tion detection rate of <70% reported in most large studies (1–4). If mutational analysis of other parts of the ATP7B gene does not proceed once homzygous mutations are found, the perils of allele dropout may remain unnoticed. In PCR-based diagnosis of WD, we recommend screening the entire ATP7B gene for mutations if allele dropout cannot be excluded.

The work described in this report was fully supported by a grant from the Research Grants Council of the Hong Kong Special Administrative Region, China (Project CUHK4084/02M). We thank the reviewers for helpful comments.

References

DOI: 10.1373/clinchem.2005.060491

Identification of mRNA Markers for Molecular Staging of Lymph Nodes in Colorectal Cancer, Liqiang Xi,1,2 William Gooding,2 Kenneth McCarty,3 Tony E. Godfrey,1,4 and Steven J. Hughes1* (Departments of 1Surgery and 3Pathology, and the 2Pittsburgh Cancer Institute, University of Pittsburgh, Pittsburgh, PA; † current affiliation: Mount Sinai School of Medicine, New York, NY; * address correspondence to this author at: University of Pittsburgh, 497 Scaife Hall, 3550 Terrace Street, Pittsburgh, PA 15261; fax 412-648-2045, e-mail Hughess2@upmc.edu)

Background: One evolving approach to improved prog nostication of cancer patients is the identification of previously occult disease by use of quantitative reverse transcription-PCR. Surprisingly, no systematic analysis of potential mRNA markers for colorectal cancer has been reported. We therefore performed an extensive mRNA marker survey for colorectal cancers.

Methods: We identified potential markers through literature and database searches. We analyzed all markers by quantitative reverse transcription-PCR on a limited set of primary tumors and benign lymph nodes. Selected markers were further evaluated on a larger tissue set with positive lymph nodes.

Results: We evaluated 43 markers and undertook further analysis of 6 in the secondary screening. Five gene markers—CDX1, carcinoembryonic antigen (CEA), CK20, TACSTD1, and Villin (VIL1)—provided perfect classification of lymph node status.

Conclusions: Several mRNA markers are capable of providing exceptionally accurate characterization of lymph node status in colorectal cancer. An automated, multimarker, quantitative reverse transcription-PCR assay for characterization of lymph nodes from colorectal cancer patients may be useful for improved staging and therapeutic decision making in colorectal cancer.

© 2006 American Association for Clinical Chemistry

A critical component of improving the treatment of patients with colorectal cancer (CRC) and other cancers is improving our ability to accurately predict an individual patient’s prognosis. The presence of metastasis to regional lymph nodes (LNs) provides the most prognostically significant information for adenocarcinomas and drives treatment decisions. There is now conclusive data for most cancers that improved LN analysis through increased sampling (multiple cross-sections) and sensitivity [immunohistochemistry (IHC)] identifies previously occult metastases (1–5). Similarly, in CRC, a sizeable percentage of patients who would be staged as node-negative by standard histologic analysis have occult LN disease that may be clinically significant (6–8).

Most published reports have used sentinel LN localization techniques and IHC to visually detect occult disease. Others have used reverse transcription (RT)-PCR to detect carcinoembryonic antigen (CEA) mRNA as a marker of occult disease (1, 9). Interestingly, studies using RT-PCR in colon cancer occult disease detection have consistently suggested that occult disease identified by this technique is clinically significant (1, 9), whereas studies using IHC have led to conflicting results and controversial conclusions (10, 11). These controversies have contributed to the failure of acceptance, even in academic settings, of sentinel LN biopsy and/or serial section analysis of LN, including IHC analysis for CRC. The lack of standardized sample preparation procedures and the labor-intensive nature of the analysis have also contributed.

We have previously reported the identification of LN metastases in other solid-organ tumors, using internally controlled, quantitative RT-PCR (QRT-PCR) assays to provide sensitive, objective, molecular staging of LNs (2, 12–14). Importantly, we recently demonstrated that this approach can be fully automated, including sample preparation (15). These studies have consistently demonstrated that the success of this approach depends on the quality of disease-specific mRNA markers. Surprisingly, no systematic analysis of potential mRNA markers for CRC has been reported. We therefore performed an extensive mRNA marker survey for CRCs. We report several new markers capable of producing highly accurate QRT-PCR analysis of LNs from a CRC patient.

