 patients from a single institution (Medical University Vienna) who underwent 3 months of neoadjuvant and 3 months of adjuvant fluoropyrimidine-based combination chemotherapy (oxaliplatin, $n = 124$; irinotecan, $n = 18$; both, $n = 7$) including

bevacizumab were enrolled, of whom 87 (58.4%) were male. The last dose of bevacizumab was administered 5 weeks before liver resection. Clinical data were obtained from a prospectively maintained database. The patients' median age was 62 years (range 30–80). For all patients, the median recurrence-free survival (RFS) was 11.7 months (95% confidence interval 10.3, 15.1). The 3-year overall survival (OS) rate was 71% (s.e. ± 4%). The median follow-up in this study was 3.9 years (range 0.02–7.7). Patient demographic data are given in Table 1. The study was approved by the local institutional review board.

Radiological response after neoadjuvant chemotherapy was assessed by two radiologists according to Response Evaluation Criteria In Solid Tumours (RECIST 1.1) using computed tomography.[12] Only patients with radiological response or disease stabilization underwent liver resection and were included in this study.

The assessment of histological response was performed according to Rubbia-Brandt *et al.*[13] Major histological response comprised tumor regression grade 1 and 2, partial histological response tumor regression grade 3 and no histological response tumor regression grade 4 and 5.[13]

After completion of treatment, clinical examinations, computed tomography scans of thorax, liver and abdomen and blood tests including carcinoembryonic antigen were routinely performed every 3 months during the first 3 years, and then every 6 months.

SNP selection and genotyping

Candidate SNPs with a minor allele frequency of ≥ 10% in Caucasians according to the Ensembl database were chosen for analyses in this study, when having functional relevance according to Queen's University F-SNP and National Institute of Environmental Health Sciences SNP Function Prediction, representing a set of other SNPs (tag) or have been previously described to be clinically relevant according to the literature if no functional data were available (Table 2).[14–22]

Genomic DNA from formalin-fixed paraffin-embedded CLM was extracted using the QIAamp DNAeasy Kit (Qiagen, Hilden, Germany). Primers were used for PCR as given in Table 2. DNA from CLM was analyzed by direct Sanger sequencing and genotype was determined using the ABI Sequencing Scanner v1.0 (Applied Biosystems, Foster City, CA, USA). Patients' clinical data were unknown to investigators involved in SNP analyses.

Statistical analyses

SNPs involved in immune response checkpoints were tested for associations with radiological and histological response, RFS and OS. The primary end point of the study was RFS. RFS was defined as the time from the day of liver resection to the first day of documented disease recurrence or death. If recurrence or death was not observed, RFS was censored on the day of the last computed tomography scan. The secondary end points were radiological and histological response and OS. OS was defined as the time from liver resection to the date of death or censored on the date last known to be alive. All possible modes of inheritance of SNPs were considered. The distribution of the SNP alleles was tested for Hardy–Weinberg equilibrium using a 1° freedom χ^2-test. Kaplan–Meier

estimation and log-rank tests were used in univariable analysis and Cox proportional hazards regression models were used in multivariable analysis to the test for associations of SNPs with RFS and OS. The multivariable analysis was adjusted for age (< 65 vs 65+ years), number of metastases (1–2 vs >2), distribution of metastases (unilobar vs bilobar) and timing of metastases (metachronous vs synchronous) because these characteristics were associated with RFS or OS at a 0.10 significance level. χ^2-tests were used to investigate associations between SNPs and reponse in the univariable analysis. In the multivariable analysis, logistic regression model were used controlling for potential predictive variables. SNP profiles for radiological and histological response, RFS and OS were investigated using recursive partitioning.

Cure rate by individual SNPs was estimated by mixture cure model.[23] The patterns of recurrence were estimated using cumulative incidences of intrahepatic only or extrahepatic recurrence using competing risks model.

SAS/STAT 12.3 (SAS Institute, Cary, NC, USA), a SAS macro (%*pspmcm*), along with rpart and cmprsk functions in R package (Version 3.1.2, R Foundation for Statistical Computing, Vienna, Austria) were used to perform all the analyses. Case-wise deletion was applied when patients with missing polymorphisms were excluded in the analyses. All tests were two-sided at a significance level of 0.05. *P*-values were not adjusted for multiple hypothesis testing. For internal validation, leave-one-out cross validation were used as previously described.[24,25]

RESULTS

Hardy–Weinberg equilibrium

All tested SNPs were in Hardy–Weinberg equilibrium except *CD24* rs8734 G >A.

Gene variants and OS

The homozygous variant genotype (A/A) of *IDO1* rs3739319 G >A was associated with shorter OS in this study (Figure 2). The 3-year OS rate was 49% (±11%) for the patients harboring an A/A genotype compared with 75% (±5%) for patients with a G/G or G/A genotypes. The hazard ratio was 2.52 (1.33, 4.80; *P* = 0.007) in univariable analysis and 2.04 (1.03, 4.03; *P* = 0.040) in multivariable analysis. The variant genotypes (G/A or A/A) of *CD24* rs8734 G > A were associated with longer OS (Figure 3). The 3-year OS rate was 76% (±7%) for G/A or A/A compared with 63% (±7%) for G/G. The hazard ratio was 0.53 (0.28, 0.97; *P* = 0.042) in univariable analysis. In multivariable analysis, *CD24* rs8734 G>A did not remain significant for OS (hazard ratio 0.55 (0.30, 1.01; *P* = 0.054). Data on OS are given in Table 3.

