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Variations in genes involved in dormancy associated with outcome in patients with resected colorectal liver metastases

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Background: Tumor dormancy has been described as a state of hibernation. Dormancy can be switched to proliferation by different pathways, which may play a critical role in tumor recurrence. In this study, we investigated genetic variations

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within genes involved in tumor dormancy and their association with recurrence and outcome in patients with colorectal liver metastases (CLM) who underwent neoadjuvant bevacizumab-based chemotherapy.

Patients and methods: Genomic DNA was extracted from resected CLM (FFPE) from 149 patients. Single-nucleotide polymorphisms (SNPs) in 14 genes associated with dormancy were analyzed by direct Sanger DNA sequencing and evaluated for response, recurrence-free survival (RFS), overall survival (OS) and recurrence patterns.

Results: *NME1* rs34214448 C>A was significantly associated with RFS in univariable analysis ($P = 0.039$) and with intrahepatic recurrence ($P = 0.014$). *NOTCH3* rs1044009 T>C and *CD44* rs8193 C>T showed a significant difference in 3-year OS rates ($P = 0.004$ and $P = 0.042$, respectively). With respect to radiological response, *CD44* rs8193 C>T variant genotypes were associated with a significantly higher response rate ($P = 0.033$). Recursive partitioning analyses revealed that *Dll4* rs12441495 C>G, *NME1* rs34214448 C>A and *NOTCH3* rs1044009 T>C were the dominant SNPs predicting histological response, RFS and OS, respectively.

Conclusion: Our data suggest that gene variations within genes involved in tumor dormancy pathways are associated with response and outcome in patients with resected CLM. These data may lead to new and more effective treatment strategies targeting tumor dormancy.

Key words: bevacizumab, colorectal liver metastases, dormancy, neoadjuvant chemotherapy, single-nucleotide polymorphisms

introduction

Dormancy is a state of hibernation, in which disseminated tumor cells (DTCs) remain until they are switched on to start growing, sometimes years or even decades after apparent cure [1, 2]. It is generally assumed that dormant cancer stem cells (CSCs) cause these late recurrences [3]. The microenvironment of CSCs appears to play a crucial role in the maintenance of dormancy. Three categories have been defined: (i) cellular dormancy of single cells caused by intrinsic and extrinsic factors, (ii) angiogenic dormancy of tumors maintained by a balance of cell proliferation and death due to insufficient blood vessels, and (iii) immune-mediated dormancy maintained by cytotoxic immunosurveillance [4].

Dormancy is playing a critical role in cancer recurrence because dormant cells are known to be refractory to adjuvant chemotherapy [5]. Recent data suggest that CSCs are disseminated very early, in contrast to the traditional doctrine that considers it to be a late step in cancer progression [6]. This may explain that recurrence may occur widespread in some distant organs (e.g. liver and lung), while these cells remain in a dormant state in other organs (e.g. bone marrow) [7].

Patients who underwent curative resection for liver-limited metastases are an interesting cohort to test whether genes involved in dormancy pathways are associated with clinical outcome since only 25% of them are cured [8]. Our hypothesis is that undetected dormant DTCs left behind may account for recurrences. After liver resection, liver regeneration with activation of multiple different pathways has been accused to be responsible for switching on dormant cancer cells left in the liver [9]. Understanding the molecular pathway leading to awakening of these dormant cells will be critical to identify new targets and development of novel treatment strategies.

