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PI3K/Akt/mTOR signaling and plasma membrane proteins are implicated in responsiveness to adjuvant dendritic cell vaccination for metastatic colorectal cancer

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Abstract

Purpose—We have previously demonstrated that metastatic colorectal cancer patients who exhibit immune responses to a dendritic cell (DC) vaccine have superior recurrence-free survival following surgery, compared to patients in whom responses do not occur. We sought to characterize the patterns of T lymphocyte infiltration and somatic mutations in metastases that are associated with and predictive of response to the DC vaccine.

Experimental Design—Cytotoxic, memory, and regulatory T cells in resected metastases and surrounding normal liver tissue from 22 patients (11 responders and 11 non-responders) were enumerated by immunohistochemistry prior to vaccine administration. In conjunction with tumor sequencing, the Combined Multivariate and Collapsing method was used to identify gene mutations that are associated with vaccine response. We also derived a response prediction score for each patient using his/her tumor genotype data and variant association effect sizes computed from the other 21 patients; greater weighting was placed on gene products with cell membrane-related functions.

Results—There was no correlation between vaccine response and intra-tumor, peri-tumor, or hepatic densities of T cell subpopulations. Associated genes were found to be enriched in the PI3K/Akt/mTOR signaling axis ($P < 0.001$). Applying a consistent prediction score cutoff over 22 rounds of leave-one-out cross-validation correctly inferred vaccine response in 21 of 22 patients (95%).

Conclusions—Adjuvant dendritic cell vaccination has shown promise as a form of immunotherapy for patients with metastatic colorectal cancer. Its efficacy may be influenced by

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Conflicts of interest:

None disclosed

somatic mutations that affect pathways involving PI3K, Akt, and mTOR, as well as tumor surface proteins.

Keywords

metastatic colorectal cancer; dendritic cell vaccine; immunotherapy; PI3K/Akt/mTOR; tumor surface proteins

Colorectal cancer (CRC) is the fourth leading cause of cancer death in the world (1). About one in three patients diagnosed with CRC dies of metastatic disease (2). The majority of metastases develop in the liver and lung. Although metastases can be completely resected in many patients, 60–80% of these patients eventually die due to the growth of small metastases that were undetectable at the time of surgery (3, 4). Currently, the addition of adjuvant chemotherapy has limited impact on patient survival (5, 6). The pressing need for better treatments and growing recognition of the immune system's role in cancer progression have prompted tremendous efforts to develop immunotherapies for CRC (7).

Immunotherapies aim to either enhance the anti-tumor immune response or prevent immune surveillance suppression by cancer cells. Whole tumor vaccines for CRC have not been shown to confer survival benefit. Since only a tiny fraction of proteins in an autologous tumor vaccine is specific to cancer cells, dilution by self-antigens is believed to make the vaccine poorly immunogenic (8). Specific CRC tumor-associated antigens have subsequently been identified and used as peptide vaccines (9–14). However, the extent of antigen presentation varies widely depending on patients' HLA type (15). Dendritic cell (DC) vaccines overcome the limitations of these two earlier vaccine approaches. Following *ex vivo* stimulation by tumor lysate, DCs are not confined to process any specific antigen chosen in advance and can also activate a tumor-specific T lymphocyte response (16).

We have previously demonstrated that metastatic CRC patients who exhibit immune responses to a DC vaccine have superior 5-year recurrence-free survival following surgical resection of liver metastases compared to counterparts in whom immune responses do not occur (63% versus 18%, $P = 0.037$) (17). Since T cell densities in the tumor microenvironment are well known to correlate with survival of patients who have either primary or metastatic CRC (18, 19), we now investigated potential relationships between DC vaccine response and densities of cytotoxic, memory, and regulatory T cells in the context of metastatic CRC. Furthermore, we pursued genomic analyses. A recent phase 2 study of pembrolizumab, an anti-programmed death 1 immune checkpoint inhibitor, for CRC showed that clinical benefit is more likely to be observed in patients with tumors carrying higher mutation burdens due to deficiency in DNA mismatch repair (MMR) (20); however, the prevalence of MMR deficiency among metastatic CRC cases is only 3.5% (21). Therefore DC vaccination may be an effective therapy for a larger portion (17) of such patients. To evaluate the feasibility of precision immunotherapy using this approach, we examined mutation patterns and constructed a vaccine response prediction model based on CRC metastasis sequencing.

