A Signature Predicting Poor Prognosis in Gastric and Ovarian Cancer Represents a Coordinated Macrophage and Stromal Response

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Abstract

Purpose: Gene-expression profiling has revolutionized the way we think about cancer and confers the ability to observe the synchronous expression of thousands of genes. The use of putative genome-level expression profiles has allowed biologists to observe the complex interactions of genes that constitute recognized biologic pathways. We used gastric and ovarian datasets to identify gene-expression signatures and determine any functional significance.

Experimental Design: Microarray data of 94-tumor and 45-benign samples derived from patients with gastric cancer were interrogated using Hierarchical Ordered Partitioning and Collapsing Hybrid analysis identifying clusters of coexpressed genes. Clusters were further characterized with respect to biologic significance, gene ontology, and ability to discriminate between normal and tumor tissue. Tumor tissues were separated into epithelial and stromal compartments and immunohistochemical analysis performed to further elucidate specific cell lineages expressing genes contained in the signature.

Results: We identified a "stromal-response" expression signature, highly enriched for inflammatory, extracellular matrix, cytokine, and growth factor proteins. The majority of genes in the signature are expressed in the tumor-associated stroma but were absent in associated premalignant conditions. In gastric cancer, this module almost perfectly differentiates tumor from nonmalignant gastric tissue and hence can be regarded as a highly tumor-specific gene-expression signature.

Conclusions: We show that these genes are consistently coexpressed across a range of independent gastric datasets as well as other cancer types suggesting a conserved functional role in cancer. In addition, we show that this signature can be a surrogate marker for M2 macrophage activity and has significant prognostic implications in gastric and ovarian high-grade serous cancer. Clin Cancer Res; 20(10); 2761–72. ©2014 AACR.

Introduction

Cancer is characterized by the invasion of malignant cells into surrounding supportive tissue and distant sites (1). It is well recognized that the consequent tissue deformation leads to an inflammatory host response with elevated levels of infiltrating inflammatory and immune cells (2). Although this host reaction could merely represent a generic response of the host to injury, the genes that control heterotypic cell interactions in wound healing and cancer stroma are subject to germ line sequence variations (3–8), suggesting that the tumor microenvironment may vary among patients exhibiting a variety of phenotypic responses. One such response was reported recently suggesting an immunosuppressive role of fibroblast activation protein α (FAP-α)—expressing cells in the tumor stroma that was tumor permissive (9). The relative contribution of stromal cells, such as tumor-associated macrophage (TAM) and carcinoma-associated fibroblasts (CAF) to the cancer phenotype in different patients is of great interest (10). More importantly, the identification of the specific stromal cell lineage responsible for cancer promotion would enable therapeutic trials aimed at patient cohorts that would best respond to an agent targeted against specific stromal components. FAP-α—expressing cells may be one example as well as TAMs that are thought to promote cancer and interact with both the cancer cells (11, 12) and other stromal cells (13).
Genomic profiling using gene-expression arrays was designed as a discovery tool aimed at increasing insight into the cause and therapy of disease. The approach proved useful to dissect molecular subtypes of malignancies, including diffuse large B cell lymphoma (14) and solid tumors such as breast cancer (15) and gastric cancer (16). Gene-expression analysis of a whole tumor is the sum of the mRNA contributions from different cell lineages, driven by cell-autonomous genomic changes as well as non–cell-autonomous cell–matrix and cell–cell interactions (17). We previously described a gene-expression signature of coexpressed genes exhibiting increased expression in malignant tissue (16, 18, 19). More recently, we found a highly related signature in a group of patients with ovarian cancer, which was associated with the poorest survival in the Australian Ovarian Cancer Study (AOCS) cohort (20).

In this study, we describe the discovery and exploration of a conserved signature of genes derived from gastric cancer. We extended the analysis to include independent gastric datasets (21, 22) as well as a broader group of malignancies, including ovarian cancer (20), colorectal cancer (23), lung cancer (24), and breast cancer (25). This novel finding of a conserved signature in diverse epithelial malignancies, the ontology of the genes in this signature (tissue remodeling), and the specific upregulation of this signature in gastric cancer over the respective premalignant tissues, all suggest that this signature reflects a cancer-specific inflammatory response. We therefore termed the signature as "stromal-response" to emphasize that it is a response to the presence of malignant epithelium. Here, we examine the factors that potentially control the expression of this gene-expression signature and show, for the first time, data that suggest that this signature arises from the stromal compartment of the cancer and is strongly associated with the presence of M2-polarized macrophages or TAMs.

