SUPPLEMENTAL DATA

Methods

Colony formation assay

Growth analysis of cells was performed by the colony formation assay described previously (1). Eighty percent confluent cells were trypsinized and single-cell suspensions were obtained. Four hundred viable cells were seeded per well in 6-well plates. Ten days later, cells were fixed with 70% ethanol and stained with 10% (v/v) Giemsa (MERCK, Damstadt, Germany). Colonies consisting of more than 50 cells were counted. Each experiment was done in triplicate and the mean values ± SEM are presented.

Matrigel invasion assay

Invasion assay was carried out as described previously (2). Conditioned medium from si-scramble, si- CPE-ΔN, or si-NEDD9 cells was placed in the lower chambers as chemo-attractants. After 22 hours in culture, the cells were removed from the upper surface of the filter by scraping with a cotton swab. The cells that invaded through the Matrigel and were adherent to the bottom of the membrane were stained with crystal violet solution. The cell-associated dye was eluted with 10% acetic acid and its absorbance at 595 nm determined. Each experiment was done in triplicate and the mean values ± SEM are presented.

Northern Blot of human cancer cells

Total RNA was extracted from MHCC97H cells using SV total RNA isolation kit (Promega, WI). RNA quality was determined by the 2100 bioanalyzer (Agilent, CA). Total RNA (10μg) was separated on a 1.5% agarose/0.7 M formaldehyde gel, transferred

to a charged nylon membrane by downward capillary transfer, and immobilized by UV cross-linking. Blots were prehybridized at 68° C for 1h in ULTRAhyb-Oligo buffer (Ambion) and probed with 3’ TEG-Biotinylated oligo probe hCPE-NB (Seq -probe CAT CCC TCC AGG TAC GCT GTA CCA AGC ACC ACC GTT GGT GGT TCC ATC). The final wash was in 0.1x standard saline citrate containing 0.1% sodium dodecyl sulfate at 60° C for 15 min. Blots were visualized by chemiluminescent nucleic acid detection module kit (Thermo Fisher Scientific) according to the manufacturer's protocol.

Animal Studies

Generation of luciferase-expressing cells

For luciferase labeling of MHCCLM3 cells, lentiviral vector containing the sequence of the firefly luciferase gene was constructed, transfected into the cells using the method described previously (3). Stable transfectants were generated from a pool of >20 positive clones, which were selected by blasticidin at a concentration of 2 µg/mL.

Bioluminescent imaging of live animals bearing tumors

Animal care and euthanasia were conducted with full approval by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong. Approximately 1 x 10^{6} MHCCLM3 cells stably expressing firefly luciferase were transduced with either si-Scr or si-CPE-ΔN and injected subcutaneously into the right flank of four-week-old male BALB/c-nu/nu mice with a 30-gauge hypodermic needle (4). The mice were imaged on day 0 and day 30 after cell inoculation. Mice were anesthetized with ketamine-xylazine mix (4:1). Imaging was done using a Xenogen IVIS 100 cooled CCD camera (Xenogen). The mice were injected with 200 µL of 15 mg/mL D-luciferin

i.p. for 15 minutes before imaging, after which they were placed in a light-tight chamber. A gray-scale reference image was obtained followed by the acquisition of a bioluminescent image. The acquisition time ranged from 3 seconds to 1 minute. The images shown are pseudoimages of the emitted light in photons/s/cm^2/steradian, superimposed over the gray-scale photographs of the animal.

Metastatic orthotopic nude mouse model

Approximately 1 x 10^6 MHCCLM3 cells (in 0.2-mL culture medium) transduced with either si-Scr or si-CPE-ΔN were injected subcutaneously into the right flank of nude mice, which were then observed daily for signs of tumor development. Once the subcutaneous tumor reached 1 to 1.5 cm in diameter, it was removed and cut into about 1- to 2-mm cubes, which were implanted into the left liver lobe of the nude mice, using the method described previously (3). The mice were imaged on the day 0 and day 35 after tumor inoculation. Mice were anesthetized with ketamine-xylazine mix (4:1). Imaging was performed using a Xenogen IVIS 100 cooled CCD camera (Xenogen) as described above and metastasis to the lung and intestines was tracked. After imaging, metastasis to these tissues was confirmed by inspection and imaging of the dissected tissues.