MATERIALS AND METHODS

Details on the preparation of DC vaccines, detection of immune response, and approval for the study of human subjects have been previously reported (17). Briefly, 22 CRC patients underwent surgical resection of liver metastases (Table 1) within 1–2 months of diagnosis at the Norris Cotton Cancer Center. Tumor lysates were derived from resected samples. These lysates were then added to immature DC cultures differentiated from autologous leukopheresed monocytes that had been supplemented with recombinant human granulocyte macrophage colony-stimulating factor and recombinant human interleukin-4. At 4 weeks, 7 weeks, and 10 weeks after surgery, tumor lysate-pulsed DCs (5×10^6 cells in 0.5 mL) were injected into two inguinal lymph nodes of each patient. All patients were confirmed to have no detectable residual disease by computed tomography (CT) scan prior to the initial vaccine injection. Development of immune response was defined as observing significantly greater secretion of interferon γ (IFN γ) (22) or induced proliferation of T lymphocytes (23, 24) by peripheral blood mononuclear cells 1 week following the third autologous tumor lysate-pulsed DC vaccination, compared to unpulsed DC stimulation.

Inspection of T cell infiltration

The resected specimens were formalin fixed, paraffin preserved, serially sectioned, and confirmed to contain metastatic tumor by hematoxylin and eosin staining. We performed immunohistochemical analysis on tumor-containing slides using the Leica BOND-MAX automated IHC system (Leica Microsystems) along with primary antibodies to human cytotoxic T cells (CD8, Novacastra #NCL-CD8-4B11), human memory T cells (CD45RO, Novacastra #NCL-L-UCHL1), and human regulatory T cells (FoxP3, BioLegend #623802). These subpopulations were specifically chosen to examine both immune-promoting (cytotoxic and memory) and immune-suppressive (regulatory) T cell roles in the tumor microenvironment. Blinded to the clinical characteristics of patients, an experienced gastrointestinal pathologist (A.S.) enumerated the positively-stained CD8, CD45RO, and FoxP3 T cells within the tumor, at the tumor periphery, and in surrounding normal liver tissue. Five high power fields with a field area of 0.24 mm^2 were counted at each location, and average counts were recorded as cells/ mm^2 . We tested for associations between response to the DC vaccine and densities of T cell subpopulations as well as other clinical metrics using logistic regression (Table 1).

Targeted gene sequencing

The 22 metastatic tumor samples were submitted to the Genomics & Molecular Biology Shared Resource at Dartmouth to sequence 541 genes (Supplementary Table S1) from the HaloPlex Cancer Research Panel (Agilent Technologies). DNA was isolated from samples using the QiaAmp DNA FFPE Tissue Kit (Qiagen) and digested with restriction enzymes to create a library of DNA fragments. Digests were hybridized to HaloPlex probes for target enrichment and sample barcoding. Targeted DNA fragments were then captured by magnetic beads, amplified, and purified using AMPure XP beads (Beckman Coulter). The final library sizes were 150–550 bp, as determined by Qubit (Thermo Fisher Scientific) after quality control by Fragment Analyzer (Advanced Analytical). Sequencing was performed using the Ion Proton System (Thermo Fisher Scientific) with the Ion PI Template OT2 200 v2 kit, the

Ion PI Sequencing 200 v2 kit, and a PI v2 chip. Sequenced reads in FASTQ file format (25) were aligned to the hg19 reference genome using TMAP (<https://github.com/iontorrent/TMAP>). The Genome Atlas Toolkit (GATK) (26) was used to call variants in VCF format (27), filtering for quality score > 100 and read depth > 30 in all 22 patients. Likely germline variants were removed from consideration by matching their position, reference allele, and alternative allele with records in the National Center for Biotechnology Information (NCBI) Short Genetic Variations database (28).

