Gene Expression Profiling of Peritoneal Metastases from Appendiceal and Colon Cancer Demonstrates Unique Biologic Signatures and Predicts Patient Outcomes

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BACKGROUND: Treatment of peritoneal metastases from appendiceal and colon cancer with cytoreductive surgery and hyperthermic intraperitoneal chemotherapy (HIPEC) shows great promise. Although long-term disease-free survival is achieved in some cases with this procedure, many patients have recurrence. Oncologists have treated such recurrences of appendiceal cancer similarly to colorectal carcinoma, which has been largely ineffective. This study uses gene expression analysis of peritoneal metastases to better understand these neoplasms.

STUDY DESIGN: From a prospectively maintained database and tissue bank, 41 snap frozen samples of peritoneal metastases (26 appendiceal, 15 colorectal) from patients undergoing HIPEC with complete cytoreduction and more than 3 years of follow-up underwent global gene expression analysis. Distinct phenotypes were identified using unsupervised hierarchical clustering based on differential gene expression. Survival curves restratified by genotype were generated.

RESULTS: Three distinct phenotypes were found, 2 consisting of predominantly low grade appendiceal samples (10 of 13 in Cluster 1 and 15 of 20 in Cluster 2) and 1 consisting of predominantly colorectal samples (7 of 8 in Cluster 3). Cluster 1 consisted of patients with good prognosis and Clusters 2 and 3 consisted of patients with poor prognosis ($p = 0.006$). Signatures predicted survival of low- (Cluster 1) vs high-risk (Cluster 2) appendiceal ($p = 0.04$) and low-risk appendiceal (Cluster 1) vs colon primary (Cluster 3) ($p = 0.0002$).

CONCLUSIONS: This study represents the first use of gene expression profiling for appendiceal cancer, and demonstrates genomic signatures quite distinct from colorectal cancer, confirming their unique biology. Consequently, therapy for appendiceal lesions extrapolated from colonic cancer regimens may be unfounded. These phenotypes may predict outcomes guiding patient management. (J Am Coll Surg 2012;214:599–607. © 2012 by the American College of Surgeons)

Peritoneal carcinomatosis (PC) from gastrointestinal malignancies has historically been associated with dismal outcomes and therapeutic nihilism, with patients progressing to death in 5 to 7 months.[1-3] However, over the last 2 decades, an aggressive approach of surgical cytoreduction and hyperthermic intraperitoneal chemotherapy (HIPEC) has emerged as a promising strategy. HIPEC has been found to be associated with long-term survival for patients with isolated peritoneal disease from gastrointestinal malignancies, including that arising from colorectal and appendiceal primaries. The long-term survivorship has never been previously reported with even the most aggressive systemic chemotherapy alone.[4-13] Key prognostic factors for patients undergoing HIPEC include primary tumor site, completeness of resection, presence of ascites, clinical performance status, and the experience of the operative team.[14]

Despite these results, many patients with PC from colorectal and appendiceal malignancies undergoing surgical

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cytoreduction and HIPEC will suffer recurrence and ultimately die from their disease. Most patients may die from locoregional peritoneal recurrence, with a minority succumbing to distant metastatic disease. These patients may benefit from advances in systemic chemotherapeutics and biologic agents for the treatment of metastatic colorectal cancer. Newer agents have resulted in median survival times as high as 24 months, although scarce data exist on their efficacy in patients with PC.[15,16] Little is known about systemic treatment options and efficacy for patients with disseminated appendiceal cancer, and these patients have traditionally simply been given agents known to be active against colorectal cancer.[14]

Gene expression profiling using DNA microarrays is a powerful tool with increasing clinical application, which allows measurement of thousands of messenger RNA (mRNA) transcripts simultaneously. Best studied in patients with breast cancer, these data can be used to create molecular signatures that predict oncologic outcomes and may even predict response to various chemotherapeutics.[15] Similarly, a gene expression signature was recently validated that may predict recurrence in patients with early stage colorectal cancer.[16]

Given the uncertainty of predicting outcomes in patients with disseminated appendiceal cancer, we sought to use the tools of gene expression profiling to better understand these rare malignancies at a molecular level in order to better predict oncologic outcomes. In addition, we compared profiles of peritoneal metastases from colorectal and appendiceal primaries to better understand whether there is biologic rationale for the similar chemotherapeutic strategies traditionally used for these different malignancies.

