Genetic Differentiation of Appendiceal Tumor Malignancy

A Guide for the Perplexed

Irvin M. Modlin, MD, PhD, Mark Kidd, PhD,* Igor Latich, BA,* Michelle N. Zikusoka, BS,* Geeta N. Eick, PhD,* Shrikant M. Mane, PhD,‡ and Robert L. Camp, MD, PhD†*

Objective: To use differential gene expression of candidate markers to discriminate benign appendiceal carcinoids (APCs) from malignant and mixed cell APCs.

Summary Background Data: Controversy exists in regard to the appropriate surgical management of APCs since it is sometimes difficult to predict tumor behavior using traditional pathologic criteria. We have identified 5 differentially expressed genes (a mitosis-regulatory gene *NAP1L1*, an adhesin *MAGE-D2*, an estrogen-antagonist, the metastasis marker *MTA1*, the apoptotic marker *NALP*, and chromogranin A) that define gut neuroendocrine cell behavior.

Methods: Total RNA was isolated using TRIzol reagent from 42 appendiceal samples, including appendiceal carcinoids identified at exploration for appendicitis (no evidence of metastasis; n = 16), appendicitis specimens (n = 11), malignant appendiceal tumors (>1.5 cm, evidence of metastatic invasion; n = 7), and mixed (goblet) cell appendiceal adenocarcinoids (n = 3), normal appendiceal tissue (n = 5), and 5 colorectal cancers. Gene expression (*CgA*, *NAP1L1*, *MAGE-D2*, *MTA1*, and *NALP1*) was examined by Q-RT PCR (Applied Biosystems) and quantified against *GAPDH*.

Results: *CgA* message was elevated (>1000-fold, $P < 0.05$) in all tumor types. *NAP1L1* was elevated (>10-fold, $P < 0.03$) in both malignant and goblet cell adenocarcinoids compared with normal and incidental lesions ($P < 0.006$). *MAGE-D2* and *MTA1* message were significantly elevated (>10-fold, $P < 0.01$) in the malignant and goblet cell adenocarcinoid tumors but not in the appendicitis-associated carcinoids or normal mucosa. The apoptotic marker, *NALP1*, was overexpressed (>50-fold, $P < 0.05$) in the appendicitis-associated and malignant appendiceal carcinoids but was significantly decreased (>10-fold, $P < 0.05$) in the goblet cell adenocarcinoids. Elevated CgA transcript and protein levels indicative of a carcinoid tumor were identified in one acute appendicitis sample with no histologic evidence of a tumor.

Conclusions: These data demonstrate that malignant APCs and goblet cell adenocarcinoids have elevated expression of *NAP1L1, MAGE-D2*, and *MTA1* compared with appendiceal carcinoids identified at surgery for appendicitis. This and the differences in *NALP1* gene expression (decreased in goblet cell adenocarcinoids) provide a series of molecular signatures that differentiate carcinoids of the appendix. *CgA* identified all appendiceal tumors as well as covert lesions, which may be more prevalent than previously recognized. The molecular delineation of malignant appendiceal tumor potential provides a scientific basis to define the appropriate surgical management as opposed to morphologic assessment alone.

(*Ann Surg* 2006;244: 52–60)

Although generally regarded as an insignificant organ, the appendix at the turn of the 19th century was a source of considerable vexation to clinicians since the diagnosis of appendicitis was often difficult and outcome commonly associated with considerable morbidity and mortality.[1] It is of interest that a century later, although the problem of appendicitis has dramatically receded in the pantheon of medical problems, the pathologic delineation and management of appendiceal tumors, particularly carcinoids, remains an area of confusion and difficulty.[2–4] Even the great medical sage, Maimonides (1135–1204), were he to add a chapter to his remarkable text, *A Guide for the Perplexed*, would have struggled to define the precise nature of the lesion or define a rational therapeutic strategy given the lack of scientific data available.[5] The histology of the tumor is often equivocal, the identification of microscopic spread often difficult to identify if infection is present, and management decisions are often made on an empiric or purely judgmental basis. This investigation seeks to identify a molecular profile that can define appendiceal malignancy and be used to provide a basis for the development of rational surgical and oncological management.

Appendiceal carcinoids rank among the commonest types of gastrointestinal carcinoid tumor,[6] are usually discovered incidentally at surgery, and often only identified postoperatively on pathologic examination. Although in most instances the tumors are derived from the enterochromaffin cell (EC), there exist a number of variants, which include mucinous (goblet cell) adenocarcinoid, goblet cell carcinoid, and mixed adenocarcinoma-carcinoid.[7] In the latter instance, the distinct signet ring cell features have occasionally led to the diagnosis of poorly differentiated adenocarcinoma.[4,7] The

From the Departments of *Surgery and †Pathology and ‡HHMI Keck Institute, Yale University School of Medicine, New Haven, CT.

Supported in part by NIH R01-CA-097050 (to I.M.M.) and by the Bruggeman Medical Foundation (to M.K. and G.E.).

Reprints: Yale University School of Medicine, New Haven, CT 06520-8062. E-mail: Imodlin@optonline.net.

ISSN: 0003-4932/06/24401-0052
DOI: 10.1097/01.sla.0000217617.06782.d5

overall 5-year survival rate (71.0%) is among the best of all types of carcinoids and reflects both the early and often serendipitous detection of the tumor as well as, in most cases, the modest biologic behavior of the lesions.[4,6,7] In contrast, the survival of patients with mucinous variants is far less propitious and is estimated overall to be ~26 ± 19 months.[8] To date, optimal surgical strategies for appendiceal carcinoid tumors have been inferred from the retrospective analysis of surgical and pathologic series and are based on a variety of criteria including, but not limited to tumor size, mitotic index, meso-appendiceal invasion, lymph node spread, and location of the lesion.[4,9,10] Nevertheless, recurrences of these tumors or pseudo myxoma peritonei occurs; the failure to accurately define the biology of the tumor or precisely predict its pathologic behavior plays a major role in these developments.[4,9,10]

The relationship between the size of a tumor and its malignancy is, in many instances, an epiphenomenon, since metastasis occurs as a result of a series of well-characterized alterations in a variety of genes that define cell adhesion, proteolysis, migration, and angiogenesis. These regulatory genes can be identified at a molecular level and may provide the basis for generating a molecular profile of individual tumors that can be then used to predict behavior and thus allow for a refinement of therapeutic strategy.[11] Histologic analysis per se (of neuroendocrine lesions especially) cannot determine if a tumor is benign or malignant,[12] and despite considerable progress in molecular biology, molecular staging has yet to be integrated into current prognostic/predictive pathologic protocols.[12] In the absence of this combinatorial synergistic approach, the biologic basis of appendiceal carcinoid malignancy and metastasis is unknown and unpredictable; hence, it is currently not possible to accurately or adequately define appropriate surgical management. This is reflected in the large SEER (NCI) database study that concluded that the most important predictor of survival was "extent" of disease and not histology.[13]