Recursive partitioning for RFS and OS

IDO1 rs3739319 G>A was the most important SNP to predict RFS and OS upon recursive partitioning analysis (Figures 4 and 5). Other SNPs predicting OS in subgroups were *NT5E* rs6922 G>T and *LAG3* (lymphocyte-activation gene 3) rs3782735 A>G. For RFS, node 1

showed a trend toward longer RFS (log rank, P = 0.098). Recursive partitioning analyses did not create decision trees for radiological and histological response.

Gene variants and pattern of recurrence

IDO1 rs3739319 G>A was associated with extrahepatic recurrence. The 2-year incidence of extrahepatic recurrence was 72% (s.e. ± 9%) for A/A and 56% (±5%) for G/G or G/A (P =0.024).

Gene variants and radiological and histological response, RFS and probability of cure

None of the SNPs investigated in this study was significantly associated with radiological or histological response, probability of cure or RFS in univariable or multivariable analysis (Tables 3 and 4).

Internal validation

Upon leave-one-out cross validation, *IDO1* rs3739319 G>A was significantly associated with OS in both univariable and multivariable analysis for the recessive model (132 of 149 leave-one-out data sets). For the dominant model of *CD24* rs8734 G>A, 101 of 149 leave-one-out data sets remained significant in univariable analysis and 43 of 149 in multivariable analysis (42 of 149 in both univariable and multivariable analysis).

DISCUSSION

We investigated a unique cohort of patients with bevacizumab-pretreated CLM who underwent liver resection with curative intent. This study confirms that the immune response influences clinical outcome and that SNPs within genes of immune checkpoints may serve as biomarkers in the treatment of colorectal cancer. The most important SNP to predict clinical outcome was *IDO1* rs3739319 G>A. *IDO1* rs3739319 G>A identified patients who benefited most from this multidisciplinary treatment approach with respect to OS.

IDO1 rs3739319 G> A is an intronic SNP and is associated with transcriptional regulation, suggesting functional relevance.[15] IDO1 is an enzyme that converts the essential amino acid tryptophan into kynurenine. Tryptophan metabolites are involved in the regulation of the immune response. Toxic kynurenine activates Tregs and inhibits cytotoxic T cells.[26,27] In turn, Tregs can also suppress T-cell activation. Moreover, tryptophan deficiency has been shown to inhibit the Mechanistic Target of Rapamycin pathway and activate the General Control Nonderepressible 2 pathway, leading to upregulation of stress response pathways, which suppresses T cells.[28–30] Under physiological conditions, these processes provide immune tolerance at the fetal–maternal interface.[31] In cancer, IDO1 is expressed by cancer cells and antigen-presenting cells and is associated with poor prognosis.[32] Due to the mechanisms of action of IDO1, the host's immune response is attenuated, which facilitates tumor growth. VEGF has been shown to increase IDO1 expression in dendritic cells, therefore anti-VEGF targeted treatment may potentially influence IDO1 and immune response.[33] The importance of *IDO1* rs3739319 G>A is further confirmed by the fact that it predicted the probability of extrahepatic recurrence. This finding that the A/A genotype was significantly associated with a higher 2-year extrahepatic recurrence rate and a lower 3-year

OS rate is in line with a previous study demonstrating that extrahepatic recurrence after liver resection of CLM is a negative prognostic marker.[34] However, the investigation of the clinical value of extrahepatic recurrence prediction warrants further studies. IDO1 is a promising drug target and its inhibition has demonstrated anti-proliferative efficacy in murine cancer models *in vitro* and *in vivo*.[35–37] Clinical studies are currently conducted to investigate the IDO1 inhibitors, INCB024360 and indoximod, in combination with immunotherapy or chemotherapy in patients with melanoma, breast cancer and pancreatic cancer (NCT01604889, NCT01792050 and NCT02077881). In liver-limited metastatic colorectal cancer, the introduction of activators of a T-cell-driven immune response, such as IDO1 inhibitors, to the treatment concept appears especially promising due to the liver's highly immunogenic nature.

Besides *IDO1* rs3739319 G>A, *CD24* rs8734 G>A was associated with OS in univariable analysis. CD24 is a cell surface glycosyl-phosphatidylinositol-anchored protein expressed on T cells and on cancer cells. In T cells, CD24 mediates homeostatic proliferation, a mechanism to replace activated and discarded lymphocytes.[38] In addition, *CD24* rs8734 G>A has previously been associated with inflammatory bowel disease susceptibility.[17] In cancer cells, CD24 expression has been associated with cancer stem cells and more aggressive cancer behavior.[39,40] Interestingly, hypoxia inducible factor-1 α is up-regulating CD24 expression and in turn CD24 salvages cell proliferation in hypoxia inducible factor-1α-depleted cancer cells, suggesting a protective effect of CD24 against hypoxia.[41] The connection of hypoxia inducible factor-1α to CD24 is not surprising, as angiogenesis has been closely linked to the immune system. In colorectal cancer, selective blockade of the VEGF-A/VEGFR axis resulted in an attenuated proliferation of T-cell-inhibiting Tregs *in vivo*.[10] Furthermore, anti-VEGF treatment was associated with a polarization of M2-immunosuppressive toward M1-antitumoral macrophages in a breast cancer model.[42] However, it is more likely that the effect of CD24 observed in this study is related to immune response and VEGF-modulation by bevacizumab treatment rather than to a cancer stem cell-related effect. This hypothesis is supported by the fact that *CD24* rs8734 G>A did not affect survival in a previous study in the adjuvant setting.[43] However, the interpretation of the results for *CD24* rs8734 G>A has to be made with caution, as this SNP was not in Hardy–Weinberg equilibrium. Moreover, the statistical significance for OS was lost in multivariable analysis.

