A Hallmark-Based Six-Gene Expression Signature to Assess Colorectal Cancer and Its Recurrence Risk

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Purpose: As part of the effort to establish a general profile for solid tumors, the aim of this study was to develop a real-time polymerase chain reaction (RT-PCR)-based assay to assess colorectal cancer (CRC) and its recurrence risk utilizing the limited amounts of tissues available from biopsies through colonoscopy.

Materials and Methods: Six candidate genes, reflecting the hallmarks of cancer cells, were identified by analyzing the gene expression profiles of primary invasive tumors in the public database. The expression of these genes in CRC and noncancerous colon tissues was quantified by RT-quantitative PCR. Classifiers were then generated to distinguish the tumors from the normal colon tissues, and to assess the risk of CRC recurrence based on the disease-free survival time, overall survival time, and metastatic status of the patients.

Results: The expression profile of a five-gene panel was utilized to build a model that is capable of distinguishing CRC cancer tissues from noncancerous colorectal tissues (p < 0.0001). A classifier based on the expression signature of four genes, three of which were included in the five-gene panel, was then developed for assessing the tumor recurrence risk. This classifier could correctly identify those with a poor likelihood of survival (high risk of recurrence) >80% of time. There was a significant difference in disease-free survival time between patients in the low recurrence group and those in the high-risk group.

Conclusion: The expression signatures of the six genes that reflect the genetic hallmarks of cancer cells could serve as a biomarker for identifying CRC and assessing the risk of recurrence with high sensitivity and specificity.

Keywords: colorectal cancer, biomarker, gene signature, metastasis

Introduction

Colorectal cancer (CRC) is one of the most common malignancies with >1.4 million new cases and ~700,000 deaths each year worldwide (Siegel et al., 2017). Noteworthy, CRC survival is highly dependent on the tumor stages at the time of diagnosis. While the 5-year survival rate for stage I patients is >90%, ~50% of these at advanced stages died within 5 years, and most of the fatal outcomes resulted from tumor recurrence and metastasis (Chen et al., 2016). Therefore, early recognition and novel effective treatment of relapsed tumors are critical for improving the prognosis of patients with CRC.

It has been documented that radical surgery alone is an effective treatment for the majority of patients (~80%) presenting with stage II colon cancer, whereas ~20% of patients will experience recurrence of the disease within 5 years (Brenner et al., 2014). Currently, clinicopathologic characters such as lymph nodes metastasis identified at surgery, tissue infiltration level and differentiation of tumor, and presence of lymphovascular invasion, bowel obstruction or perforation have been used as poor prognostic factors. However, it is still controversial whether patients can benefit from adjuvant chemotherapy with or without these poor prognostic features (O’Connor et al., 2011; Ejaz et al., 2017). A significant number of stage II patients who have low recurring risk are often overtreated and underwent unnecessary suffering. Therefore, identification of the patients who have high risk of relapse and likely response to chemotherapy is urgently needed for precision treatment of patients with CRC.

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The applications of microarray, RNA-seq, and whole-genome sequencing have led to the identification of large numbers of DNA mutations, dysregulated gene expression, and other genomic alterations. The term predictive genomics was coined to use these genomic data for predicting tumor clinical phenotypes and prognosis (Wang et al., 2015). It has been revealed that there are hundreds of mutations in each CRC cell, which lead to alteration of RNA and protein functions as well as their timing and level of expression. Among them, the dysfunctions of the Wnt pathway, DNA repair, growth factor receptors, and their signal transduction play major roles in the pathogenic process (Cancer Genome Atlas Network, 2012). As a result, cancer cells usually possess characteristic hallmarks that include sustaining proliferative signaling, evading growth suppressors, resisting cell death, inducing angiogenesis, activating invasion and metastasis, reprogramming energy metabolism, and escaping immune destruction (Hanahan and Weinberg, 2011).