At this time, no molecular signature exists to differentiate between a malignant and benign carcinoid of the appendix. Such information would be of considerable clinical importance when appendiceal tumors are identified, and the need for further surgery is uncertain, given that current management strategies are based on relatively simplistic macroscopic criteria and light microscopy.[14] Our group has identified the following candidate genes to be differentially overexpressed in a variety of gastrointestinal neuroendocrine cells, including EC and ECL cells: *Chromogranin A* (*CgA*), the mitotic regulatory gene *NAP1L1*, the adhesion gene *MAGE-D2*, the malignancy marker gene, *MTA1*, and the caspase-3 activating apoptosis gene, *NALP1*.[15] In previous studies, we have demonstrated that the differential expression of these genes enables the delineation of localized nonmetastatic (type I/II) gastric carcinoids from aggressive sporadic or neuroendocrine carcinoma type tumors (type III/IV).[16] Based upon the differential expression of these genes in gastric carcinoid tumors, we hypothesize that these genes will enable discrimination between different types of appendiceal tumors (nonmalignant and those identified incidentally at surgery during routine [en passant] or acute appendectomy versus aggressive and metastatic).

Small intestinal EC tumors (carcinoids) and appendiceal carcinoids are derived from the EC cell, although overall the former behave more aggressively than appendiceal lesions.[7] Nevertheless, both can exhibit local spread, lymph node metastasis and distant (liver) metastases, while the appendiceal goblet cell variants may produce myxoma peritonei and ovarian implantation lesions.[17,18]

To establish and verify the clinical utility of a PCR-based protocol for the tissue resource, we initially examined archival material. For this, we used paraffin-embedded tissue and archival samples, which constitute the majority of bankable tissue available for analysis. We examined archival paraffin-embedded samples (collected between 1965 and 2003 by the Yale Department of Pathology) to evaluate the expression of the marker genes of interest, and we correlated their expression with clinical data, tumor size, and the presence of clinically and histologically documented metastasis. Thereafter, we prospectively examined gene expression in surgically collected appendiceal samples, largely from patients with acute appendicitis, to establish the utility of this molecular approach in readily available samples.

METHODS

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

Patients and Samples

Tissue Specimens

Paraffin-embedded tumor tissue blocks were collected from 25 patients (M:F = 8:17; median age, 40 years; range, 11–95 years) with histologically proven appendiceal carcinoid tumors who had undergone surgical resection for acute appendicitis or a primary tumor between 1965 and 2004 in the Yale University Department of Surgery. Control tissue included colorectal adenocarcinomas (n = 5), and normal tissue samples from adjacent, macroscopically normal, nontumor mucosa (n = 5) were also examined.

Appendiceal samples were prospectively collected from twelve patients (M:F = 8:4; median age, 19 years; range, 6–38 years) with acute or suppurative appendicitis (n = 11) and one histologically proven invasive appendiceal carcinoid tumor, including mucosa (n = 2), omental (n = 3), and liver metastases (n = 1) who had undergone emergency surgical resection in 2004 at the Yale University Department of Surgery, and snap-frozen in liquid nitrogen.

Tissue Techniques

RNA Isolation

Paraffin blocks were deparaffinized and digested as previously described.[19,20] Total RNA was isolated from paraffin-blocks or frozen sections using TRIzol reagent (Invitrogen, Carlsbad, CA) as described.[21] RNA was then dissolved in DEPC water, measured spectrophotometrically and an aliquot analyzed on a denaturing gel using electrophoresis to check the quality of RNA isolated.

Q RT-PCR

Fifty-two samples were examined by quantitative real-time PCR using the Assays-on-Demand approach (Applied Biosystems) since this system identifies RNA of 60 to 150 base pairs in length and is thus particularly suitable for paraffin-tissue examination.[20] Messages from *Chromogranin A*, *NAP1L1*, *MAGE-D2*, *MTA1*, *NALP1* and the housekeeping gene, *GAPDH*, were quantitatively measured as described.[21] Q RT-PCR was performed using the ABI 7900 Sequence Detection System. Total RNA from each sample was reverse transcribed using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. Quantitative real-time PCR analysis was then performed in triplicate. Briefly, cDNA in 7.2 μL of water was mixed with 0.8 μL of 20× Assays-on-Demand primer (*CgA* = Hs00174938; *NAP1L1* = Hs00748775, *MAGE-D2* = Hs00374760, *MTA1* = Hs00183042, *NALP1* = Hs00248187, *GAPDH* = Hs99999905) and probe mix, 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, then 95°C for 10 minutes, followed by 40 cycles at 95°C/0.15 minutes and 60°C /1 minute. A standard curve was generated for each gene using cDNA 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 and calculated using the relative standard curve method (ABI, User Bulletin #2).

Immunostaining of Appendicitis Specimens

Triple-color immunostaining was performed on tissue sections using monoclonal antibodies against CgA to identify the cellular location of this marker.[21,22] For antigen retrieval purpose, sections were initially immersed in citrate buffer (10 mm sodium citrate, pH 6.0) and subjected to 1 × 10 minutes high temperature-high pressure treatment followed by treatment with 0.3% H_2O_2 in methanol for 30 minutes at 37°C to inactivate endogenous peroxidase. Slides were then incubated for 24 hours at 4°C with a 1:1000 dilution of the anti-CgA mouse monoclonal antibody (DAKO Corp, Carpinteria, CA) and rabbit anticytokeratin antibody cocktail (AE1/AE3; DAKO Corp) (to identify tumor carcinoid cells). Goat anti-mouse antibodies conjugated to a horseradish peroxidase-decorated dextran polymer backbone (Envision; DAKO Corp) were used as a secondary reagent for CgA, and goat anti-rabbit antibodies conjugated to Alexa-488 fluor (DAKO Corp) were used to identify cytokeratin. CgA staining was visualized with a fluorescent chromogen (Cy-5-tyramide; NEN Life Science Products, Boston, MA) and nuclei were visualized by 4′, 6-diamidino-2-phenylindole (DAPI). A pathologist (R.L.C.) examined staining expression.

Statistical Analysis

Results are expressed as mean ± SEM; n indicates the numbers of patients in each study group. Statistical significance was calculated by the two-tailed Student's test for paired and unpaired values as appropriate, with $P < 0.05$ representing significance. Linear regression analysis was performed to evaluate the relationship between CgA levels and tumor size.