METHODS

Patient tumor samples

One hundred thirteen samples were obtained for genomic analysis from a prospectively maintained database and tissue bank. Samples of 104 total peritoneal metastases (colon [n = 52] and appendiceal [n = 52]) were collected under a protocol (Protocol BGO1−372) approved by the Institutional Review Board at Wake Forest University Baptist Medical Center. Neuroendocrine sources of metastatic disease were excluded. All of the specimens from Wake Forest underwent a complete cytoreduction (R0 or R1) and had at least 3 years of follow-up before analysis. They were kept in a prospectively maintained tumor/tissue bank until the time of analysis. A total of 9 primary colon (n = 4) and primary appendiceal (n = 5) samples were collected under a protocol approved by the Institutional Review Board at Duke University (Protocol Pro00002435). All patients had tissue obtained at the time of cytoreductive surgery and HIPEC.

Our techniques for HIPEC have been described elsewhere,[8,10,14] but briefly consisted of cytoreductive surgery with a goal of complete extirpation of all gross disease. After cytoreductive surgery and while the patient was still in the operating room, the HIPEC treatment was performed. Two inflow cannulae were inserted with tips placed beneath the hemidiaphragms and 2 outflow cannulae were directed into the pelvis. The abdominal incision was closed temporarily with running skin sutures. A crystalloid prime (3 L of lactated Ringer's solution) was instilled in order to establish a closed perfusion circuit. Mitomycin C 30 mg (total dose) was added to the circuit once inflow temperatures exceeded 38.5°C and another 10 mg (total dose) was added after 60 minutes of perfusion. Inflow and outflow temperatures were monitored continuously. Plateau inflow temperatures were restricted to 42.5°C with the modified cardiothoracic equipment and circuit used in this study. The perfusion was run for a total of 2 hours with a flow rate of 1 L/minute and a target outflow temperature of 40.0°C. The abdomen was gently massaged for the entire perfusion period to improve drug distribution. The HIPEC was followed by washout with several liters of lactated Ringer's solution. The abdomen was reopened for inspection, removal of cannulae, and completion of operation. Patients were monitored for 24 hours in the surgical ICU. After hospital discharge, patients were followed with examination and CT, at 6-month intervals for 5 years, and as clinically indicated thereafter.

Tumor sample and microarray data processing

Tumor samples from Wake Forest University were snap frozen at time of resection. Tumor samples from Duke University were frozen at optimal cutting temperature. Before isolation of RNA, frozen sections of tumor samples were stained with hematoxylin and eosin and pathologically reviewed to ensure that the samples contained at least 10% tumor. Of note, the majority of the samples were >60% mucin. Several of the snap frozen specimens were difficult to prepare in this fashion, which led to them being rejected for further analysis.

For RNA extraction, tumor samples were placed in RNA lysis buffer (Applied Biosystems) and homogenized using FastPrep-24 (MP Biomedicals) apparatus. RNA was extracted using the mirVana miRNA isolation kit (Applied Biosystems). The integrity and quantity of the RNA was assessed with an Agilent 2100 Bioanalyzer using the RNA 6000 nanochip Kit (Agilent Technologies). Samples not meeting Agilent quality control standards (distinct 18S/28S peaks with minimal background signal) were discarded. RNA from 61 tumor samples met initial quality

control along with RNA from 10 randomly chosen matching normal samples (5 appendiceal, 5 colorectal).

A total of 2 μg of total RNA from each sample meeting quality control was biotin labeled with the Ovation Biotin System (Nugen) and hybridized to Affymetrix Human Genome U133A 2.0 arrays (Affymetrix). Samples were subsequently analyzed using a Gene Array Scanner (Affymetrix) following the manufacturer's instructions at the Duke University Institute for Genome Sciences and Policy Microarray Core Facility.

Gene expression from microarray data was generated using RMA and MAS5 (Affymetrix) algorithms.[17] After additional quality control (% P > 45%, scaling factor < 20, background < 1 SD above average and glyceraldehyde-3-phosphate dehydrogenase [GAPDH] 3'/5' < 1 SD above average), 55 tumor samples and 10 normal tissue samples were subsequently used for analysis.