We tested whether variations in a comprehensive panel of genes involved in dormancy pathways will predict clinical outcome in patients with colorectal liver metastases (CLM) treated with neoadjuvant bevacizumab-based chemotherapy. This panel included CSC- and angiogenesis-related genes involved in regulating cell proliferation through integrin signaling via the extracellular matrix (*ITGA5*, *ITGB1*), genes regulating integrin receptor activation (*PLAU*, *PLAUR*, *EGF*, *EGFR*), genes regulating angiogenic dormancy (*DLL4*, *NOTCH3*), genes suppressing metastasis-associated aspects, such as invasion and survival (*NME1*), and genes associated with CSCs (*TCF7L2*, *AXIN2*, *CD44*, *LGR5*, *ALDH1A1*) [10–14]. A better understanding of genes and pathways relevant for dormancy may help to identify predictive and/or prognostic biomarkers and potential targets for future therapies.

patients and methods

One hundred forty-nine consecutive patients [87 (58.4%) male, median age 62 years (range 30–80 years)] were enrolled (Table 1). Median follow-up was 3.9 years (range 0.02–7.7 years). Patients received 3 months of neoadjuvant and adjuvant fluoropyrimidine-based combination chemotherapy [oxaliplatin ($N = 124$), irinotecan ($N = 18$), both ($N = 7$)] including bevacizumab and curative liver resection (2005–2011). Last dose of bevacizumab was administered 5 weeks before surgery. Clinical data were provided from a prospectively maintained database. The study was approved by the local ethics committee.

Patients with progressive disease (<5%) under neoadjuvant chemotherapy according to Response Evaluation Criteria In Solid Tumours (RECIST 1.1) did not undergo liver resection and were not enrolled [15].

According to the classification by Rubbia-Brandt et al., tumor regression grade (TRG) 1 and 2 were categorized as major histological response, TRG 3 as partial histological response and TRG 4 and 5 as no histological response [16].

To detect disease recurrence, clinical examination, computed tomography scans of thorax, liver and abdomen and blood tests including carcinoembryonic antigen were carried out every 3 months for 3 years, and then every 6 months.

single-nucleotide polymorphisms selection and genotyping

Genes (*DLL4*, *NOTCH3*, *PLAU*, *PLAUR*, *ITGA5*, *ITGB1*, *EGF*, *EGFR*, *TCF7L2*, *AXIN2*, *CD44*, *LGR5*, *ALDH1A1*, *NME1*) were selected because of their association with dormancy (supplementary Table S1, available at *Annals of Oncology* online) [10–14]. Candidate single-nucleotide

Table 1. Baseline characteristics ($N = 149$)

	N	%
Age		
Median (range)	62 (30–80)	
<65 years	90	60.4
≥65 years	59	39.6
Sex		
Male	87	58.4
Female	62	41.6
Timing of metastases		
Metachronous	55	36.9
Synchronous	94	63.1
Number of metastases[a]		
1–2	88	59.5
>2	60	40.5
Size of metastases[a]		
1–50 mm	127	85.8
>50 mm	21	14.2
Distribution of metastases		
Unilobar	76	51.0
Bilobar	73	49.0
Primary tumor site[a]		
Right colon	39	26.4
Left colon	60	40.5
Rectum	49	33.1
KRAS status		
Wild-type	97	66.9
Codons 12, 13 or 61 mutant	48	33.1

[a]One patient had missing information.

polymorphisms (SNPs) were selected for analyses when having a minor allele frequency of ≥10% in Caucasians according to the Ensembl database and when having functional relevance for transcriptional regulation, protein coding or splicing according to Queen's University F-SNP and National Institute of Environmental Health Sciences SNP Function Prediction. Polymorphisms described in previous studies were also included [17–19].

The QIAamp DNAeasy Kit (Qiagen, Hilden, Germany) was used to extract genomic DNA from formalin-fixed paraffin-embedded CLM. DNA analyses were carried out by direct Sanger sequencing. Supplementary Table S1, available at *Annals of Oncology* online, lists primers used for polymerase chain reaction (PCR). To test primers for quality control, PCRs were carried out on known test DNA samples extracted from whole blood. The ABI Sequencing Scanner v1.0 (Applied Biosystems, Foster City, CA) was used to analyze DNA sequences. Investigators performing DNA analyses were blinded to patients' clinical data.

