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\textsuperscript{a} Cancer Immunology and Immunotherapy Centre; School of Cancer Sciences; University of Birmingham; Birmingham, UK

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An immunogenomic stratification of colorectal cancer: Implications for development of targeted immunotherapy

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Keywords: colorectal cancer, immune signature, microsatellite instability, RAS, stratification

Abbreviations: CIRC, Co-ordinate Immune Response Cluster; CMS, consensus molecular subtypes; CRC, colorectal cancer; CRCSC, colorectal cancer subtyping consortium; MSI-H, microsatellite unstable (high); MSI-L, microsatellite unstable (low); MSS, microsatellite stable; TCGA, The Cancer Genome Atlas; TILs, tumor-infiltrating lymphocytes

Although tumor infiltrating lymphocyte (TIL) density is prognostic and predictive in colorectal cancer (CRC), the impact of tumor genetics upon colorectal immunobiology is unclear. Identification of genetic factors that influence the tumor immunophenotype is essential to improve the effectiveness of stratified immunotherapy approaches. We carried out a bioinformatics analysis of CRC data in The Cancer Genome Atlas (TCGA) involving two-dimensional hierarchical clustering to define an immune signature that we used to characterize the immune response across key patient groups. An immune signature termed The Co-ordinate Immune Response Cluster (CIRC) comprising 28 genes was coordinately regulated across the patient population. Four patient groups were delineated on the basis of cluster expression. Group A, which was heavily enriched for patients with microsatellite instability (MSI-H) and POL mutations, exhibited high CIRC expression, including the presence of several inhibitory molecules: CTLA4, PDL1, PDL2, LAG3, and TIM3. In contrast, RAS mutation was enriched in patient groups with lower CIRC expression. This work links the genetics and immunobiology of colorectal tumorigenesis, with implications for the development of stratified immunotherapeutic approaches. Microsatellite instability and POL mutations are linked with a high mutational burden and high immune infiltration, but the coordinate expression of inhibitory pathways observed suggests combination checkpoint blockade therapy may be required to improve efficacy. In contrast, RAS mutant tumors predict for a relatively poor immune infiltration and low inhibitory molecule expression. In this setting, checkpoint blockade may be less efficacious, highlighting a requirement for novel strategies in this patient group.

Introduction

The density of TILs and the expression of certain immune-related genes are of prognostic and predictive value in CRC. However, the factors that determine a patient’s immune phenotype are unclear, and few systematic analyses have investigated the somatic and germline molecular drivers of immune infiltration. Although, microsatellite unstable (MSI-H) cancers are known to be associated with increased TIL density, the nature of the immune infiltration and the molecular drivers of the immune phenotype in microsatellite stable (MSS) CRC are poorly understood. In particular, it is unclear whether defined molecular subsets (RAS mutant, BRAF mutant, PIK3CA mutant, quadruple wildtype (BRAF, PIK3CA, KRAS, NRAS all wildtype)) are associated with high or low immune infiltration. In addition, in both MSI-H and MSS cancers, the extent to which therapeutically tractable inhibitory immune checkpoint receptors are represented is unclear and is of substantial interest, particularly considering recent checkpoint blockade failures in CRC. Given the prognostic and predictive relevance of CRC immunophenotype, a clearer understanding of the link between immunophenotype with tumor genotype is crucial.

In this study, we carried out a bioinformatics analysis of CRC data in The Cancer Genome Project (TCGA). Our analyses defined a co-regulated cluster of immune related genes with a distinct Th1 bias, expression of which defines four patient subgroups. The findings have implications for our understanding of the immunobiology of colorectal carcinogenesis, and for development of stratified immunotherapy approaches for CRC.
Results

Coordinate expression of immune response-related genes in colorectal cancer

We interrogated expression of immune response-related genes in the CRC dataset in the TCGA (n = 195). Preliminary analyses focused on an initial gene list (Table S1) based partly on previous studies on bio-molecular networks incorporating immune genes linked with disease-free survival in CRC.1,4,5 These included those associated with Th1 subset function (STAT1, IRF1, IFNG, TBX21, IL18RAP, ICOS, GNLY), certain chemokines (CX3CL1, CXCL9, CXCL10), adhesion molecules (ICAM and MADCAM), and an array of class II genes. Also, we included a number of immune checkpoint genes (PD-1, PD-L1, PD-L2, LAG3, TIM3, CTLA-4), two of which (PD-L1 and PD-L2) were previously associated with outcome.9 Finally, our initial gene list was supplemented with class I genes and additional class II genes and genes involved in T cell activation, together with NKG2D ligands and related genes relevant to innate immune activation (e.g. ULBPs, PROCR15).