Histopathology

To confirm that metastasis to the lungs occurred, the animal was autopsied as soon as the original signal was recorded. Lungs were examined and imaged with the Xenogen camera to confirm the bioluminescence of this tissue and then fixed by intrabranchial perfusion of 10% neutralized formalin solution. Paraffin-embedded sections (4 μm) were cut and stained with H&E.

Supplementary References

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3. Lee, T.K., Poon, R.T., Wo, J.Y., Ma, S., Guan, X.Y., Myers, J.N., Altevogt, P., and Yuen, A.P. 2007. Lupeol suppresses cisplatin-induced nuclear factor-kappaB activation in head and neck squamous cell carcinoma and inhibits local invasion and nodal metastasis in an orthotopic nude mouse model. *Cancer Res* 67:8800-8809.
4. Fu, X.Y., Besterman, J.M., Monosov, A., and Hoffman, R.M. 1991. Models of human metastatic colon cancer in nude mice orthotopically constructed by using histologically intact patient specimens. *Proc Natl Acad Sci U S A* 88:9345-9349.

REMARK compliance for CPE-ΔN biomarker for HCC

INTRODUCTION

Marker examined: Carboxypeptidase E-ΔN, (CPE-ΔN)

Study objective: To determine if CPE-ΔN is a valuable prognostic marker for predicting future extra- or intra-hepatic metastasis (heretofore referred to as recurrence) of HCC.

Hypothesis: That using the ratio of CPE-ΔN in tumor vs. surrounding normal tissue (T/N), with a cutoff of 2 derived from a subset of pilot patients, would predict recurrence in the resected primary tumor in HCC patients during a two year (or possibly 3 year) follow-up period.

MATERIALS AND METHODS

Patients

Source: Dept. of Surgery, University of Hong Kong, China. Patients underwent resection for HCC from 2000-2005.

Stage: Patients were primarily (78%) stages 2 and 3, split evenly between them; an additional 20% were stage 4, and the final 2% were stage 1.

Inclusion criteria: Selected HCC patients with sufficient clinical data before surgery and follow up data after liver tumor resection, who had viable tumor/normal tissue specimens available for assay

Exclusion criteria: HCC patients who received treatment prior to tumor resection

3. Treatment received for HCC patients: None beyond the tumor resection.

Specimen characteristics

4. Resected tumor (HCC) and non-tumorous tissues situated at a distance from the tumors were dissected, removed, and immediately frozen in liquid nitrogen and stored at -80°C.

Assay methods

5. Method of RNA extraction of the biospecimens and quantitative RT-PCR (qRT-PCR) of CPE-ΔN mRNA and reagents are described in detail in the methods section of the text. Quality control and reproducibility were assessed by randomly selecting tumor samples and reassaying them in a different laboratory, or on another day using a different

batch of PCR kit, PCR machine, and personnel doing the work. Results all fell within a 10% range of the original value. Assays were blinded to the person conducting the tissue extraction and CPE-ΔN mRNA assay to the end-point.

Study design

6. Case selection: from an initial set of resected HCC patients that were eligible, 100 were retrospectively selected to provide patients for both the Training (Pilot) and Test sets of patients. They were chosen in an unmatched "case-control" fashion, with 50 of them having recurrence and 50 remaining disease-free during their follow-up periods; different patients had different lengths of follow-up, ranging from a few days to over 9 years. Patients were chosen based on diagnosed HCC regardless of stage of malignancy. Although not explicitly matched, patients from each group had similar median ages and gender ratios. The cases were from 6/2000 to 7/2005. The end of the follow-up period for the last patient was 9/2009. The median follow-up time for patients, to either recurrence or censoring, was 25.4 months.

7. Clinical end-points examined: HCC patients were followed up monthly in the first year and thereafter quarterly. Regular monitoring was done for recurrence by serum α-fetoprotein level and ultrasonography or computerized tomographic (CT) scans of the liver. The diagnosis of recurrence was based on typical imaging findings on CT or arteriography, and if necessary, percutaneous fine-needle aspiration cytology. Recurrence of the disease was analyzed without further delineation into intrahepatic or extrahepatic. Disease-free survival was measured from the date of hepatic resection to the date when recurrent disease was diagnosed or, in the absence of detectable tumor, to the date of

death or last follow-up. Actuarial survival was measured from the date of hepatic resection to the date of death or last follow-up.