Gene associations and pathway enrichment

Sequencing genotype data facilitate the calling of both common and rare variants. In order to identify associations between genes containing these variants and a given outcome, collapsing approaches that test for the collective presence of variants within genes have been developed to optimize the detection of rare variant effects (29, 30). We identified associated genes using the Combined Multivariate and Collapsing (CMC) method, which has been shown to be more powerful than previous single-marker tests and more robust to misclassification of relevant variants than previous multiple-marker tests (30). The method ranks genes by computing Hotelling's T^2 statistics and corresponding P -values (Supplementary Table S2). Given the small number of subjects in this study, we set a liberal cutoff for choosing genes (41 genes with $P < 0.1$) to evaluate statistical enrichment of biologic pathways. For every curated BioCarta, KEGG, PID, and Reactome pathway (set C2) in the Molecular Signatures Database (MSigDB) (31), the hypergeometric test was used to assess the probability that 41 random genes from 8,384 genes in 1,291 pathways (in MSigDB at the time of this analysis) would better represent the pathway than our top 41 genes. These probabilities can be interpreted as P -values, and have been adjusted for Benjamini-Hochberg false discovery rate in multiple hypothesis testing (32). In addition, we accounted for spurious enrichment due to pathway size bias and the fact that most subsets of genes on the HaloPlex Cancer Research Panel should by default be overrepresented in cancer-related pathways. Null distributions of pathway enrichment were generated by randomly sampling 41 genes from the original panel gene list and applying the hypergeometric test over 1000 permutations. We retained only pathways (Table 2) whose observed enrichment values surpassed the 95th percentile of their respective null distributions (33).

Prediction of dendritic cell vaccine response

Although the CMC method is superior to single-marker tests at detecting gene-level associations, the former masks association direction by combining variants that both positively and negatively influence the outcome of interest (34). Therefore examining individual markers is more informative for predicting vaccine response. We derived a response prediction score for each patient using his/her tumor genotype data and variant association effect sizes computed from the other 21 patients:

$$S_i = \sum_{k=1}^n t_k w_k x_{k,i} \quad (\text{Equation 1})$$

S_i is the prediction score for individual i , t_k is the t -statistic for difference in allele frequency of variant k between vaccine responders and non-responders excluding individual i , all n variants chosen for consideration have t_k with corresponding P -value < 0.005 , w_k is an assigned weight for variant k , and $x_{k,i}$ is the allele count (0, 1, or 2) of variant k carried by individual i . Under this construction, S_i is more positive for individuals with tumors that have an abundance of variants associated with vaccine response; conversely, S_i is more negative for individuals with tumors that have an abundance of variants associated with lack of vaccine response. We then performed 22 rounds of leave-one-out cross-validation. Inference of response to the DC vaccine for each individual i was determined by whether $S_i > C$, a consistent cutoff applied to every round of cross-validation. Vaccine response prediction accuracy (percent correct out of 22 predictions) was recorded as the highest achieved accuracy through incrementing C along the range of 22 S_i values. We repeated this procedure for various weighting schemes of w_k . First, we set $w_k = 1$ for all variants. With respect to each curated MSigDB pathway, we then differentially weighted variants according to pathway membership of their spanning genes ($w_k = 8$ for inclusion, $w_k = 1$ otherwise). The choice of 8 was arbitrary, as results do not change for integer weights between 4 and 15. Finally, pathways were ranked based on the prediction accuracy of their annotation-guided S_i calculations, with top findings outlined in Table 3. Since leave-one-out cross-validation requires determining variant associations using only 21 of the 22 total individuals, effect sizes for some variants fluctuate between rounds of cross-validation. The up-weighted components of each pathway listed in Table 3 have association effect sizes that are significant ($P < 0.005$) and carry the same sign in all 22 rounds of cross-validation.

RESULTS

Patient characteristics of DC vaccine responders and non-responders are presented in Table 1. Of the 11 patients who had immune responses, 8 patients displayed greater IFN γ secretion, 5 patients displayed greater T cell proliferation, and 2 patients displayed both. None of the measured attributes were associated with vaccine response. The Fong clinical risk score (3), a popular prognostic tool for CRC patients undergoing liver metastasis resection (constructed from indicators for preoperative carcinoembryonic antigen blood level > 200 ng/ml, size of largest metastasis ≤ 5 cm, more than 1 metastasis, lymph node-positive primary tumor, and fewer than 12 months between primary tumor resection and liver metastasis diagnosis), was not useful for vaccine response inference. Recruitment of cytotoxic and memory T cells to metastases was significantly elevated relative to surrounding liver abundances ($P < 0.001$). However, vaccine response among metastatic CRC patients did not correlate with these tumor T cell densities. The mutation burden within targeted sequencing regions of tumor samples used to form the DC vaccine also did not differ between vaccine responders and non-responders. Nevertheless, sequencing of the metastatic tumors revealed other important insights.