In addition to univariable and multivariable analyses, we performed recursive partitioning analyses that identified SNPs that were associated with RFS and OS in population subgroups in an unbiased, hierarchical manner. *IDO1* rs3739319 G>A was the strongest predictor of RFS and OS, whereas *NT5E* rs6922 G>T and *LAG3* rs3782735 A>G predicted OS in subgroups.

NT5E is an ecto-nucleotidase expressed on cancer cells and Tregs that converts extracellular adenosine monophosphate to adenosine. Adenosine binds to its receptor A2AR on T cells leading to their suppression and also to the A2AR receptor on Tregs leading to their stimulation.[44] By suppression of T cells and stimulation of Tregs, NT5E inhibits immune response. In previous studies, NT5E expression has been shown be affected by hypoxia inducible factor-1 a and, VEGF expression by CD74 and adenosine demonstrating its

relevance for angio- genesis.[45,46] These results suggest that *NT5E* rs6922 G>T may be associated with outcome in patients treated with anti-VEGF targeted treatment.

In patients harboring any G allele in *NT5E* rs6922 G>T, *LAG3* rs3782735 A>G separated patient with intermediate risk for death. LAG3, which is expressed on T cells and Tregs and binds to class II major histocompatibility complex, has been associated with inhibited immune response. LAG3 leads to T-cell suppression and Treg stimulation.[47] Anti-LAG3 targeted therapy has demonstrated anti-tumor activity in a murine model by interrupting its immunosuppressive effect.[48] So far, LAG3 has not been associated with VEGF and angiogenesis, therefore it is unknown if the differences in OS found in one of the three analysis subgroup can be considered to be treatment related.

The findings from recursive partitioning analyses demonstrate that some genes may not be relevant for all patients but only for some patients in distinct subgroups. This statistical approach allows stratifying patients according to their clinical outcome in a comprehensive manner and may help to develop genetic risk profiles for future studies. However, the identified risk groups require further validation in prospective trials before they can be introduced into clinical practice. The fact that a recursive partitioning tree was created for *IDO1* rs3739319 G>A for RFS while the results from the log-rank test for this SNP were not statistically significant can be explained with the different statistical methods used. Nevertheless, as the two methods yielded different results, the interpretation of the association should be performed with caution. Moreover, no trees were created for radiological and histological response. This can also be explained by the statistical method that defines the terminal nodes (responders vs non-responders) depending on the patient majority. The method creates a tree only when, in one genotype group, the majority of patients are responders and non-responders in the other. However, in all genotype groups for all investigated SNPs, the majority were responders.

Interestingly, the SNPs investigated in this study were related to OS and not to response or RFS. However, it is unclear whether these SNPs are prognostic or predictive, as the differences seen between the groups may be a treatment effect of bevacizumab and subsequent immune modulation. The hypothesis that these findings are due to a treatment effect is supported by the results from the pivotal study that investigated the anti-CTLA4 antibody ipilimumab and led to its approval in melanoma. Patients receiving ipilimumab had prolonged OS, but not an improved RFS compared with those receiving glycoprotein 100.[49]

A limitation of this study is that the results could not be validated in an independent patient population as the presented cohort underwent a unique treatment regimen. However, the results for *IDO1* rs3739319 G>A could be validated in internal cross validation. Nevertheless, when interpreting the results, it has to be considered that no correction for multiple testing has been performed.

In conclusion, this study shows for the first time that variations within genes involved in immune response checkpoints predict clinical outcome in this uniformly treated patient cohort. As currently most of the patients who undergo liver resection will develop recurrent disease, biomarkers that predict cure are needed. The results of this study may help to

identify those patients who can be cured by this aggressive treatment concept and, moreover, may help to identify those who do not benefit and should therefore be referred to other treatment strategies. Furthermore, the close link of angiogenesis and immune response may make these SNPs potentially relevant biomarkers in the treatment of metastatic colorectal cancer with anti-VEGF targeted treatment. Apart from the identification of SNPs as biomarkers, these results may be relevant to identify targets for novel treatment strategies to enhance immune response.