Materials and Methods

Patients and samples

Patients diagnosed with operable gastric cancer were included in the study as part of a large cohort study in which clinical, demographic, and surgical pathology information was collected (Peter MacCallum Cancer Centre [PMCC] cohort; Supplementary Table S1). Histology of specimens was assessed by an independent pathologist (S. Lade). Patients were recruited from six metropolitan hospitals in Melbourne with written informed consent. All procedures were ethically approved by individual hospital Institutional Review Boards (IRB) and were overseen by the Peter MacCallum Cancer Centre IRB. Cases of ovarian cancer were obtained through the AOCS as previously described (20).

Microarray experiments

Fresh-frozen tissue was collected at the time of surgery as previously described (16). Whole tissue sections from 94 tumors and 45 benign tissues were profiled. All tissues required pathology evaluation and the minimum acceptable percentage of tumor cells was 60%. Total RNA was isolated by acid phenol extraction (Trizol; Invitrogen) and column chromatography (RNeasy; Qiagen). RNA quantity and quality were assessed by spectrophotometer (Nanodrop; ThermoScientific) and Bioanalyser (Agilent Technologies). Microarrays were hybridized using Affymetrix U133 +2 chips (Affymetrix) according to the protocol described in the manual. Microarrays were scanned using the Genechip Scanner (Affymetrix). Data have been submitted to Gene Expression Omnibus (GEO) (Series GSE51105).

Additional/validation microarray datasets

Details of additional and validation datasets are provided in the Supplementary Materials and Methods.

Laser capture microscopy

Laser capture microscopy was performed for 4 cases of gastric cancer and 5 cases of ovarian cancer. A total of 10-μm sections were placed into membrane-coated slides (MDS Analytical Technologies) and stained with cresyl violet (Ambion). Tumor and stromal compartments were identified by a pathologist and individually isolated using laser capture microdissection (LCM; Arcturus Veritas; MDS Analytical Technologies). RNA isolation, quantitation, and Affymetrix arrays were run as described for microarray experiments.

In situ hybridization and immunohistochemistry

Gastric cancer and premalignant tissues were ethanol fixed, paraffin embedded and used to create tissue microarrays (TMA), which were subsequently used for in situ hybridization (ISH) and immunohistochemistry (IHC). Details of relevant protocols are described in the Supplementary Materials and Methods.

Statistical analysis

Differentially expressed genes between gastric cancer and normal gastric epithelium were identified using the
empirical Bayes method available in the R-package limma. A false discovery rate (FDR) of 5% was used as the cutoff to select differentially expressed genes. Hierarchical Ordered Partitioning and Collapsing Hybrid (HOPACH) analysis was performed on the gastric dataset using the R statistical package, which builds a hierarchical tree of maximally homogeneous clusters by recursively partitioning a dataset while ordering and collapsing clusters at each level (26). Eisen cluster was used for hierarchical clustering using average linkage (27). The statistical significance of coexpression of a gene set was computed using a Monte Carlo procedure. The average correlation of the pairwise correlation of the set of genes was noted. The average correlation of a randomly selected set of the same number of genes within the same dataset was computed N times. The proportion of times the randomly selected genes had higher equal correlation to that of the original set is used as the statistical significance of the correlation. Gene set enrichment analysis (28) was used for assessment of gene enrichment across Australian and Hong Kong datasets as well as the tumor/normal datasets referred to above. The Kaplan–Meier analysis was performed for the progression-free survival and log-rank statistics was used to analyze the data. The mean expression level of the cluster of genes was used to stratify samples into high and low status for that particular cluster. The top and bottom 33% of the samples were classified as high and low, respectively.