Genes identified to be associated with DC vaccine response by the CMC method demonstrate statistical enrichment of pathways that primarily involve the PI3K/Akt/mTOR signaling axis (pathway #s 1, 2, 4, and 7 in Table 2). In brief, phosphatidylinositol 3-kinase (PI3K) is induced by extracellular signals through receptor tyrosine kinases to phosphorylate and activate Akt, which then promotes mammalian target of rapamycin (mTOR) to localize

to the nucleus and alter gene transcription (35). A diversity of transcription repertoire can then be affected, influencing many mechanisms such as cell survival, proliferation, motility, and metabolism. These also intersect with functions of the other highlighted transcription factor pathways (pathway #s 5 and 6 in Table 2). Although it is intriguing that disturbance to B lymphocyte receptor signaling is implicated as well (pathway #3 in Table 2), this finding may be a statistical artifact due to the pathway's considerable overlap with the actions of Akt and mTOR, including another shared downstream effector of Akt, tuberous sclerosis complex 2 (TSC2) (35). The anti-tumor response provoked by antigen presentation has been characterized to be predominantly T cell-mediated, while the roles of B cells are still being clarified (16).

Table 3 presents the pathways that optimally guide variant weights for prediction of DC vaccine response using Equation 1. Prediction scores of the 21 training set individuals and leave-one-out test set individual across all rounds of cross-validation for these top pathways are plotted in Supplementary Figure S1. Not surprisingly, training set scores cluster well into distinguishable groups of vaccine responders and non-responders, since their differences in allele frequencies were used to determine standardized variant association effects t_k ($P < 0.005$) in Equation 1. Among test set individuals, applying equal weights to associated variants correctly predicted 73% of vaccine responses (16 of 22). Differential weighting guided by top pathways increased accuracy up to 86% (19 of 22). In the absence of restrictions for pathway definitions, placing greater weight on variants within genes that comprise any top pathway further improved accuracy to 95% (21 of 22) (Figure 1); the only misclassified DC vaccine response was that of Individual 10. Even so, the corresponding prediction score became more positive with pathway-based variant weighting compared to equal weighting, which indicates adjustment in the correct direction as Individual 10 was a vaccine responder.

There is a conspicuous theme underlying up-weighted variants found to improve the accuracy of vaccine response prediction (Table 3). They tend to reside within genes that encode plasma membrane-related proteins. Many are proteins that interact with the extracellular matrix (integrins, collagens, fibronectin, and cluster of differentiation 36). Some are receptor tyrosine kinases that relay signals from outside of the cell to inside (vascular endothelial growth factor receptor, platelet-derived growth factor receptor, transforming growth factor β receptor, and insulin-like growth factor receptor). Other proteins are downstream effectors (phosphatidylinositol 3-kinases and mitogen-activated protein kinases) of cascades initiated by the aforementioned classes of transmembrane proteins. Two important contributors to prediction using Equation 1 and distinct outliers to this trend are variants within genes for glutaminase (GLS2) and methionine synthase (MTR). Normally enzymes involved in amino acid metabolism (pathway #10 in Table 3), GLS2 and MTR are suspected to promote tumorigenesis when their under-expression or impaired function compromises regulation of oxidative stress (36, 37). Furthermore, the majority of association directions across highlighted variants is negative (Table 3 and asymmetry of prediction scores with respect to zero seen in Figure 1). In other words, response to adjuvant DC vaccination is more likely to be observed in a CRC patient who has liver metastases that carry the reference genome allele instead of the alternative allele, for most somatic mutations with association $P < 0.005$ identified by our tumor sequencing.