ACKNOWLEDGMENTS

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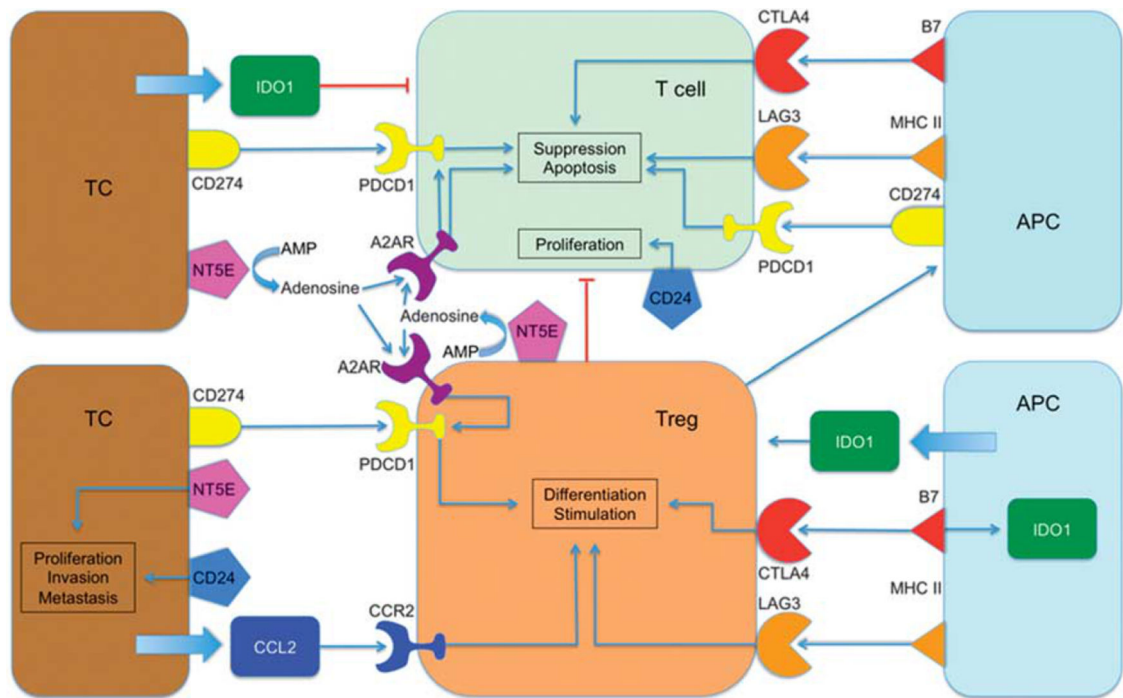
Figure 1.
Network of action of the investigated genes. A2AR, adenosine A2A receptor; AMP,
adenosine monophosphate; APC, antigen-presenting cell; B7, B-lymphocyte-activation
antigen B7; CCL2, chemokine (C-C motif) ligand 2; CCR2, C-C chemokine receptor 2;
CTLA4, cytotoxic T-lymphocyte antigen 4; MHC II, major histocompatibility complex II;
PDCD1, programmed cell death 1; TC, tumor cell; Treg, T-regulatory cell.
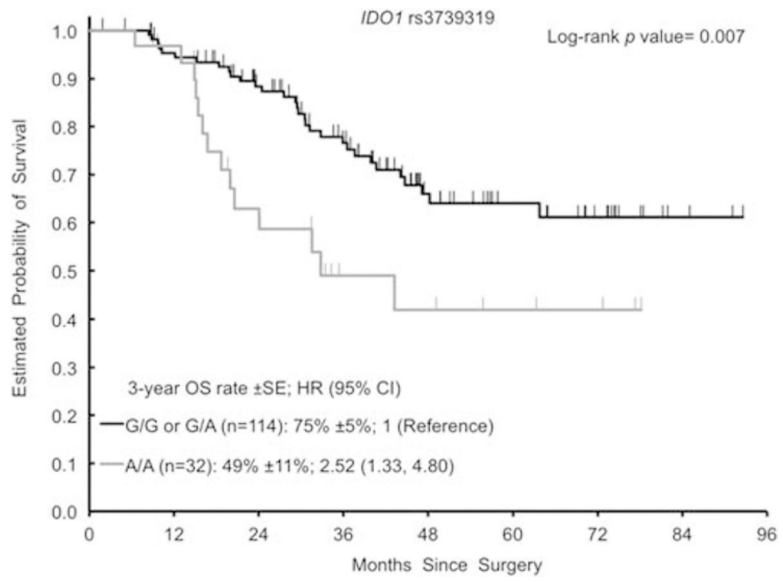
Figure 2.
Kaplan–Meier curves for *IDO1* rs3739319 G>A for OS. CI, confidence interval; HR, hazard ratio; OS, overall survival.
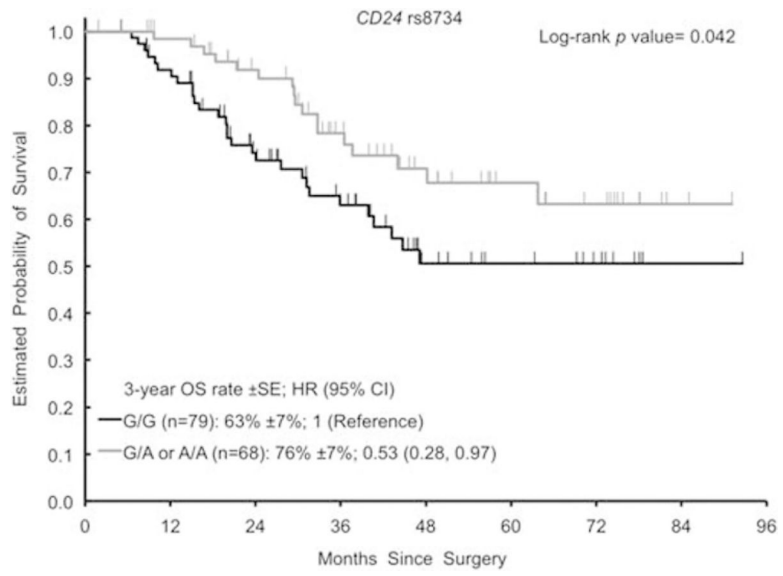
Figure 3.
Kaplan–Meier curves for *CD24* rs8734 G>A for OS. CI, confidence interval; HR, hazard ratio; OS, overall survival.
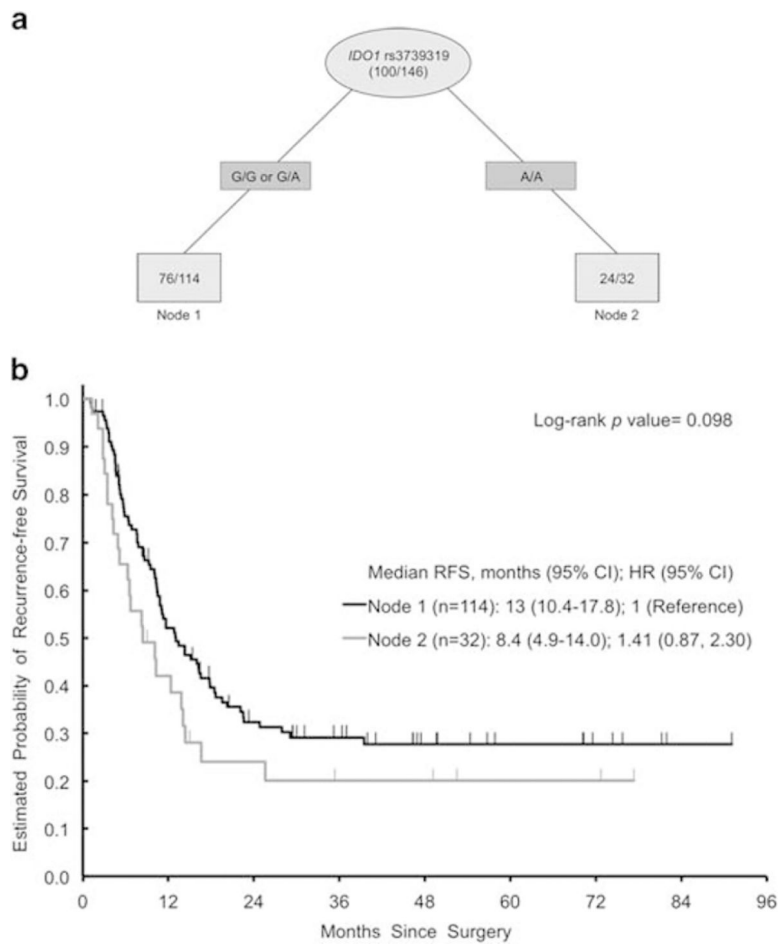
Figure 4.