Data analysis

Data were analyzed using the open-source R platform (http://www.r-project.org/) with the Bioconductor bioinformatics package (http://www.bioconductor.org/) and with GenePattern (http://www.broadinstitute.org/cancer/software/genepattern/). R was used to generate RMA data and to perform expression data filtering using the Coefficient of Variation method (c_v = standard deviation/mean) in addition to unsupervised hierarchical clustering using the Spearman correlation metric. Gene Pattern was used to perform supervised Class Neighbors analysis. Statistical significance was defined as a p value of <0.05. Gene set enrichment analysis was performed to identify differentially regulated pathways between 2 phenotypes (http://www.broad.mit/gsea/).[18] Gene sets were first preprocessed to exclude gene sets with less than 10 and more than 500 genes. Ten thousand iterations were then performed per analysis with a signal to noise metric used to rank genes based on their differential expression across the 2 classes. For discovery, gene sets with a normalized p value < 0.05 were identified.

Kaplan-Meier mortality curves and their significance level were generated to evaluate the prognostic role of the individual clusters of patients with peritoneal metastasis using the graph pad software. The log-rank test was used to assess the differences between the survival curves and to calculate the nominal p values between groups. We defined a p value of < 0.05 as statistically significant for the purposes of this manuscript.

RESULTS

Patient tumor samples

From a prospectively maintained database and tissue bank, a total of 113 peritoneal colon (n = 56) and peritoneal appendiceal (n = 57) samples were collected at Wake Forest University and Duke University. After initial histologic review, 61 samples were deemed adequate for RNA isolation. Of the 61 samples from which RNA was isolated, 55 passed quality assurance/quality control for generation of gene expression data. In order to check for normal contamination in the tumor samples, both unsupervised hierarchical clustering and supervised Class Neighbors analysis was performed on the entire data set of 55 tumor samples and 10 normal tissue samples. Tumor samples that clustered with the normal tissue samples and had similar Class Neighbors expression profiles as the normal tissue samples were considered to be normal contaminated and removed from further analysis. Using this method, 50 tumor samples were found not to have significant normal contamination.

Clinical outcomes data were queried for the 50 remaining tumor samples, and of these, 41 samples had analytic clinical outcomes data. The other samples were not of appendiceal/colorectal origin, were lost to follow-up, or the patient did not receive HIPEC. Within this final data set of 41 samples there were 24 men and 17 women. Twenty-six cancers were primary appendiceal and 15 were primary colorectal. All but 2 of the appendiceal cancers were of low histologic grade. Patient ages ranged from 38 to 76 years, with a mean of 53 years.

Unsupervised analysis

Expression data from the 41 samples was filtered using R with the Coefficient of Variation method (c_v = standard deviation/mean) with a cutoff of $c_v = 0.8$. This filtered the number of probes down from 22,215 to 4,443. Using R, unsupervised hierarchical clustering was then performed on the filtered samples using the Spearman correlation metric. This clustering produced 3 main clusters (Fig. 1); 2 clusters consisted of predominantly primary appendiceal samples (Clusters 1 and 2), and the third consisted of predominantly primary colorectal samples (Cluster 3). Furthermore, the distribution of low grade appendiceal tumors was similar between Clusters 1 and 2. Specifically, Cluster 1 had 10 of 13 appendiceal cancers, Cluster 2 had 15 of 20, and Cluster 3 had 1of 8. Mean follow-up for the survivors is 39 months, 33 months, and 18 months for Clusters 1, 2, and 3, respectively.

Survival analysis

Using the 3 clusters generated from the filtered unsupervised analysis as phenotypes, survival data were plotted to each cluster. Kaplan-Meier survival curves were then generated which revealed 3 distinct survival curves (Fig. 2A). The survival curve with the worst prognosis consisted of predominantly colorectal samples with no survival at 5 years. The survival curve with the best prognosis consisted of predomi-

