Colorectal cancer (CRC) is the fourth most common noncutaneous malignancy in the United States and the second most frequent cause of cancer-related death. In 2007, an estimated 153 760 cases of CRC will be diagnosed, and 52 180 people will die from the disease (Jemal et al, 2006). The most important determinant of colon cancer survival is stage. The tumour–node–metastasis system, as defined by the American Joint Committee on Cancer, is the most commonly used cancer staging system and classifies colon cancer into four stages based on the depth of invasion of the bowel wall (T), extent of regional lymph node involvement (N), and presence of distant sites of metastatic disease (M) (Greene et al, 2002). Stage I includes T1 and T2 tumours without nodal or distant metastases and most patients with this disease will be cured with segmental colectomy alone. The overall 5-year survival (OS) of this stage is 93.2%. Stage II is subdivided into two classes (IIA and IIB; OS = 84.7 and 72.2%, respectively) and includes T3 and T4 tumours, respectively. Like stage I, nodal or distant metastasis is absent in stage II disease. Although many patients with stage II colorectal cancer will be cured by surgical resection alone, many patients with completely resected stage II disease will ultimately die from colon cancer (Jemal et al, 2006). Stage III disease includes tumours that do contain nodal disease but do not contain distant metastases. After complete surgical resection, these patients face a 50–60% chance of developing recurrent disease. A survival benefit from adjuvant 5-fluorouracil-based chemotherapy has been firmly established in these patients, and recent data have shown further efficacy through the inclusion of oxaliplatin into adjuvant treatment programmes (Chung and Saltz, 2007; Wolpin et al, 2007).

At present, the standard procedure for determining the spread of metastatic disease to lymph nodes is pathological examination of ~20 resected lymph nodes stained with haematoxylin and eosin (H&E). We reasoned that an assay that was able to identify patients with metastatic disease by measuring RNA expression levels of select genes would be helpful for making clinical decisions. In the current study, we investigated whether expression levels of 14 cancer-associated genes in the primary tumour were correlated with lymph node metastases. These 14 genes are derived from a set of 22 that our laboratory has previously identified as being overexpressed in various cancers (Reed et al, 2007).

In addition to the set of 14 genes, we also chose to examine the expression of β2-microglobulin (B2M). Our rationale for the
inclusion of B2M was based on the following observations: (1) 32% of B2Mnull × IL-8null mice develop adenocarcinoma in the proximal half of the colon between 6 and 12 months (Shah et al., 1998), (2) out of 19K genes screened from 25 matched CRC tissue and normal mucosa, B2M was the most highly down-regulated gene in CRC (Bianchini et al., 2006), (3) oncogenic K-ras mutations (which are present in the majority of CRC) inhibit the expression of B2M and other interferon (IFN)-responsive genes (Klampfer et al., 2003), (4) downregulation of B2M in CRC has been confirmed by real-time RT–PCR (Bianchini et al., 2006), and (5) the level of expression of B2M is very high and can be reliably measured in formalin-fixed paraffin-embedded (FFPE) tissues (Chen et al., 2007).

MATERIALS AND METHODS

Patients and tissues

This study was approved by the Institutional Review Boards at the Medical University of South Carolina and by the Mayo Clinic College of Medicine in Jacksonville. Metastatic and benign lymph nodes from colon cancer patients. Medical records were searched for patients who underwent surgical resection and who did (n = 7) and did not (n = 7) have associated lymph node metastases at the Mayo Clinic. A 50-μm-thick section was cut for real-time RT–PCR studies and a 5-μm-thick section was used for H&E staining. The presence of metastatic disease in lymph nodes identified as positive was confirmed by the study pathologist. Primary tumour specimens. Medical records were searched for colon cancer patients who had at least one metastatic lymph node (n = 20) or no metastatic lymph nodes (n = 18). Duplicate 50-μm-thick sections were cut for real-time RT–PCR studies and a 5-μm-thick section was used for H&E staining.