Results

The stromal-response signature is a set of coexpressed genes that discriminates gastric cancer from benign gastric tissues

We and others have used gene-expression profiling to classify cases according to groups of coexpressed gene signatures (14, 16, 19). Often, such signatures are associated with histopathological and clinical phenotype, such as drug response, metastatic spread, or overall survival. To use this information to instruct patient management, further mechanistic insight about the coregulation of the signature genes is required. We have expanded on our previously published gene-expression profiling of gastric cancers with the addition of an extra 29 tumor samples (16). All samples in the cohort were repurposed this time using Affymetrix U133+2 gene chips. The HOPACH (29) method was used to identify "clusters" of genes that are strongly coexpressed within a given dataset, irrespective of the pathology of the sample. All genes with a cluster membership value of \(<0.3\) were disregarded. This left nine major clusters, each including at least 34 genes, which showed significant coexpression with an FDR smaller than \(10^{-6}\) (Table 1; see Supplementary Table S2 for gene lists).

Five of these HOPACH clusters (clusters 27, 21, 12, 0, and 26) included a significant proportion of genes (over 60% of genes in the cluster) that were also found to be statistically overrepresented as discriminatory between tumor and normal based on empirical Bayes tests (Table 1; Supplementary Table S3).

Figure 1A shows hierarchical clustering of genes using Pearson correlation as a similarity matrix and average linkage method in our Affymetrix data. The relative positions of the clusters from Table 1 are also indicated. Cluster 27 not only exhibited the most significant coexpression, but also contained the highest percentage of differentially expressed genes. Figure 1B shows Eisen clustering of all 144 probes (representing 100 nonredundant genes; Supplementary Table S4) in cluster 27, which clearly differentiates normal and tumor samples.

To confirm that this signature is only expressed in tumor samples, a separate HOPACH analysis was also performed using all normal and premalignant samples in the cohort. None of the clusters generated in this analysis significantly overlapped with cluster 27 (data not shown), suggesting a tumor-specific signature, which is not seen in normal or inflamed premalignant tissue.

Biologic significance of the HOPACH clusters

To gain more biologic insight into the mechanism of the coexpression observed for each of the clusters, we performed GeneGo MetaCore analysis on each of the top nine cancer-specific tightly coexpressed clusters. The top network from each of those clusters in GeneGo analysis was used to give a representative ontogenic title to each cluster (Table 1). While cluster 14 represented recognized stomach function, without an obvious assignment to which cell expresses those genes (tumor or stroma), some clusters were composed of cell-autonomous ontologies, such as cell cycle (clusters 21 and 28). By contrast, some clusters represented reactive stroma, either of immune cell origin (clusters 26 and 30) or with fibrotic characteristics (cluster 27).

Cluster 27—termed the SRC—was predominantly an extracellular matrix signature as shown in Fig. 2A. Other major processes represented by cluster 27 genes include cell adhesion and development pathways (Fig. 2B), which are driven essentially by WNT-TCF, VEGF-anoxia, and TGF-β signaling.

The stromal-response signature genes arise from stromal components specifically juxtaposed to cancer

The input RNA hybridized onto the Affymetrix arrays described in the previous analysis was derived from whole tumor pieces, which ultimately included both tumor and stromal compartments. SRC could represent reactive stroma, but could also represent a mesenchymal state of the cancer cells, such as EMT (epithelial–mesenchymal transition). For example, the serum response signature of cell lines was originally expected to represent reactive fibroblasts, but was later found to be expressed by the epithelial cells (30, 31). Using species-specific expression profiling, we and others found that some genes in stromal reaction signatures are in fact expressed by epithelial cancer cells (32, 33).

To better determine the specific cellular compartment of the tumor expressing the SRC, we performed LCM of four cases of gastric cancer and five cases of high-grade serous...
Table 1. Top-ranking HOPACH clusters and differentially expressed genes in PMCC_gastric dataset