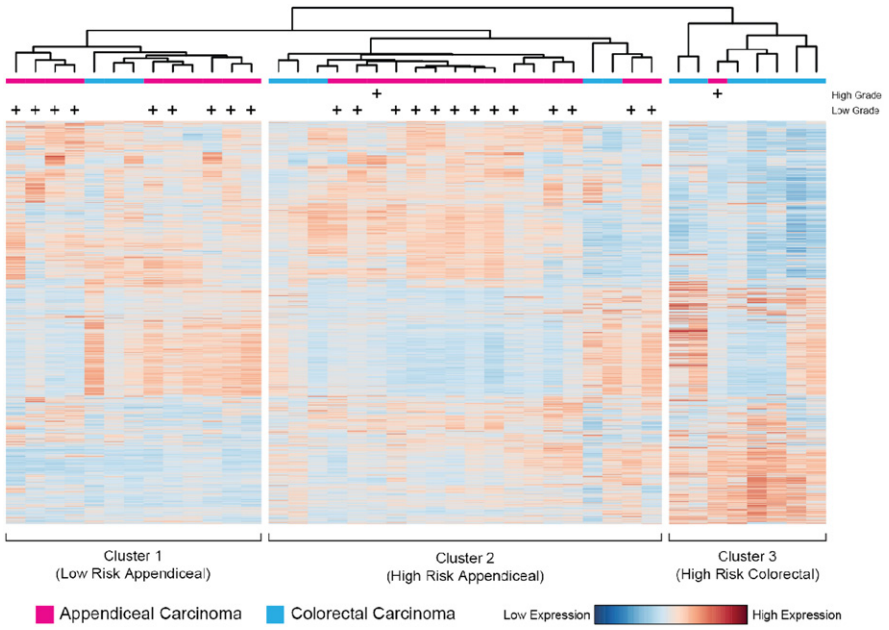

Figure 1. Global gene expression comparison of peritoneal samples. Unsupervised hierarchical clustering on 41 samples of peritoneal metastasis (26 appendiceal, 15 colorectal) revealed 3 distinct clusters.

nantly appendiceal samples with approximately 70% survival at the 116-month mark (the latest data point). The remaining survival curve consisting of predominantly appendiceal samples had about 25% survival at the 116-month mark. These curves were given the following labels: low-risk appendiceal (Cluster 1), high-risk appendiceal (Cluster 2), and high-risk colorectal (Cluster 3), respectively. Comparison of the high-risk colorectal curve with the low-risk appendiceal curve was shown to be statistically significant ($p = 0.0060$) (Fig. 2B). Comparison of the high-risk appendiceal curve with the low-risk appendiceal curve was not statistically significant ($p = 0.143$), but a trend toward survival separation can be seen, so the lack of statistical significance may be due to modest sample size ($n = 26$) (Fig. 2C). However, if only the appendiceal samples were analyzed between the high-risk appendiceal cluster and the low-risk appendiceal cluster, there was a statistical significance between the two groups ($p = 0.0459$) (Fig. 2D).

Supervised analysis

To characterize the biologic differences between the low-risk appendiceal (Cluster 1), high-risk appendiceal (Cluster 2), and high-risk colorectal (Cluster 3), we first used a supervised analysis using 1-versus-all *t*-tests and permutation testing to identify individual genes with expression significantly associated with the site of the primary ($p < 0.05$) (Fig. 3). Genes associated with worse prognosis in the appendiceal tumors included mucin-related genes such as mucin 5, mucin 2, and trefoil factors 1 and 2.

We next used gene set enrichment analysis between the low-risk appendiceal (Cluster 1) and high-risk appendiceal (Cluster 2)[18] to identify biologic processes and pathways associated with the poor prognosis (Fig. 4). This revealed multiple pathways known to be involved in advanced disease (immune pathways, oncogenic pathways such as *src* and *myc*, transforming growth factor [TGF]-β, and resistance to chemotherapy).

DISCUSSION

The appendix is, of course, part of the colon. Therefore, it seems sensible to use systemic chemotherapy regimens extrapolated from cancer of the colon for cancer of the appendix.[19] Currently, there is no standard approach for systemic therapy for appendiceal cancer. Given the rarity of appendiceal neoplasms, the lack of prospective randomized trials should not be surprising, nor should the limited data available on systemic therapy to this approach (typically 5-fluorouracil-based).[19-22] This study represents the first use of gene expression profiling for appendiceal cancer and demonstrates genomic signatures quite distinct from colorectal cancer, confirming their unique biology. Consequently, therapy for appendiceal lesions extrapolated from colonic cancer may be unfounded. These phenotypes may predict outcomes guiding patient management.