RNA isolation from paraffin sections

mRNA extraction followed the method of Specht et al. (2001). Briefly, paraffin-embedded tissue sections were deparaffinised twice with 1 ml of xylene at 37°C or room temperature for 10 min. The pellet was subsequently washed with 1 ml of 100, 90, and 70% of ethanol and air-dried at room temperature for 2 h. The pellet was resuspended in 200 μl of RNA lysis buffer (2% lauryl sulphate, 10 mmol l⁻¹ Tris-HCl (pH 8.0), and 0.1 mmol l⁻¹ EDTA) and 100 μg of proteinase K and incubated at 60°C for 16 h. RNA was extracted using 1 ml of phenol/chloroform (5:1) solution (Sigma, St Louis, MO, USA). The aqueous layer containing RNA was transferred to a new 1.5 ml tube. Phenol/chloroform extraction was performed a total of three times. RNA was precipitated with an equal volume of isopropanol, 0.1 volume of 3 mol l⁻¹ sodium acetate, and 100 μg of glycogen at −20°C for 16 h. After centrifugation at 12 000 r.p.m. for 15 min (4°C), the RNA pellet was resuspended in 200 μl of DEPC water and treated with DNase before complementary DNA (cDNA) synthesis as described in the text.

Complementary DNA synthesis and real-time RT–PCR

Complementary DNA was made from 6 μl of RNA described above, 200 U of M-MLV reverse transcriptase (Promega, Madison, WI, USA), and a panel of truncated gene-specific primers (Table 1). Real-time RT–PCR was performed on a PE Biosystems Gene Amp® 7300 or 7500 Sequence Detection System (Foster City, CA, USA). With the exception of the SYBR Green I master mix (purchased from Qiagen, Valencia, CA, USA), all reaction components were purchased from PE Biosystems. Standard reaction volume was 10 μl and contained 1 × SYBR RT–PCR buffer, 3 mM MgCl₂, 0.2 mM each of dATP, dCTP, and dGTP, 0.4 mM dUTP, 0.1 U UNG Erase enzyme, 0.25 U AmpliTaq Gold, 0.35 μl cDNA template, and 50 nM of oligonucleotide primer. Initial steps of RT–PCR were 2 min at 50°C for UNG Erase activation, followed by a 10 min hold at 95°C. Cycles (n = 40) consisted of a 15 s melt at 95°C followed by a 1 min annealing/extension at 60°C. The final step was a 60°C incubation for 1 min. All reactions were performed in triplicate. Before cDNA synthesis, RNA was treated with or without DNase as described in the text.

Gene expression and statistical analysis

To quantitate gene expression, the ΔCt method was used. As an internal reference, we used either the mean Cₜ value of all genes or the median value as described in the text. A primary tumour sample was considered to have sufficient mRNA if its mean Cₜ value was < 35.2 (38 out of 38 samples; mean ± s.d. of all samples = 27.5 ± 4.06). Area under the curve (AUC) measurements were performed for single gene analysis using MedCalc software (Mariakerke, Belgium); patients were dichotomised according to lymph node metastasis status. For AUC analysis of B2M/gene X expression ratios, ΔCt values of 14 different gene combinations were obtained by subtracting the Cₜ value of B2M from the other genes. Area under the curve analysis was then performed using MedCalc software. Associations between categorical values were assessed using Fisher’s exact test. For ordinal variables (e.g. T-stage, pathologic stage), we also used r-test to compare mean levels across lymph node expression categories due to concern over sparseness. Correlation coefficient analysis of potential reference genes was performed using Microsoft Excel software.

| Table 1 Primers used for real-time RT–PCR |
|-----------------------------------------|
| Gene | Accession number | Sequence 5’–3’ | Fragment length (bp) |
| AGR2 | NM_006408 | GCCGACGACCTTGTGCTCCTTCA | 76 |
| B2M | NM_004048 | GCCGTGAGATGCTGACCTT | 97 |
| CDH1 | NM_004360 | CACCGCAGTACAAGGTC | 94 |
| CDH3 | NM_001793 | ACCATGCTGGCCAGAGCAT | 166 |
| CEA6 | NM_002483 | ATGATGCACTGCGTTCG | 104 |
| CK19 | NM_002276 | AACGCGCGATGAGGTTGG | 204 |
| Claudin7 | NM_001307 | TGGCCATCAGATTGTCACAGAC | 88 |
| E1B | NM_004433 | TCTCCACACCGGATGTTT | 124 |
| EpCAM1 | NM_002354 | CGGACGTCAGAAGAATGGTG | 88 |
| EpCAM2 | NM_002353 | ACCGCGGACGAGGATGTTT | 100 |
| GPX2 | NM_002083 | GGCACCTAGAGGAAGCA | 127 |
| MAL2 | NM_052886 | CTCATGGTACGGGGCAGAAC | 103 |
| MAP7 | NM_003980 | CAGGAAAGCACCAAGACAA | 87 |
| S100P | NM_005980 | GACGCTTCTCGCACTATCGGG | 127 |
| Spint2 | NM_021102 | GTCCCTATCAAGATGGTCGG | 81 |