RESULTS

Clinical Results

Sixteen of the 25 paraffin-embedded appendiceal tumors were carcinoids identified incidentally postoperatively with no evidence of serosal invasion or lymph node metastasis (Table 1). The mean size (±SEM) of these tumors was 0.68 ± 0.075 cm. The mean age of the patients at diagnosis was 36.9 years and the follow-up was 113 months. None of the patients subsequently developed lymph node or liver metastases and was considered disease-free.

Nine of the remaining tumors presented with local invasion and liver or lymph node metastases. Three exhibited a goblet-cell phenotype and were considered to be appendiceal adenocarcinoids. The mean size of the 9 tumors was significantly greater than the 16 incidentally identified lesions (2.7 ± 0.4 vs. 0.7 ± 0.08 cm; $P < 0.00002$). The mean age of these patients at diagnosis was 57 years and the follow-up was 199 months. One patient subsequently developed liver metastases. All patients in this group were considered overtly disease-positive.

The 11 fresh frozen samples had suppurative appendicitis (n = 3 samples with peri-appendicitis) with no pathologic evidence of carcinoid tumor.

RNA Isolation

RNA isolated from twenty-five paraffin-embedded appendiceal carcinoid tumor specimens and 10 control samples had concentrations ranging from 0.02 to 0.14 μg/μL. Using Assays-on-Demand (Applied Biosystems), *GAPDH* was amplified in all samples using Q RT-PCR (Fig. 1). These results confirm, as previously determined, that this approach is suitable for paraffin-tissue examination.[20]

TABLE 1. Clinical Evaluation of 25 Patients With Appendiceal Carcinoids

Group	n	Age (yr) [Median (Range)]	Gender (M:F)	Tumor Size (cm)	Presence of Metastases	Follow-Up (mo) [Median (Range)]	Subsequent Pathology
Incidental	16	30 (11–73)	6:10	0.7 ± 0.08	None	33 (8–468)	None
Malignant	9	59 (39–95)*	2:7	2.7 ± 0.4	LI: n = 3 LNM: n = 5 LVM: n = 1	113 (29–443)	n = 1

*P = 0.053 versus [incidental.]
LI indicates locally invasive; LNM, lymph node metastases; LVM, liver metastases.

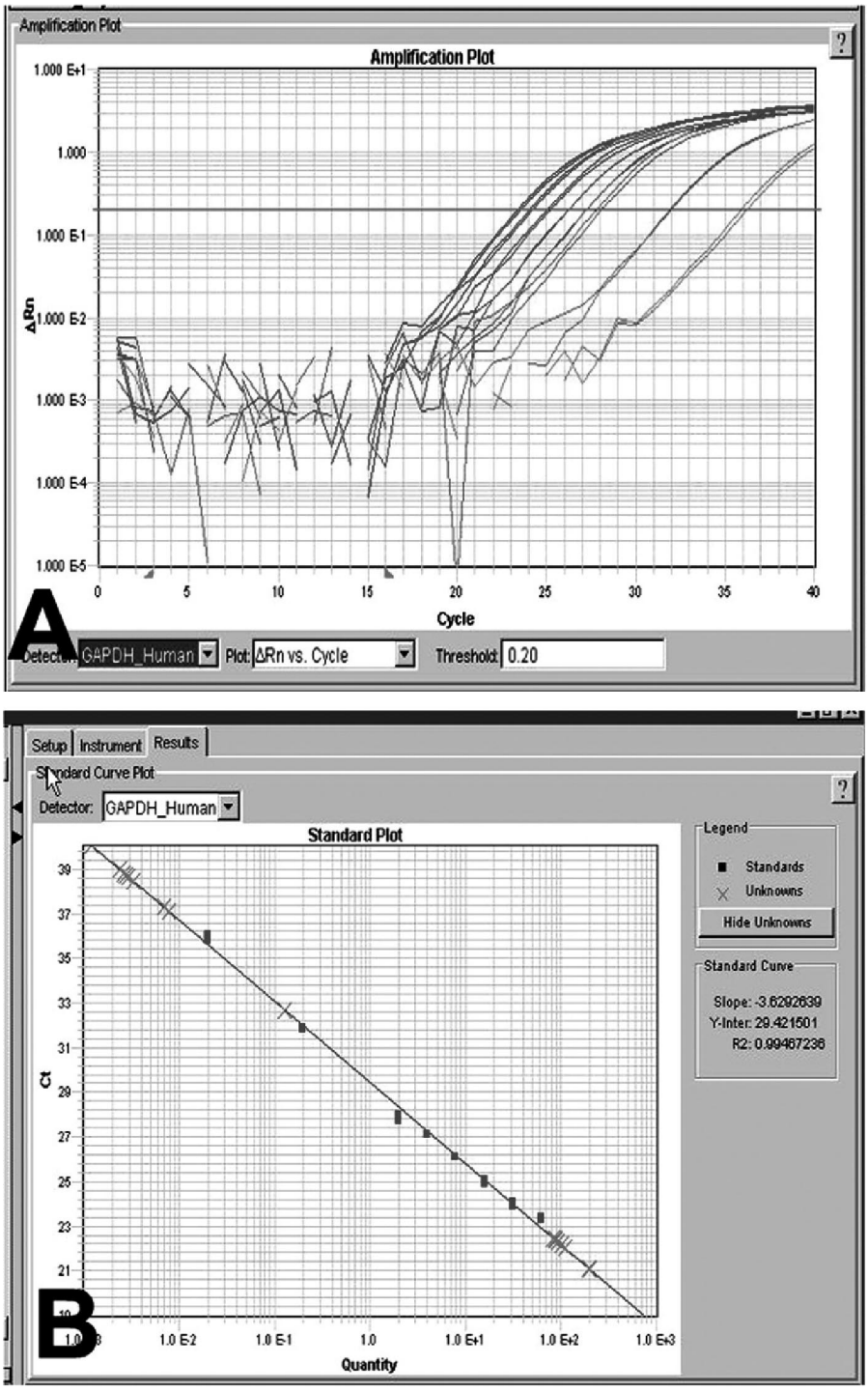

FIGURE 1. Real-time PCR plots using the *Assays-on-Demand* approach (Applied Biosystems) of the housekeeping gene, *GAPDH*, in paraffin-embedded appendiceal carcinoid tissue. A, Amplification plot of PCR fluorescence versus cycle number for the pooled carcinoid samples. This demonstrates concentration-dependent amplification of *GAPDH*. B, Standard curve of *GAPDH* (C_T values plotted vs. the log of the initial amount of cDNA) derived from A. The level of gene expression in a sample is calculated from the C_T and standard curve. C_T, threshold cycle.