Enlightened by the pioneering works from Wang’s laboratory, which indicated that the genes of signature and their interacting partners are effective and robust prognostic markers for cancers (Li et al., 2010; Zaman et al., 2013), we asked whether it is possible to establish a generalized profiling for solid tumors. In this study, we selected genes that are related to these hallmarks and dysregulated in breast cancer by data mining. Through quantifying their expression with real-time quantitative polymerase chain reaction (RT-qPCR) in CRC, a six-gene panel was generated. Their expression signatures could be used to evaluate the presence of CRC tissue and its metastasis and recurrence potentials.

Materials and Methods

Gene selection

The whole human genome gene expression profiles of 249 primary invasive breast tumors with survival time after surgery were extracted from the publicly available GEO database (GSE4922) (Wilhite and Barrett, 2012). Among 288 patients in the set, 149 patients with disease-free survival time (DFS) <2 years or >5 years were selected for developing cancer recurrence risk candidate gene set. We randomly selected whole-genome gene expression signature of 99 tumors as the training set, and reserved the data from the rest 50 tumors as the validation set.

Patients with survival time >5 years after the surgery were assigned to the high-risk group, the rest were assigned to the high-risk group. Significance analysis of microarrays was used to determine the significance of changes between expressions of high and low breast cancer recurrence risk groups (Witten and Tibshirani, 2008). Twenty-five genes with their biological functions mapped to the six hallmarks of cancer-related biology based on the gene ontology were found to have ~1.25-fold (false discovery rate [FDR] ≤5%) difference in the mean gene expression levels between the high-risk and low-risk groups. This gene set was further enriched and evaluated by building a statistical model for prediction recurrence risk in breast cancer patients.

Classification models were developed with seven different algorithms implemented in BRB ArrayTools, which includes Compound covariate, Linear discriminant, 1 Nearest Neighbor, 3 Nearest Neighbors, Nearest Centroid, Support Vector Machine, and Bayesian Compound Covariate. Prediction error of each model was estimated using leave-one-out cross-validation (Ramdaswamy et al., 2001; Radmacher et al., 2002; Tibshirani et al., 2002; Yang and Naiman, 2014). The 1000 permutation tests were performed for each model to determine if the cross-validated misclassification rate is lower than would be expected by chance (FDR). A summary model considering both robustness and the similarity in its algorithm with voting mechanisms was used to generate a single recurrence risk score based on prediction outcomes of the seven models. Six genes, ERBB2 (Shimada et al., 2017), BCL2 (Hector and Prehn, 2009), FGF18 (Sonvilla et al., 2008), INHBA (Okano et al., 2013), MKI67 (Li et al., 2016), and PGR (Liu, 2016), that involved in biological functions for sustaining cell proliferation, resisting cell death, inducing angiogenesis, activating invasion and metastasis were found to be able to separate breast tumors into two groups related to the survival time of the patients.

Study population and tissue samples

The human CRC and paired paracancer tissues were collected from 65 patients enrolled into the Department of Colorectal Surgery, Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine from January 2008 to December 2012. The investigation was approved by the ethics committee of Xinhua hospital, and informed consents were obtained from all the patients.

RNA preparation and quantitative RT-PCR

Total RNA was extracted from CRC and noncancerous tissues using RNAiso Plus (TaKaRa, Dalian), and the reverse transcription was carried out using the PrimeScript™ TM RT Master Mix Kit (TaKaRa). Quantitative RT-PCR (qRT-PCR) was performed using SYBR® Premix (TaKaRa) on a Roche LightCycler 480. The specific primers used are detailed in Supplementary Table S1. The level of β-actin mRNA served as an internal control. The thermal profile was optimized as follows: 95°C for 2 min, 40 cycles of PCR cycling steps involving 95°C for 15 s and 60°C for 30 s.

Statistical analysis

All data were analyzed with SPSS 17.0 software package. For Student’s t-test, p < 0.05 was considered as significant.