We conducted an extensive literature and public database survey to identify any potential markers relevant to CRC, followed by 2 phases of screening with QRT-PCR. All potential markers entered the primary screening
phase, and expression was analyzed in 6 primary colon tumors and in 10 benign LNs obtained from patients without cancer. Markers that showed good characteristics for LN metastasis detection (i.e., high expression in primary tumors and low expression in benign LNs) passed into the secondary screening phase. The secondary screen consisted of expression analysis on 28 primary tumors, 12 histologically positive LNs, and 21 benign LNs from 21 patients without cancer (see Table 1 in the Data Supplement that accompanies the online version of this Technical Brief at www.clinchem.org/content/vol52/issue3). All tumors and positive LN samples were from different patients. Primary tumor and benign LN specimens were obtained from tissue banks at the University of Pittsburgh Medical Center through protocols approved by the Institutional Review Board. Positive LNs from patients with colon cancer were purchased from Ardais. We performed all quantitative PCRs on the ABI Prism 7700 Sequence Detection Instrument (Applied Biosystems). Relative expression of the marker genes was calculated by use of previously described delta-CT methods (16) and with β-glucuronidase (GUS) as the endogenous control gene. The details of RNA preparation, reverse transcription, and quantitative PCR have been described in previous reports (17, 18). Primers and probes were designed with use of the ABI Primer Express software (Ver. 2.0), and where possible, amplicons spanned exon junctions to provide cDNA specificity. In addition, all primer pairs were tested empirically for amplification from 100 ng of genomic DNA, and primers were redesigned when necessary. With the exception of CK19, cDNA-specific primer sets were successfully identified for all genes in this study. The sequences of primers and probes (purchased from IDT) for genes evaluated in the secondary screen are listed in Table 1.

Our literature and database surveys identified a total of 43 genes for evaluation in the primary tumor marker screen. All of these genes were analyzed for expression in 6 primary colon tumors and in 10 benign LNs. By calculating median expression in the tumors and lowest tumor/highest benign node and median tumor/highest benign node ratios, we selected 6 genes that we judged to have expression characteristics suitable for detection of LN metastases (see Table 2 in the online Data Supplement). Five of these genes—CDX1, CEA, CK19, TACSTD1, and Villin1 (VIL1)—have median tumor/highest benign node ratios >300 and therefore have the potential to detect small foci of tumor while still differentiating negative nodes. In addition, we selected CK20 based on the

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotide</th>
<th>Sequence, 5'→3'</th>
<th>Annealing Tm, °C</th>
<th>Sensitivity with 100% specificity,</th>
<th>Fold difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GUS</td>
<td>Forward primer</td>
<td>CTCATTTGGAATTTTGCCGATT</td>
<td>81</td>
<td>12/12 (100)</td>
<td>96.3</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>CCGATGGAGATCCCTTAATTA</td>
<td>60</td>
<td></td>
<td>286.1</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>TGAAACGATCCCGACGAGTGCTGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDX1</td>
<td>Forward primer</td>
<td>CGGTGGCAAGCAGGTAAGAC</td>
<td>109</td>
<td>12/12 (100)</td>
<td>96.3</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>GATTGTGATGTAACGGCTGTAATG</td>
<td>62</td>
<td></td>
<td>286.1</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>ACCAGGACAGATCAGGTTGCTACA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CEA</td>
<td>Forward primer</td>
<td>AGACAATCTACAGTCTTCGGGA</td>
<td>77</td>
<td>12/12 (100)</td>
<td>474.4</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>ATCCTTGGTCTCTCCACGGGTT</td>
<td>64</td>
<td></td>
<td>1349.8</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>CAAGGCCTCTCATCTCAGCAAAACT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CK19</td>
<td>Forward primer</td>
<td>AGATCGACAACCGCCCGT</td>
<td>64</td>
<td>10/12 (83.3)</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>AGAGCCTTGTCCGTCTTCAAA</td>
<td>60</td>
<td></td>
<td>14.6</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>TGCTCGCAGATGCTCCGGACCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CK20</td>
<td>Forward primer</td>
<td>CACCTCCAGAGCCTTTGAGAT</td>
<td>86</td>
<td>12/12 (100)</td>
<td>48.3</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>GGCCCTTGTCCTTCTCAGAG</td>
<td>60</td>
<td></td>
<td>8025.8</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>CCATCTGAGCATGAGAATGGTCTT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TACSTD1</td>
<td>Forward primer</td>
<td>TCAATTGGTCACAAGGGTCCTG</td>
<td>82</td>
<td>12/12 (100)</td>
<td>959.0</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>GGTTTTGGTCCTCTCCCAAATTTT</td>
<td>64</td>
<td></td>
<td>2025.0</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>AAATTGGCTGTAGGAAAGGCGACGAAATGAATGG</td>
<td>12/12 (100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VIL1</td>
<td>Forward primer</td>
<td>TGGTTCTCCTGGCTTGGGAT</td>
<td>74</td>
<td>12/12 (100)</td>
<td>29.0</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>TGCCCGACCTCCTCGGCTTCTCT</td>
<td>62</td>
<td></td>
<td>90.4</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>TCAAGTGAGTAACCAAAATCCCTATGAGGACC</td>
<td>12/12 (100)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. mRAN marker design and detection sensitivity.