Quantitative Real-Time PCR

Chromogranin A

Chromogranin A was amplified in all appendiceal tumor samples and was significantly elevated (100–1000-fold; $P < 0.05$) in the incidental and malignant appendices and ~50-fold in the goblet cell adenocarcinoids compared with normal mucosa and to colorectal adenocarcinomas (Fig. 2). Malignant tumors also had elevated *CgA* levels compared with incidental lesions (98 ± 41 vs. 1.02 ± 0.6, $P = 0.048$). The fact that the *CgA* levels of the goblet cell adenocarcinoids were comparable to the serendipitously identified lesions might be considered to reflect neuroendocrine cell number. An examination of the relationship between tumor size and *CgA* message levels, however, only identified a moderate correlation between these 2 parameters ($R^2 = 0.304$, $P = 0.063$). Although it has been suggested that a relationship

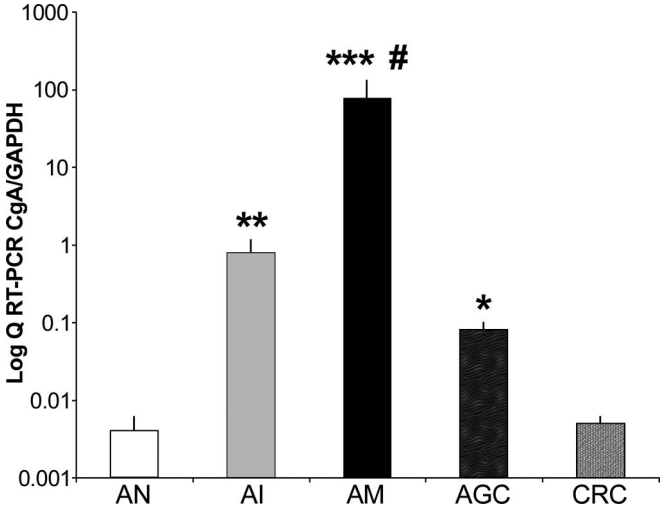

FIGURE 2. Message levels of *CgA* determined by Q RT-PCR. Levels of *CgA* were significantly overexpressed (~100 times) in incidental (benign) appendiceal carcinoids (AI), malignant appendiceal carcinoids (AM; >1000 times), and appendiceal carcinoids with goblet cell morphology (AGC; ~20 times) as compared with normal mucosa (AN). Malignant carcinoids also had elevated *CgA* levels compared with incidental and goblet cell carcinoids. No differences were noted between colorectal cancer (CRC) samples and normal mucosa (AN). $^{\#}P = 0.05$. $^{*}P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.005$. Data are mean ± SEM.

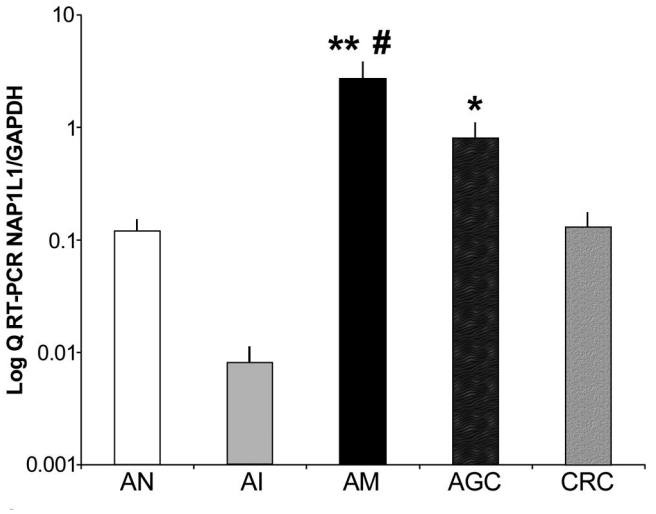

FIGURE 3. Message levels of *NAP1L1* determined by Q RT-PCR. Levels of *NAP1L1* were significantly overexpressed in malignant appendiceal carcinoids (AM; ~15 times), and in appendiceal carcinoids with goblet cell morphology (AGC; ~8 times) compared with normal mucosa (AN). Malignant carcinoids also had elevated *NAP1L1* levels compared with incidentally identified carcinoids (AI). No significant differences were noted between either incidental (benign) appendiceal carcinoids or colorectal cancer (CRC) samples and normal mucosa. $^{*}P = 0.03$. $^{**}P < 0.01$. $^{\#}P = 0.006$. Data are mean ± SEM.

exists between plasma CgA and tumor size,[23] our data suggest that tumor size and mRNA levels may not be as closely correlated as previously considered. This is consistent with other reports indicating that cellular secretory product levels may have little relationship to plasma values.[24]

NAP1L1

NAP1L1 is a nuclear protein involved in chromatin assembly and DNA replication.[25] Messenger RNA levels of *NAP1L1* were elevated >10-fold ($P < 0.03$) in malignant appendiceal carcinoid tumors and in goblet cell adenocarcinoids compared with normal mucosa. Levels were also elevated >100-fold ($P < 0.006$) in malignant carcinoids compared with the incidentally identified lesions (Fig. 3). Levels in colorectal adenocarcinomas were not different to normal mucosa.

MAGE-D2

MAGE-D2 is an adhesion gene and potential predictive marker of colorectal liver metastases.[26] Levels of *MAGE-D2* were elevated 10- to 100-fold ($P < 0.01$) in the malignant appendiceal carcinoids, goblet cell adenocarcinoids, and colorectal adenocarcinomas compared with normal mucosa (Fig. 4). Both malignant appendiceal tumors and colorectal tumors had elevated expression levels of *MAGE-D2* compared with incidentally identified carcinoids. No differences in expression were noted between the latter and normal mucosa.

MTA1

MTA1 is an estrogen-antagonistic breast cancer malignancy gene that has been used for the identification of progressive (metastatic) disease in a range of tumors including breast, hepatocellular, esophageal, gastric, and colorectal carcinomas.[27–31] Message levels of *MTA1* were elevated 20- to 1000-fold, ($P < 0.01$) in the malignant appendiceal carcinoids, goblet cell adenocarcinoids, and colorectal adenocarcinomas compared with normal mucosa (Fig. 5). Both malignant appendiceal tumors and colorectal tumors had elevated levels of *MTA1* compared with incidental carcinoids. No differences in expression were noted between the incidental tumors and normal mucosa.

NALP1

The apoptotic marker, *NALP1*, was overexpressed ~50- to 100-fold ($P < 0.05$) in the incidentally identified ("benign") and malignant appendiceal carcinoids compared with normal mucosa (Fig. 6). *NALP1* was significantly decreased ($P < 0.05$) in the goblet cell adenocarcinoids and colorectal adenocarcinomas compared with normal mucosa. In addition, malignant carcinoids had significantly elevated expression compared with all other tumor types.