statistical analysis

The primary objective was to explore the predictive value of SNPs of 14 genes involved in the dormancy pathways. The primary end point was recurrence-free survival (RFS), and secondary end points were overall survival (OS) and radiological and histological responses. RFS was calculated as the interval from the day of liver resection to the first day of documented disease recurrence or death. In patients without recurrence and who were alive at the last follow-up, RFS was censored on the day of the last CT scan. OS was calculated as the time from liver resection to the date of death or censored on the date of the last follow-up. A 1-degree-freedom χ^2 test was used to test if the allelic distribution of each dormancy polymorphism was within Hardy–Weinberg equilibrium. The associations between polymorphisms and RFS and OS were investigated using Kaplan–Meier estimation and log-rank tests in univariable analysis. A Cox proportional hazards regression model was used in multivariable analysis. The following baseline characteristics were adjusted in the multivariable analysis: age (<65 versus ≥65 years), number (1–2 versus >2), distribution (unilobar versus bilobar) and timing of resectable liver metastases (metachronous versus synchronous) and KRAS mutation status because they were associated with RFS or OS at a 0.10 significance level. To assess relationships between polymorphisms and response, χ^2 tests were used in univariable analyses and logistic regression model controlling for potential predictive variables were used in multivariable analyses. Recursive partitioning analyses were used to explore and identify profiles of polymorphism associated with RFS, OS and radiological and histological responses.

A mixture cure model was used to assess the cure rate by individual polymorphisms [20]. The recurrence patterns (intrahepatic only or extrahepatic) were assessed using cumulative incidence of recurrence in the competing risks model.

Leave-one-out cross validation, an internal validation method, was carried out to assess the process of polymorphism selection to predict clinical outcomes.

All analyses were carried out using SAS/STAT 12.3 (SAS Institute, Cary, NC), a SAS macro (%*pspmcm*), along with rpart and cmprsk functions in R package. All analyses were carried out with two-sided tests at a significance level of 0.05. *P* values were not adjusted for multiple hypothesis testing.

results

Hardy–Weinberg equilibrium

All SNPs were in Hardy–Weinberg equilibrium except *PLAUR* rs2302524 A>G and *ITGB5* rs7306692 C>T.

gene variants and recurrence-free survival, overall survival and response

The A/A genotype of *NME1* rs34214448 C>A was associated with significantly shorter RFS compared with other genotypes [any C 12.8, A/A 8.3 months; hazard ratio (HR) 1.72 (1.02, 2.91), $P = 0.039$]. This difference did not remain significant in multivariable analysis (HR 1.58 (0.84, 2.99), $P = 0.16$) (supplementary Figure S1, available at *Annals of Oncology* online). For *NOTCH3* rs1044009 T>C, variant genotypes were associated with a significantly lower 3-year OS rate (T/T 73%, any C 55%; univariable HR 2.22 (1.19, 4.16), $P = 0.010$; multivariable HR 2.62 (1.37, 5.04), $P = 0.004$) (supplementary Figure S2, available at *Annals of Oncology* online). For *CD44* rs8193 C>T, variant genotypes were associated with nonsignificantly higher 3-year OS rate in univariable analysis [C/C 60%, any T 74%; HR 0.63 (0.35, 1.14); $P = 0.12$], which became significant in multivariable analysis [HR 0.53 (0.29, 0.98); $P = 0.042$] (supplementary Figure S3, available at *Annals of Oncology* online). Data on RFS and OS are presented in supplementary Table S2, available at *Annals of Oncology* online. In keeping with the OS data, *CD44* rs8193 C>T variant genotypes were associated with a significantly higher radiological response rate compared with the C/C genotype (C/C 72%, any T 88%, $P = 0.033$). None of the SNPs was associated with histological response (supplementary Table S3, available at *Annals of Oncology* online).

gene variants and recurrence patterns

Patients with an A/A genotype of *NME1* rs34214448 C>A had a significantly higher 2-year intrahepatic recurrence rate than those harboring any C (27% (±11%) versus 8% (±3%), $P = 0.014$). Other polymorphisms were not associated with intra- or extrahepatic recurrence (data not shown).

