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The Role of Genetic Markers—*NAP1L1*, *MAGE-D2*, and *MTA1*—in Defining Small-Intestinal Carcinoid Neoplasia

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Background: Standard clinical and immunohistochemical methods cannot reliably determine whether a small intestinal carcinoid (SIC) is indolent or aggressive. We hypothesized that carcinoid malignancy could be defined by using quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR) and immunohistochemical approaches that evaluate potential marker genes.

Methods: Candidate marker gene expression (nucleosome assembly protein 1–like 1 [*NAP1L1*], melanoma antigen D2 [*MAGE-D2*], and metastasis-associated protein 1 [MTA1]) identified by Affymetrix transcriptional profiling was examined by QRT-PCR in SIC, liver, and lymph node (LN) metastases, colorectal carcinomas, and healthy tissues. Immunohistochemical expression levels of MTA1 were analyzed quantitatively by a novel automated quantitative analysis in a tissue microarray of 102 gastrointestinal carcinoids and in a breast/prostate carcinoma array.

Results: Affymetrix transcriptional profiling identified three potentially useful malignancy-marker genes (out of 1709 significantly altered genes). By QRT-PCR, *NAP1L1* was significantly ($P < .03$) overexpressed in SIC compared with colorectal carcinomas and healthy tissue. Increased levels ($P < .05$) were identified in both liver and LN metastases. Levels in colorectal carcinomas were the same as in healthy mucosa. *MAGE-D2* and MTA1 were increased ($P < .05$) in primary tumors and metastases and overexpressed in carcinomas. Automated quantitative analysis demonstrated the highest levels of MTA1 immunostaining in malignant primary SICs and in metastases to the liver and LN. These were significantly increased ($P < .02$) compared with nonmetastatic primary tumors. MTA1 was overexpressed in breast and prostate carcinomas ($P < .05$).

Conclusions: SICs overexpress the neoplasia-related genes *NAP1L1* (mitotic regulation), *MAGE-D2* (adhesion), and MTA1 (estrogen antagonism). The ability to determine the malignant potential of these tumors and their propensity to metastasize provides a biological rationale for the management of carcinoids and may have prognostic utility.

Key Words: Automated quantitative analysis—Carcinoid—Malignancy—*NAP1L1*—Polymerase chain reaction—Tissue microarray.

Small-intestinal enterochromaffin cell–derived carcinoids are currently the most frequent type of carcinoid tumor (approximately 29%).[1–3] At the time of diagnosis, 58% to 64% of patients have nonlocalized disease, and the 5-year survival rate is 60.5%.[1,4–7] In contrast, carcinoids of the stomach or rectum exhibit far better survival rates (> 80%).[1,5,8,9] The poor prognosis of small-intestinal carcinoids (SICs) reflects the inherent clinical difficulty in identifying small-bowel malignancies in a timely fashion,

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as well as the intrinsically malignant nature of the tumor with dissemination to both the lymph nodes (LNs) and the liver.[1,5,10]

Currently, the biological basis of SIC malignancy and metastasis is unknown, and it is not possible to predict tumor behavior or devise a biologically rational basis for surgical management. Diagnosis is based on symptoms, serum values of chromogranin A, and serotonin, and the therapeutic strategy reflects identification of metastasis (somatostatin receptor scintigraphy and computed tomographic scan).[5,11,12] At surgery, visual evidence of metastasis coupled with pathologic confirmation of metastatic tissue provides the only clue to the degree of malignancy or the extent of the disease.[13,14] A lesion of >2 cm has long been proposed as indicative of tumor aggression,[15] but exceptions to this exist.[16–18] Pathologic staging or grading of carcinoids includes angioinvasion and differentiation but is limited,[19] whereas sentinel LN mapping is not readily undertaken. The utility of Ki-67 as a prognostic index in neuroendocrine tumors has been documented,[20,21] but this marker provides limited predictive information.[22] Overall, therefore, determinants of malignancy and metastasis in SIC are lacking, and what is known reflects the quantification of disease extent, as opposed to delineation of the biological behavior of the tumor. Indeed, no technique exists to identify whether or when a SIC tumor is malignant and to determine whether it has already developed, or is likely to develop, metastases.

Novel strategies for the identification of when a primary tumor becomes metastatic are based on molecular profiling of the tumor. In the gastrointestinal tract, methods including gene expression analyses (e.g., oligonucleotide arrays) and molecular polymerase chain reaction (PCR) analysis of specific genes (e.g., *p53*) have been undertaken in colorectal tumors.[23,24] In this study, we found that nucleosome assembly protein 1–like 1 (*NAP1L1*), melanoma antigen D2 (*MAGE-D2*), and metastasis-associated protein 1 (MTA1) are potential marker genes of carcinoid malignancy. *NAP1L1* has been demonstrated to be upregulated in hepatoblastomas compared with nondiseased adult livers,[25] *MAGE-D2* has been identified as a molecular marker predictive of colorectal liver metastases and is overexpressed in $>75\%$ of primary tumors with metastases,[26] and overexpression of MTA1 messenger RNA (mRNA) and protein correlates with tumor invasion and metastasis in a variety of tumors, including breast, hepatocellular, esophageal, gastric, and colorectal carcinomas.[27–31] These marker genes have not previously been examined in SIC tumors.