Clinical Relationship Between Gene Expression Levels and Appendiceal Disease

Two of the 25 patients included in this study were lost to follow-up; both of these patients belonged to the cohort of 16 patients with "incidental" tumors. None of the remaining 14 patients with incidental tumors for whom follow-up information was available was subsequently identified with lymph node or liver metastases (mean follow-up, 113 months; range, 8–372 months) (Table 1). In the group of 9 patients diag-

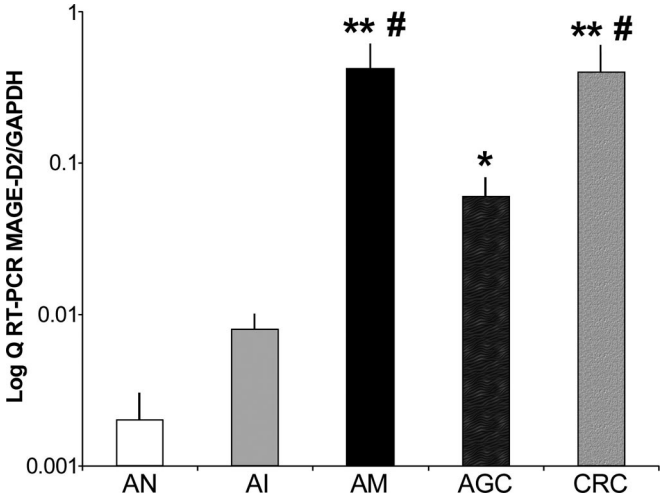

FIGURE 4. Message levels of *MAGE-D2* determined by Q RT-PCR. Levels of *MAGE-D2* were significantly overexpressed in malignant appendiceal carcinoids (AM; ~100 times), in appendiceal carcinoids with goblet cell morphology (AGC; ~12 times), and in colorectal cancer (CRC; ~100 times) samples compared with normal mucosa (AN). No significant differences were noted between incidental (benign) appendiceal carcinoids (AI) or normal mucosa. Malignant carcinoids and CRC tumors had elevated *MAGE-D2* levels compared with incidental carcinoids. $^{*}P < 0.01$. $^{\#}P < 0.005$. $^{**}P < 0.001$. Data are mean ± SEM.

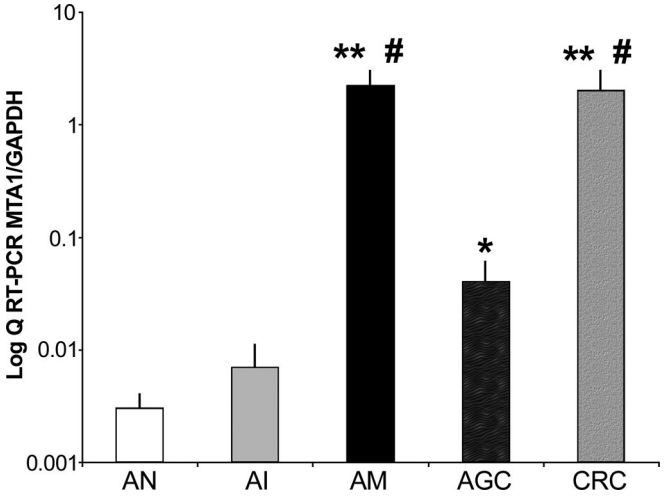

FIGURE 5. Message levels of *MTA1* determined by Q RT-PCR. Levels of *MTA1* were significantly overexpressed in malignant appendiceal carcinoids (AM; ~1000 times), in appendiceal carcinoids with goblet cell morphology (AGC; ~15 times), and in colorectal cancer (CRC; ~1000 times) samples compared with normal mucosa (AN). No significant differences were noted between incidental (benign) appendiceal carcinoids (AI) or normal mucosa. Malignant carcinoids and CRC tumors had elevated *MTA1* levels compared with incidental carcinoids. $^{*}P < 0.01$. $^{\#}P < 0.005$. $^{**}P < 0.001$. Data are mean ± SEM.

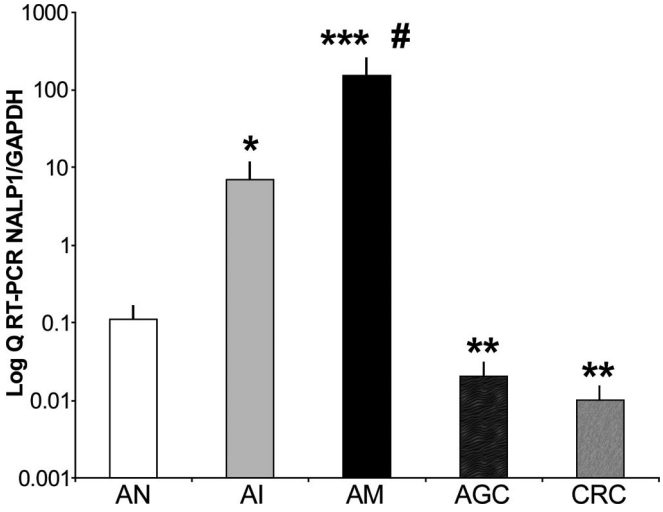

FIGURE 6. Message levels of *NALP1* determined by Q RT-PCR. Levels of *NALP1* were significantly overexpressed (~100 times) in incidental (benign) appendiceal carcinoids (AI) and in malignant appendiceal carcinoids (AM; >1000 times) compared with normal mucosa (AN). Levels were significantly decreased in appendiceal carcinoids with goblet cell morphology (AGC; ~15 times) and in colorectal cancer (CRC; ~1000 times) samples compared with normal mucosa (AN). Malignant carcinoids had elevated *NALP1* levels compared with incidental carcinoids, goblet cell carcinoids, and CRC tumors. $^{\#}P = 0.05$. $^{**}P = 0.05$. $^{*}P < 0.01$. $^{***}P < 0.005$. Data are mean ± SEM.

nosed with malignant tumors, 1 patient developed liver metastases (mean follow-up for this group, 199 months; range, 33–468 months). The small number of patients precludes a robust statistical analysis of this data.

Pathologically, the "malignant group" tumors could be separated into tumors with local invasion (n = 3), tumors with lymph node metastases (n = 5), and a tumor with a liver metastasis (n = 1). An examination of gene expression levels in these categories demonstrated that 4 of the 5 candidate genes could be associated with lymph node or liver metastases. Thus, levels of *CgA, NAP1L1, MAGE-D2*, and *MTA1* were ~100 fold higher in the tumors that had pathologic evidence of metastases compared with appendiceal carcinoids that were locally invasive. Interestingly, gene expression levels in the tumors classified as locally invasive were not different to the 16 patients with incidental tumors suggesting a threshold of expression may be required prior to the development of metastatic disease. The lack of overlap in gene expression levels between tumors that were classified as incidental (ie, apparently disease-free) and tumors classified as malignant (ie, overtly disease-positive) indicates that the measurement of gene levels (four of the 5 markers) has potential clinical utility.