recursive partitioning for recurrence-free survival, overall survival and histological response

NME1 rs34214448 C>A was the dominant SNP to predict RFS. *ALDH1A1* rs1342024 G>C and *PLAUR* rs2302524 A>G were predictive in subgroups (Figure 1). *NOTCH3* rs1044099 T>C was the dominant SNP to predict OS and *PLAU* rs4065 A>G, *CD44* rs8193 C>T and *ITGA5* rs7306692 C>T in subgroups (supplementary Figure S4, available at *Annals of Oncology*

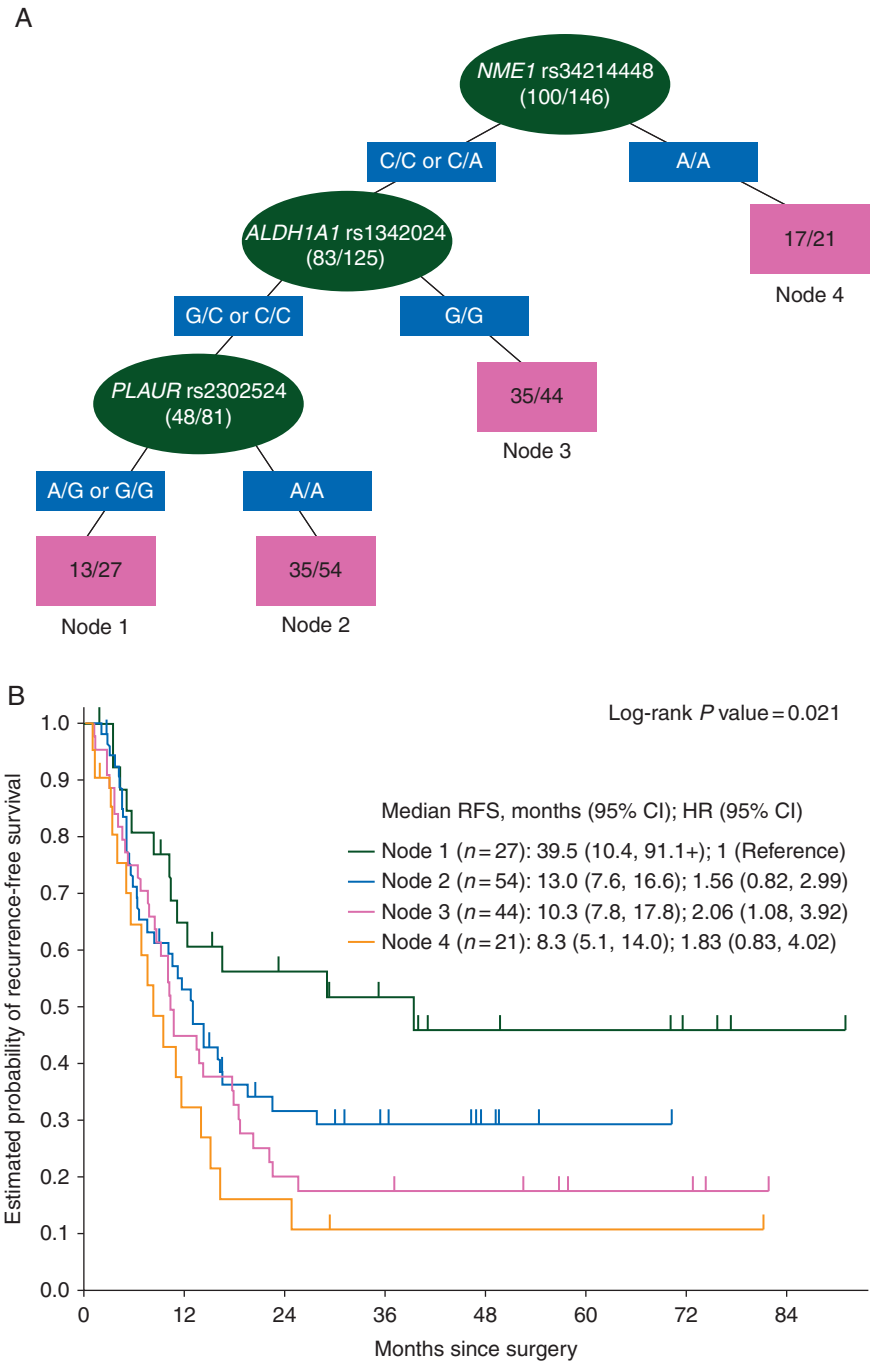

Figure 1. (A) Recursive partitioning tree for RFS. Light grey (green online) ovals represent intermediate subgroups; light grey (pink online) squares represent terminal nodes. Dark grey (blue online) rectangles indicate predictive polymorphism. Fractions within nodes indicate patients who relapsed/total patients with that node. Node 1 represents low-risk patients. Node 2 and 3 represent intermediate risk patients. Node 4 represents high-risk patients. (B) Kaplan–Meier curves for terminal nodes.

online). *DLL4* rs12441495 C>G was the dominant SNP to predict histological response, and *EGF* rs4444903 T>C, *DLL4* rs4923888 G>C and *ITGA5* rs7306692 C>T in subgroups (supplementary Figure S5, available at *Annals of Oncology* online). None of the SNPs was associated with radiological response.

internal validation

Among 149 sets, all *P* values were <0.05 for both univariable and multivariable OS analyses for *NOTCH3* rs1044099 T>C. For *CD44* rs8193 C>T, in none of 149 sets, *P* value was <0.05 in univariable OS analysis; in 122 of 149 sets, *P* value was <0.05 in multivariable OS analysis. For *NME1* rs34214448 C>A, in 139 of 149 datasets, *P* value was <0.05 in univariable RFS analysis, but in none of them in multivariable analysis. For *CD44* rs8193 C>T, in 136 of 149 datasets, *P* value was <0.05 for radiological response. For *NME1* rs34214448 C>A, 144 of 149 LOOCV datasets showed a *P* value of <0.05 for intrahepatic recurrence.

discussion

The goal of liver resection in patients with CLM is cure; however, the majority of patients develop disease recurrence within the liver. We tested whether variations of dormancy-related genes in a unique cohort of patients who underwent neoadjuvant bevacizumab-based chemotherapy and curative liver resection will predict tumor recurrence.