(**a**) Recursive Partitioning for RFS. Light gray ovals represent intermediate subgroups; light gray squares represent terminal nodes. Dark gray rectangles indicate predictive polymorphism. Fractions within nodes indicate patients who relapsed per total patients with that node. Node 1 represents intermediate-risk patients. Node 2 represents high-risk patients. (**b**) Kaplan–Meier curves for RFS nodes. HR, hazard ratio; RFS, recurrence-free survival.
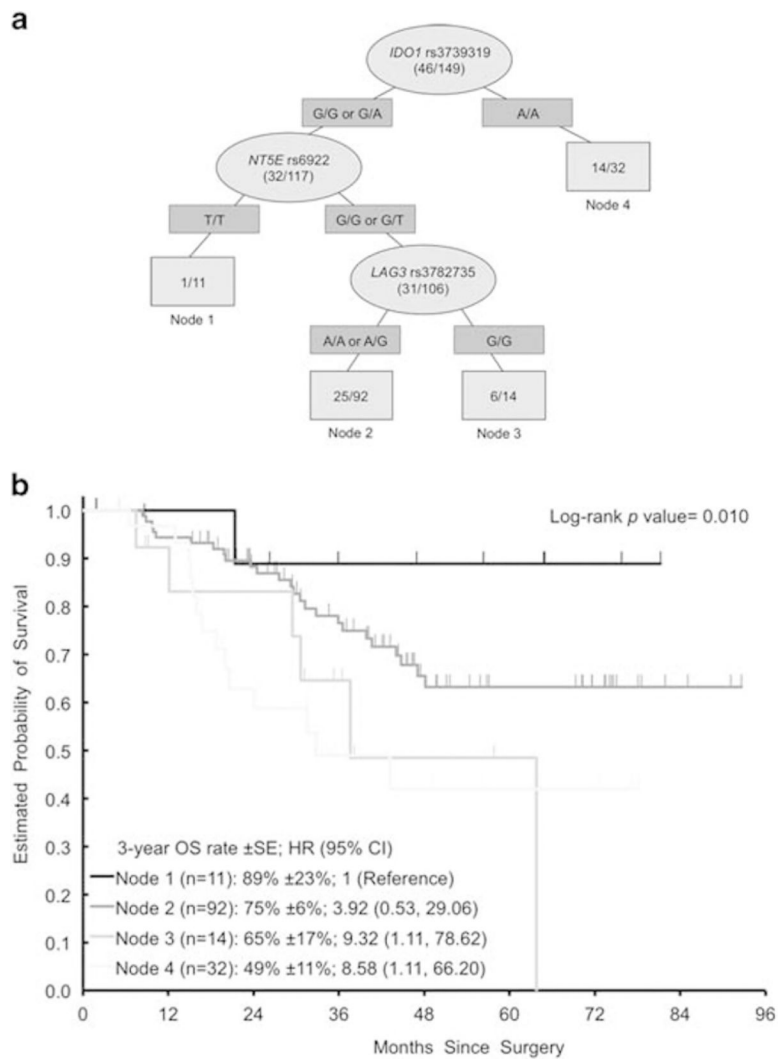
Figure 5.
(**a**) Recursive Partitioning for OS. Light gray ovals represent intermediate subgroups; light gray squares represent terminal nodes. Dark gray rectangles indicate predictive polymorphism. Fractions within nodes indicate patients who died per total patients with that node. Node 1 represents low-risk patients; Node 2 represents intermediate-risk patients. Node 3, 4 represent high-risk patients. (**b**) Kaplan–Meier curves for OS nodes. HR, hazard ratio; OS, overall survival.