Results

Expression of the putative biomarkers in CRCs

Based on the analysis of the whole human genome gene expression profiles of 149 primary invasive breast tumors with survival time, 25 genes, which affect cancer cell proliferation, invasion, metastasis, metabolism, escaping immune destruction, and angiogenesis, were found to be expressed differentially ~1.25-fold (FDR <5%) between the high-risk and low-risk groups. We further evaluated whether these genes can be utilized to predict recurrence risk in the database by building statistical models. Interestingly, six genes, ERBB2, BCL2, FGF18, INHBA, MKI67, and PGR (Table 1), contributed most weight in the model that separates low-risk and high-risk breast cancers. They were then selected as the candidate gene set for developing a qPCR-based diagnostic/prognostic assay for CRC.
The expression of the six genes was assessed in 10 paired CRC and paracancer tissues using the specific primers (Supplementary Table S1). Similar to their expression in breast cancers, these genes were detected in CRCs and often upregulated compared with those in the paracancer tissues (Fig. 1). We then used the RT-qPCR to examine the expression of BCL-2, ERBB2, FGF18, INHBA, MKI67, and PGR in additional 55 CRC tissues, among which 34 had paired paracancer samples. The demographic and clinicopathologic characters of these patients are shown in Table 2.

### Development and validation of a classification model for identifying CRC

We first evaluated the gene expression profiles against the clinicopathologic characters (Table 2) using Student’s t-test. No single gene was found to be statistically significantly associated with patient’s age and gender, tumor volume, clinical staging, pathological staging, number of positive lymph nodes found, or p53 status. We then randomly selected 30 CRC and paired 19 noncancerous tissues from this collection, and developed a normal logistic model that was fitted with the related expression levels of the five genes, ERBB2, MKI67, FGF18, BCL2, and PGR. As shown in Figure 2A, the model yielded area under curve = 0.91 in the receiver operating characteristic plot. We further tested this model with the reserved gene expression sets from 35 CRC and 25 paracancer tissues. It was found that the model separated CRC from normal tissue with a sensitivity of 94.1% and specificity of 88.5% (Fig. 2B).

### A classification model for assessing CRC recurring risk

Understanding the risk of recurrence is often critical for the treatment of CRCs. To develop a model that predicts the recurrence risk, 55 of the patients with known DFS and overall survival time (Table 3) were assigned to either low-risk or high-risk group, who had DFS \( \geq 28 \) months with or without tumor metastasis. Data from 25 patients (13 in low-risk, 12 in high-risk group) were allocated to a training set. A normal logistic risk

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**Table 1. Six Genes Found Through Data Mining as Putative Biomarkers for Tumor Recurrence**

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>GenBank acc. no.</th>
<th>Gene Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCL2</td>
<td>NM_000633</td>
<td>B-cell lymphoma 2 Apoptosis inhibitor</td>
</tr>
<tr>
<td>ERBB2</td>
<td>NM_004448</td>
<td>Erythroblastic leukemia viral oncogene homolog 2 Growth-promoting protein tyrosine kinase</td>
</tr>
<tr>
<td>FGF18</td>
<td>NM_003862</td>
<td>Fibroblast growth factor 18 Growth factor</td>
</tr>
<tr>
<td>INHBA</td>
<td>NM_002192</td>
<td>Inhibin beta A Common subunit of TGFβ family proteins activin and inhibin</td>
</tr>
<tr>
<td>MKI67</td>
<td>NM_002417</td>
<td>Marker of proliferation Ki-67 Nuclear protein associated with cell proliferation</td>
</tr>
<tr>
<td>PGR</td>
<td>NM_000926</td>
<td>Progesterone receptor Steroid-activated transcription factor</td>
</tr>
</tbody>
</table>

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**FIG. 1.** Expression of the six genes in CRCs and controls. RNAs were extracted from 10 pairs of CRC and paracancer tissues. The mRNAs of the six genes were quantified using real-time polymerase chain reaction after reverse transcription with specific primers. \( \beta \)-actin expression in each sample was utilized as the control. CRC, colorectal cancer.
A classification model based on the expression profiles of ERBB2, BCL2, FGF18, and INHBA was fitted to predict the probability of being high risk and low risk (Fig. 3A). All subjects in the high-risk group were correctly classified as high risk, and 10 of 13 low-risk subjects were classified as low risk in the training set. This model was further validated with the expression data from the rest 30 patients. Among them, 14 of 16 high-risk patients and 11 of 14 low-risk patients were correctly classified (Fig. 3B), indicating that it has good sensitivity and specificity for identifying recurrence risk.