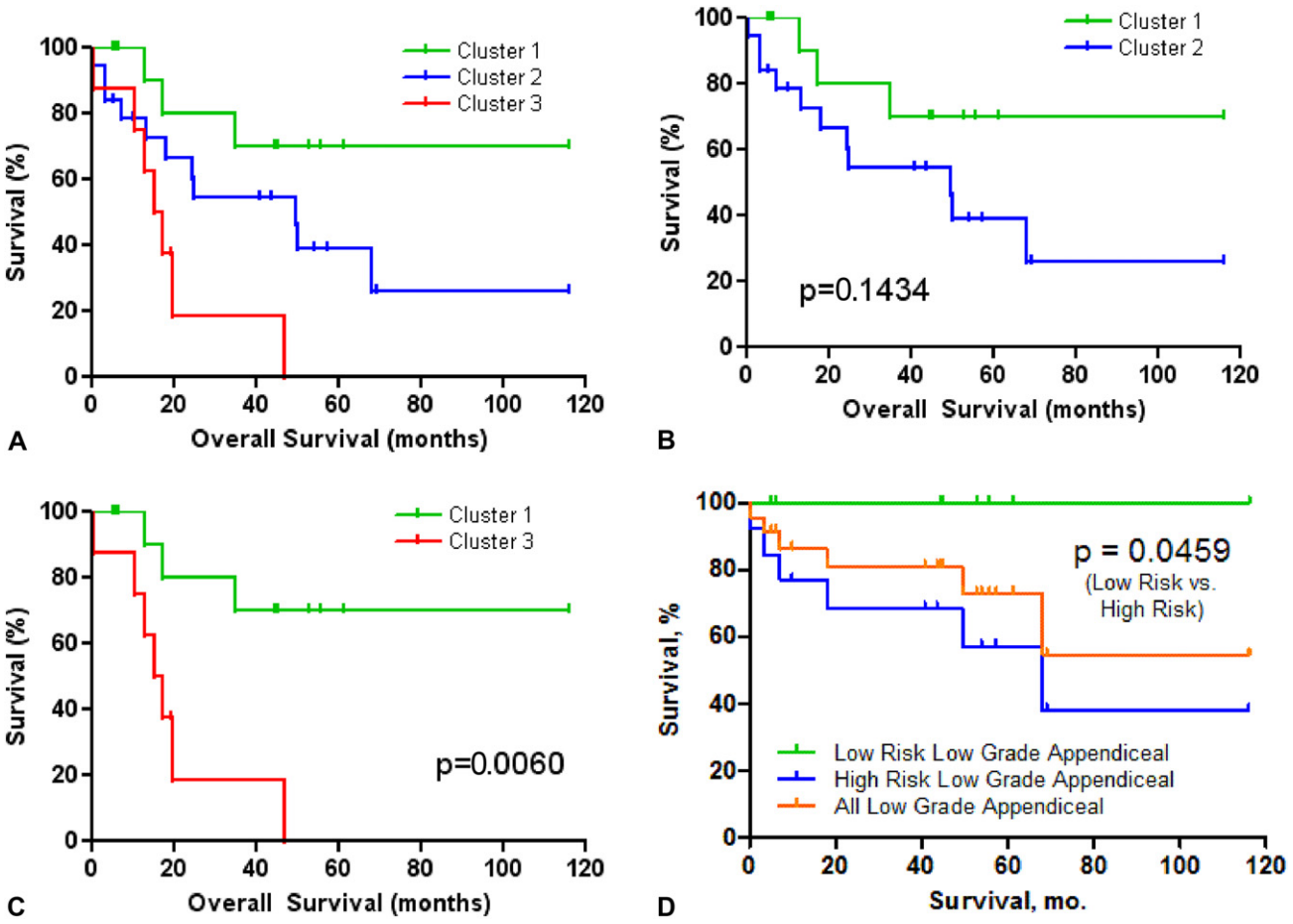

Figure 2. Kaplan-Meier survival curves of peritoneal samples. (A) Kaplan-Meier survival curves of the low-risk appendiceal (Cluster 1), high-risk appendiceal (Cluster 2), and high-risk colorectal (Cluster 3) groups revealed 3 distinct survival curves. (B) Comparison of the high-risk appendiceal curve (Cluster 2) and the low-risk appendiceal curve (Cluster 1) was not statistically significant ($p = 0.1434$); however, a trend toward survival separation can be seen, and the lack of statistical significance may be due to modest sample size ($n = 26$). (C) Comparison of the high-risk colorectal curve (Cluster 3) and the low-risk appendiceal curve (Cluster 1) was shown to be statistically significant ($p = 0.0060$). (D) Comparison of only the appendiceal samples between the high-risk appendiceal curve and low-risk appendiceal curve revealed a statistical significance between the 2 groups ($p = 0.0459$).

Histologic examination of appendiceal tumors has long been known to have great prognostic value. Grading of the lesions clearly stratifies prognosis; however, even with low-grade lesions there are a minority of patients who fail quickly.[20,22] The gene expression profiles clearly have prognostic value and were found to be prognostic without stratification by grade as 24 of the 26 appendiceal cases were low grade. So, we have identified, via the first genetic analysis of this disease that we are aware of, a prognostic signature for appendiceal cancer. This breaks low-grade appendiceal disease (by histology) into 2 separate groups with a 5-year survival difference of nearly 50% (Fig. 2D). In addition to pure prognostication, this has potential value in selecting patients most likely to benefit from emerging adjuvant therapies. Clearly, not all low grade appendiceal disease has a good prognosis.

Furthermore, clearly defining the genetic features that segregate the low- from the high-risk appendiceal subset is important because this could lead to development of both a clinically relevant prognostic and predictive marker. This can potentially change our treatment paradigm in the treatment of peritoneal metastasis by deciding who should get operation vs chemotherapy based on the biology of the tumor. In our initial analysis to look for the top differential genes between the high-risk (Cluster 2) and low-risk appendiceal (Cluster 1) groups, trefoil factors 1 and 2 and mucin-related genes were consistently observed in the top 10. Trefoil factors 1 and 2 are small, compact proteins coexpressed with mucins in the gastrointestinal tract.[23,24] The trefoil factors have been found in a variety of cancers and appear to induce tumor genesis in gastrointestinal cancers.[24] Furthermore, MUC-2 and MUC-5AC are clearly related to prognosis in disseminated appendiceal cancer