Table 1.

Patient and tumor characteristics

	n	%
Sex		
Male	87	58.4
Female	62	41.6
Age		
Median (range)	62 years (30–80)	
< 65 years	90	60.4
≥ 65 years	59	39.6
Size of metastases[a]		
1–50 mm	127	85.8
> 50 mm	21	14.2
Number of metastases[a]		
1–2	88	59.5
> 2	60	40.5
Distribution of metastases		
Unilobar	76	51.0
Bilobar	73	49.0
Timing of metastases		
Metachronous	55	36.9
Synchronous	94	63.1
Primary tumor site[a]		
Right colon	39	26.4
Left colon	60	40.5
Rectum	49	33.1

[a]One patient had missing information.

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Table 2.

Genes and SNPs of immune checkpoints

rs number	MAF[a]	Base change	Location	Function	Primer sequence
CCL2					
rs4586	39%	A > G	Exon 2	Protein coding, splicing regulation and post translation	F: 5′-TCAATGCCCAGTCACCT-3′ R: 5′-TCACATCACAGCTTCTTTGGG-3′
LAG3					
rs3782735	42%	A > G	Intronic	Tag	F: 5′-GGACTCCCCTGCTCTCAATT-3′ R: TCACTCCTCCCAAATCCAGG-3′
NT5E					
rs6922	33%	G > T	3′-UTR	Transcriptional regulation	F: 5′-TTGTTGCAGCAAAATAATAGCC-3′ R: 5′-CCCTATTTTACTGGCCAAGTGT-3′
PDCD1					
rs10204525	12%	C > T	3′-UTR	Tag	F: 5′-GGTGTTGGGAGGGCAGAA-3′ R: 5′-GTGTGGATGTGAGGAGTGGA-3′
CCR2					
rs3092964	22%	T > C	Near 5′-UTR	Transcriptional regulation	F: 5′-CCTGTATCTCCGCCTTCACT-3′ R: 5′-AATGCCTACAACTCACCAGC-3′
IDO1					
rs9657182	45%	A > G	Upstream	NA	F: 5′-GGTCATAAAAGGAGAAAATGACC-3′ R: 5′-TTGCAAAGAGCTTTCAGAAAAA-3′
rs3739319	40%	G > A	Intronic	Transcriptional regulation	F: 5′-GACCTTGACCTCAGTGAATGC-3′ R: 5′-ATGTCCTGGAGGAACTGAGC-3′
CD274					
rs2297137	27%	G > A	Intronic	Transcriptional regulation	F: 5′-ATTCTCCTGCCCCTCACC-3′ R: 5′-AAGGGGTGAGAATTGGATCA-3′
CTLA4					
rs231777	15%	C > T	Intronic	Transcriptional regulation	F: 5′-TGACACCAGGTTTGTTACACG-3′ R: 5′-TCTTCTCCAGTAAAAATTAAATCATCA-3′
CD24					
rs8734	32%	G > A	Exon 1	Protein coding, splicing regulation and post translation	F: 5′-TGGAGGTAGCTGTGATGTGG-3′ R: 5′-ACCACGAAGAGACTGGCTGT-3′

Abbreviations: A, adenine; C, cytosine; G, guanine; MAF, minor allele frequency; NA, not available; rs, reference SNP number; T, thymine; 3′-UTR, 3′-untranslated region; 5′-UTR, 5′-untranslated region.

[a] According to the Ensembl database (phase 1 of the 1000 Genomes Project) for Europeans.

Table 3.