Finally, the Kaplan–Meier survival analysis was performed for this group of patients. Nine deaths were recorded before the last follow-up visit among the 17 patients predicted to have high tumor recurring risk in the validation set. This patient group had estimated mean DFS of 23 months (standard deviation [SD] = 1.87) based on the Kaplan–Meier survival analysis. For the low tumor recurring risk group, we observed 6 deaths among 13 patients at the last follow-up visit with mean DFS = 28 months (SD = 2.11). The difference in the mean survival time was statistically significant (median DFS: 30 vs. 26 months, \( p = 0.049 \)) (Fig. 4). Thus, despite the very small number of patients in the groups and limited follow-up time, there was a significant difference in the low-risk and high-risk groups that were identified by using the four-gene expression profiles.

### Discussion

Despite significant progress in understanding cancer genomes and signal transduction, precision treatment of early stages of CRCs remains a significant challenge in clinic. It has been shown by a number of studies from Wang’s laboratory that signature-associated genes are effective prognostic markers for breast cancer (Li et al., 2010; Zaman et al., 2013). By analyzing the whole human genome gene expression profiles of primary invasive breast tumors, we identified 25 putative gene biomarkers related to cancer survival and recurrence status. A six-gene set was then selected based on high statistical significance and biological relevance to cancer progression. We then developed the CRC classification and recurrence risk assessment models based on RT-qPCR analysis of the tumor and paratumor tissues, and the combination of various statistical methods. The models can identify CRC tissues with high sensitivity and specificity, and separate CRC at high risk of recurrence from those at low risk. Consistent with the result, DFS of CRC patients in this cohort was closely associated with clinical stages, tumor metastasis, and recurrence, but not the sites of the tumor and positive lymph nodes identified (Supplementary Table S2). Thus, the signature appears to be an early and effective prognostic marker. It is conceivable that this diagnostic and prognostic method could be utilized to analyze the very small amount of biopsy specimen from colonoscope for detecting potential tumor and for determining if aggressive therapies are needed. Further prospective studies appear merited.

Being one of the most commonly diagnosed cancers worldwide, CRC treatment was largely based on the tumor, lymph node, and metastasis (TNM) criteria, which often causes substantial overtreatment of the patients. Significant efforts have been made to find efficient biomarkers that could

### Table 2. Clinicopathologic Features of Enrolled Patients

<table>
<thead>
<tr>
<th>Characteristics of patients</th>
<th>Training set (n=30)</th>
<th>Validation set (n=35)</th>
<th>Total (n=65)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at screening, years</td>
<td>Mean (SD)</td>
<td>62.3 (13.75)</td>
<td>61.7 (18.63)</td>
</tr>
<tr>
<td>Gender, n (%)</td>
<td>Male</td>
<td>17 (56.7)</td>
<td>19 (54.3)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>13 (43.3)</td>
<td>16 (45.7)</td>
</tr>
<tr>
<td>Tumor location, n (%)</td>
<td>Colon</td>
<td>15 (50.0)</td>
<td>19 (54.3)</td>
</tr>
<tr>
<td></td>
<td>Rectum</td>
<td>15 (50.0)</td>
<td>16 (45.7)</td>
</tr>
<tr>
<td>Clinical stage, n (%)</td>
<td>I</td>
<td>3 (10.0)</td>
<td>2 (5.7)</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>17 (56.7)</td>
<td>15 (42.9)</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>8 (26.7)</td>
<td>18 (51.4)</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>2 (6.7)</td>
<td>0</td>
</tr>
<tr>
<td>Differentiation, n (%)</td>
<td>Poorly differentiated</td>
<td>1 (3.3)</td>
<td>5 (14.3)</td>
</tr>
<tr>
<td></td>
<td>Moderately differentiated</td>
<td>27 (90.0)</td>
<td>28 (80.0)</td>
</tr>
<tr>
<td></td>
<td>Highly differentiated</td>
<td>2 (6.7)</td>
<td>2 (5.7)</td>
</tr>
<tr>
<td>p53 status, n (%)</td>
<td>-</td>
<td>12 (43.4)</td>
<td>17 (48.6)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>10 (33.3)</td>
<td>10 (28.6)</td>
</tr>
<tr>
<td></td>
<td>++</td>
<td>4 (13.3)</td>
<td>3 (8.6)</td>
</tr>
<tr>
<td></td>
<td>+++</td>
<td>3 (10.0)</td>
<td>5 (14.3)</td>
</tr>
<tr>
<td>Tumor volume, mm(^3)</td>
<td>Mean (SD)</td>
<td>757.3 (1064.55)</td>
<td>477.2 (500.84)</td>
</tr>
<tr>
<td>Number of positive lymph nodes</td>
<td>Mean (SD)</td>
<td>1 (2.7)</td>
<td>2 (4.1)</td>
</tr>
</tbody>
</table>