8. List of candidate variables: None

9. Rationale for sample size, including effect size to detect and power:

This study was designed as a retrospective study, to look at the ratio of CPE-ΔN levels in resected tumor (T) vs. those in adjacent normal tissue (N). Starting with a population of resected HCC patients who met inclusion/exclusion criteria, unmatched case-control sampling was used to acquire equal numbers of patients who recurred and who didn't recur during their respective periods of follow-up (which were variable due to the date of initial surgery and the date each patient was last seen or who had recurrence). Approximately 35-40% of these patients were to be used as a TRAINING set ("Pilot") to establish criteria for CPE-ΔN ratios for predicting recurrences (n=37 of the 99 useable patients were ultimately used in this Pilot part of the study). The remainder of the patients serve as the TEST set, or confirmatory set, for independently validating the usefulness of this biomarker (n=62). The rationale for the total sample size of 100 derived from two considerations: (1) as noted, we wanted to use 35-40% of the patients in the TRAINING set; (2) we hoped that with an appropriate cutoff derived from results in the Pilot/Training set, that CPE-ΔN would potentially be a very good classifier of say 2 year recurrence; specifically we expected that the proportions of patients in the 2 year recurrer and non-recurrer groups being "positive" by the CPE-ΔN criterion would differ substantially, by 40% or more — e.g. we expected 30% positives in the non-recurring patients and 70% in

the recurrers. If such a difference were true, then the EXACT power, using Fisher's exact test to compare independent proportions at the standard 2-sided 0.05 level of significance with a TOTAL of 55 evaluable patients, is 81.3%, just slightly better than the power of 80% designed for. However a small percentage (~10-15%) of patients in the population would either not have sufficient follow-up to be evaluable for 2 year recurrence or would be unusable due to either a poor mRNA sample or the matching surrounding non-tumorous tissue not being available.

Thus, to have 55 evaluable patients in the TEST set, we needed to start with 60-65 patients. Back-calculating from these considerations, it was determined that we should start with a cohort of N=100 patients, split evenly between recurrers and non-recurrers. This number would allow for 35-40 patients being used as a Pilot/Training set; the other 60-65 patients would be used for the Test set, with roughly 55 of them being evaluable for a 2 year recurrence endpoint.

Statistical analysis methods

10. Only one marker was used. Since there were no other variables, there was no selection procedure and no model building. However, for item #17 regarding the relationship between the marker and standard prognostic variables – in this case just stage of tumor – we used a simple logistic regression model for predicting 2 year recurrence using these 2 variables as predictors. The primary statistical methods used were exact tests and confidence intervals for the odds ratio for recurrence between those with high (>2) or low (<2) CPE-ΔN T/N ratios at 2 years of follow-up. The analyses were also done at 3 years of follow-up and very similar ORs and P-values obtained. Since the sampling

was of a case-control design, and NOT a simple random sample of the population of eligible patients, using odds ratios and associated analysis methods (including Fisher's exact test, which is conditional on the marginals) insures the VALIDITY of the results (cf. "Applied Logistic Regression, 2nd ed., by Hosmer & Lemshow, pp. 218ff for a thorough discussion of the applicability of OR-based methods and logistic regression under a VARIETY of sampling schemes, including a case-control design like this one). In this study , using 2 yr recurrence (or 3 yr) as an endpoint, 92 of the 99 patients that were analyzable (out of the initial sample of N=100) had sufficient follow-up to use for 2 yr recurrence analysis; in the Pilot/Training subset 33/37 were analyzable, while in the Confirmatory subset 59/62 were analyzable. However this amount of follow-up did capture 41 of the 50 recurrences (82%), so only a small amount of information was lost (and the complementary analyses using 3 year recurrence captured 47 to the 50 recurrences). In this study the associated small loss of power is really irrelevant, however, since the exact primary P-values were around 10^{-7}. As an adjunct to these fully valid analyses of 2 year recurrence rates, we also present Kaplan-Meier plots, and the associated (approximate) logrank P-value for disease-free survival. These are visually striking and easier to understand than 2 year recurrence proportions and make full use of all the follow-up information we have on each patient. However, due to the case-control sampling design, they have the potential for being misleading (they can give biased estimates of the curves and associated P-values can be incorrect; for example see "Evaluation of Sampling Strategies for Modeling Survival of Uveal Malignant Melanoma," by Tero Kivela and Patrician Grambsch in "Investigative Ophthamology & Visual Science," August 2003, pp. 3288-3293, for a Monte Carlo study based on actual