We hypothesized that these candidate malignant marker genes would be overexpressed and provide a basis to establish a predictive biological characterization of SIC and would thus enable a viable method to be developed that would effectively delineate malignancy. We used a real-time PCR approach to identify the presence of the marker genes of interest in primary tumors and metastases and confirmed the expression, utility, and specificity of these markers against healthy mucosa and colorectal carcinomas. We then examined the expression of MTA1, which has well-defined commercially available antibodies, to assess and compare the utility of an immunohistochemical approach.

METHODS

These studies were approved by the Human Investigations Committee at Yale University School of Medicine.

Patients and Samples

Tissue Specimens

Tumor tissue was collected from 24 patients (16 men and 8 women; median age, 54 years; range, 36–78 years) with histologically proven SIC tumors or colorectal carcinomas who had undergone resection of the primary tumor between 1997 and 2003 in either the Yale University Department of Surgery or the Cooperative Human Tissue Network, which is funded by the National Cancer Institute. Eight primary carcinoid tumors, seven liver metastases from carcinoids, four carcinoid LN metastases, and nine primary colorectal tumors were studied. Paired healthy tissue samples were also obtained from adjacent, macroscopically normal, nontumor mucosa ($n = 14$), liver ($n = 3$), or lymph ($n = 4$) nodes in 17 patients.

Tissue Microarray Immunostaining, Image Acquisition, and Data Analysis

A carcinoid tissue microarray (TMA) containing 55 SICs (Yale Tumor Microarray; YTMA60) and a second array, YTMA25, that included breast and prostate carcinomas and healthy tissue samples from each of these sites were examined by automated quantitative analysis (AQUA) after immunohistochemical staining. For YTMA60, the following

clinically relevant information was available: 28 men and 27 women; median age, 62 years; range, 40−89 years; median tumor size, 15 mm; range, 2−55 mm; 30% of tumors had developed liver metastases; 67% were associated with LN metastases; and the patient follow-up was a median of 43 months (range, 1−456 months). All metastases were synchronous tumors. Histospots on the array included 55 primary SICs, 14 matched (synchronous) LN metastases, and 7 matched (synchronous) liver metastases. YTMA25 was used as a control array because MTA1 is overexpressed in both breast[31] and prostate[32] cancers. On this array, histospots included primary tumors from patients with synchronous distant metastases.

Tissue Techniques

RNA Isolation

Total RNA was isolated from frozen SIC tumor tissue, metastases, and colorectal carcinomas (n = 28) and from healthy tissues (n = 20) by using TRIzol reagent (Invitrogen, Carlsbad, CA) as described previously.[33] RNA was dissolved in diethyl pyrocarbonate water and measured spectrophotometrically, and an aliquot was analyzed on a Bioanalyzer (Agilent Technologies, Palo Alto, CA) to assess the quality of the RNA.

Affymetrix Transcriptional Profiling

High-throughput gene expression analysis was undertaken to identify genes that were specifically altered in SICs compared with normal mucosa. Material from 12 tissue samples (normal mucosa, n = 4; malignant carcinoids with histological identification of liver and LN metastases, n = 8) was examined. Labeled chromosomal RNA was prepared and hybridized to human U133A arrays by using the guidelines of the Affymetrix resource facility at Yale University (http://keck.med.yale.edu/affymetrix). The Affymetrix U133A array consists of >500,000 unique oligonucleotide sequences that represent approximately 22,000 genes (http://www.affymetrix.com/products/arrays/specific/hgu133.affx). The hybridized arrays were scanned by using a confocal laser fluorescence scanner (Agilent Microarray Scanner; Agilent Technologies). Arrays were scaled to an average intensity of 500 and analyzed independently by using Microarray Suite 5.0 software (Affymetrix, Santa Clara, CA). The hybridization intensity data were converted into presence or absence of calls for each gene. DNA Chip Analyzer (dCHIP) Version 1.3[34] was used for downstream analysis by using Microarray Suite 5.0−generated CEL files, normalizing, and modeling to generate model-based estimates of expression for each probe set. Data analysis comprised sample comparison to identify differentially altered genes by using the lower 90% confidence bound of n-fold change[35] (lower bound fold change ≥1.2-fold) and the unpaired t-test ($P < .05$), and hierarchical clustering based on Pearson correlation was used for measuring the similarity between samples.