We performed unsupervised two-dimensional hierarchical clustering to assess the extent to which gene expression was co-ordinate or independent across the patient cohort. Visual analysis of the clustering highlighted a 28-gene subset (Table 1) which formed a clear gene grouping, expression of which was coordinately regulated (Fig. S1). Gene-tree analysis of the dendrogram confirmed the validity of this grouping, identifying a subset of 24 highly coordinated genes (distance threshold 0.46).

Table 1. Genes within the coordinate immune response cluster (CIRC). These genes are presented in the order of the CIRC signature.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>HLA-DQA1</th>
<th>HLA-DQA2</th>
<th>HLA-DQB5</th>
<th>CTLA4</th>
<th>PDCD1LG2</th>
<th>ICAM1</th>
<th>CD274</th>
<th>STAT1</th>
<th>IFNG</th>
<th>GNL1</th>
<th>TBX21</th>
<th>CCL5</th>
<th>LAG3</th>
<th>CD247</th>
<th>ICOS</th>
<th>IL18RAP</th>
<th>CXCL9</th>
<th>CXCL10</th>
<th>HLA-DPB1</th>
<th>HLA-DPA1</th>
<th>HLA-DMB</th>
<th>HLA-DRA</th>
<th>CD80</th>
<th>HLA-DOA</th>
<th>CD4</th>
<th>HAVCR2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
</tbody>
</table>

Of the 28 genes in the CIRC, 20 have previously been associated with outcome based on experimental data.1,4,5 We show here that these prognostic immune response genes are highly coordinately expressed in CRC and correlate with other biologically associated genes including HLA-DQA1, HLA-DRB5, HLA-DPB1, LAG3, TIM3, CTLA4, and CCL5. The degree of correlation between immune checkpoint receptor/ligand gene expression was notable (Table S2). Particularly striking was the correlation between PD1 and LAG3 (r² = 0.62, Fig. 1). Given the Th1 bias of the cluster we performed separate unsupervised hierarchical clustering analysis of Th2 cytokines IL2, IL3, IL4, IL5, and IL6 and transcription factor GATA3 (data not shown), revealing that these genes were excluded from the CIRC. We also note that the Th17 cytokine IL17A was also excluded from the cluster (Fig. S1).

Molecular determinants of the co-ordinate immune response cluster

Two-dimensional hierarchical clustering was performed for the entire gene list (Table S1) incorporating expression data together with molecular and clinical characteristics (KRAS, BRAF, NRAS, TP53, PIK3CA, and PTEN mutations; microsatellite status, methylation subtype, tumor stage, tumor site, and recurrence data). The microsatellite status of the total patient population was as follows: 11.8% were MSI-H, 15.9% were MSI-L (microsatellite-low), and 71.8% were MSS. 6.7% of patients were BRAF mutant (MT)/MSI-H, and 3.1% were BRAF MT/MSS. The mutational status of the cohort was as follows – KRAS MT (39.5%), NRAS MT (8.2%), BRAF MT (9.7%), PIK3CA MT (18.4%), and TP53 MT (51.8%). 38.5% of patients were quadruple wild type (BRAF WT, KRAS WT, NRAS WT and PIK3CA WT). We also investigated mutations in the novel genes POLE (7.2% of patients) and POLD1 (2.6% of patients), which have recently been highlighted as key drivers of colorectal carcinogenesis for a minority of CRC patients.16

