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

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Abstract

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 for some cases with this procedure, many patients recur. Oncologists have treated such recurrences of appendiceal cancer similarly to colorectal carcinoma, which has been largely ineffective. This study utilizes 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 >3 years of follow up underwent global gene expression analysis. Distinct phenotypes were identified using unsupervised hierarchical clustering based upon differential gene expression. Survival curves restratified by genotype were generated.

Results—Three distinct phenotypes were found, two consisting of predominantly low grade appendiceal samples (10/13 in Cluster 1 and 15/20 in Cluster 2) and one consisting of predominantly colorectal samples (7/8 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 ($p=.04$) and low risk appendiceal (Cluster 1) vs. colon primary (Cluster 3) ($p=.0002$).

Conclusions—This study represents the first use of gene expression profiling for appendiceal cancer, and demonstrates genomic signatures quite distinct from colorectal cancer, confirming

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their unique biology. Consequently, therapy for appendiceal lesions extrapolated from colonic cancer regimens may be unfounded. These phenotypes may predict outcomes guiding patient management. HIPEC, hyperthermic intraperitoneal chemotherapy PC, peritoneal carcinomatosis OTC, optimal cutting temperature GSEA, gene set enrichment analysis

Introduction

Peritoneal carcinomatosis (PC) from gastrointestinal malignancies has historically been associated with dismal outcomes and therapeutic nihilism, with patients progressing to death in 5–7 months (1–3). However, over the last two 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 cytoreduction and HIPEC will recur 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, though 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 utilizing DNA microarrays is a powerful tool with increasing clinical application that 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 utilized for these different malignancies.

Materials and Methods

Patient Tumor Samples

A total of 113 samples were obtained for genomic analysis from a prospectively maintained database and tissue bank. 104 total peritoneal metastases; colon (n = 52) and appendiceal (n = 52) samples 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 prior to 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. Following cytoreductive surgery and while still in the operating room, the HIPEC treatment was performed. Two inflow cannulae were inserted with tips placed beneath the hemidiaphragms and two outflow cannulae were directed into the pelvis. The abdominal incision was closed temporarily with running skin sutures. A crystalloid prime (3 liters 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 two hours with a flow rate of 1 liter/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 surgery. Patients were monitored for 24 hours in the surgical intensive unit. Following hospital discharge, patients were followed examination and CT, at 6-month intervals for five 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 in OTC (Optimal Cutting Temperature). Prior to 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 lead 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, Solon, OH) 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, Santa Clara, CA). Samples not meeting agilent quality control standards (distinct 18/28S 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, San Carlos, CA) and hybridized to Affymetrix Human Genome U133A 2.0 arrays (Affymetrix, Santa Clara, CA). 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 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 ($c_v = \text{standard deviation} / \text{mean}$) 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 < .05. Gene set enrichment analysis (GSEA) was performed to identify differentially regulated pathways between two phenotypes (<http://www.broad.mit.edu/gsea/>) (18). Gene sets were first preprocessed to exclude gene sets with <10 and >500 genes. Ten thousand iterations were then performed per analysis with a signal to noise metric used to rank genes based upon their differential expression across the two 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 <.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 histological review, 61 samples were deemed adequate for RNA isolation. Of the 61 samples from which RNA was isolated, 55 passed quality assurance/quality control (QA/QC) 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 both 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 outcome data was queried for the 50 remaining tumor samples, and of these, 41 samples had analytic clinical outcome data. The other samples were either not of appendiceal/colorectal origin, were lost to follow up, or the patient did not receive intraperitoneal hyperthermic chemotherapy (HIPEC). Within this final data set of 41 samples there were 24 males, 17 females, 26 were primary appendiceal and 15 were primary colorectal. All but 2 of the appendiceal cancers were of low histologic grade. The ages ranged from 38–76 years with a mean of 53.

Unsupervised Analysis

Expression data from the 41 samples was filtered using R with the Coefficient of Variation method ($c_v = \text{standard deviation} / \text{mean}$) 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 three main clusters (Figure 1); two 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 1 of 8 in Cluster 3. The 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 three clusters generated from the filtered unsupervised analysis as phenotypes, survival data was plotted to each cluster. Kaplan-Meier survival curves were then generated and three distinct survival curves (Figure 2A). The survival curve with worst prognosis consisted of predominantly colorectal samples with no survival at the 5 years. The survival curve with the best prognosis consisted of predominantly appendiceal samples with ~70% survival at the 116 month mark (the latest data point). The remaining survival curve consisting of predominantly appendiceal samples had ~25% survival at the 116 month mark. These curves were given 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 to the Low-Risk Appendiceal curve was shown to be statistically significant ($p = 0.0060$) (Figure 2B). Comparison of the High-Risk Appendiceal curve to Low-Risk Appendiceal curve was not statistically significant ($p = 0.143$), however a trend towards survival separation can be seen, thus the lack of statistical significance may be due to modest sample size ($n = 26$) (Figure 2C). However, if only the appendiceal samples were analyzed between High Risk Appendiceal cluster and Low Risk Appendiceal cluster, there was a statistical significance between the two groups ($p = 0.0459$) (Figure 2D).

Supervised Analysis

To characterize the biological 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 one-versus-all t-tests and permutation testing to identify individual genes with expression significantly associated with site of primary ($p < 0.05$) (Figure 3). Gene 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 (GSEA) between the Low-Risk Appendiceal (Cluster 1) and High-Risk Appendiceal (Cluster 2) (18) to identify biological processes and pathways associated with the poor prognosis (Figure 4). This revealed multiple pathways known to be involved in advanced disease (immune pathways, oncogenic pathways such as *src* and *myc*, TGF- β , and resistance to chemotherapy).

Discussion

The appendix is, of course, part of the colon. Therefore, it seems sensible to utilize 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, and 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.

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. Thus, 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% (Figure 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 as this could lead the 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 surgery versus 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 ten. 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 and have been previously related to outcomes with peritoneal surface disease, and its overexpression was certainly expected as these two 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 vaccines 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 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 for 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. While 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 upon 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 upon 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 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).

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 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, are valuable, but are limited. The identification of genetic signatures associated with better outcomes has the clear potential to help define better candidates for this (and other) procedure. Given the significant morbidity attendant to HIPEC procedures we believe that additional evaluation of gene expression profiling must be continued and expanded.

Glossary

HIPEC	hyperthermic intraperitoneal chemotherapy
PC	peritoneal carcinomatosis
OTC	optimal cutting temperature
GSEA	gene set enrichment analysis

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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.

Figure 2. Kaplan-Meier Survival Curves of Peritoneal Samples

A. Kaplan-Meier survival curves of the Low-Risk Appendiceal (Cluster 1) and High-Risk Appendiceal (Cluster 2) and High-Risk Colorectal (Cluster 3) revealed three distinct survival curves

B. Comparison of the High-Risk Colorectal curve to the Low-Risk Appendiceal curve was shown to be statistically significant ($p = 0.0060$)

C. Comparison of the High-Risk Appendiceal curve to Low-Risk Appendiceal curve was not statistically significant ($p = 0.1434$), however a trend towards survival separation can be seen, and the lack of statistical significance may be due to modest sample size ($n = 26$)

D. Comparison of only the appendiceal samples between High Risk Appendiceal curve and Low Risk Appendiceal curve revealed a statistical significance between the two groups ($p = 0.0459$).

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 three clusters. Red- high gene expression; Blue- low gene expression.

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.