Our data showed for the first time that SNPs in cellular and angiogenic dormancy-related (*NOTCH3* and *NME1*) and CSC-related genes (*CD44*) are associated with response, recurrence patterns and clinical outcome in patients with CLM. These findings suggest that SNPs in these genes may be predictive biomarkers to identify patients who benefit most from these resections. These findings also identify potential novel targets and hopefully lead to new drug development strategies, which might increase cure rates.

NME1 rs34214448 C>A was associated with RFS and intrahepatic recurrence. Although the exact mechanism of action of the metastasis suppressor NME1 (NME/NM23 nucleoside diphosphate kinase 1) to promote dormancy remains elusive, various roles have been described. NME1 may induce dormancy through inhibition of Ras/Raf/MAPK (mitogen-activated protein kinase) signaling via phosphorylation and thereby inactivation of KSR1 (kinase suppressor of Ras 1), a scaffold protein in this pathway [12]. Furthermore, NME1 negatively regulates LPAR1 (lysophosphatidic acid receptor 1), which is an activator of the MAPK ERK (extracellular signal regulated kinase) 1/2 [21]. This suppression of LPAR1 leads to a reduction ERK1/2 signaling and to activation of stress-activated kinase p38 signaling, which has been described to facilitate dormancy [22]. Another mechanism of ERK/p38 signaling is modulation of angiogenic dormancy via proangiogenic vascular endothelial growth factor (VEGF) and antiangiogenic thrombospondin [23]. These data suggest that mechanisms of cellular and angiogenic dormancy are overlapping and that targeting one pathway may have effects on multiple categories of dormancy. The finding that *NME1* rs34214448 C>A was associated with only intrahepatic recurrence is of special interest as these patients may be candidates for repeat resection. However, one has to be aware of the fact that statistical significance for RFS was lost in multivariable analysis.