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Cluster_27</th>
<th>Cluster_21</th>
<th>Cluster_12</th>
<th>Cluster_0</th>
<th>Cluster_14</th>
<th>Cluster_26</th>
<th>Cluster_28</th>
<th>Cluster_15</th>
<th>Cluster_30</th>
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<tbody>
<tr>
<td>MainGeneGo biologic process or network</td>
<td>ECM remodeling (stromal response)</td>
<td>the metaphase checkpoint</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Number of genes in cluster</td>
<td>100</td>
<td>77</td>
<td>73</td>
<td>68</td>
<td>64</td>
<td>34</td>
<td>91</td>
<td>37</td>
<td>71</td>
</tr>
<tr>
<td>Number of differentially expressed genes</td>
<td>83</td>
<td>67</td>
<td>58</td>
<td>54</td>
<td>45</td>
<td>27</td>
<td>40</td>
<td>23</td>
<td>28</td>
</tr>
<tr>
<td>Percentage of differentially expressed genes</td>
<td>83.00</td>
<td>87.01</td>
<td>79.45</td>
<td>79.41</td>
<td>70.31</td>
<td>79.41</td>
<td>43.96</td>
<td>62.16</td>
<td>39.44</td>
</tr>
<tr>
<td>P value</td>
<td>3.75E-61</td>
<td>1.42E-51</td>
<td>2.32E-40</td>
<td>1.35E-37</td>
<td>1.22E-27</td>
<td>4.00E-19</td>
<td>1.00E-14</td>
<td>6.84E-13</td>
<td>2.21E-09</td>
</tr>
<tr>
<td>FDR*</td>
<td>5.25E-60</td>
<td>9.92E-51</td>
<td>1.08E-39</td>
<td>4.72E-37</td>
<td>3.41E-27</td>
<td>9.34E-19</td>
<td>2.00E-14</td>
<td>1.20E-12</td>
<td>3.44E-09</td>
</tr>
</tbody>
</table>

Statistical significance of the clusters in other cancer types


NOTE: Clusters in italics indicate those with >60% total genes differentially expressed between normal and tumor.

*FDR is based on the percentage of differentially expressed genes among the total number of genes in the cluster.
ovarian cancer. Epithelial and stromal components were separated by LCM and Affymetrix expression analysis on RNA derived from the two areas was performed independently. Overall, 370 genes were overexpressed in the epithelial compartment of the gastric samples and 654 in the stromal compartment. The greatest ontological enrichment in the epithelial compartment was by genes identified in the regulation of gene metabolism (bile acid cluster, cluster 14) and the cell cycle (the metaphase checkpoint cluster, cluster 21). The greatest enrichment for stromal expression was exhibited by the cell-cycle regulation of G1–S (cluster 28) and the SRCs (cluster 27; Supplementary Table S5).

A heatmap was generated showing expression of the SRC genes (cluster 27). Preferential clustering was observed in the stromal fraction for both gastric cancer and ovarian cancer (Fig. 2C). We then further analyzed the distribution of the 100 unique stromal-response genes with respect to both the epithelial and stromal compartments of gastric cancer and ovarian cancer. We observed that most (gastric cancer, n = 38; ovarian cancer, n = 54) of the SRC genes are predominantly expressed in the gastric cancer and ovarian cancer stroma and only 2 or 4 were highly expressed in the epithelium of gastric cancer and ovarian cancer, respectively (Fig. 2D and E). Interestingly, a large number of genes (gastric cancer, n = 60 and ovarian cancer, n = 42) are not specifically upregulated in stroma or epithelial compartments (Fig. 2D and E), suggesting that they are equally expressed in both compartments and show a dynamic interaction at the epithelial–stromal interface (see Supplementary Table S6 for the compartmental location for each gene). Genes expressed by the epithelial cells, which are tightly coexpressed with SRC, serve as evidence that variation in expression of SRC is the product of heterotypic cancer–stromal cross-talk (34).

The stromal-response signature is coexpressed in multiple carcinoma types

To determine whether each of the clusters is universal across multiple cancer types, we interrogated a variety of publicly available datasets as outlined in Table 1. A permutation approach (Monte Carlo method) was used to
compute the likelihood that these clusters of coexpressed genes were also overrepresented in other solid cancers. The datasets we used were colorectal carcinoma (23), lung carcinoma (24), breast carcinoma (25), and two independent gastric carcinoma cohorts (21, 22). We found that many tumor datasets that are publicly available had few, if any, nonmalignant tissue profiled as part of the dataset limiting the number of datasets available for the analysis to determine if the cluster was tumor specific.

Across all cohorts, several clusters, including cluster 27 (the SRC), were tightly coexpressed. This general concordance between the cluster ontology and the robustness of each cluster expression in different organ further supports our contention that stromal response is a robust, universal, and consistent aspect of carcinoma gene expression.