Quantitative Reverse Transcriptase-PCR

Twenty-three primary SIC tumors or metastases to the liver or LNs, 9 colorectal carcinomas, and 20 control tissues (small intestine, liver, and LN) were examined by quantitative reverse transcriptase-PCR (QRT-PCR). *NAP1L1*, *MAGE-D2*, *MTA1*, and glyceraldehyde phosphate dehydrogenase (*GAPDH*) messages were quantitatively measured as previously described.[33] QRT-PCR was performed with the ABI 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). Total RNA from each sample was subjected to reverse transcription with the High Capacity cDNA Archive Kit (ABI, Foster City, CA) by following the manufacturer's directions. Briefly, 2 μg of total RNA in 50 μL of water was mixed with 50 μL of 2× RT mix containing Reverse Transcription Buffer, deoxynucleotide triphosphate solution, random primers, and Multiscribe Reverse Transcriptase. The RT reaction was performed in a thermal cycler for 10 minutes at 25°C followed by 120 minutes at 37°C. Real-time PCR analysis was then performed in triplicate. Briefly, complementary DNA in 7.2 μL of water was mixed with .8 μL of 20× Assays-on-Demand primer (*NAP1L1*, Hs00748775; *MAGE-D2*, Hs00374760; *MTA1*, Hs00183042; *GAPDH*, Hs99999905) and probe mix and 8 μL of 2× TaqMan Universal Master mix in a 384-well optical reaction plate. The following PCR conditions were used: 50°C for 2 minutes and then 95°C for 10 minutes, followed by 40 cycles at 95°C for .15 minutes and 60°C for 1 minute. A standard curve was generated for each gene by using complementary DNA obtained by pooling equal amounts from each sample. The expression level of target genes was normalized to internal *GAPDH*. Data were analyzed using Microsoft Excel (Microsoft Corp., Redmond, WA) and calculated by using the relative standard curve method (ABI, User Bulletin 2).

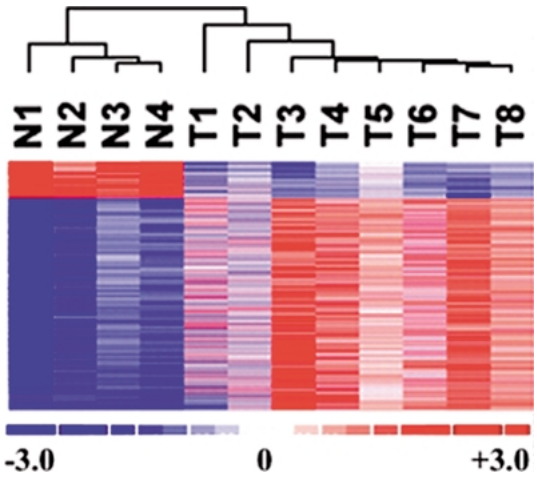

FIG. 1. Hierarchical clustering of 12 Affymetrix chips including normal small intestinal mucosa (N; n = 4) and malignant small intestinal carcinoid (SIC) tumors (T; n = 8). There is a clear separation between normal mucosal genes and tumor genes. A total of 1709 genes were altered in the malignant SIC tumors compared with normal small-intestinal mucosa. Of these, 1451 were significantly upregulated, and 258 transcripts were significantly downregulated. The color scheme refers to the expression relative to the mean expression of a gene across all samples. Red represents expression levels above the mean expression of a gene across all samples, white represents mean expression, and blue represents expression lower than the mean. Expression levels of -3.0 to +3.0 are indicated below.

TMA Immunostaining, Image Acquisition, and Data Analysis

The immunohistochemical expression of MTA1 in gastrointestinal carcinoids was examined by immunohistochemistry and AQUA quantitation of staining intensity in YTMA60 and YTMA25. Expression values of these proteins were correlated with clinical evidence of disease (liver or LN metastases).

Carcinoid TMA slides were stained as previously described.[33,36] For antigen retrieval, sections were immersed in citrate buffer (10 mM of sodium citrate; pH 6.0) and subjected to 10 minutes of high-temperature/high-pressure treatment followed by treatment with .3% H_2O_2 in methanol for 30 minutes at 37°C to inactivate endogenous peroxidase. Slides were incubated for 24 hours at 4°C with a 1/100 dilution of the anti-MTA1 (Santa Cruz; A11) antibody as previously described in a prostate TMA.[32] Goat anti-mouse antibodies conjugated to a horseradish peroxidase–decorated dextran polymer backbone (Envision; Dako Corp., Carpinteria, CA) were used as a secondary reagent. For automated analysis, neuroendocrine tumor cells or normal mucosal epithelia were identified by the use of a fluorescently tagged anti-cytokeratin antibody cocktail (AE1/AE3; Dako), nuclei were visualized by 4′,6-diamidino-2-phenylindole, and targets were visualized with a fluorescent chromogen (Cy-5-tyramide; NEN Life Science Products, Boston, MA).[33,37] Briefly, monochromatic, high-resolution (1024 × 1024 pixels; .5 μm) images were obtained of each histospot. Areas of tumor or normal epithelia from stromal elements were distinguished by creating a mask from the cytokeratin signal. Coalescence of cytokeratin at the cell surface localized the cell membranes, and 4′,6-diamidino-2-phenylindole was used to identify nuclei. The MTA1 signal from the nucleus of tumor cells or epithelial cells was automatically scored and expressed as signal intensity divided by the nuclear area. Disk scores from the same tumor were averaged to produce a single score. Histospots containing <10% tumor, as assessed by mask area (automated), were excluded from further analysis.