data of the possible effects of a case-control sampling scheme like ours.) Although the case-control sampling scheme may introduce some bias and give logrank P-values that are in error, we believe that in this study, due to the characteristics of the sample, that these problems are very modest. To confirm this we looked at a representative sample of N=317 patients drawn from the full population of HCC cases; in this sample, 48.2% recurred at 3 years compared to 52.5% in our sample; at 4years the numbers are 56.7% and 52.5%, respectively. Thus our case-control sample has recurrences and non-recurrences in proportions very similar to the entire population, and hence any bias in the Kaplan-Meier curves should be on the order of at most a few percent (similar to what Grambsch found). This small potential bias is swamped by the enormous differences between the 2 survival curves in Figure 7B; the computed (approximate) logrank P-value for these curves is 5 x 10-10; this likely is too small, due both to the bias caused by sampling and because the chi-square approximation is overly significant; but the valid exact odds ratio P-value for 2 year recurrence is 1.7 x 10-7 and the 3 year value is 2.2 x 10-6, so a valid logrank P-value would be in a similar range. However we do not have available the detailed information that could be used to “adjust” the nominal logrank test to account for the case-control sampling done. So to be ultra-conservative we simply report the logrank P-value to be <0.0001. Since there was minimal missing data for analyses (besides the one non-recurrer who could not be analyzed (see item #12), the only missing data was for 3 patients who had missing cancer stage), no specific techniques for missing data (such as imputation methods) were used: in the analyses involving this missing data, only patients with complete data (96 of 99) were used. Thus for the logistic regression analysis of the N=62 patients in the Test set using stage and the

biomarker (also Table 1-iii), 56 patients could actually be used – 3 were not useable because of lack of 2 year follow-up and 3 more because of lack of stage information.

11. How are marker values handled including methods for cut point determination?

For prediction of recurrence, CPE-ΔN mRNA from resected primary tumor (T) and surrounding normal tissue (N) from a Pilot/Training subset of 37 of these 99 patients was used. Because of lack of 2 year follow-up in 4 of these, the 2 year recurrence outcomes for 33 of these patients was used to evaluate several possible cutoff; 15 of these had recurrence within 2 years of resection and 18 were disease-free at 2 years. Based on the sensitivity/specificity results at several thresholds, a threshold T/N value of 2 was established – values at or above this are taken to predict tumor recurrence within 2 years. Among potential cutoff values evaluated, a value of 2 had both high sensitivity (100%) and specificity (83%) value (see table). At cutoffs of 2.5 or higher, the sensitivity appeared to decline somewhat, without any concomitant improvement in the specificity. This fact, combined with the greater importance we gave to sensitivity, pointed to 2 being a good cutoff.

HCC fold cut-off for 95% CI	Sensitivity	Specificity	PLR	NLR
2.00	100.0	83.3	6.0	0.00
2.50	73.3	83.3	4.4	0.32
3.00	73.3	83.3	4.4	0.32
3.50	66.7	83.3	4.0	0.40

Cl- Coefficient level, PLR- Positive likelihood ratio, NLR- Negative likelihood ratio.

RESULTS

Data

12. Patients with HCC that had recurrence or were disease-free were chosen retrospectively from a population of HCC-resected eligible patients. One patient from the non-recurrence group had to be dropped due to missing surrounding non-tumorous tissue. As mentioned in #9, we had designed our sample size to handle the possibility of a few such cases and still maintain the designed-for 80% power. As noted in #10, the 3 and 4 year recurrence rates in the entire population and in our subset of 99 patients were very similar, indicating the selection indeed worked well on the critical endpoint of recurrence. The selection of the Pilot/Training and Test subsets from among these 99 patients also worked well – the 2 year recurrence rates are, respectively, 45.5% and 44.1%.

13. Distribution of basic demographic characteristics: Group of patients that showed recurrence: 41 males, 9 females, median age 52.5, average age 54.0. Group of patients with no recurrence: 41 males, 8 females, median age 54 and average age 55.1

Analysis and presentation

14. The relation of the marker to the standard prognostic variable of cancer stage, was analyzed; see Table 3 that details the results stage by stage, including odds ratios and exact associated P-values. An overall logistic regression was also done to summarize all these results – see #17 below.