DISCUSSION

Through histologic enumeration of T lymphocyte subpopulations, targeted gene sequencing of CRC liver metastases, and subsequent pathway-based genomic analyses, we explored the potential biologic mechanisms that confer greater likelihood of response to an adjuvant DC vaccine among CRC patients following metastasis resection. Identification of biomarkers that predict response to immunotherapies is critical for optimizing their use in treating patients (38). Previous investigations have shown that cytotoxic and memory T cell infiltration of primary and metastatic CRC tumors is positively associated with patient survival after surgical resection (18, 19). Conversely, depletion of regulatory T cells enhances the tumor antigen-specific immune response (39). However, in the present study, none of these microenvironment T cell parameters that characterize the tumor interior, periphery, and surrounding of CRC liver metastases trended with DC vaccine response or survival.

Our pathway analyses implicate signaling by PI3K/Akt/mTOR and a variety of plasma membrane-related proteins as factors that may influence vaccine response among metastatic CRC patients. Although these inferences seemingly differ based on whether we performed multiple-variant joint association testing (the CMC method) or single-variant association testing, respectively, the findings are not contradictory but rather complementary. The CMC method is more statistically robust and conservative in identifying gene associations, but the generated results suffer from absence of association direction. And while single-variant association testing is more liable to identify false positives, it is able to produce association magnitudes and directions that can be directly used in prediction modeling. Biologically, the PI3K/Akt/mTOR axis (Table 2) is in fact a convergence point for upstream signaling from integrins, receptor tyrosine kinases, and apoptosis regulators Shp2 and the caspase family (Table 3) (35, 40).

These cellular mechanisms can plausibly account for tumor attributes that promote DC vaccine resistance and poorer survival. First, impaired sensitivity to molecular cues for cell death maintains tumor survival (pathway #s 4, 5, and 10 in Table 3). Second, tumor surface proteins interact with the extracellular matrix to re-purpose the microenvironment for optimal growth (pathway #s 1, 3, 6, 8, and 9 in Table 3). Reciprocal signaling from the extracellular matrix to transmembrane proteins that is improperly relayed to the rest of the cell can stimulate unwarranted growth as well (10, 41). Specifically, integrins on the cell membrane regulate migration, invasion, and proliferation, especially through endothelium interactions during metastasis and tumor-initiated angiogenesis (pathway #7 in Table 3) (40, 42). Integrin $\alpha_v\beta_3$ expression in CRC liver metastases (pathway #3 in Table 3) has been shown to be nearly double the level in non-metastatic primary CRC tumors (43). Tumors can also hijack the vascular migration apparatus of innate immune cells (pathway #2 in Table 3) by inducing them to release membrane vesicles that contain integrin $\alpha_M\beta_2$ (pathway #8 in Table 3) for fusion with tumor cells, ultimately facilitating metastasis (44). Moreover, integrin $\alpha_M\beta_2$ is involved in monocyte development and differentiation (45). Substantial loss of integrin $\alpha_M\beta_2$ from monocytes may compromise their ability to secrete IFN γ or prime T lymphocytes following antigen presentation, the principal anti-tumor action of DC vaccines.

Third, the directions of variant association effects in Table 3 offer additional clues. As mentioned earlier, the abundance of negative effects suggest that collective loss of function among some pathway components leads to pathway dysregulation and may give rise to a more aggressive cancer phenotype. Every other variant with a positive effect is contained within a gene that encodes a plasma membrane-related or secreted protein. It has been shown that although an intracellular tumor-specific protein can trigger both cell-mediated and humoral immune responses, no appreciable tumor eradication is observed, likely a consequence of the protein's intracellular expression (46). In contrast, considerable exposed changes in peptide sequence would be expected for membrane proteins linked to the vaccine response-predisposing variants in Table 3; most are frameshift indels. Even the remaining variants are still missense mutations that cause amino acid substitutions. Despite their potential deleterious effects on pathway functions, the positive associations between these variants and DC vaccine response may be due to their gene products' cell surface immunogenicity, especially in the face of proactive tumor efforts to hide foreign antigens through under-expressing major histocompatibility complex class I (MHC-I) molecules or displaying MHC-I surrogates (47).