Statistical Analysis

Results were expressed as mean ± SEM; n indicates the number of patients in each study group. Statistical significance was calculated by the two-tailed Student's *t*-test for paired and unpaired values, as appropriate. $P < .05$ represented significance. The Biostatistics Resource at the W. M. Keck Foundation Biotechnology Resource Laboratory (Yale University) undertook all analyses for the Affymetrix profiling experiments.

RESULTS

Affymetrix Gene Profiling

There were 1709 genes that were significantly altered in the malignant SIC tumors compared with normal small-intestinal mucosa. Of these, 1451 were upregulated, and 258 transcripts were significantly downregulated (Fig. 1). Data examination demonstrated alterations in transcripts of a variety of oncoproteins and positive and negative growth regulators. Of these 46 altered genes, *NAP1L1* (+3.57-fold vs. normal mucosa; $P = .0002$), *MAGE-D2* (+2.7; $P = .013$), and the breast cancer estrogen-antagonistic malignancy gene, *MTA1* (+2.1; $P = .011$), were examined further as potential marker genes of carcinoid malignancy. These three genes were selected because they have previously been characterized and have established neoplastic regulatory roles in other tumor types.[25–31]

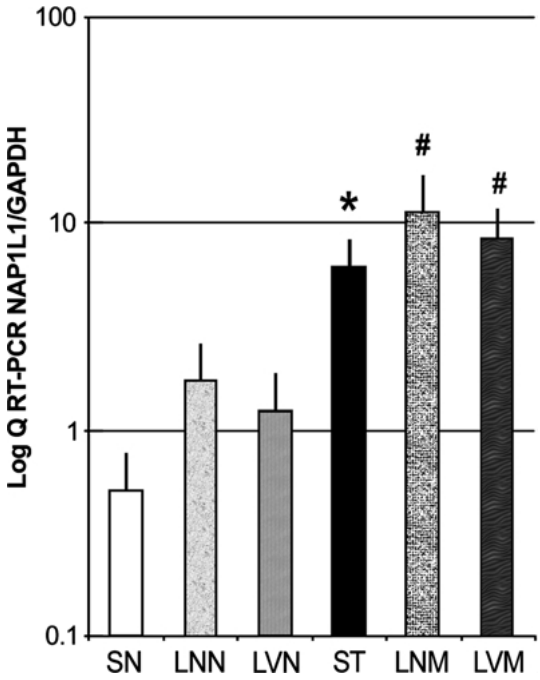

FIG. 2. Message levels of nucleosome assembly protein 1−like 1 (*NAP1L1*) determined by quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR). Levels of *NAP1L1* were significantly overexpressed (12×) in small intestinal carcinoid (SIC) tumor (ST; n = 6) samples compared with normal mucosa (SN; n = 8), in liver metastases (LVM; n = 7) compared with normal liver (LVN; n = 3), and in LN metastases (LNM; n = 4) compared with normal LNs (LNN; n = 4; $^{*}P = .03$; $^{\#}P = .05$). Data are mean ± SEM. LN, lymph node; GAPDH, glyceraldehyde phosphate dehydrogenase.