To identify the specific stromal cell types expressing genes from the SRC, we performed ISH for the SPARC gene on a TMA comprising gastric cancer and its premalignant counterparts. SPARC was chosen as it is among the top 50 most significantly differentiated genes between tumor and normal and acted as a surrogate marker for the metagene. Representative images showing SPARC ISH on normal and tumor samples obtained from the same patient are shown in Fig. 3A. As expected, staining was predominantly observed in stromal areas and while most stromal cell types expressed these genes to varying extents, fibroblast cells were preferentially stained. SPARC ISH was indicative of the stromal-response signature and was highly expressed in stroma juxtaposed to tumor epithelium. SPARC expression was not high in nonmalignant stroma despite the presence of significant amounts of stroma in our tissue sections and despite considerable inflammation in nonmalignant tissues. This suggests a tumor-specific function for the stromal-response genes.

Figure 2. A, GeneGo schematics illustrating the top-ranking pathway for the HOPACH-generated gene list associated with cluster 27. This pathway is primarily associated with ECM and cell adhesion as has been termed the "stromal-response" cluster. Thermometer icon to the right of the gene name indicates altered expression, with red and blue indicating up- and downregulation, respectively. B, the top 10 GeneGo pathways associated with cluster 27 (based on P value) are enriched for cell adhesion-related processes. C, LCM of four gastric and five ovarian cancer samples where stroma (Str) and epithelium (Epi) compartments were dissected and profiled on Affymetrix U133þ2 arrays independently. Stromal-response genes show higher expression in stroma of gastric and ovarian cancers compared with the epithelial compartments. D, Venn diagram showing the interaction of genes overexpressed in gastric epithelium and stroma with the SRC. Here, both epithelial and stromal components of gastric cancers are intersected with stromal-response genes and many (n = 60) do not fall into epithelial or stromal compartments specifically suggesting they may be shared between them. E, Venn diagram of the stromal-response genes (cluster 27) with ovarian stroma and epithelium-enriched genes. In this instance, 42 genes are equally expressed by both compartments.
The stromal-response signature is correlated with macrophage infiltrates

Although the stromal-response signature was exceptionally specific and consistent in all gastric cancer cases, the overall degree of expression was nevertheless variable across multiple cases. Across the gastric cancer and benign gastric tissues, the expression of SPARC in the microarray and the SPARC ISH signals were highly correlated. We therefore aimed to exploit this variation to determine which stromal cell type most tightly correlates with the overall expression level of the stromal-response signature, which was defined by SPARC ISH as a surrogate of the signature.

Serial sections of the TMA were prepared. One section was stained by ISH for SPARC as described above. The remaining sections were stained with antibodies for specific markers of stromal cell lineages; anti-smooth muscle actin (anti-SMA), anti-CD31, anti-CD45, and anti-CD68 as markers of connective tissue, endothelial, leukocyte, and macrophage cells, respectively. We then correlated the degree of ISH staining for SPARC and abundance of specific cell lineage(s) in the tumor, based on the markers listed above. Figure 3B shows an example of SPARC ISH on a representative tumor sample with IHC stains performed on serial sections for cell markers described. The association of expression of SPARC ISH and cell markers is summarized in Table 2 for all cases, including the nonmalignant ones.

We find that the expression of the SPARC correlated best with CD31- and CD68-positive cells when assessed using the Fisher exact test ($P = 3.2 \times 10^{-6}$ and $P = 3.7 \times 10^{-8}$, respectively). Indeed, when we assess the tumors specifically, then CD68-positive cells demonstrate the most significant association (Table 2, right; $P = 0.007$) with the...
expression of the SPARC ISH. While we do not propose that the stromal response genes themselves are being produced by the macrophages, these results suggest that macrophage (CD68-positive cell) presence in the tumor microenvironment is important for the expression of the SRC signature locally in the setting of invasive cancer. Macrophage activation in vitro leads to elongated fibrous cell morphology of these cells, hindering the identification of the cell type. Alternatively, it is possible that cross-talk between fibroblasts, the main cells expressing the SRC signature and macrophages, is a critical modulator of the overall stromal-response gene signature level of expression.

Macrophage polarization in the cases that exhibit high stromal-response signature

Macrophages, which originate from monocytes, can be activated or polarized into two phenotypically distinct subtypes, each with distinct cytokine secreting profiles and functions. M1 macrophages are said to be classically activated by the Th1 cytokine IFN-γ in the absence or presence of lipopolysaccharide. M1 macrophages are proinflammatory and also promote apoptosis and ECM destruction. M2 macrophages, in contrast, are alternatively activated and are known to promote proliferation, invasion, and ECM construction.