- **GUS** Forward primer: CTCATTTGGAATTTTGCCGATT
- **CDX1** Forward primer: CGGTGGCAAGCAGGTAAGAC
- **CEA** Forward primer: AGACAATCTACAGTCTTCGGGA
- **CK19** Forward primer: AGATCGACAACCGCCCGT
- **CK20** Forward primer: CACCTCCAGAGCCTTTGAGAT
- **TACSTD1** Forward primer: TCAATTGGTCACAAGGGTCCTG
- **VIL1** Forward primer: TGGTTCTCCTGGCTTGGGAT

- **Abbreviations:**
  - **GUS:** Glucuronidase
  - **Tm:** Melting temperature
  - **Sensitivity:** Calculated based on absolute cutoff on highest expression in benign LNs
  - **Fold difference:** Calculated from the fold changes of expression of each marker in positive LNs based on the maximum expression in benign LNs.

- **Clinical Chemistry** 52, No. 3, 2006 521
finding that it was moderately expressed in tumors but undetectable in benign nodes.

The relative expression profiles of the markers selected for the secondary screen are shown in Fig. 1. As the data indicate, all markers are expressed in positive LNs as well as in primary tumors, indicating that metastatic tumor cells continue to express these genes. Of the 6 markers analyzed, all except CK19 provided perfect classification of positive and benign LNs with 100% sensitivity and 100% specificity. CK19 had poor sensitivity (83.3%) because of relatively high background in benign LNs (Table 1). Although our primer design for CK19 does not avoid the known CK19 pseudogenes, all samples were DNase-treated, and “no-reverse transcriptase” controls were consistently negative, indicating that the background expression observed was not a result of contaminating DNA. On the basis of expression results in positive nodes and distribution of expression in benign nodes, TACSTD1 expression was, at a minimum, 595-fold higher in positive nodes than the highest expression in benign nodes. Expression of the existing markers, CEA and CK20, was 474- and 48-fold higher, respectively, in positive nodes than in benign nodes. Expression of CDX1 and VIL1 was relatively low in positive nodes compared with other markers, but the expression rates were 96- and 29-fold higher, respectively, in benign nodes.

Despite promising data from the literature or gene expression databases, we found that the expression characteristics of most of the 43 marker genes evaluated in the primary screen were not suitable for use in CRC. Most had either very low or infrequent expression in the neoplasms, or they had relatively high expression in healthy or benign LNs. We were, however, able to identify 6 markers that we believed had good potential for identification of CRC in LNs. These markers (CEA, CK19, CDX1, CK20, TACSTD1, and VIL1) are mostly epithelial-cell related markers that have absent or low expression in healthy LNs. TACSTD1, also known as EPCAM, KS1/4, GA-733-2, and MIC-18, is a human cell surface antigen that is detected by the monoclonal antibodies AUAI and Ber-EP4, and it has been used in several studies to detect LN micrometastases in a variety of tumor types via both IHC and RT-PCR (19–21). Although TACSTD1 was expressed at easily detectable concentrations in all positive LNs in our study, it was very highly and consistently expressed in CRC samples. As with CEA, high expression of TACSTD1 in tumors facilitates the identification of even small amounts of metastatic tumor in LNs when a quantitative assay is used.