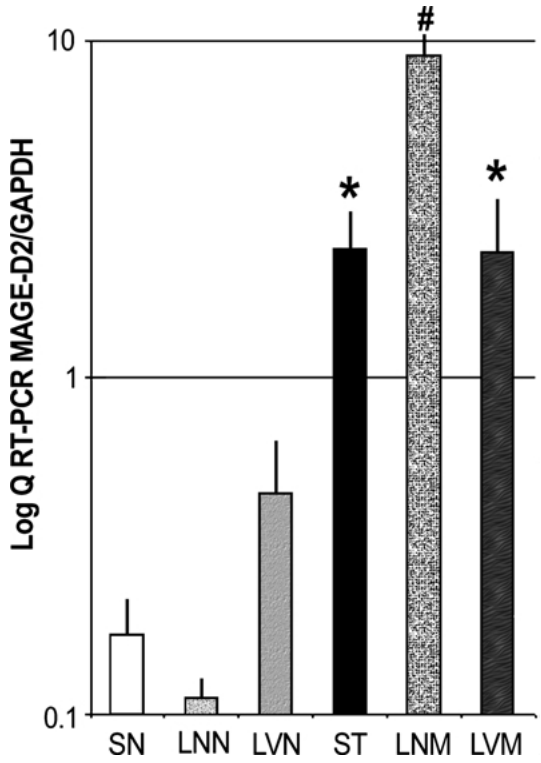

FIG. 3. Message levels of melanoma antigen D2 (*MAGE-D2*) determined by quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR). Levels of *MAGE-D2* were significantly overexpressed (approximately 10×) in small intestinal carcinoid (SIC) tumor (ST; n = 6) samples compared with normal mucosa (SN; n = 8), in liver metastases (LVM; n = 7) compared with normal liver (LVN; n = 3), and in LN metastases (LNM; n = 4) compared with normal LNs (LNN; n = 4; $^{*}P < .01$; $^{\#}P = .05$). Data are mean ± SEM. LN, lymph node; GAPDH, glyceraldehyde phosphate dehydrogenase.

Real-Time PCR

NAP1L1

NAP1L1 is a nuclear protein that is involved in chromatin assembly and DNA replication and is upregulated in hepatoblastomas.[25] Messenger RNA levels of *NAP1L1* were increased (> 10-fold; $P = .03$) in malignant SIC tumors compared with normal mucosa (Fig. 2). Message levels were similarly increased ($P < .05$) in liver and LN metastatic tissue compared with healthy liver and healthy LN material, respectively.

MAGE-D2

MAGE-D2 is an adhesion gene and potential predictive marker of colorectal liver metastases.[26] Levels of *MAGE-D2* were increased (10-fold; $P < .01$) in malignant SIC tumors compared with normal mucosa (Fig. 3). Message levels were increased in liver metastases (5-fold; $P < .01$) and LN metastatic tissue (8-fold; $P < .05$) compared with healthy liver and healthy LN material, respectively.

MTA1

MTA1 is an estrogen-antagonistic breast cancer malignancy gene that has been used to identify progressive (metastatic) disease in a range of tumors.[27–32] Message levels were increased in liver metastases
(3-fold; $P < .05$) and LN metastatic tissue (5-fold; $P < .05$; Fig. 4). In addition, mRNA levels of *MTA1* were increased (5-fold; $P < .05$) in malignant SIC tumors compared with normal mucosa.

Specificity of NAP1L1, MAGE-D2, and MTA1 as Marker Genes for SIC Tumors Compared With Colorectal Carcinomas

Having identified that the gene expression levels of *NAP1L1*, *MAGE-D2*, and *MTA1* were increased in SIC tumors, we examined their expression in nine colorectal carcinomas by using the same technology. Message levels of both *MAGE-D2* and *MTA1* were

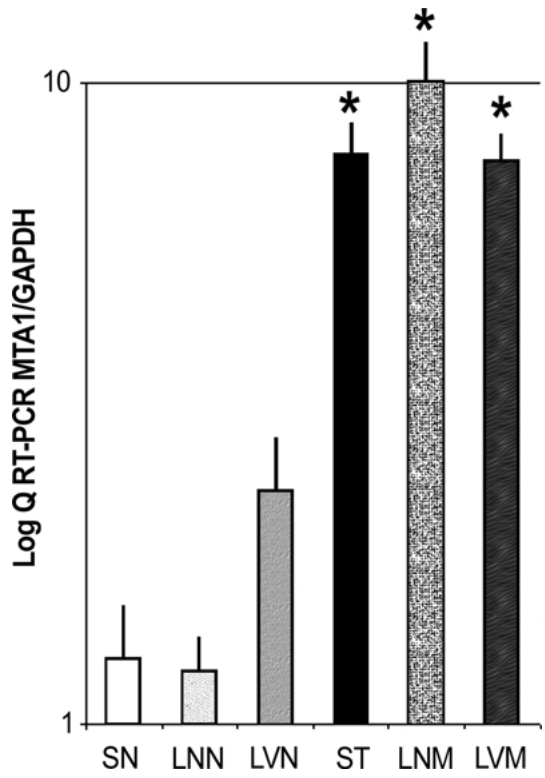

FIG. 4. Message levels of metastasis-associated protein 1 (*MTA1*) determined by quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR). Levels of *MTA1* were significantly overexpressed (5×) in small intestinal carcinoid (SIC) tumor (ST; n = 6) samples compared with normal mucosa (SN; n = 8), in liver metastases (LVM; n = 7) compared with normal liver (LVN; n = 3), and in LN metastases (LNM; n = 4) compared with normal LNs (LNN; n = 4; $^{*}P < .05$). Data are mean ± SEM. LN, lymph node; GAPDH, glyceraldehyde phosphate dehydrogenase.