RT–PCR = reverse transcription–PCR. *Gene-specific primer sequences used for cDNA synthesis are underlined.
RESULTS

Using a novel microarray/bioinformatics approach, previously we identified a set of 22 genes that were predicted to be overexpressed in multiple cancers (Reed et al., 2007). To investigate whether these genes were overexpressed in metastatic CRC, we selected 14 genes and measured their level of expression in lymph nodes obtained from CRC patients who were positive (n = 7) and negative (n = 7) for metastatic disease by H&E staining. We next performed AUC analysis, the most commonly used statistical method for determining accuracies of diagnostic tests (Henderson, 1993). Receiver–operator characteristic (ROC) curve analysis is based on a plot of sensitivity as a function of 1−specificity. The area under the ROC curve (W) is a measure of diagnostic (or prognostic; see below) accuracy such that values between 0.5 and 0.7 indicate low accuracy, values between 0.7 and 0.9 indicate moderate accuracy, and values greater than 0.9 indicate high accuracy (Swets, 1988).

We observed that the AUC values for detection of metastatic disease of 11 out of 14 (79%) genes were greater than 0.80 (Table 2 and Figure 1). We conclude from this experiment that the set of 14 genes is highly overexpressed in metastatic CRC and hypothesise that one or more genes in this set may be prognostic for lymph node metastases.

To determine whether expression levels of molecular markers might correlate with lymph node metastases, RNA was isolated from FFPE primary tumour sections as described in Materials and Methods and analysed for the expression of the 14 cancer-associated genes listed in Table 1. For reasons stated in the introduction section, we also included in our marker panel the B2M gene. Characteristics of the patients with (n = 20) and without (n = 18) lymph node metastases are shown in Table 3. As anticipated, we observed a significant association between lymph node metastases and T-stage, pathologic stage, and tissue differentiation (continuous).

To evaluate potential prognostic values of the genes, we simply used as an internal reference the mean C value of all 15 genes (Figure 2). Our rationale for this approach was two-fold. First, an ideal internal reference gene in cancer prognostics is one that adds value should be a reliable measure of tumour content. Second, the number of genes we used for reference was 15, a number sufficiently high to avoid potential problems caused by outliers. In addition to the analysis using the mean C value of the 15-gene set as an internal reference, a separate analysis was also performed using the median C value.

Table 2 Diagnostic accuracies of cancer-associated genes for detection of metastatic disease in lymph nodes derived from CRC patients

| Gene       | AUC    | 95% CI     |
|------------|--------|------------|
| Map7       | 0.980  | 0.734–1.000|
| AGR2       | 0.939  | 0.674–0.987|
| MAL2       | 0.939  | 0.674–0.987|
| EpCAM2     | 0.939  | 0.674–0.987|
| EpCAM1     | 0.939  | 0.674–0.987|
| CEA6       | 0.918  | 0.647–0.988|
| CDH1       | 0.918  | 0.647–0.988|
| S100P      | 0.837  | 0.548–0.972|
| ELF3       | 0.837  | 0.548–0.972|
| Sprint2    | 0.816  | 0.525–0.964|
| Claudin7   | 0.816  | 0.525–0.964|
| GPX2       | 0.714  | 0.419–0.914|
| CDH3       | 0.694  | 0.399–0.903|
| CK19       | 0.531  | 0.255–0.792|

AUC = area under the curve; CRC = colorectal cancer; CI = confidence interval.

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Figure 1 Real-time RT–PCR analysis of metastatic and benign lymph nodes from colon cancer patients. Real-time PCR analyses of seven benign lymph nodes (left side of each matched data set; open triangles) and seven metastatic lymph nodes (right side of each matched data set; closed diamonds) were performed as described in Materials and Methods using primer pairs for the indicated genes. C values for each gene were determined from triplicate reactions. ∆C values were obtained by subtracting the mean C value of B2M (which is highly expressed in normal lymph node tissue) from the mean C value for each respective gene. Note: The mean B2M C value of the metastatic lymph nodes was slightly lower (i.e. B2M gene expression was slightly higher) but not significantly different from that of benign tissue (17.8 ± 2.1 vs 20.2 ± 2.1, respectively).