Hierarchical clustering delineated four distinct patient groups (Fig. 2, Table 2). Group A patients demonstrated strong CIRC expression (mean expression 0.98). Notably, all of the MSI-H patients were included in this group, who constituted 82.1% of all group A patients, whereas only 10.7% were MSS and 7.1% were MSI-L. Across the entire cohort, expression of the CIRC signature was significantly higher in the MSI-H cancers vs. MSS (p < 0.001) and MSI-L cancers (p < 0.001). Multivariate analysis revealed that expression of HLA-A, HLAB, and HLAC were all significantly less in MSI-H than MSS cancers (p = 0.027, p =
0.017, and $p = 0.018$, respectively for HLA-A, B and C, consistent with previous observations. Group A was characterized by the CpG island methylator phenotype (CIMP-H) (67.9%), right-sided tumor site (82.1%), TP53 wild type (65%) and KRAS wild type (82.1%). 50% of patients in this group were BRAF mutant and of these BRAF mutants, 92.9% were MSI-H. Notably, POLE and POLD1 mutant tumors were associated with higher expression of the CIRC ($p < 0.05$). 42.9% of patients in group A were either POLE or POLD1 MT, and 70.6% of all patients with POLE or POLD1 mutations were found in group A: 100% of POLD1 mutants were assigned to group A. Of the non-MSI-H patients in group A, 3/5 were POLE mutant, two of which were MSS and one MSI-L.

We then analyzed patient groups B, C, and D (Fig. 2), which displayed lower expression of the CIRC signature than group A, to determine whether any molecular characteristics were associated with low CIRC expression. Group D comprised cancers with the lowest expression of the cluster (mean expression −0.62) and represented 43% of the entire patient population; group B had a lower mean cluster expression (0.13) than group C (0.50). Significantly, RAS mutation (KRAS/NRAS), occurring in 47.7% of patients, was associated with lower CIRC expression ($p < 0.001$). This was confirmed in the provisional TCGA data set, in which mutation and expression data for a further 30 patients (225 total) is available ($p < 0.001$). The frequency of RAS mutation was significantly higher in group B and D than other patient groups ($p = 0.01$), with group D having the highest frequency (D (61.9%) > B (51.0%) > C (28.1%) > A (21.4%)), ($p = 0.01$). Consistent with these observations, within MSS cancers (enriched in groups B–D), there was a strong trend for RAS mutation to be associated with low CIRC expression ($p = 0.076$). In group A, 83.3% of RAS mutations were MSI-H and 16.7% were MSS (Table 2). In RAS mutant MSI-H patients, the expression of HLA-DRA was lower (0.518) than in RAS wild types (1.027) but this difference did not reach significance ($p = 0.209$). Finally, multivariate analysis indicated that KRAS and NRAS mutation was associated with decreased CD4 expression ($p < 0.001$) (Table S3). The association of low expression of the cluster and RAS mutation was particularly strong in the case of NRAS mutation where 76.9% of these cancers were in group D and over 90% of these tumors clustered in the two lowest expression groups (B and D). Thus RAS mutation, unless linked with microsatellite instability, is commonly associated with low levels of Th1 infiltration and activation, class II expression and inhibitory checkpoint expression. Table 3 shows the distribution of key mutations.

Several groups have identified molecular subtypes of CRC based on expression data. In an effort to create consensus, The
Figure 2. Two-dimensional hierarchical clustering delineates distinct immunological CRC patient groups. Gene expression (yellow, high expression; black, intermediate; blue, low expression) was clustered together with mutation data of key genes (TP53, KRAS, BRAF, NRAS, PIK3CA and PTEN (yellow, mutant; blue, wildtype)) and clinical data (microsatellite status (yellow, MSI-H; black, MSI-L; blue, MSS), recurrence data (yellow, recurred/progressed; blue, disease-free), tumor site (yellow, left sided; blue, right sided), tumor stage (yellow, stage III/IV; blue, stage I/II), methylation subtype (yellow, CIMP-H; black, CIMP-L; blue, CIMP-negative). Clustering was performed by genes/mutations/clinical data (rows) and patients (columns) using the Pearson algorithm. Red boxes indicate groups of patients with strong clustering of the coordinate immune response cluster. Patients were delineated into four distinct groups (A–D) on the basis of the dendrogram and the cluster expression.
Table 2. Characteristics of patient groups. For microsatellite status, methylation and tumor side, the most frequent result is stated. Tumor side refers to the right or left side of the colon. The cluster expression pattern displays the expression pattern of the CIRC cluster in each patient group