In constructing an accurate prediction model for DC vaccine response among patients with metastatic CRC following surgery (95% correct, 21 of 22 patients), we implicated several mechanisms of cancer proliferation and immune system evasion that are likely to impact immunotherapy success in this disease context. A weakness of our study is its small sample size. Substantially larger sample sizes were used to identify the survival-T lymphocyte relationships reported in previous studies (18, 19) that could not be replicated here for DC vaccine response. Having more than 22 CRC patients with liver metastases can allow the derivation of stronger and more reliable variant association effects. It would be interesting to assess whether the prediction score cutoff shown in Figure 1 can maintain the current prediction accuracy when applied to somatic genotype data from an independent cohort of patients.

Given the discovery nature of this study, targeted gene sequencing was performed in favor of whole-genome and whole-exome sequencing. With the latter two approaches in future studies, new pathway suspicions may be raised. Furthermore, whole-genome sequencing would be able to reveal outliers in total mutation burden which limited gene panel sequencing cannot (Table 1). Perhaps uncommon metastatic CRC patients with orders of magnitude more tumor mutations due to deficient MMR (21) will have greater tendency to benefit from DC vaccination, as they do for immune checkpoint inhibition (20). Interestingly, this within-cancer phenomenon has been shown to be untrue for DC vaccination across cancers, as clinical benefit relative to placebo is lower among melanoma patients (more mutations) than glioma and renal cell carcinoma patients (fewer mutations) (48). Another important difference compared to checkpoint inhibitors is that no toxicities were noted (17), so it is conceivable that even higher doses of DC vaccine may be indicated. Sequenced variants were also called relative to the publically available hg19 reference genome instead of paired normal colorectal tissue genomes. Although we removed germline variants based on annotations in the NCBI database (28), we may have still captured noise signals that are not relevant to CRC given their undiscerned presence in normal tissue.

In addition to more comprehensive bioinformatics studies, companion experimental pursuits are warranted. Our genomic analyses serve to not only construct a meaningful DC vaccine response prediction model, but also suggest novel directions for molecular investigations that may lead to an increase in the response prevalence or absolute efficacy of this immunotherapy. If metastatic CRC resistance to DC vaccination is truly influenced by the display of tumor surface neoantigens and aberrant transmembrane signaling through integrins and the PI3K/Akt/mTOR axis, combination immunotherapy with pharmacologic therapy may synergistically enhance tumor-specific immune responses and also lower the required dose, plus corresponding adverse effects, of each regimen. Among CRC patients, PI3K inhibitors and integrin inhibitors have already been separately shown in clinical trials to be well-tolerated anti-cancer agents, in particular with superior effects for metastatic disease (49, 50). Findings in the present study merit further work to evaluate the potential benefits of DC vaccine co-administration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of translational relevance

It has been well established that the immune system can effectively and specifically destroy cancer cells. Immunotherapies are intended to augment this natural aptitude for targeting tumor antigens. Dendritic cells serve as one of the body's most important antigen presenters that stimulate the adaptive immune system. Among metastatic colorectal cancer (CRC) patients, approximately half respond to a dendritic cell vaccine in the adjuvant setting following surgical resection of metastases. These patients have better recurrence-free survival compared to patients who do not respond. In the present study, resected CRC liver metastases were sequenced to identify likely mechanisms driven by somatic mutations that influence response to the vaccine. Our bioinformatic implication of PI3K/Akt/mTOR signaling and various plasma membrane proteins provides both a useful model for vaccine response inference in precision medicine (95% accurate) and new experimental directions for exploring cancer resistance against immunologic elimination.

Figure 1.

Pathway-guided vaccine response prediction. Response prediction scores for 22 rounds of leave-one-out cross-validation are presented. The test set individual in each round is denoted by a black asterisk, while training set responders and non-responders are denoted by blue dots and red dots, respectively. Test set individuals with scores above/below the dashed line were predicted to be vaccine responders/non-responders. Of the variants found to be associated with vaccine response among training set individuals ($P < 0.005$), those within genes that are listed in Table 3 were up-weighted in the prediction model.