Prospective Q RT-PCR Analysis of Fresh Frozen Appendices

Levels of *CgA* were used to determine whether any covert appendiceal tumors could be identified in 11 prospectively collected surgical acute appendicitis cases. Levels were

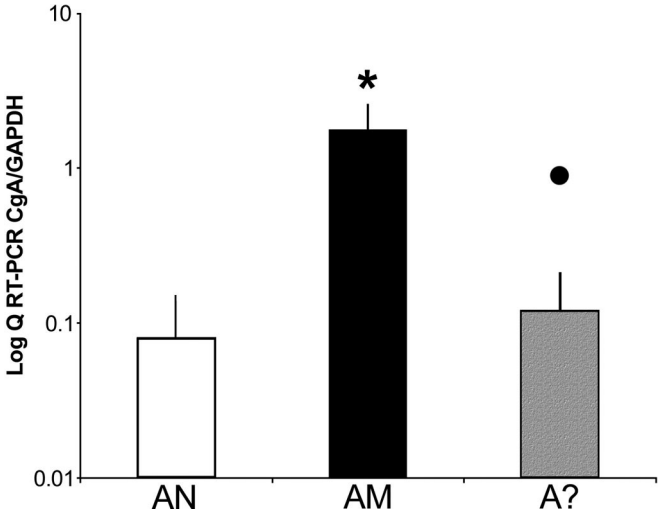

FIGURE 7. Message levels of *CgA* determined by Q RT-PCR. Levels of *CgA* were significantly overexpressed (~15 times) in the malignant appendiceal tumor and its liver and omental metastases (AM) compared with normal mucosa (AN). Levels were not different from normal mucosa in 10 of the acute appendicitis specimens (A). One acute sample had elevated *CgA* message. ● = acute appendicitis sample with abnormally elevated CgA gene expression. *$P < 0.005$. Data are mean ± SEM.

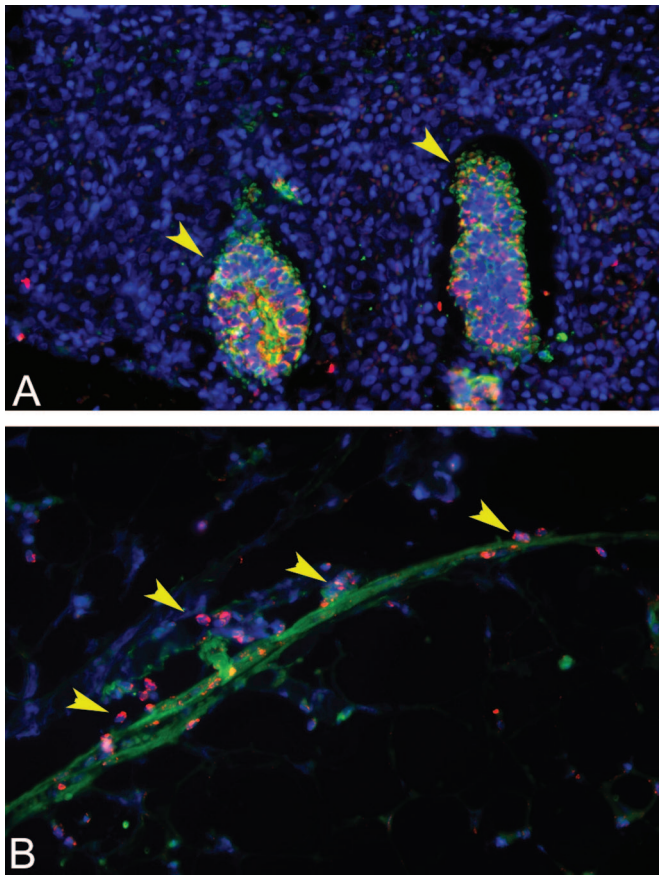

FIGURE 8. Expression levels of CgA determined by immunohistochemistry in a specimen of acute suppurative appendicitis with elevated CgA transcript levels. Tri-color imaging of this section demonstrated significant overlap between cytoplasmic CgA and cytokeratin staining in discrete areas. These included the area adjacent to the lumen (A) where CgA-positive cells forming glandular-type structures were noted and in fatty areas where individual CgA-positive cells could be noted (B). Yellow arrow heads identify CgA-positive cells. Blue, nuclei (DAPI); green, cytokeratin (Alexa488); red, CgA (Cy5). Dual CgA and cytokeratin staining (red and green) results in a yellow color (original magnification ×100).

compared with normal appendiceal samples and with a highly malignant appendiceal tumor with liver and omental metastases. *CgA* levels were elevated in the positive controls compared with normal mucosa ($P < 0.0008$) and 10 of the 11 appendicitis specimens ($P < 0.005$) (Fig. 7). Levels of *CgA* were low in appendicitis samples, except for 1 case (acute suppurative appendicitis with peri-appendicitis) that exhibited *CgA* levels at ~10 times the levels present in other tissues. This was significantly ($P < 0.02$) elevated compared with both the normal mucosa and other appendicitis specimens. Expression levels of the other 4 marker genes (*NAP1L1*, *MAGE-D2*, *MTA1*, and *NALP1*) were not elevated in this sample and levels were not different to expression levels in the 16 "incidental" carcinoids (examined above). Staining of this appendiceal specimen demonstrated the presence of a cluster of CgA immunopositivity (Fig. 8). This was absent in samples without elevated CgA gene expression. Based on these observations, it is plausible that one of the 11 surgically resected appendiceal specimens is worthy of consideration to be upgraded to a covert appendiceal tumor.

DISCUSSION

These data demonstrate, using a Q RT-PCR approach in paraffin-embedded tissue, that malignant appendiceal carcinoids, which like small intestinal carcinoids are derived from the EC cell, have elevated expression of *CgA*, *NAP1L1*, *MAGE-D2*, and *MTA1* compared with incidentally identified appendiceal carcinoids. Goblet cell adenocarcinoids, which are a mixed cell tumor type that also includes neuroendocrine cells, also expressed elevated *CgA*, *NAP1L1*, *MAGE-D2*, and *MTA1* compared with normal mucosa. These levels were not as elevated as in the malignant EC-derived carcinoid tumors. Incidentally identified tumors, like overt malignant carcinoids, had elevated *CgA* and elevated *NALP1* expression. In contrast, adenocarcinoids had significantly decreased *NALP1* expression. The difference in *NALP1* expression (elevated in appendiceal carcinoids, decreased in goblet cell adenocarcinoids) provides a molecular marker to differentiate between carcinoids and adenocarcinoids of the appendix. Previous studies have not identified specific genetic differences in EC cell-derived appendiceal tumors compared with other appendiceal tumors or to normal mucosa. In one study, no mutations were identified in K-*ras*, β-*catenin*, or *DPC4* in goblet cell carcinoids, and *p53* was not elevated,[32] while another study determined that mucinous and nonmucinous carcinomas of appendix had similar genetic alterations.[8] The current study, which uses a defined panel of biologically-relevant

marker genes, can distinguish different neuroendocrine tumor types found in the appendix.