<table>
<thead>
<tr>
<th></th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Group D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsatellite status</td>
<td>MSI-H (82%)</td>
<td>MSS (86%)</td>
<td>MSS (94%)</td>
<td>MSS (75%)</td>
</tr>
<tr>
<td>Methylation</td>
<td>CIMP-High (68%)</td>
<td>CIMP-Neg (77%)</td>
<td>CIMP-Neg (66%)</td>
<td>CIMP-Neg (69%)</td>
</tr>
<tr>
<td>Side of tumour</td>
<td>Right (82%)</td>
<td>Left (63%)</td>
<td>Left (94%)</td>
<td>Left (82%)</td>
</tr>
<tr>
<td>Stage I–II</td>
<td>73%</td>
<td>55%</td>
<td>72%</td>
<td>49%</td>
</tr>
<tr>
<td>TPS3 MT</td>
<td>35%</td>
<td>65%</td>
<td>62%</td>
<td>48%</td>
</tr>
<tr>
<td>BRAF MT</td>
<td>50%</td>
<td>4%</td>
<td>3%</td>
<td>1%</td>
</tr>
<tr>
<td>KRAS MT</td>
<td>18%</td>
<td>47%</td>
<td>22%</td>
<td>49%</td>
</tr>
<tr>
<td>NRAS MT</td>
<td>0%</td>
<td>4%</td>
<td>9%</td>
<td>13%</td>
</tr>
<tr>
<td>PIK3CA MT</td>
<td>39%</td>
<td>14%</td>
<td>9%</td>
<td>18%</td>
</tr>
<tr>
<td>Quadruple WT</td>
<td>18%</td>
<td>39%</td>
<td>69%</td>
<td>33%</td>
</tr>
<tr>
<td>Percentage of patients</td>
<td>14%</td>
<td>26%</td>
<td>16%</td>
<td>43%</td>
</tr>
<tr>
<td>Mean cluster expression z-score</td>
<td>+0.98</td>
<td>+0.13</td>
<td>+0.50</td>
<td>−0.62</td>
</tr>
</tbody>
</table>

Table 3. Frequencies of mutations in total patient population and distribution of mutations across patient groups A–D. We classified patients initially on the basis of KRAS status, followed by NRAS, BRAF, and finally PIK3CA. According to these criteria, KRAS mutations may also have NRAS, BRAF, and PIK3CA mutations (KRAS MT +/-NRAS MT +/-BRAF MT +/-PIK3CA MT). NRAS mutations are KRAS wildtype but may have mutations in BRAF and PIK3CA (KRAS WT + NRAS MT +/-BRAF MT +/-PIK3CA MT). BRAF mutants are KRAS and NRAS wildtype but may have mutations in PIK3CA (KRAS WT + NRAS WT + BRAF MT +/-PIK3CA MT). PIK3CA mutants are wildtype for KRAS, NRAS, and BRAF. Quadruple wildtype patients are KRAS, NRAS, BRAF, and PIK3CA wildtype. TPS3 mutation status is independent of other mutations

<table>
<thead>
<tr>
<th></th>
<th>% of total patient population</th>
<th>% of total in patient group A</th>
<th>% of total in patient group B</th>
<th>% of total in patient group C</th>
<th>% of total in patient group D</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPS3 MT</td>
<td>51.8%</td>
<td>6.9%</td>
<td>32.7%</td>
<td>19.8%</td>
<td>40.6%</td>
</tr>
<tr>
<td>BRAF MT</td>
<td>9.2%</td>
<td>72.2%</td>
<td>11.1%</td>
<td>5.5%</td>
<td>11.1%</td>
</tr>
<tr>
<td>KRAS MT</td>
<td>41.0%</td>
<td>7.5%</td>
<td>30%</td>
<td>10%</td>
<td>52.5%</td>
</tr>
<tr>
<td>NRAS MT</td>
<td>6.7%</td>
<td>0%</td>
<td>15.4%</td>
<td>7.7%</td>
<td>76.9%</td>
</tr>
<tr>
<td>KRAS/NRAS MT</td>
<td>47.7%</td>
<td>6.5%</td>
<td>28.0%</td>
<td>9.7%</td>
<td>55.9%</td>
</tr>
<tr>
<td>PIK3CA MT</td>
<td>4.1%</td>
<td>50%</td>
<td>37.5%</td>
<td>0%</td>
<td>12.5%</td>
</tr>
<tr>
<td>Quadruple wildtype</td>
<td>39.0%</td>
<td>6.6%</td>
<td>26.3%</td>
<td>28.9%</td>
<td>38.2%</td>
</tr>
</tbody>
</table>
signature we defined stems from the work of Galon and colleagues, who first established the prognostic impact of T cell infiltration in CRC, initially highlighting seven co-modulated genes principally associated with Th1-associated immunity that correlated with outcome.\(^4\) Subsequently, they demonstrated that the Th1 genes, TBX21, IFNG, IRF1 and STAT1 were all individually associated with outcome.\(^4\) Other independent immune gene predictors in that analysis were IL18RAP, ICOS, PD-L1, PD-L2 and PD-1. We extended this gene list by adding the class I and class II genes, further immune checkpoint genes and a number of other genes related to innate immune recognition and T cell activation, generating an initial gene list of > 50 genes. Unsupervised hierarchical clustering then highlighted a core cluster of 28 tightly co-regulated genes comprising the CIRC signature. Importantly, 20 of the 28 genes had previously been highlighted by Galon and colleagues\(^{14,5}\) as either prognostically or predictively relevant, including five of the original prognostic seven gene cluster (with only CD8A (for which microarray data was unavailable) and GZMB not represented). In contrast, genes associated with Th2 and Th17 profiles were excluded from the cluster. Thus, applying a different approach to an independent data-set our analysis verified the high level of correlation of a number of genes based on Th1-associated immunity, and also highlighted involvement of additional immune genes.