SD, standard deviation.
A model based on the expression of ERBB2, MKI67, FGF18, BCL2, and PGR identifies CRC from normal tissues. (A) ROC curve of the 30 CRC and paired 19 noncancerous tissues training samples. The relative expression levels of five genes, ERBB2, MKI67, FGF18, BCL2, and PGR, were used to build a linear regression model for classifying samples into CRC and normal tissue groups. (B) Comparison of the actual tissue type versus the classified tissue type of 60 validation samples. AUC, area under curve; C, CRC; N, normal tissue; ROC, receiver operating characteristic.

**Table 3. Colorectal Cancer Patients with Recurrence Information**

<table>
<thead>
<tr>
<th>Patients</th>
<th>Training set</th>
<th>Validation set</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low risk (n=10)</td>
<td>High risk (n=15)</td>
</tr>
<tr>
<td>Age at screening, years</td>
<td>Mean (SD)</td>
<td>58.5 (16.11)</td>
</tr>
<tr>
<td>Disease-free survival time, months</td>
<td>Mean (SD)</td>
<td>27.4 (1.43)</td>
</tr>
<tr>
<td>Overall survival time, months</td>
<td>Mean (SD)</td>
<td>28.2 (4.59)</td>
</tr>
<tr>
<td>Clinical stage, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1 (10.0)</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>7 (70.0)</td>
<td>8 (42.9)</td>
</tr>
<tr>
<td>III</td>
<td>2 (20.0)</td>
<td>6 (51.4)</td>
</tr>
<tr>
<td>IV</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Metastasis status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metastasis occurred</td>
<td>6 (60.0)</td>
<td>11 (73.3)</td>
</tr>
<tr>
<td>Metastasis not observed</td>
<td>4 (40.0)</td>
<td>4 (26.7)</td>
</tr>
</tbody>
</table>

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FIG. 3. A model based on the expression of ERBB2, BCL-2, FGF18, and INHBA is able to assess the risk of CRC recurrence. (A) ROC curve of the 25 training samples. The relative expression levels of four genes, ERBB2, BCL-2, FGF18, and INHBA, were used to build a linear regression model for classifying samples into low-risk and high-risk groups. (B) Comparison of the actual risk versus the classified risks of 30 validation patients.

FIG. 4. The Kaplan–Meier plot of the time to death in the independent validation sample. The predicted low- and high-risk groups have significant different mean survival times. Patients predicted to be at high risk in the validation set have significantly shorter DFS than the subjects at the low-risk class as estimated by the Kaplan–Meier survival analysis, and the difference in the mean survival time is statistically significant. DFS for patients alive at the last follow-up visit was censored at the visit date. DFS, disease-free survival time.
be utilized in clinic for precision treatment (Reimers et al., 2013). It has been shown that the fecal DNA test, which examines K-ras mutation and methylation of the promoters of BMP and NDRG4, is effective in CRC screening, and has been approved and recommended to use in clinical practice (Imperiale et al., 2014; Rex et al., 2017). Noteworthy, DNA tests that examine 29 gene expressions or methylation of Septin 9 promoter in plasma have been developed for CRC screening (Payne, 2010; Ciarnloni et al., 2016). While the sensitivity and specificity of the 29-gene test remain to be further validated, the Septin 9 assay has not been recommended due to lack of sensitivity (Rex et al., 2017).