On the basis of the results described in Table 2, we investigated whether M1 or M2 macrophages were more highly enriched in samples with high representation of the SRC genes and analyzed our data with respect to previously published M1 and M2 gene signatures (35).

Table 2. Association between SPARC and IHC markers

<table>
<thead>
<tr>
<th>Normal and tumor</th>
<th></th>
<th>Tumor only</th>
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<tr>
<td></td>
<td>SPARC ISH</td>
<td>P value</td>
<td>SPARC ISH</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1+</td>
<td>2+</td>
</tr>
<tr>
<td>α-SMA</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td></td>
<td>1+</td>
<td>23</td>
<td>8</td>
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<td></td>
<td>2+</td>
<td>11</td>
<td>8</td>
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<td></td>
<td>3+</td>
<td>7</td>
<td>3</td>
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<tr>
<td>α-CD31</td>
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<td>0</td>
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<td>2+</td>
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<td></td>
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<td>α-CD45</td>
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<td></td>
<td>1+</td>
<td>29</td>
<td>9</td>
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<td></td>
<td>1+</td>
<td>13</td>
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<td>3+</td>
<td>2</td>
<td>3</td>
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First, samples were ranked according to their overall stromal-response signature, and classified them as either SRC high or low (see Materials and Methods). The expression values for each M1 signature gene were then determined for all samples in the cohort and the fold-change expression of each gene between SRC high and low was determined. A similar analysis was performed for M2 signature genes. The box plot in Fig. 3C shows that M2 genes are more positively correlated with SRC-high samples (P = 0.032). The absence of M2 genes in the SRC signature can be attributed to the small cellular contribution of macrophages to the overall tumor microenvironment and expected dilution of expression of these genes in the context of the tumor and other stromal components.

In more-advanced stages of tumor development, it is generally well accepted that TAMs have an M2 phenotype based on their cytokine secretion profile with high levels of CD163 expression being a key marker. Using the remaining serial section from the TMA, we then performed IHC for CD163 and correlated its expression with SPARC ISH as before (Fig. 3D). Table 2 (bottom) shows a significant correlation (P = 0.002; Fisher’s exact test) when both normal and tumor samples were considered. There was no significant correlation of SPARC ISH/CD163 staining in tumors.
most likely due to a large number of missing data, which can be attributed to TMA exhaustion. These results suggest that CD163⁺ M2 macrophages may be driving the SRC signature.

**High-level expression of the stromal-response signature predicts poor outcome**

Comprehensive clinical data available for the PMCC and Ooi and colleagues gastric, as well as the AOCS ovarian cohort allowed us to compare the association of each of the cluster expression levels with patient outcome (progression-free survival), using Kaplan–Meier analyses (Supplementary Table S7 and Fig. 4). We analyzed the core group of genes by using a metagene expression signature and determined cases that express high and low levels of the signature based upon the median expression of each metagene. In all datasets, elevated expression of genes in SRC and cluster 28 was correlated with worse outcome (Fig. 4A and B). Consistently, across all cohorts, expression of clusters 21, 0, and 26 was not significantly predictive of outcome (data not shown). High expression of cluster 30 was associated with poor outcome only in ovarian cancer (Fig. 4C; \( P = 0.007 \)). We then used the PMCC dataset to show that these survival differences were not associated with the American Joint Committee on Cancer stage of the patients, which in itself is a known prognostic factor for gastric cancer (Supplementary Table S8).

These observations suggest that the metagene, which may be driven by M2 polarization, has a prognostic effect. Similarly, other cancer-intrinsic features, such as cell cycle, or other stromal responses do not seem to be reflective of overall patient survival.