CDX1 is a member of the caudal-type homebox family of genes. These are cognates of the caudal gene in Drosophila, which is required for anterior-posterior regional identity. Homologous genes have been found in the mouse, rat, chicken, and Xenopus. Bonner et al. (22) isolated the human CDX1 gene from a small-intestine cDNA library, using a murine CDX1 cDNA probe. Later, Northern analysis indicated that expression of CDX1 in adults is limited to the intestine and colon. As such, CDX1 appeared to be a good potential marker for CRC. Our screening results showed that expression of CDX1 is at least 96-fold higher in positive nodes than in benign nodes. When combined with other markers, CDX1 is likely to provide a good assay to detect colon tumor cells in LNs.

Finally, Villin1 (VIL1) is a major structural component of the brush border cytoskeleton. It is a calcium-regulated, actin-binding protein that is specifically expressed in simple epithelia of some tissues of the gastrointestinal and urogenital tracts. Because CRC arises from a columnar epithelium, the finding that VIL1 is a potentially useful marker for this disease is not surprising.

In summary, the current TNM staging system for CRC is flawed, and one approach to improved prognostication is the identification of previously occult disease through techniques such as sentinel LN mapping, IHC, and RT-PCR. We have identified several new mRNA markers that are capable of providing exceptionally accurate character-

![Fig. 1](image-url)
ization of LN status in CRC. An automated, multimarker QRT-PCR assay for characterization of LNs from CRC patients could be very useful for improved staging and treatment decision-making in CRC.

This work was supported in part by Grant CA-01958 from the National Institutes of Health (to S.J.H.), and by a cooperative research and development grant from Cepheid (to T.E.G.).

References


Cell-Free Plasma DNA: A Marker for Apoptosis during Hemodialysis, Johanna Atamaniuk,1* Katharina Ruzicka,1 Karl M. Stuhlmeter,2 Alireza Karimi,1 Manfred Eigner,3 and Mathias M. Mueller4 (1 Institute of Laboratory Diagnostics, and 2 First Medical Department, Dialysis Unit, Kaiser Franz Josef Hospital, Vienna, Austria; 3 Ludwig Boltzmann Institute for Rheumatology, Vienna, Austria; 4 address correspondence to this author at: Institute of Laboratory Diagnostics, Kaiser Franz Josef Hospital, Kundratstrasse 3, A-1100, Vienna, Austria; fax 43-60191-3309, e-mail johanna.atamaniuk@wienkav.at)

Background: We evaluated whether cell-free plasma DNA might be an appropriate marker for cell damage during hemodialysis (HD) and whether it correlated with annexin V expression and 7-aminocyclomycin D (7AAD) nuclear staining of blood leukocytes.

Methods: Circulating DNA, annexin V, and 7AAD were measured in HD patients before HD, 20 min after start of HD, and after HD had ended. Healthy volunteers provided control measurements. Necrosis and apoptosis were monitored by gel electrophoresis.

Results: Plasma DNA concentrations were not significantly different between controls and patients before HD. Circulating DNA increased significantly (P <0.05) after 20 min of treatment with HD. Post-HD concentrations of DNA were significantly higher compared with pre-HD and controls (P <0.005). Agarose gel electrophoresis showed ladders typical of apoptosis in post-HD samples. Two subpopulations of CD45+ leukocytes were defined by flow cytometry: annexin V+/7AAD+ population for apoptosis, and annexin V+/7AAD− for early apoptosis. Compared with healthy controls, mean fluorescence (MF) of 7AAD+ apoptotic cells in the annexin V+/7AAD+ subpopulation before HD was not significantly increased. HD increased MF of 7AAD+ cells in the annexin V+/7AAD+ subpopulation. In this subpopulation, MF of annexin V+ cells was significantly higher (P <0.01). MF of annexin V+ cells in the annexin V+/7AAD+ subpopulation increased during HD.

Conclusions: During HD, cell-free plasma DNA concentrations, annexin V expression, and 7AAD uptake in