The clinical relevance of this strategy is highlighted by the observation that none of the patients with low expression levels developed metastasis. Nevertheless, the relatively short follow-up (113 months, although follow-up in 5 of the 16 patients extended >19 years) indicates that at this stage a degree of caution is necessary in interpreting these results. Patients with high expression levels had preexisting malignant disease or subsequently developed metastases irrespective of the length of follow-up. This group, however, was 2 decades older than patients with incidental tumors, although the difference in age was not statistically significant ($P = 0.053$, 2-tailed). Clearly, a prospective study with longer follow-up in appropriate sex- and age-matched patients is required to definitively evaluate the relationship between gene expression of these markers and disease progress in appendiceal carcinoids.

While histologic examination is clearly useful in staging appendiceal disease, it is limited since a pattern-recognition technique is vulnerable when early cellular transformation events are occurring and can only broadly predict biologic outcome once obvious changes are evident. The ability to identify at the molecular level gene regulators that govern proliferation and invasion has obvious potential advantages. In this respect, the objective quantification of gene expression levels, particularly genes with defined biologic functions, is of potential considerable clinical advantage. Thus, in patients where a carcinoid tumor of the appendix is identified and the need for further surgical intervention is uncertain since the criteria of tumor size, location, and light microscopy are either inconsistent or provide ambiguous information, it is likely that the determination of gene expression may offer novel predictive information of considerable clinical relevance. Currently, available information on which therapeutic strategy is based requires the exercise of clinical judgment, a commodity both quite variable and sometimes dubious in its application as opposed to objectively quantifiable molecular data.

In the current study, using a PCR-based approach, CgA expression was detected in one of 11 histologically negative fresh-frozen appendicitis samples. Light microscopic examination of tissue sections, (4 μm thickness), by a pathologist (R.L.C.) failed to identify a carcinoid tumor. Subsequent immunostaining of this section with anticytokeratin and anti-CgA followed by tyramide amplification of the CgA signal identified clusters of dual-stained cells both adjacent to the lumen and within appendiceal peri serosal fat. The former appeared to have an epithelial morphology but were intensely CgA-positive. The latter were consistent with microcarcinoids. It is possible that injury or inflammation may be implicated in endocrine cell differentiation and that such events represent cytokine mediated phenomena.[33] Alternatively, such agents with well-defined growth factor-like bioactive properties may cause appendiceal endocrine cell hyperplasia. The latter phenomenon has not been carefully examined in the appendix but is well described in association with chronic bronchopulmonary inflammation.[34,35] In addition, chronic atrophic gastritis is also associated with enterochromaffin cell hyperplasia and may well reflect a similar series of inflammation-mediated events.[36] It is noteworthy that prolonged infection and chronicity are key requirements in such circumstances. If either of these 2 etiologies were responsible for the elevated *CgA* noted in our study, we would expect all samples from the 11 patients with suppurative appendicitis to express elevated levels of this marker. This was not the case. We therefore propose that the single patient with elevated *CgA* message and CgA protein expression is an authentic example of a covert appendiceal tumor detected using a molecular targeted strategy. Additional genetic examination of this specimen, using gene expression of *NAP1L1*, *MAGE-D2*, *MTA1*, and *NALP1*, identified that levels of these markers were all within normal range. This serves to support the opinion that this specimen was nonmalignant (no expression of malignancy-associated genes) and could potentially be categorized as an incidental nonmalignant appendiceal carcinoid tumor.

This observation suggests that in acute appendiceal samples obtained at surgery covert carcinoid tumor not readily identifiable by standard light microscopy can be identified using a molecular screen. Indeed, our previous demonstration that approximately 25% of histologically normal lymph nodes in small bowel carcinoid resections are *CgA*-PCR-positive (indicative of covert metastasis) suggests that this technique will be of similar utility in the identification of covert appendiceal neuroendocrine tumors.[37] In general, the detection rate for appendiceal carcinoids using standard histologic techniques in appendectomy samples is approximately 1%.[38] Our study, using a more sensitive PCR molecular genetic approach, suggests that this may well be higher.

RNA isolation from paraffin-blocks is becoming an increasingly acceptable method for examining gene expression. In the current study, RNA was isolated from all samples and the genes of interest were readily amplified. This confirms the utility of this technique in appendiceal carcinoid samples as has been previously demonstrated for other tumor types and tissue samples including Barrett's esophageal adenocarcinomas and breast tumors.[19,20,39] Furthermore, *CgA* transcript levels from these paraffin blocks could be related to protein expression levels identified on a tissue microarray.[37] Correlating *CgA* transcript from the current study with protein levels of CgA measured by AQUA in the same appendiceal tumors demonstrated these were significantly related: $R^2 = 0.40$, $P < 0.03$. The absence of an absolute correlation may either reflect a degree of RNA degradation or more likely the well-described discrepancy between mRNA and protein expression profiles due to differences in transcript processing and protein stability.[40]

CONCLUSION

Our data demonstrate overexpression of *CgA* and *NALP1* in appendiceal carcinoids, overexpression of *NAP1L1*, *MAGE-D2*, and *MTA1* in malignant appendiceal carcinoids and mixed cell (goblet cell) adenocarcinoids, and decreased expression of *NALP1* in the latter tumor type. We therefore propose that this evaluation supports the utility of the measurement of such biomarkers to differentiate appendiceal tumor types both in

paraffin-embedded and fresh frozen samples. The ability to identify occult carcinoid tissue by *CgA* expression with such amplified sensitivity also indicates that this technique may have application in the detection of appendiceal tumors or their metastasis, which cannot be identified by conventional pathologic techniques. The implications for altering staging and hence therapeutic strategy are of clear clinical relevance.