Gene variants and recurrence-free survival and overall survival

			Recurrence-free survival			Overall survival	
	n	Median (95%CI)	Univariable HR (95% CI)	Multivariable HR (95% CI)	3-year rate	Univariable HR (95% CI)	Multivariable HR (95% CI)
CCL2 rs4586							
A/A	55	11.7 (8.3, 16.0)	1 (reference)	1 (reference)	0.63 ± 0.08	1 (reference)	1 (reference)
A/G	68	13.5 (10.1, 22.5)	0.87 (0.57, 1.35)	1.05 (0.67, 1.64)	0.72 ± 0.07	0.84 (0.45, 1.59)	1.08 (0.56, 2.07)
G/G	22	10.1 (4.6, 14.3)	1.31 (0.73, 2.34)	1.47 (0.80, 2.69)	0.83 ± 0.14	0.61 (0.21, 1.79)	1.05 (0.38, 2.89)
P-value[a]			0.37	0.44		0.83	0.97
LAG3 rs3782735							
A/A	58	11.2 (8.4, 18.5)	1 (reference)	1 (reference)	0.66 ± 0.08	1 (reference)	1 (reference)
A/G	70	13.0 (9.5, 16.5)	0.92 (0.60, 1.40)	0.89 (0.58, 1.37)	0.77 ± 0.07	0.82 (0.42, 1.59)	0.96 (0.50, 1.85)
G/G	20	11.0 (4.1, 81.2)	0.99 (0.53, 1.84)	0.97 (0.51, 1.85)	0.59 ± 0.14	1.61 (0.72, 3.58)	1.68 (0.74, 3.83)
P-value[a]			0.92	0.87		0.35	0.38
NT5E rs6922							
G/G	61	13.0 (9.2, 18.6)	1 (reference)	1 (reference)	0.72 ± 0.08	1 (reference)	1 (reference)
G/T	72	10.3 (8.4, 16.3)	1.11 (0.73, 1.68)	0.89 (0.58, 1.37)	0.66 ± 0.07	1.28 (0.68, 2.40)	1.09 (0.58, 2.07)
T/T	15	14.0 (6.5, 16.6)	1.02 (0.51, 2.05)	0.87 (0.42, 1.79)	0.75 ± 0.17	0.74 (0.22, 2.52)	0.64 (0.18, 2.24)
P-value[a]			0.89	0.85		0.54	0.70
PDCD1 rs10204525							
C/C	119	11.1 (9.5, 14.3)	1 (reference)	1 (reference)	0.69 ± 0.05	1 (reference)	1 (reference)
C/T	25						
T/T	3						
C/T or T/T	28	16.0 (9.2, 77.3)	0.68 (0.39, 1.17)	0.69 (0.40, 1.21)	0.74 ± 0.13	0.56 (0.22, 1.41)	0.61 (0.24, 1.58)
P-value[a]			0.16	0.20		0.17	0.31
CCR2 rs3092964							
T/T	90	10.8 (8.4, 14.4)	1 (reference)	1 (reference)	0.68 ± 0.06	1 (reference)	1 (reference)
T/C	47	14.3 (10.4, 22.1)	0.85 (0.55, 1.29)	0.87 (0.56, 1.36)	0.78 ± 0.08	0.91 (0.47, 1.77)	0.82 (0.42, 1.61)
C/C	10	7.8 (4.0, 46.3)	1.17 (0.53, 2.55)	1.09 (0.49, 2.41)	0.40 ± 0.15	2.17 (0.83, 5.66)	1.79 (0.66, 4.85)

	n	Median (95%CI)	Recurrence-free survival Univariable HR (95% CI)	Multivariable HR (95% CI)	3-year rate	Overall survival Univariable HR (95% CI)	Multivariable HR (95% CI)
P-value[a]			0.64	0.78		0.24	0.38
IDO1 rs9657182							
A/A	48	11.7 (7.6, 27.9)	1 (reference)	1 (reference)	0.66 ± 0.09	1 (reference)	1 (reference)
A/G	75	11.0 (8.7, 15.1)	1.19 (0.76, 1.88)	1.00 (0.63, 1.59)	0.70 ± 0.07	0.86 (0.44, 1.68)	0.77 (0.40, 1.50)
G/G	26	16.5 (6.5, 29.1)	0.96 (0.53, 1.76)	0.80 (0.41, 1.54)	0.75 ± 0.13	0.76 (0.31, 1.90)	0.80 (0.32, 2.01)
P-value[a]			0.64	0.74		0.75	0.74
IDO1 rs3739319							
G/G or G/A	114	13.0 (10.5,17.8)	1 (reference)	1 (reference)	0.75 ± 0.05	1 (reference)	1 (reference)
G/G	43						
G/A	71						
A/A	32	8.4 (4.9,14.0)	1.48 (0.93,2.34)	1.13 (0.68,1.88)	0.49 ± 0.11	2.52 (1.33,4.80)	2.04 (1.03,4.03)
P-value[a]			0.093	0.64		0.007	0.040
CD274 rs2297137							
G/G	88	12.3 (10.1, 16.3)	1 (reference)	1 (reference)	0.71 ± 0.06	1 (reference)	1 (reference)
G/A	49						
A/A	8						
G/A or A/A	57	11.2 (8.4, 19.5)	0.87 (0.57, 1.31)	0.84 (0.54, 1.31)	0.66 ± 0.08	1.08 (0.58, 2.00)	1.11 (0.59, 2.10)
P-value[a]			0.50	0.44		0.76	0.74
CTLA4 rs231777							
C/C	111	12.8 (10.1, 15.1)	1 (reference)	1 (reference)	0.67 ± 0.06	1 (reference)	1 (reference)
C/T	33						
T/T	4						
C/T or T/T	37	11.7 (6.7, 18.6)	1.17 (0.75, 1.81)	1.11 (0.70, 1.75)	0.80 ± 0.10	0.85 (0.42, 1.73)	0.65 (0.31, 1.36)
P-value[a]			0.48	0.65		0.55	0.25
CD24 rs8734							
G/G	79	10.8 (7.7, 14.3)	1 (reference)		0.63 ± 0.07	1 (reference)	1 (reference)
G/A	64						
A/A	4						

	n	Recurrence-free survival			Overall survival		
		Median (95%CI)	Univariable HR (95% CI)	Multivariable HR (95% CI)	3-year rate	Univariable HR (95% CI)	Multivariable HR (95% CI)
G/A or A/A	68	11.7 (10.1, 22.6)	0.79 (0.53, 1.17)	0.74 (0.49, 1.13)	0.76 ± 0.07	0.53 (0.28, 0.97)	0.55 (0.30, 1.01)
P-value[a]			0.24	0.17		0.042	0.054