An important feature of the CIRC signature is that it includes essentially all class II MHC loci, as well as CD4, whereas in contrast, expression of class I MHC molecules, CD8B and also GZMB are all excluded from the signature. In addition to the critical role of T helper cells for CD8\(^+\) cell priming\(^39,20\) and expansion,\(^21,22\) CD4\(^+\) cells have also been suggested to be major mediators of immunological tumor cell death.\(^23-25\) Adoptive CD4\(^+\) T cell transfer has been found to upregulate class II expression on tumor cells mediating protection from tumor progression.\(^26\) Class II upregulation was mediated via IFN\(\gamma\) and protection was attenuated using anti-IFN\(\gamma\) antibodies. Recent adoptive transfer approaches involving autologous CD4\(^+\) T cells have met with clinical success. A durable complete response was obtained in a patient with melanoma after infusion of NY-ESO-1 specific CD4\(^+\) cells recognizing an HLA-DP4 restricted epitope.\(^26\) Additionally, a durable response was obtained in a patient with cholangiocarcinoma on infusion of autologous mutation-specific CD4\(^+\) cells which adopted a poly-functional Th1 phenotype.\(^27\) These studies highlight the potential for CD4\(^+\) cells to mediate clinically potent anti-tumor responses via Th1 mechanisms. The CD4-centric nature of our CIRC highlights that CD4\(^+\) T cells may be important in CRC anti-tumor immunity.

The CIRC included the major immune checkpoint molecules. Not only PD-L1, PD-L2 but also LAG3, TIM3 and CTLA4 were all represented in the CIRC and there was a high degree of correlation between inhibitory checkpoint gene expression. This is consistent with the expected feedback sequence of a pronounced Th1 infiltrate. IFN\(\gamma\) is the canonical cytokine associated with Th1 T helper cells and expression of PD-L1 is significantly augmented by IFN\(\gamma\).\(^28\) IRF1 is of primary importance in the constitutive expression of PD-L1 and in IFN\(\gamma\)-driven upregulation.\(^29\)

In animal models, significant percentages of infiltrating CD4\(^+\) and CD8\(^+\) cells co-express high levels of both PD-1 and LAG-3.\(^30\) Dual anti-LAG3/anti-PD-1 immunotherapy strikingly enhanced survival in the MC38 colon cancer model compared with animals treated with single antibodies alone.\(^30\) Our data provide justification for trialing combinations of checkpoint blockade agents in CRC.

One of the key aims of this study was to investigate the somatic factors associated with the immune response in CRC. Group A, which exhibited strong expression of the CIRC signature, was dominated by MSI-H tumors, all of which fell in this grouping. This complements very well the work of Biossière-Michot and colleagues, who revealed that MSI-H tumors have a high density of Th1-positive Th1 T cells relative to MSS tumors, and higher expression of the chemokines CCL5, CXCL9, and CXCL10, all of which are found in the CIRC.\(^12\) The Th1 response may be driven through activation of the CXCL9/CXCL10 signaling axis.\(^12\) Previous studies have also shown that MSI-H phenotype is highly associated with class II expression.\(^31\) Strongly DR positive tumors had a significantly higher TIL density than those with absent or weak staining and survival was significantly better in patients with high DR expression. Consistent with this, Bindea and colleagues demonstrated that the expression of several class II genes, including HLA-DR, are individually associated with improved disease-free survival.\(^5\) High DR expression in MSI-H CRC contrasts with that of class I molecules, which are completely lost in 60% of sporadic MSI-H cases and only 16.7% of right sided MSS cancers.\(^17\) Thus, the immune landscape of MSI-H CRC, which is characterized by a high mutational burden including frame-shift mutations,\(^10\) is dominated by T helper cell infiltration and activation, class II expression and coordinated upregulation of a range of immune checkpoint genes. These patients may therefore have greater responses to checkpoint blockade therapies. Similarly, POLE/POLD1 mutant tumors, which also have a high mutational burden,\(^16\) were also associated with high CIRC expression. These data support the hypothesis that high