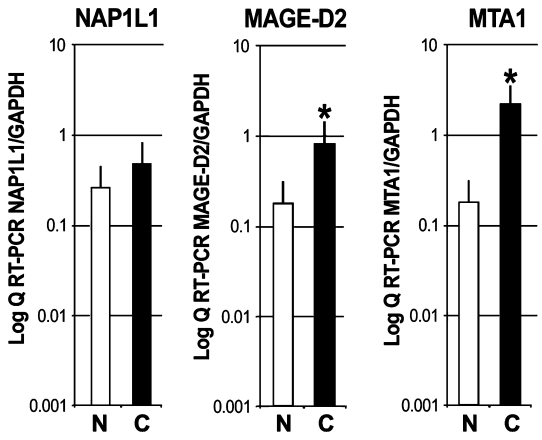

FIG. 5. Message levels of nucleosome assembly protein 1–like 1 (*NAP1L1*), melanoma antigen D2 (*MAGE-D2*), and metastasis-associated protein 1 (*MTA1*) determined by quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR) in colorectal carcinomas and normal colorectal mucosa. Both *MAGE-D2* and *MTA1* were increased in colorectal carcinomas compared with normal mucosa. Levels of *NAP1L1* were not increased in these samples ($^{*}P < .01$). Data are mean ± SEM. N, normal mucosa (n = 6); C, colorectal carcinoma (n = 9); GAPDH, glyceraldehyde phosphate dehydrogenase.

significantly increased (approximately 10-fold; $P < .01$) in these adenocarcinomas compared with normal mucosa (Fig. 5). In contrast, *NAP1L1* was not increased in colorectal carcinomas, although it was significantly overexpressed in SICs.

Protein Immunohistochemistry of MTA1 in Gastrointestinal Carcinoids

The immunohistochemical expression of MTA1 in gastrointestinal carcinoids was examined by immunohistochemistry by AQUA quantitation of staining intensity with YTMA60. Expression values of the MTA1 protein were correlated with clinical evidence of disease (liver or LN metastases).

MTA1 was identified using anti-MTA1 (Santa Cruz; A11; 1/100) as previously described in a prostate TMA.[32] Expression levels of MTA1 on YTMA60 were increased in primary carcinoid tumors that developed liver or LN metastases compared with both primary tumors that had not metastasized (did not develop liver or LN metastases; $P < .02$) and normal mucosa ($P < .001$; Fig. 6). Similarly, levels of MTA1 were increased in liver metastatic tissue ($P < .001$) and LN metastatic tissue ($P < .001$) compared with healthy tissues (Fig. 7). Levels of MTA1 in primary tumors that were nonmetastatic were increased compared with normal mucosa ($P < .002$) but were significantly lower than MTA1 levels in liver ($P < .02$) and LN metastases ($P < .02$). Analysis of the SIC tumor groups individually demonstrated that MTA1 levels were significantly increased ($P < .001$) in all metastatic types compared with nonmetastatic carcinoids and normal mucosa.

On YTMA25, expression levels of MTA1 were increased in metastatic breast tumors ($P < .05$) and in malignant prostatic tumors ($P < .05$) compared with normal mucosa (Fig. 7). These data are consistent with previous reports that identified MTA1 as a marker of malignancy for breast and prostatic cancer.[31,32] MTA1 is therefore a marker of tumor malignancy irrespective of the cell of origin (epithelial or neuroendocrine).

DISCUSSION

These data demonstrate that with a QRT-PCR approach in malignant SIC tumors and metastatic tissue samples, the candidate marker genes *NAP1L1*, *MAGE-D2*, and *MTA1* were significantly