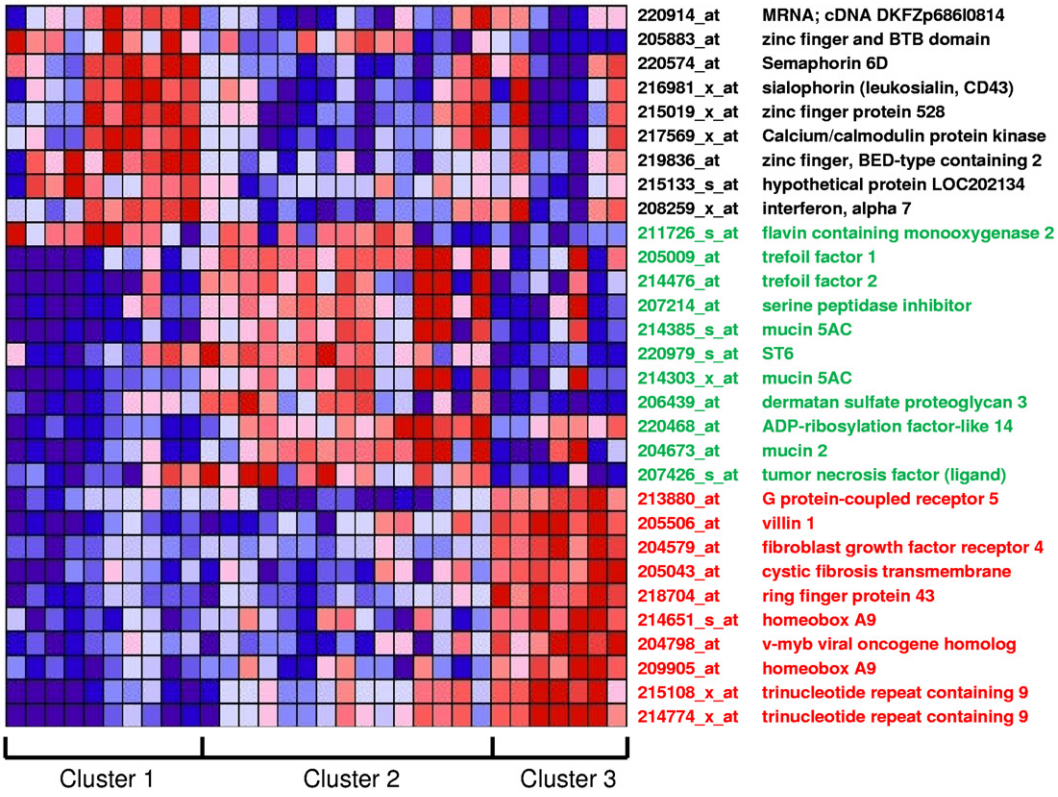

Figure 3. Supervised analysis of low-risk appendiceal (Cluster 1) and high-risk appendiceal (Cluster 2) and high-risk colorectal (Cluster 3) based on gene expression identifies the top differentially regulated genes between the 3 clusters. Red, high gene expression; blue, low gene expression.

and have been previously related to outcomes with peritoneal surface disease, and its overexpression was certainly expected because these 2 mucins confer the physicochemical property of being gel-forming, a property exhibited by pseudomyxoma peritonei grossly.[25,26] The presence of these genes suggests the importance of mucin-related pathogenesis in the prognosis of these cancers.

Although the first step is to identify groups of patients with poor prognosis, the next step is to determine potential therapeutic options for them. It is reassuring that the gene set enrichment analyses (which are derived experimentally and computationally) yielded redundant results in terms of pathways identified. Using gene set enrichment analysis, we identified the src, TGF-β, and immune-related pathways that are differentially regulated in the high-risk appendiceal group (Cluster 2). Src inhibitors such as dasatinib and vaccine-related therapy are already in clinical trials for colorectal cancer and our findings suggest the potential of using similar drugs for the treatment of appendiceal cancers.

It has long been theorized that metastases at a single site are homogeneous and similar in behavior. Gene expression profiling has the potential to clarify this issue for peritoneal metastasis specifically as well as for other sites of metastasis generally. Further study evaluating the expression patterns of separate metastatic deposits would clearly be of value and may be helpful in guiding therapy. Peritoneal surface disease is an excellent model to evaluate this approach in light of the number and distribution of metastases commonly encountered.

We are cognizant of the weaknesses of this analysis. First, nearly half of the tissue specimens submitted for analysis were not cellular enough to be analytic. Although this clearly could affect the result, it must be kept in mind that low grade appendiceal cancer is predominantly mucin, making any cellular analysis challenging. Second, the study is based on a small number of patients. This is clearly so, but appendiceal cancer is a rare disease with a long natural history. We are unaware of any other large snap frozen tissue/dataset for these patients, which would demand either the analysis be performed on formalin-fixed, paraffin-embedded tissue or that fresh tissue start being collected for analysis, years from now. Further, we would like to confirm our findings with an additional set of tissues for validation. Unfortunately, we are unaware of a similar set of snap frozen tissues with follow-up of similar duration. Finally, the

Pathway	p Value (<0.05)
TNFALPHA_30MIN_UP	<0.00001
BRUNO_IL3_DN	0.00218
RESISTANCE_XENOGRAFTS_UP	0.0148
FERNANDEZ_MYC_TARGETS	0.0217
MYC_ONCOGENIC_SIGNATURE	0.0279
EMT_DN	0.0314
JECHLINGER_EMT_DN	0.0340
PGC	0.0386
PASSERINI_ADHESION	0.0388
LEE_E2F1_UP	0.0391
SRC_ONCOGENIC_SIGNATURE	0.0404
CIS_XPC_UP	0.0438
TGFBETA_ALL_UP	0.0442
LEE_MYC_TGFA_UP	0.0454
KENNY_WNT_DN	0.0462

Figure 4. Gene set enrichment analysis of low-risk appendiceal (Cluster 1) and high-risk appendiceal (Cluster 2) identifies pathways associated with poor prognosis.