Table 1

Characteristics of dendritic cell vaccine responders and non-responders

	Responders (N = 11)	Non-responders (N = 11)	P
Age	61.6 (7.9)	62.5 (11.2)	0.835
Male	64%	82%	0.346
Carcinoembryonic antigen level, ng/ml	32.1 (57.4)	18.3 (28.5)	0.483
Size of largest metastasis, cm	3.1 (1.8)	4.6 (3.4)	0.220
Number of metastases	1.5 (0.7)	1.6 (0.7)	0.519
Lymph node-positive primary tumor	55%	45%	0.670
Synchronous metastasis detection	45%	73%	0.200
Fong clinical risk score	1.7 (1.0)	2.3 (1.2)	0.252
Cytotoxic (CD8) T cells, per mm ²			
Tumor interior	13.3 (12.4)	9.0 (6.8)	0.323
Tumor periphery	44.1 (13.0)	37.4 (16.2)	0.289
Surrounding non-adjacent liver	10.5 (4.4)	8.5 (2.9)	0.201
Memory (CD45RO) T cells, per mm ²			
Tumor interior	24.8 (11.5)	17.5 (11.9)	0.159
Tumor periphery	80.4 (19.6)	70.9 (24.3)	0.311
Surrounding non-adjacent liver	15.9 (9.1)	12.9 (4.8)	0.337
Regulatory (FoxP3) T cells, per mm ²			
Tumor interior	6.1 (4.3)	5.5 (3.2)	0.693
Tumor periphery	8.1 (3.3)	7.6 (5.3)	0.777
Surrounding non-adjacent liver	0.3 (0.5)	0.2 (0.4)	0.613
Mutations per megabase	39.1 (2.3)	40.6 (2.1)	0.135

NOTE: Continuous and categorical patient characteristics are displayed as means (standard errors in parentheses) and percentages, respectively. "Mutations per megabase" refers to the mutation burden across all targeted sequencing gene regions. *P*-values indicate the predictive utility of each characteristic for dendritic cell vaccine response, as evaluated by univariate logistic regression.

Table 2

Pathway enrichment results

Pathway Name	Size	<i>P</i> -value	Randomization Rank	Implicated Components
1. KEGG: MTOR signaling pathway	52	6.25×10^{-5}	0.964	<i>MTOR, AKT3, RPS6KA2, TSC2</i>
2. REACTOME: PI3K cascade	56	7.31×10^{-5}	0.977	<i>MTOR, AKT3, FGFR2, TSC2</i>
3. REACTOME: Signaling by the B cell receptor	126	1.04×10^{-4}	0.967	<i>MTOR, AKT3, MALT1, CD79B, TSC2</i>
4. REACTOME: PIP3 activates AKT signaling	29	1.34×10^{-4}	0.972	<i>MTOR, AKT3, TSC2</i>
5. BIOCARTA: PITX2 pathway	15	6.31×10^{-4}	0.968	<i>TRRAP, CTNNB1</i>
6. PID: AR pathway	61	1.04×10^{-3}	0.985	<i>NCOA4, CTNNB1, AR</i>
7. REACTOME: PKB mediated events	29	2.41×10^{-3}	0.966	<i>MTOR, TSC2</i>

NOTE: Genes identified to be strongly associated with DC vaccine response by the CMC method were assessed for enrichment ("Implicated Components") of curated pathways in MSigDB using the hypergeometric test. "*P*-values" have been adjusted using the Benjamini-Hochberg false discovery rate method. "Size" describes the total number of genes comprising each pathway. "Randomization Rank" denotes the fraction of 1000 null enrichment values, computed using randomly sampled genes from the sequencing panel, that are less than the observed enrichment. Results have been filtered for Randomization Rank > 95th percentile.

Abbreviations: AR, androgen receptor; MTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PITX2, paired-like homeodomain transcription factor 2; PKB, protein kinase B which is also synonymous with AKT.