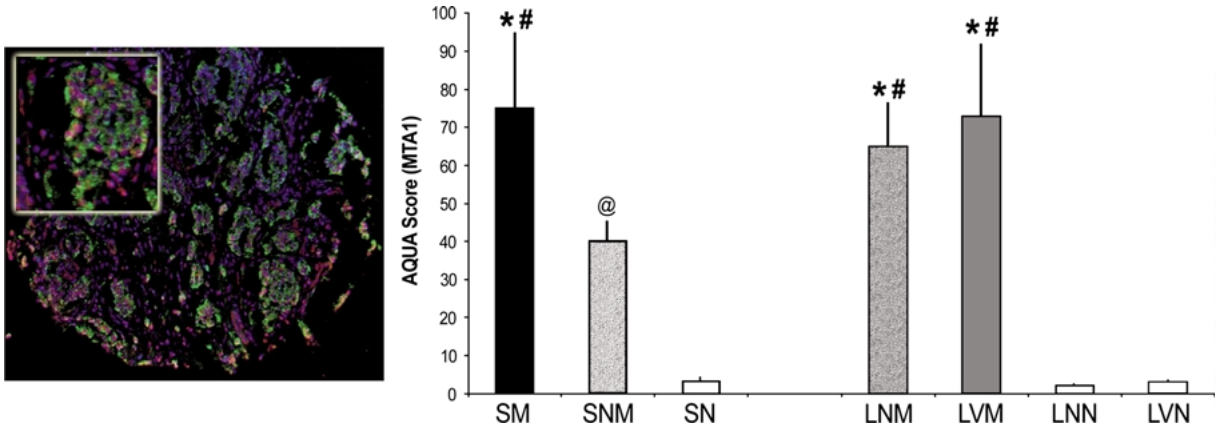

FIG. 6. Expression levels of metastasis-associated protein 1 (MTA1) determined by immunohistochemistry and automated quantitative analysis (AQUA) quantitation (*left panel*) in gastrointestinal carcinoids (n = 55), liver metastases (n = 12), and lymph nodes (LNs) (n = 21) and in normal tissue (n = 55) on YTMA60 (*right*). *Left panel*: Pseudo 3-color image of a small intestinal carcinoid (SIC). Immunostaining of MTA1 is invariably localized to the nucleus. Blue, nuclei (4′,6-diamidino-2-phenylindole; DAPI); green, tumor mask (cytokeratin; Alexa488); red, *MTA1* (Cy5). Dual nuclear staining (red and blue) results in purple (original magnification, ×100). Significant overlap is shown between DAPI and nuclear MTA1 staining (*inset*). *Right panel*: Levels of MTA1 were significantly overexpressed both in primary malignant SIC tumors that had developed metastases (SM) and in primary nonmetastatic SIC tumors (SNM) compared with normal small-intestinal mucosa (SN). *MTA1* was also overexpressed in primary malignant SIC tumors compared with nonmetastatic tumors. Expression levels of MTA1 were increased in LN metastatic tissue (LNM) and in liver metastases (LVM) compared with normal LNs (LNN) and normal liver (LVN), respectively. In addition, MTA1 levels were increased in liver metastases and LN metastatic tissue compared with primary nonmetastatic SIC tumors ($^{*}P < .001$; $^{\#}P < .002$; $^{@}P < .02$). Data are mean ± SEM.

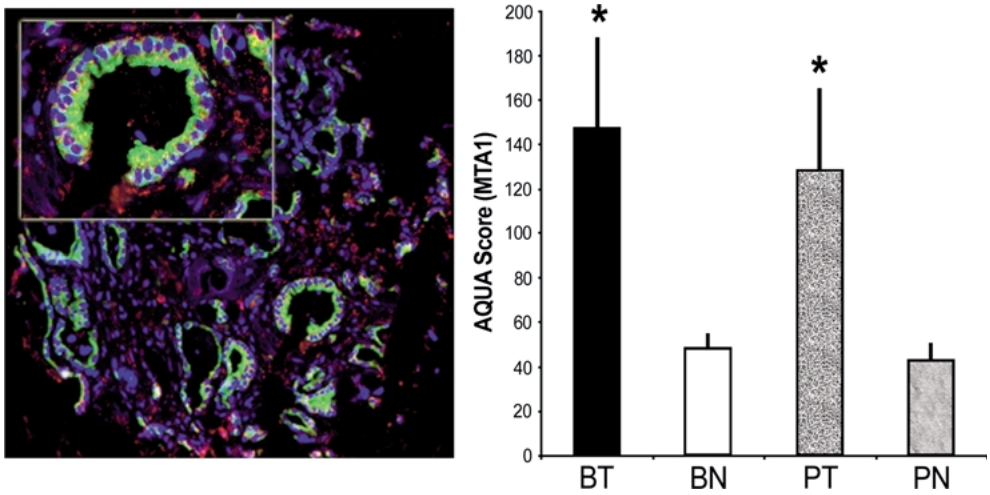

FIG. 7. Expression levels of metastasis-associated protein 1 (MTA1) determined by immunohistochemistry and automated quantitative analysis (AQUA; *left*) in adenocarcinomas on YTMA25 (*right*). *Left*: Pseudo 3-color image of a prostatic adenocarcinoma. Immunostaining of MTA1 was invariably nuclear localized. Blue, nuclei (4′,6-diamidino-2-phenylindole; DAPI); green, tumor mask (cytokeratin; Alexa488); red, MTA1 (Cy5). Dual nuclear staining (red and blue) results in purple (original magnification, ×100). Significant overlap is shown between DAPI and nuclear *MTA1* staining (*inset*). *Right*: Levels of MTA1 were significantly overexpressed in malignant breast tumors (BT; n = 10) and in prostate tumors (PT; n = 10) compared with normal matched tissue, normal breast (BN; n = 7), and normal prostate (PN; n = 7; $^{*}P < .05$). Data are mean ± SEM.

overexpressed in all samples. Increased gene expression levels identified both malignant primary tumors and their metastases. These data confirm the utility of both *MAGE-D2* and *MTA1* as candidate markers of metastases and identify that *NAP1L1* may be a novel marker of SIC tumor metastasis.