subset of patients with analytic tissue from a colonic primary had a poorer survival than we would have predicted from our previous experience. This could also have had an impact on the survival analyses, although we would predict a small one.[8,12,27]

CONCLUSIONS

Despite the favorable outcomes found with cytoreductive surgery and HIPEC for appendiceal cancer, the optimal treatment for peritoneal dissemination from cancer of the appendix continues to be debated.[3-7,19-22,27,28] The utility of systemic chemotherapy is not well defined, but is clearly limited at present. Whether the HIPEC improves outcomes compared with cytoreductive surgery alone cannot be discerned from this analysis. However, the utility of cytoreductive surgery seems clear. Several clinical prognostic features are well defined and are valuable, but are limited. Identification of genetic signatures associated with better outcomes has the clear potential to help define better candidates for this procedure (and others). Given the significant morbidity attendant to HIPEC procedures, we believe that additional evaluation of gene expression profiling must be continued and expanded.

Author Contributions

Study conception and design: Levine, Hsu
Acquisition of data, Levine, Stewart, Shen, Blazer, Guy, Kim
Analysis and interpretation of data: Hsu, Levine, Guy, Kim
Drafting of manuscript: Levine, Hsu, Blazer
Critical revision: Levine, Hsu

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Discussion

DR DOUGLAS TYLER (Durham, NC): Hyperthermic intraperitoneal chemotherapy (HIPEC) and cytoreduction surgery have been the focus of a lot of controversy as a therapeutic intervention for carinomatosis. As evidenced by a recent New York Times article and a national trial that was recently closed due to failure to accrue patients, there is clearly a lack of consensus regarding the role of this intervention within the context of our armamentarium of therapies for patients with this form of metastatic disease, especially in the context of colorectal cancer.

Frequently, the debate segregates surgeons on one side from medical oncologists on the other. As our therapies of gastrointestinal malignancies are increasingly driven by genomic and genetic factors, it's nice to see surgeons taking a lead to bring some rigorous correlative science to this unique type of regional therapy, and ultimately developing some objective data on which consensus therapeutic decisions can be developed. But therein lie some of my concerns and questions regarding the data presented in this manuscript.

First, I wonder about the utility or value of a predictive genomic test that includes only 41 samples from an initial pool of 113 that were obtained. What strategies can the authors describe that can be used to improve the yield of biopsies from which correlative information can be processed? And do we know anything about the outcomes of the patients in the group that did not have tissue that could impair the analysis?

Second, what is the genomic variability within the abdomen of tumor samples? This is of significant importance, as obviously there are many areas that can be biopsied in these individuals. Defining not only the tumor heterogenicity within a given patient but also the reproducibility or variability of gene signatures from multiple biopsies obtained in a single area of a tumor in a given patient can be extremely important.

Third, while the initial data set identified some genes associated one way or another with survival, have you attempted to validate these gene signatures and determine whether they hold up using a second validation set not used in the primary analysis? The importance of this exercise is that anytime you sample 40,000 genes, you will, by chance, find significant associations. And to try to validate or determine which ones are really important will be key, with a secondary set that's not used in the first.

Finally, associating gene signatures with pre-HIPEC cytoreduction and survival can be difficult, especially in colon cancer, where many of these patients will have received other forms of treatment, either before or after their HIPEC cytoreduction. And that could clearly confound the analysis. Has there been any attempt to associate genomic profiles with a short-term outcome, like response to HIPEC and cytoreduction?

This obviously would push the group to more clearly define how to grade response and outcomes to this procedure in the relative short-term and think about how to use the test to decide who should, and potentially more importantly, who shouldn't get this form of treatment.

Overall, I enjoyed the paper and like where this group is going. And I would encourage them to continue expanding their study so that we can hopefully learn more about how to optimally manage this group of patients in a multidisciplinary manner.

DR CHARLES SCOGGINS (Louisville, KY): I have a few questions for the authors.

1. You noted in the manuscript that gene expression profiles may be linked to response to specific chemotherapy agents. Do you have any data from your study that look at the association between response to intraperitoneal chemotherapy and specific gene expression profiles?
2. Because many, if not most, of these patients will also get systemic chemotherapy, what about a link between gene profile and response to systemic therapy?
3. Finally, you noted a better outcome for low-grade appendiceal