Figure 2 Real-time PCR analyses of FFPE primary tissue from CRC patients. Real-time RT–PCR was performed on patients who did (n = 18; right side of each matched data set; filled circles) and who did not have (n = 18; left side of each matched data set; open circles) metastatic lymph nodes from CRC as described in Materials and Methods using primer pairs for the indicated genes. C values for each gene were determined from triplicate reactions.
Using a Change in expression (ΔCt) of 4.5 for a threshold for marker positivity, we observed that the sensitivity for nodal disease detection was 85% (17 out of 20 stage III and stage IV patients were correctly classified; see Table 3), whereas the specificity was 83% (15 out of 18 stage I and stage II patients were correctly classified; see Table 3). Interestingly, all apparent 'false positives' in the node-negative groups were derived from stage I patients. The relevance of this finding is discussed in further detail below.

To investigate whether one or two genes from the panel could substitute for the entire set, we analysed our expression results in two manners. First, we calculated the expression ratios of all B2M/gene X pairs to determine whether a particular pair exhibited high prognostic accuracy. These calculations revealed that the expression ratio of B2M/Spint2 had the highest prognostic accuracy of all possible pairs (AUC = 0.87; 95% CI = 0.71–0.96). Second, we performed a correlation coefficient analysis and determined that the mean Ct value of the 15-gene set was most highly correlated with Spint2 (R² = 0.97; Figure 3). The results of these analyses suggest that Spint2 can be used as an internal reference control gene.

To assess the reproducibility of the B2M/Spint2 expression ratio, the analysis described above was repeated using duplicate tissue sections and RNA that was treated with DNase. In this second analysis, we measured the expression values of B2M, Spint2, GPX2, Elf3, CDH1, CDH3, EpCAM1, and CEAD6. We observed that the prognostic accuracy of the B2M/Spint2 expression ratio as determined by AUC analysis was within 6% of the value observed in the first study (AUC = 0.91; 95% CI = 0.73–0.98; data not shown). These results provide evidence that the B2M/Spint2 expression ratio is a reliable indicator of nodal metastases in CRC patients. Further, using the eight genes as internal reference, we observed that the prognostic accuracy of B2M was 0.82 (95% CI = 0.63–0.94; data not shown). In a separate study conducted with a small set of tissues (n = 14), we observed that B2M maintained its high prognostic accuracy for lymph node metastases (AUC = 0.79) when more classical reference control genes TBP (Ohl et al., 2006) or UBP (Andersen et al., 2004) were substituted for Spint2 (not shown).

| Variable | Categories | LN+ | P-value | Low expression of B2M* | P-value |
|----------|------------|-----|---------|-----------------------|---------|
| Gender   | Male       | 12/20 (60%) | 0.52 | 14/20 (70%) | 0.03 |
|          | Female     | 8/18 (44%) |       | 6/18 (33%)   |         |
| Site     | Right colon | 11/24 (46%) | 0.63 | 11/24 (46%) | 0.63 |
|          | Left colon  | 2/2 (100%) |       | 2/2 (100%)  |         |
|          | Rectum     | 4/7 (57%)  |       | 4/7 (57%)   |         |
|          | Sigmoid    | 2/3 (67%)  |       | 2/3 (67%)   |         |
|          | Left colon+rectum | 1/1 (100%) |       | 1/1 (100%) |         |
|          | transverse | 0/1 (0%)   |       | 0/1 (0%)    |         |
| Type     | Adeno      | 15/30 (50%) | 0.70 | 17/30       | 0.44 |
|          | Adeno, mucinous | 5/8 (63%)  |       | 3/8        |         |
| Differentiation | Well | 3/10 (30%) | 0.13 | 6/10 (60%) | 0.82 |
|          | Well to moderate | 0/1 (0%)  |       | 0/1 (0%)   |         |
|          | Moderate   | 14/24 (58%) |       | 13/24 (54%) |         |
|          | Moderate to poor | 2/2 (100%) |       | 1/2 (50%)  |         |
|          | Poor       | 1/1 (100%) |       | 0/1 (0%)   |         |
| Type     | Continuous | <0.001 | <0.001 | 3/12 (25%) |         |