NAP1L1 is the human counterpart of the yeast NAP-I protein, a histone-binding factor required for the maintenance of cumulative nucleosome formation in vivo.[38] As a transcription factor,[38] it is involved in regulating mitosis in yeast,[39] and in humans, *NAP1L1* is a nuclear protein involved in chromatin assembly

and DNA replication.[40] Increased expression of *NAP1L1* may be related to the progression of cell growth, because levels of both *NAP1L1* mRNA and protein increase rapidly in conjunction with the induction of cellular proliferation in a T-lymphoid cell model.[40] *NAP1L1* has been examined in the clinical setting by using high-density oligonucleotide DNA arrays and serological identification of antigens by recombinant expression cloning technology. In genome-wide profiling by using Affymetrix technology, *NAP1L1* has been identified to be overexpressed in fetal liver compared with adult liver[41] and in hepatoblastomas compared with nondiseased adult livers.[25] Serological identification of antigens by recombinant expression cloning technology, which is used to search for genes whose products elicit antibody production in the patient, has identified *NAP1L1* to be a potential serological antigen in a subset (< 5%) of breast, renal, and colorectal cancer patients, but the mRNA study results were largely negative.[42] Our current study demonstrates that mRNA for *NAP1L1* is upregulated in small-intestinal neuroendocrine cell–derived neoplastic tissue rather than epithelial-derived tumor tissue in the gastrointestinal tract. In addition, metastases from primary SIC also demonstrated an increased *NAP1L1* signal. This confirms that *NAP1L1* is a marker of carcinoid tumor metastasis.

MAGE-D2 encodes one of the cancer testes families of genes.[43] The putative ancestral *MAGE* gene is the *MAGE-D* gene family,[44] but unlike the testis- and tumor-specific expression of many MAGE genes, *MAGE-D2* mRNA is also expressed in healthy human tissues and in most cell types examined.[43] It does not encode any of the known *MAGE* antigenic peptides[43] and cannot therefore be considered a classic cancer testes antigen. *MAGE-D2* has been examined in the clinical setting by using high-density oligonucleotide DNA arrays and has been identified as a molecular marker to predict liver metastases from colorectal tumors.[45] It is overexpressed in > 75% of primary colon tumors with metastases.[45] The function of *MAGE-D2* is unknown, but its similarity to troponin indicates that it is involved in cell adhesion,[46] and increased expression is thought to facilitate the adhesion of cancer cells to vascular epithelium.[45] The overexpression of *MAGE-D2* in malignant primary SIC tumors and metastases demonstrates that assessment of this marker has utility as a component of a panel for identifying and predicting the malignant and metastatic behavior of carcinoid tumors.

MTA1 is a component of the nucleosome remodeling and histone deacetylation complex, which is associated with adenosine triphosphate–dependent chromatin remodeling and histone deacetylase activity.[47] Functionally, *MTA1* is also involved in the transcriptional repression of methylated DNA,[48] and in breast tissue, *MTA1* represses estrogen receptor–mediated transcription and is therefore estrogen antagonistic.[49] In breast tissue, the presence of estrogen receptors is usually associated with less aggressive tumors[50]; in vitro analysis of *MTA1* expression in estrogen receptor–positive cells is associated with increased proliferation and a more aggressive phenotype.[49] *MTA1* is normally expressed at low levels in various tissues but, like the cancer testes antigens, is more highly expressed in the testis.[51] On DNA arrays, *MTA1* is selectively overexpressed in metastatic prostate cancer compared with clinically localized prostate cancer and benign prostate tissue.[32] In a prostate cancer TMA, a strong relationship between MTA1 expression and prostate cancer progression has been identified.[32] The close correlation between mRNA and protein levels of *MTA1* and the utility of this marker to identify progressive disease in a range of tumors indicate that this marker will be useful in identifying and predicting the metastatic behavior of SIC tumors.

In conclusion, our data demonstrate overexpression of *NAP1L1*, *MAGE-D2*, and *MTA1* mRNA and MTA1 protein in tumor and metastatic SIC tissue, indicate the utility of these markers for identifying metastatic gastrointestinal carcinoid cells (tissue) in both liver and LNs, and demonstrate that *NAP1L1* may be a neuroendocrine tumor–specific marker. A biomarker panel assessment of SIC tumors that uses these genes may thus have utility as a predictor of clinical behavior and may redefine diagnostic and therapeutic strategies, as well as facilitate prognostic assessment.

The traditional clinical, morphological, and pathologic criteria for defining malignancy in SICs are of limited utility. This study has identified several novel and known genes with well-characterized biological functions that identify malignant primary small-intestinal tumors and metastases. The ability to define when a tumor is malignant and will develop metastases will provide a biological rationale for the surgical management of these poorly characterized tumors.

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