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**Table 4.** Distribution of Consensus Molecular Subtype (CMS) groups across CIRC patient groups. The percentage of each CMS group that fall within each CIRC patient group. 5.1% of TCGA patients did not have CMS classification data available.

<table>
<thead>
<tr>
<th>CMS</th>
<th>Percentage of total patient population</th>
<th>CIRC Group A</th>
<th>CIRC Group B</th>
<th>CIRC Group C</th>
<th>CIRC Group D</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMS1</td>
<td>13.8%</td>
<td>81.5%</td>
<td>7.4%</td>
<td>3.7%</td>
<td>7.4%</td>
</tr>
<tr>
<td>CMS2</td>
<td>40.0%</td>
<td>0.0%</td>
<td>15.4%</td>
<td>23.1%</td>
<td>61.5%</td>
</tr>
<tr>
<td>CMS3</td>
<td>10.3%</td>
<td>0.0%</td>
<td>30.0%</td>
<td>20.0%</td>
<td>50.0%</td>
</tr>
<tr>
<td>CMS4</td>
<td>18.5%</td>
<td>5.6%</td>
<td>63.9%</td>
<td>13.9%</td>
<td>16.7%</td>
</tr>
<tr>
<td>Unclassified</td>
<td>12.3%</td>
<td>12.5%</td>
<td>25.0%</td>
<td>12.5%</td>
<td>50.0%</td>
</tr>
</tbody>
</table>
mutational burden may translate into a strong immune response, possibly due to neoantigen presentation, leading to better outcomes. The finding that the gene encoding TGFβR undergoes frameshift mutation in 90% of MSI-H CRC, giving rise to a highly immunogenic promiscuous class II peptide, is entirely in keeping with this hypothesis.32

**RAS** mutation was significantly associated with lower CIRC expression, with over 60% of cancers in the group exhibiting very low expression (group D) being **RAS** mutant. **KRAS** and **NRAS** mutant CRC had significantly lower levels of CD4⁺ T cells on multivariate analysis. Although 21.4% of cancers in group A were **RAS** mutant, over 80% of these were MSI-H. This is the first analysis to our knowledge to clearly define the immunological landscape of **RAS** mutant CRC. Ogino and colleagues examined the interaction between T cell infiltration and **KRAS**, **BRAF**, and **PIK3CA** mutation status and found no significant associations with **KRAS** mutation.33 but this study was limited to the density of CD3⁺, CD8⁺, CD45RO⁺, and FoxP3⁺ cells. Morris and colleagues demonstrated that transfection of mutant **KRAS** into fibroblasts abrogated the IFNγ-mediated upregulation of class II expression: effects on class I expression were minimal.34 **RAS** transfection inhibited proliferation and IFNγ production of alloreactive T cells, an effect mediated by loss of class II expression on target cells.35 These data suggest a possible mechanism for our observed paucity of class II expression and hence **Th1**-related gene expression in **RAS** mutant CRC. Our data complement previous studies demonstrating widespread abnormalities of class I presentation in **RAS** mutant CRC.36 In MSI-H tumors, it is likely that any inhibitory effect of **RAS** mutation is overcome by the strong immunity resulting from neoantigens linked to mutations induced by microsatellite instability. Thus MSI-H **RAS** mutant tumors retain strong **Th1** immunity, despite having numerically lower **HLA-DRA** expression than MSI-H **RAS** wildtype tumors. In sum, these data suggest that the micro-environment of **RAS** mutant CRC is relatively immunologically unfavorable to conventional αβ T cell responses.