Biomarkers derived from gene expression analysis may also be utilized for precision treatment of CRC. It has been shown that chemoresistance of CRC was associated with hypermethylation and decreased expression of gene encoding transcription factor AP-2 epsilon (Ebert et al., 2012). A 12-gene signature was found capable of assessing the sensitivity of CRC cells to 5-FU, a commonly used drug for CRC patients. Retrospective analysis indicated that the overall survival of patients responded well to 5-FU according to the 12-gene signature did not improve upon the combination TOP 1 inhibitor irinotecan, whereas these who predicated did not respond to 5-FU survived better with the combination therapy (Paquet et al., 2015). Further multicenter perspective studies will certainly help determine the usefulness of these markers.

Identifying CRC patients who may benefit from adjuvant chemotherapies has been the focus of many investigations. It was reported that the expression signature of six miRNAs is a reliable prognostic and predictive tool for CRC recurrence in stage II patients, and might be able to predict which patients benefit from adjuvant chemotherapy (Zaman et al., 2013). By analyzing gene expression signature of progenitor/stem cells, it was found that lack of CDX2 expression identified a subgroup of patients who had high-risk stage II CRC and appeared to benefit from adjuvant chemotherapy (Dalerba et al., 2016).

Interestingly, a quantitative multigene RT-qPCR assay (Onco-type DX) has been developed, which was validated through both retrospective and prospective studies in assessing the recurrence risk of stage II CRC patients after surgery and providing prognostic value that complements TNM criteria (Gray et al., 2011). Additional platforms such as ColDx, ColoPrint, Previstage, and a seven-gene signature have also been described, which all proved the principle that it is possible to assess the recurrence risk of CRC by using gene expression profile (Chee and Meropol, 2014; Chen et al., 2016, 2017).

However, these tests appear not very effective in predicting response to chemotherapeutics (You et al., 2015). Noteworthily, a retrospective analysis demonstrated that, in addition to estimating recurrence risk, it is possible to predict chemotheraphy benefits in stage II CRC by using the expression profiles of 240 genes associated with the 8 hallmarks of cancer cells (Gao et al., 2016), provided a start for further exploring the use of gene expression signatures is needed. It is conceivable that we can use RNA-seq and the hallmark-based gene panels in a future prospective study to determine whether patients with CRC could be treated more precisely and effectively.

Conclusion

In this study, through data mining, RT-qPCR, and statistical analysis, we demonstrated that the expression signature of the six genes that reflect the genetic hallmarks of cancer cells is an informative biomarker for identifying CRC and assessing the risk of recurrence with high sensitivity and specificity. To our knowledge, this is a profile assay utilizing the least number of genes for CRCs. It is conceivable that the signature could be used to analyze the small amounts of samples from biopsies, determining whether it contains tumor cells and the risk of recurrence, laying the foundation for personalized treatment and care.

Acknowledgments

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Authors’ Contributions

H.L., Y.T., Z.W., H.H., and X.C. designed and performed the RT-qPCR experiments. K.Z., Q.Z., and D.L. carried out data mining and statistical analysis. G.W. and L.C. undertook tumor sample and clinical pathological data collection and analysis. Y.Y. designed the study and wrote the article with Q.Z.

Author Disclosure Statement

No competing financial interests exist.

Supplementary Material

Supplementary Table S1
Supplementary Table S2

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Ejaz A, Casadaban L, Maker AV (2017) Utilization and impact of adjuvant chemotherapy among patients with resected stage