LN+ = lymph node positive. *Low B2M-expressing sample was defined by \( C_{\text{t mean}} - C_{\text{B2M g}} > 4.5 \).
and Koch, 1989; Momburg et al, 1989). Loss of these class 1 antigens is associated with decreased histological differentiation in colon cancer (Gattoni-Celli et al, 1992), as well as increased malignancy in a number of neoplasms, including B-cell lymphoma (Moller et al, 1987) and melanoma (Paschen et al, 2003). Interestingly, loss of the native HLA-A,B,C/B2M complex appears to be sporadic in nature; in some cases, the loss is localised to certain portions of the tumour, whereas in others, loss of B2M is evident across the entire tumour (Momburg and Koch, 1989). As MHC class 1 antigens are required for the host to mount a tumour response, the loss of these antigens allows a tumour to escape recognition by the immune system.

Despite the known biological properties of B2M, this gene has been inadvertently used as an internal reference control in studies involving IFN signalling (Einau et al, 2005; Tamassia et al, 2007), as well as real-time PCR studies of colon cancer prognosis (Takeuchi et al, 2003). Interestingly, in the study performed by Takeuchi et al, the investigators examined 36 tumours and observed that the mRNA copy numbers of B2M in T3/T4 cases (mean 1.78 × 10^5 copies) had a tendency to be lower than that in T1/T2 cases (mean 4.44 × 10^5 copies; P = 0.16), but were not highly correlated with another reference gene. Owing to the later observation, the authors correctly concluded that B2M should not be used as an internal reference control. However, the investigators failed to recognise that the B2M gene itself might serve as a prognostic marker.

In this study, we observed that Spint2 was a reliable internal reference control gene for CRC. This result is consistent with the study of Kataoka et al (2000), who found no relationship between Spint2 mRNA and tumour stages in CRC. Further, during the course of acetic acid-induced experimental colitis in an in vivo mouse model, Spint1 but not Spint2 was upregulated in the recovery phase (Itoh et al, 2000), a process that requires cellular regeneration. This result further supports the concept that mRNA expression levels of Spint2 may remain constant during tumour progression in CRC.

Spint1 and Spint2 are Kunitz-type serine protease inhibitors that regulate hepatocyte growth factor (HGF) activity through inhibition of HGF activator (HGFA), matriptase and hepsin (Parr and Jiang, 2006). Matriptase, urokinase-type activator, HGFA, and hepsin are the main factors responsible for converting inactive pro-HGF into active HGF, which is mainly secreted by stromal fibroblasts. Hepatocyte growth factor is known to play a number of roles in cancer metastasis and tumour growth. Thus, because Spint1 and Spint2 serve to inhibit the activity of HGF, these genes have been characterised as tumour suppressors (Morris et al, 2005). Further, in a study of 41 ovarian cancer patients, low expression of Spint2 was determined to be an independent factor of poor prognosis (P = 0.013; hazard ratio, 2.30; Tanaka et al, 2003). Interestingly, the suppression of metastatic behaviour (e.g. cell motility) by Spint2 can be abrogated in vitro by treatment with extracellular signal-regulated kinase/mitogen-activated protein kinase and phospholipase C-β inhibitors (Morris et al, 2005), suggesting that the suppressive effects of this gene can be bypassed.

Of the 14 cancer-associated genes used in the current study, none proved to be prognostic for lymph node metastases at an AUC value > 0.80. This was rather surprising, as several of these genes have been previously shown to be prognostic for various cancers. For example, immunohistochemical studies have shown that low expression of E-Cadherin is associated with poor survival in a number of cancers including pancreatic (Shimamura et al, 2003), thyroid (Scheuerman et al, 1995), gall bladder (Hirata et al, 2006), breast (Park et al, 2007), lung (Nogawa et al, 2006), hepatic (Wu et al, 2006), endometrial (Scholten et al, 2005), and colon (Pena et al, 2005) cancer. Further, we were also surprised to find that EpCAM2/TROP2 was not a prognostic factor, as overexpression of this gene has been shown to be associated with liver metastases and survival in CRC patients.
In this study, we observed that 3 out of 18 patients who were node negative by pathological assessment had low levels of B2M. The clinical significance of this finding is not known. However, in a subsequent study performed on untreated stage II tissues obtained from a separate institute, we discovered that low B2M expression was an independent prognostic marker for poor patient survival (Blum et al., 2008). Thus, the results described here may ultimately prove to be of benefit to a significant number of CRC patients.

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