An important consideration is how the CIRC signature relates to immunosuppressive pathways. Though **FOXP3** was not in the hierarchical clustering due to an absence of microarray data, we analyzed RNA sequencing data which showed that **FOXP3** expression is highest in patient group A and lowest in group D (data not shown). Additionally, the highly immunosuppressive cytokine IL10 also exhibited high expression in this patient group (data not shown), both consistent with increased inhibitory immunoregulation following establishment of a **Th1**-polarized microenvironment. We also analyzed expression of **STAT3**, which has been linked to enhanced levels of tumor-associated macrophages, myeloid-derived suppressor cells, and poor T cell responses.37,38 Although not strongly linked to the CIRC, we found increased **STAT3** expression was associated with **PIK3CA** mutation (Table S3). Conditioned media from **PIK3CA** mutant cells has been found to drive **STAT3** activation39; Aspirin decreases **STAT3** activation in murine CRC models and promotes apoptosis. Our findings may therefore partly explain previous molecular epidemiology studies40-42, which have demonstrated that aspirin usage reduces the risk of CRC recurrence following resection in patients with CRC harboring **PIK3CA** mutations.

The CIRC molecular associations relate to the CRCSC CMS and their immune and molecular characteristics.18 The majority of CMS1 patients (MSI high and immune activated) fall within CIRC group A. 82.5% of **KRAS** mutated patients were in CIRC groups D and B (the lowest two CIRC expression groups) and almost the same percentage of CMS3 patients, characterized by **KRAS** mutations and no significant immune infiltration and activation, were present in CIRC groups D and B.

In conclusion, we have shown that MSI-H and POL mutant CRC is associated with high-level expression of a coordinated immune response cluster characterized by T helper cell and class II related genes together alongside a range of chemokines and immune inhibitory checkpoint molecules. In contrast, **RAS** mutant tumors have significantly lower expression of this cluster. These findings have potential therapeutic implications. MSI-H tumors may be particularly amenable to CD4⁺ cell expansion and adoptive transfer approaches. Second, although there is interest in the use of immune checkpoint inhibitors in CRC, the coordinated upregulation of immune checkpoint genes suggests that a combinatorial approach is likely to be more successful. Finally, any immunologically based therapy in MSS CRC aimed at **RAS** mutant patients must take into account the relatively immunologically quiescent status of the micro-environment in the majority of **RAS** mutant CRC.

**Methods**

**Gene identification**

Genes associated with innate and adaptive tumor immunity in cancer1,4 and genes relevant to established colon cancer pathways were shortlisted as initial genes. This list was expanded using molecular network pathway analysis.33-45 This revealed further molecules and genes with known genetic, pathway and functional associations with our initial genes. Genes revealed through this approach were screened for inclusion into our final gene list based on known immunological relevance (Table S1).

**Data extraction**

Normalized Agilent microarray and RNAseq z-score data for initial genes and mutation data for **TP53**, **KRAS**, **BRAF**, **NRAS**, **HRAS**, **PIK3CA** and **PTEN** were extracted from TCGA colorectal data set46 using the cBioportal tool.43-45 Where expression data were unavailable, genes were excluded from the analysis. Clinical data for these patients were retrieved from the TCGA portal (https://tcga-data.nci.nih.gov/tcga/) and tabulated with genetic data. CMS data were obtained from the CRCSC through the Synapse platform (https://www.synapse.org/, Sage Bionetworks).

**Data analysis**

Data were tabulated in Excel (Microsoft Corp.) and unsupervised two-dimensional hierarchical clustering was performed using MeV (Dana-Farber Cancer Institute, Boston, MA, USA) and the Pearson correlation. Patient and gene clusters were
identified by varying gene-tree distance-thresholds and visual analysis. Normality of distributions was confirmed with the Anderson–Darling test. Pearson Coefficient of determination (R²) values were calculated in Excel and MiniTab (Minitab Inc.) to investigate correlations in gene expression. Gene expression was correlated against β actin as a control gene. T tests were performed for univariate analyses of normally distributed data, and Mann–Whitney U tests for non-normally distributed data. Multivariate linear regression analyses of gene expression against mutations in key tumor associated genes (P53, KRAS, BRAF, NRAS, HRAS, PIK3CA, PTEN) were completed in Stata 12.2 (Statacorp.). For two by two comparisons, a Chi-squared test was used; if any groups contained less than five values, the Fisher’s Exact test was used in preference.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
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