**Discussion**

Gene signatures derived from gene-expression data have previously been used by us and others to diagnose gastric cancer (36), predict patient prognosis (19, 37, 38), and distinguish cancer stage and grade (39). Indeed, through our collaborators, we have previously published on a stromal signature derived from a Singapore cohort and validated on a group of Australian samples from our laboratory (19). A number of stromal signatures have been described in different cancer contexts (30, 31, 40), which have distinct gene lists. Indeed, when those gene-expression signatures are derived from whole tumor mass, it is...
impossible to exclude the possibility that the genes are expressed by the cancer, and not the stromal cells. We have previously addressed this concern by profiling xenografted human cancer cells grown in a mouse host (33). Bioinformatic analysis allowed a species-specific profiling of both the host and cancer cell within a heterogeneous tumor sample, but, such experiments do not confirm that the observation is physiologically relevant in the human patient body. The study described here used a discovery-based approach to identify clusters of coexpressed genes specifically cosegregated with gastric cancers in a more comprehensive dataset. The most significant cluster in terms of coexpression and number of differentially expressed genes was SRC. We termed this the "stromal-response" cluster as it is rich in genes that seemed to be derived from the stromal elements of the tumor as determined by annotation, ISH, and LCM experiments, and was specifically associated with a juxtatumoral microenvironment and is not found in benign conditions. This is a key advantage of our study, which better distinguishes what genes are expressed from what cell types.

The SRC comprises genes that are predominantly involved in the normal process of wound healing. These complex coordinated processes involve extensive intercellular cross-talk between stromal cells (macrophages, endothelial cells, lymphocytes, and fibroblasts) and the epithelial cells. Normal wound healing proceeds in three steps, initially mounting an innate immune rejection of foreign invaders, often associated with injury and inflammation (41–43). This is followed by proliferation and tissue remodeling. These stages involve angiogenesis (17, 44), ECM deposition, and stem cell recruitment (17, 45, 46). Genes involved in the processes of matricellular remodeling, cell movement, and angiogenesis are highly represented in the SRC, supporting the well-established paradigm that chronic wounds and/or inflammation are predisposing factors for tumorigenesis (42, 47, 48).

The multicellular nature of wound healing impairs the identification of a single critical stromal cell that would coordinate this reaction in cancer. In prostate cancer, it is recognized that CAFs may influence tumor behavior by encouraging growth of the primary tumor (45). In models of breast cancer, it was found that the stromal components derived from mesenchymal stem cells play an important role in divesting metastatic properties to the primary cancer (49). Macrophages are known to play a role in cancer development and progression. In the early stages of tumor initiation, M1 macrophages are believed to be activated in response to inflammation and eliciting Th1/Th17 responses, a pathway that was found to be associated with cluster 26. TAMs are a primary source of EGF in breast cancers (43, 50, 51) and hence putatively growth promoting. Cancer-related inflammation, when innate immune cells are mobilized to assist in tumor formation, has recently been described as a tumor-enabling consequence, which acts in support of the multiple hallmarks of cancer by providing the necessary factors and enzymes to aid invasion, metastasis, angiogenesis, and EMT (52).

Our data showed a strong correlation between SRC expression and the presence of macrophages, more specifically those of the M2 subclass when all samples were considered. This suggests that M2 macrophages may act as a master regulator of the stromal response. There are examples in human cancer where the immune infiltrate in a carcinoma is a beneficial factor and engenders good prognosis. For instance, lymphocytic infiltrates of breast, ovarian, and colorectal cancer (41, 53) are all positive prognostic variables. On the other hand, the complex interaction between CAFs and M2 macrophages and tumor cells has been attributed to an increase in cancer cell motility and ultimately in poor prognosis in prostate cancer (54). Our findings show a ubiquitously expressed signature of genes derived primarily from tumor stroma that seems to have negative prognostic implications for both gastric and ovarian cancer.

It is conceivable that using the stromal-response signature as a marker for M2 activity in patient biopsies provides tangible criteria for the selection of patients to treat with antiinflammatory drugs. M2 macrophages are known to secrete interleukin-10, platelet-derived growth factor, and TGF-β cytokines. Each of those cytokines is a potential target for novel cancer drugs, making those drugs potential tools in the improvement of patient outcome, by eliminating the observed stromal-response signature in suitable patients. Thus, the association between the recognized M2 macrophage polarity and this signature sheds light on the physiologic and clinical significance of M2 macrophages. Our data suggest that M2-polarized macrophages may influence stromal response to the presence of cancer cells in patients and, consequently, may accelerate cancer cell survival and attenuate cancer cell immune surveillance.

Disclosure of Potential Conflicts of Interest
A. Boussioutas has received speakers bureau honoraria from AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

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