Table 3

Top pathway-guided predictions of dendritic cell vaccine response

Pathway name	Accuracy	Up-weighted pathway components
No pathway weighting	73%	All weights in Equation 1 were set to 1
1. KEGG: Extracellular matrix receptor interactions	77%	<i>CD36</i> (7:80299307/G>GT/+), <i>ITGA10</i> (1:145527997/AG>A/-), <i>ITGA10</i> (1:145534180/A>AT/+), <i>ITGB3</i> (17:45367575/AC>A/-), <i>ITGB3</i> (17:45367575/AC>A/-), <i>COL1A1</i> (17:48271806/AC>A/-), <i>COL1A1</i> (17:48275531/AG>A/-), <i>FNI</i> (2:216288075/GC>G/-)
2. KEGG: Natural killer cell mediated cytotoxicity	77%	<i>LCK</i> (1:32739904/GC>G/-), <i>PIK3R1</i> (5:67522514/A>G/+), <i>PIK3CD</i> (1:9775549/TC>T/-), <i>PIK3CD</i> (1:9775564/TG>T/+), <i>ITGB2</i> (21:46309974/CG>C/-), <i>ITGB2</i> (21:46323312/G>C/+)
3. PID: $\alpha_v\beta_3$ integrin pathway	77%	<i>VEGFR2</i> (4:55971036/G>C/+), <i>PIK3R1</i> (5:67522514/A>G/+), <i>GPR124</i> (8:37693120/TC>T/-), <i>IGF1R</i> (15:99459983/TG>T/-), <i>IGF1R</i> (15:99478596/GA>G/+), <i>ITGB3</i> (17:45367575/AC>A/-), <i>ITGB3</i> (17:45367575/AC>A/-), <i>COL1A1</i> (17:48271806/AC>A/-), <i>COL1A1</i> (17:48275531/AG>A/-), <i>FNI</i> (2:216288075/GC>G/-), <i>TGFBR2</i> (3:30715604/C>CG/+)
4. PID: Caspase pathway	77%	<i>MAP3K1</i> (5:56179436/G>C/-), <i>NUMA1</i> (11:71726363/AG>A/-), <i>SREBF1</i> (17:17720826/TG>T/-)
5. PID: SHP2 pathway	77%	<i>VEGFR2</i> (4:55971036/G>C/+), <i>LCK</i> (1:32739904/GC>G/-), <i>PIK3R1</i> (5:67522514/A>G/+), <i>PDGFRB</i> (5:149506102/AG>A/-), <i>PDGFRB</i> (5:149515110/GC>G/-), <i>BDNF</i> (11:27722583/TG>T/-), <i>IGF1R</i> (15:99459983/TG>T/-), <i>IGF1R</i> (15:99478596/GA>G/+)
6. REACTOME: Cell surface interactions at the vascular wall	77%	<i>LCK</i> (1:32739904/GC>G/-), <i>PIK3R1</i> (5:67522514/A>G/+), <i>ITGB3</i> (17:45367575/AC>A/-), <i>ITGB3</i> (17:45367575/AC>A/-), <i>COL1A1</i> (17:48271806/AC>A/-), <i>COL1A1</i> (17:48275531/AG>A/-), <i>ITGB2</i> (21:46309974/CG>C/-), <i>ITGB2</i> (21:46323312/G>C/+), <i>FNI</i> (2:216288075/GC>G/-)
7. REACTOME: PPAR α activates gene expression	77%	<i>CD36</i> (7:80299307/G>GT/+), <i>NCOA2</i> (8:71069295/GC>G/-), <i>CREBBP</i> (16:3842089/TG>T/-), <i>SREBF1</i> (17:17720826/TG>T/-), <i>ANGPTL4</i> (19:8434086/A>AC/+)
8. PID: $\alpha_M\beta_2$ integrin pathway	82%	<i>LCK</i> (1:32739904/GC>G/-), <i>ITGB2</i> (21:46309974/CG>C/-), <i>ITGB2</i> (21:46323312/G>C/+)
9. PID: Integrin 1 pathway	82%	<i>ITGA10</i> (1:145527997/AG>A/-), <i>ITGA10</i> (1:145534180/A>AT/+), <i>COL1A1</i> (17:48271806/AC>A/-), <i>COL1A1</i> (17:48275531/AG>A/-), <i>FNI</i> (2:216288075/GC>G/-)
10. REACTOME: Metabolism of amino acids and derivatives	86%	<i>GLS2</i> (12:56868601/TC>T/-), <i>MTR</i> (1:237025593/GA>G/-)
Union of above pathways	95%	Union of above components

NOTE: Of the variants found to be associated with vaccine response among training set individuals ($P < 0.005$), those within genes that participate in the pathways above were up-weighted in the prediction model. The variants' chromosomal position, reference allele, alternative allele, and direction of association with vaccine response are shown in parentheses. Responses of the 22 test set individuals in cross-validation were inferred based on whether their prediction scores exceed the cutoffs displayed in Supplementary Figure S1 and Figure 1. Corresponding prediction accuracies are also presented.