In addition, *NOTCH3* rs1044009 T>C was associated with OS. NOTCH3 and its ligand DLL4 (delta-like 4), who are important regulators of CSCs, have been shown to be involved in escape to dormancy by upregulation of DLL4 on endothelial cells [11, 24]. This escape was facilitated by activation of antiapoptotic and survival-promoting nuclear factor kappa-light-chain-enhancer of activated B cells signaling downstream of NOTCH3 [11, 25]. These data demonstrate that the vasculature is not only mediating angiogenic dormancy, but also modulating cellular dormancy via direct NOTCH3/DLL4-mediated contact of endothelial cells and cancer cells. Interestingly, NOTCH3 signaling may be influenced by the anti-VEGF treatment in our patient population as DLL4 expression is upregulated by VEGF-A [26]. These data suggest that bevacizumab may also modulate dormancy through DLL4/NOTCH4 signaling. In summary, the DLL4/NOTCH3 axis appears to be an attractive target to block escape from dormancy and potentially prevent recurrence, and NOTCH inhibitors are in early clinical trials.

Our data also demonstrated that *CD44* rs8193 C>T C/C was associated with inferior radiological response, OS and a trend toward shorter RFS. These data are consistent with a previous study that demonstrated an association of *CD44* rs8193 C/C genotype with shorter time-to-recurrence in stage II and III patients receiving adjuvant treatment [14]. CD44 has been described as CSC marker in colorectal cancer (CRC) that drives cell proliferation via phosphatidylinositol-4,5-bisphosphate 3-kinase/AKT (protein kinase B) signaling and is a target gene for the Wnt pathway [27]. CSCs show abilities of self-renewal, chemoresistance and are considered the true tumor dormancy. The C/C genotype of *CD44* rs8193 C>T may be associated with higher transcriptional activity of this gene as predicted by F-SNP [18] consistent with the poor prognosis in these patients.

The identification of variations in genes involved in promoting dormancy provides further insights into the facilitation of dormancy in the clinical impact. Thereby, novel treatment targets may be identified to develop new treatment strategies. A potential treatment strategy could be keeping cancer cells in a dormant state to avoid regrowth of metastases after curative resection [28]. Some authors favor this concept as the concept of awakening dormant cells to make them susceptible to cytotoxic treatment harbors potential risks as they may activate alternative pathways to increase chemoresistance [4, 29].

Recursive partitioning showed which genes were relevant in subgroups and that the resulting genetic profile was associated with substantial differences in survival. These analyses revealed that different SNPs predict histological response, RFS and OS. The observation that some biomarkers may be predictive for one clinical end point, while others may be predictive for other end points may be owed to the different underlying biological mechanisms.

A limitation of this study is the absence of a validation cohort as the investigated cohort is very unique; however, the results appear to be valid due to their consistency in various clinical end points. Another limitation is the lack of a preclinical rationale of investigating dormancy in CRC as dormancy data come predominantly from breast and prostate cancers. However, the fact that dormancy is closely linked to CSCs that facilitate

recurrence make it worthwhile to investigate these pathways in resected CLM.

In summary, this study shows that variations in genes and pathways involved in the regulation of dormancy are associated with treatment efficacy and clinical outcome in patients with CLM. These insights may help to identify predictive and/or prognostic biomarkers to guide treatment strategies. These biomarkers could help to exclude patients who do not benefit from this multidisciplinary treatment approach, which is complex and bears potential complications. Moreover, a deeper knowledge of the dormancy implications in cancer recurrence may contribute to find new dormancy-related treatment targets to improve the therapy of CRC.

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disclosure

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