REFERENCES

1. Modlin I. *History of Gastroenterology at the Millenium*. Milan: MultiMed, 2000.
2. Sandor A, Modlin IM. A retrospective analysis of 1570 appendiceal carcinoids. *Am J Gastroenterol*. 1998;93:422–428.
3. Bernick PE, et al. Neuroendocrine carcinomas of the colon and rectum. *Dis Colon Rectum*. 2004;47:163–169.
4. Goede AC, Caplin ME, Winslet MC. Carcinoid tumour of the appendix. *Br J Surg*. 2003;90:1317–1322.
5. Simon SR. Moses Maimonides: medieval physician and scholar. *Arch Intern Med*. 1999;159:1841–1845.
6. Modlin IM, Lye KD, Kidd M. A 5-decade analysis of 13,715 carcinoid tumors. *Cancer*. 2003;97:934–959.
7. Modlin IM, Kidd M, Latich I, et al. Current status of gastrointestinal carcinoids. *Gastroenterology*. 2005;128:1717–1751.
8. Kabbani W, Houlihan PS, Luthra R, et al. Mucinous and nonmucinous appendiceal adenocarcinomas: different clinicopathological features but similar genetic alterations. *Mod Pathol*. 2002;15:599–605.
9. Schnirer II, Yao JC, Ajani JA. Carcinoid: a comprehensive review. *Acta Oncol*. 2003;42:672–692.
10. Varisco B, McAlvin B, Dias J, et al. Adenocarcinoid of the appendix: is right hemicolectomy necessary? A meta-analysis of retrospective chart reviews. *Am Surg*. 2004;70:593–599.
11. Bashyam MD. Understanding cancer metastasis: an urgent need for using differential gene expression analysis. *Cancer*. 2002;94:1821–1829.
12. Timar J, Csuka O, Orosz Z, et al. Molecular pathology of tumor metastasis: II. Molecular staging and differential diagnosis. *Pathol Oncol Res*. 2002;8:204–219.
13. McCusker ME, Cote TR, Clegg LX, et al. Primary malignant neoplasms of the appendix: a population-based study from the surveillance, epidemiology and end-results program, 1973–1998. *Cancer*. 2002;94:3307–3312.
14. Bucher P, Mathe Z, Demirag A, et al. Appendix tumors in the era of laparoscopic appendectomy. *Surg Endosc*. 2004;18:1063–1066.
15. Kidd M, et al. The role of genetic markers, NAP1L1, MAGE-D2 and MTA1, in defining small intestinal carcinoid neoplasia. *Ann Surg Oncol.* 2006;13:253–262.
16. Kidd M, et al. The utility of molecular genetic signatures in the delineation of gastric neoplasia. *Cancer.* 2006;106:1480–1488.
17. Moran BJ, Cecil TD. The etiology, clinical presentation, and management of pseudomyxoma peritonei. *Surg Oncol Clin North Am*. 2003;12:585–603.
18. van Ruth S, et al. Pseudomyxoma peritonei: a review of 62 cases. *Eur J Surg Oncol*. 2003;29:682–688.
19. Korbler T, Grskovic M, Dominis M, et al. A simple method for RNA isolation from formalin-fixed and paraffin-embedded lymphatic tissues. *Exp Mol Pathol*. 2003;74:336–340.
20. Specht K, et al. Quantitative gene expression analysis in microdissected archival formalin-fixed and paraffin-embedded tumor tissue. *Am J Pathol*. 2001;158:419–429.
21. Kidd M, et al. Microsatellite instability and gene mutations in transforming growth factor-beta type II receptor are absent in small bowel carcinoid tumors. *Cancer*. 2005;103:229–236.
22. Camp RL, Chung GG, Rimm DL. Automated subcellular localization and quantification of protein expression in tissue microarrays. *Nat Med*. 2002;8:1323–1327.
23. Eriksson B, Oberg K, Stridsberg M. Tumor markers in neuroendocrine tumors. *Digestion.* 2000;62(suppl)1:33–38.
24. Eriksson B, et al. Chromogranins: new sensitive markers for neuroendocrine tumors. *Acta Oncol*. 1989;28:325–329.
25. Simon HU, et al. Molecular characterization of hNRP, a cDNA encoding a human nucleosome-assembly-protein-I-related gene product involved in the induction of cell proliferation. *Biochem J*. 1994;297(Pt 2):389–397.
26. Li M, et al. Genes associated with liver metastasis of colon cancer, identified by genome-wide cDNA microarray. *Int J Oncol*. 2004;24:305–312.
27. Toh Y, Pencil SD, Nicolson GL. A novel candidate metastasis-associated gene, mta1, differentially expressed in highly metastatic mammary adenocarcinoma cell lines: cDNA cloning, expression, and protein analyses. *J Biol Chem*. 1994;269:22958–22963.
28. Toh Y, Pencil SD, Nicolson GL. Analysis of the complete sequence of the novel metastasis-associated candidate gene, mta1, differentially expressed in mammary adenocarcinoma and breast cancer cell lines. *Gene*. 1995;159:97–104.
29. Toh Y, et al. Overexpression of the MTA1 gene in gastrointestinal carcinomas: correlation with invasion and metastasis. *Int J Cancer*. 1997;74:459–463.
30. Toh Y, et al. Expression of the metastasis-associated MTA1 protein and its relationship to deacetylation of the histone H4 in esophageal squamous cell carcinomas. *Int J Cancer*. 2004;110:362–367.
31. Moon WS, Chang K, Tarnawski AS. Overexpression of metastatic tumor antigen 1 in hepatocellular carcinoma: relationship to vascular invasion and estrogen receptor-alpha. *Hum Pathol*. 2004;35:424–429.
32. Stancu M, et al. Genetic alterations in goblet cell carcinoids of the vermiform appendix and comparison with gastrointestinal carcinoid tumors. *Mod Pathol*. 2003;16:1189–1198.
33. Shia J, et al. Increased endocrine cells in treated rectal adenocarcinomas: a possible reflection of endocrine differentiation in tumor cells induced by chemotherapy and radiotherapy. *Am J Surg Pathol*. 2002;26:863–872.
34. Aguayo SM, et al. Brief report: idiopathic diffuse hyperplasia of pulmonary neuroendocrine cells and airways disease. *N Engl J Med*. 1992;327:1285–1288.
35. Kerr KM. Pulmonary preinvasive neoplasia. *J Clin Pathol*. 2001;54:257–271.
36. Solcia E, et al. Gastric carcinoids and related endocrine growths. *Digestion*. 1986;35(suppl 1):3–22.
37. Kidd M, Modlin I, Mane S, et al. Q RT-PCR detection of chromogranin A: a new standard in the identification of neuroendocrine tumor disease. *Ann Surg.* 2006;243:273–280.
38. Connor SJ, Hanna GB, Frizelle FA. Appendiceal tumors: retrospective clinicopathologic analysis of appendiceal tumors from 7,970 appendectomies. *Dis Colon Rectum*. 1998;41:75–80.
39. Man YG, et al. Direct and repeat uses of tissue sections as templates for liquid-phase polymerase chain reaction amplification: applications and implications. *Appl Immunohistochem Mol Morphol*. 2004;12:266–270.
40. Mehra A, Lee K, Hatzimanikatis V. Insights into the relation between mRNA and protein expression patterns: I. Theoretical considerations. *Biotechnol Bioeng*. 2003;84:822–833.