Abbreviations: A, adenine; C, cytosine; CI, confidence interval; G, guanine; HR, hazard ratio; T, thymine.

[a] Based on log-rank test in the univariable analysis and Wald test in the multivariable analysis within Cox regression model. Multivariable Cox regression model was adjusted for age ($<$ 65 vs $\geq$ 65 years), number of metastases (1–2 vs $>$ 2), size of metastases (1–50 mm vs $>$ 50 mm), and time since diagnosis of CRC ($<$ 6 months, 6–12 months, 1–2 years and $>$ 2 years).

Table 4.

Gene variants and radiological and histological response

	n	Radiological response		Histological response		
		PR	SD	MjHR	PHR	NHR
CCL2 rs4586						
A/A	55	42 (76%)	13 (24%)	20 (37%)	14 (26%)	20 (37%)
A/G	68	56 (85%)	10 (15%)	25 (38%)	21 (32%)	20 (30%)
G/G	22	20 (95%)	1 (5%)	9 (41%)	4 (18%)	9 (41%)
P-value[a]			0.14			0.87
LAG3 rs3782735						
A/A	58	44 (77%)	13 (23%)	19 (34%)	19 (34%)	18 (32%)
A/G	70	60 (87%)	9 (13%)	26 (38%)	17 (25%)	26 (38%)
G/G	20	16 (84%)	3 (16%)	10 (50%)	4 (20%)	6 (30%)
P-value[a]			0.35			0.64
NT5E rs6922						
G/G	61	51 (85%)	9 (15%)	21 (34%)	18 (30%)	22 (36%)
G/T	72	57 (81%)	13 (19%)	27 (39%)	18 (26%)	24 (35%)
T/T	15	13 (87%)	2 (13%)	7 (47%)	4 (27%)	4 (27%)
P-value[a]			0.85			0.67
PDCD1 rs10204525						
C/C	119	97 (83%)	20 (17%)	41 (35%)	34 (29%)	41 (35%)
C/T	25					
T/T	3					
C/T or T/T	28	22 (81%)	5 (19%)	14 (50%)	6 (21%)	8 (29%)
P-value[a]			0.79			0.23
CCR2 rs3092964						
T/T	90	76 (85%)	13 (15%)	32 (36%)	29 (33%)	28 (31%)
T/C	47	36 (80%)	9 (20%)	20 (43%)	8 (17%)	18 (39%)
C/C	10	7 (70%)	3 (30%)	3 (33%)	2 (22%)	4 (44%)
P-value[a]			0.36			0.87
IDO1 rs9657182						
A/A	48	37 (77%)	11 (23%)	14 (29%)	15 (31%)	19 (40%)
A/G	75	62 (83%)	13 (17%)	32 (44%)	18 (25%)	23 (32%)
G/G	26	22 (96%)	1 (4%)	10 (40%)	7 (28%)	8 (32%)
P-value[a]			0.14			0.35
IDO1 rs3739319						
G/G or G/A	114	92 (83%)	19 (17%)	44 (39%)	30 (27%)	39 (35%)
G/G	43					
G/A	71					
A/A	32	27 (84%)	5 (16%)	12 (40%)	9 (30%)	9 (30%)

		Radiological response		Histological response		
	n	PR	SD	MjHR	PHR	NHR
P-value[a]			1.00			0.75
CD274 rs2297137						
G/G	88	74 (85%)	13 (15%)	28 (33%)	28 (33%)	30 (35%)
G/A	49					
A/A	8					
G/A or A/A	57	44 (80%)	11 (20%)	26 (46%)	11 (20%)	19 (34%)
P-value[a]			0.49			0.31
CTLA4 rs231777						
C/C	111	92 (85%)	16 (15%)	42 (39%)	27 (25%)	40 (37%)
C/T	33					
T/T	4					
C/T or T/T	37	29 (78%)	8 (22%)	14 (39%)	12 (33%)	10 (28%)
P-value[a]			0.44			0.57
CD24 rs8734						
G/G	79	62 (79%)	16 (21%)	26 (34%)	27 (35%)	24 (31%)
G/A	64					
A/A	4					
G/A or A/A	68	57 (86%)	9 (14%)	28 (42%)	13 (19%)	26 (39%)
P-value[a]			0.38			0.98

Abbreviations: A, adenine; C, cytosine; G, guanine; MjHR, major histological response; NHR, no histological response; PHR, partial histological response; PR, partial response; SD, stable disease; T, thymine.

[a]Based on the Cochran–Mantel–Haenszel test for histological response and Fisher's exact test for radiological response.