15. Effect of tumor marker on a time- to- event outcome was analyzed for the Test subset of 62 HCC patients and presented as a Kaplan-Meier plot (see Fig 7B). Also Table

1 shows the relationship of 2 year recurrence to marker prediction in the analyzable 59 of 62 patients in this subset. For Kaplan-Meier and its associated logrank test, see extensive discussion in #10 above regarding its validity and how its results compare to those from odds-ratio analyses.

16. Multivariable analyses: Not applicable, except see item #17.

17. The relation of the marker and the standard prognostic variable of cancer stage is presented in Table 3). We did a logistic regression, for predicting the dichotomous outcome of 2 year recurrence, with stage alone in the model and then with both stage and CPE -ΔN as predictors, to assess the additional predictiveness that CPE-ΔN provides beyond cancer stage. The odds ratio for CPE-ΔN (in the model with stage also included), its confidence interval, and P-value are presented in the paper; the OR= 37.6 and was extremely significant (P=1.8 x 10-7), indicating that it provides a great deal of additional predictive power.

18. Item #10 above describes, for survival-based analyses, a number of additional analyses that indicate that, although the analyses may be slightly biased, they are quite consistent with the standard odds ratio analyses that are valid for the case-control sampling scheme used. Further evidence that the overall, ultra-significant result is plausible and consistent comes from Table 1-iii, showing separate results by cancer stage: for Stages 2 thru 4 the odds ratios for the CPE-ΔN T/N ratio as a predictor are all very large and are either statistically significant at P=0.05 or the one for Stage 4, with only 12 patients, is borderline (the OR is nominally an impressive 24, but with only a total of 12 patients one has low power for detecting anything but a perfect prediction). An additional analysis that also is fully consistent is the fact that of the 8 patients that recurred very

early – within 3 months – all 8 had elevated T/N ratios; and of the 30 patients recurring within the 1st year, 28/30 had elevated T/N ratios.

DISCUSSION

19. Our results from the retrospective study of the cohort of HCC patients supports our hypothesis that CPE-ΔN is a powerful prognostic marker for predicting future extra- and intra- hepatic metastasis (recurrence) based on levels in the primary tumor. Currently there is no known biomarker that accurately predicts future metastasis/recurrence for HCC. EpCAM, α-fetoprotein levels, and pathological staging have been used for prognosis in a subset of HCC patients, but these procedures have demonstrated poor accuracy. Thus to date no marker exists for predicting future recurrence in HCC patients with such high specificity and sensitivity as CPE-ΔN.

20. Our studies thus far show CPE-ΔN to have major clinical implications in providing a powerful biomarker for predicting future metastasis and recurrence in HCC patients. HCC is one of the most common cancers worldwide and the use of CPE-ΔN mRNA will greatly facilitate treatment stratification. Future research will be to carry out a large prospective study on HCC patients.

REMARK compliance for CPE-ΔN for pheochromocytoma (PHEO) and paraganglioma (PGL)

INTRODUCTION

1. Marker examined: Carboxypeptidase E-ΔN, (CPE-ΔN)

Study objective: To determine if CPE-ΔN is a valuable diagnostic and prognostic marker for predicting future metastasis or recurrence in PHEO/PGL patients.

Hypothesis: High levels of CPE-ΔN in the resected primary tumor are correlated with poor prognosis: future metastasis or recurrence in PHEO/PGL patients.

MATERIALS AND METHODS

Patients

2. Source: Fourteen resected tumors were obtained from the National Institutes of Health. Thirteen patients were followed up at the NIH but patient M15 was not followed up due to address change shortly after surgery. Tumors were resected between 2002-2008

Inclusion criteria: All patients who were previously diagnosed with PHEO/PGL regardless of tumor location or its malignant or recurrence status. A group of patients with high risk of metastasis (SDHB-related PHEO/PGL) and a group of patients with low risk of metastasis (SDHD- and MEN2A-related PHEO/PGL) were chosen. The patients were chosen based on these additional criteria: a) frozen tumor availability; b) those with metastatic disease had either a pathological confirmation of PHEO/PGL (e.g. from biopsy or removal of a metastatic lesion) or were positive on PHEO/PGL-specific imaging studies, together with positive biochemistry confirming the diagnosis of PHEO/PGL.

Exclusion criteria: No tumor availability; no pathology report on a tumor; no genetic testing available.

Treatments received: Patient S31 had the tumor removed in December 2003 at NIH and the tumor was assayed for CPE-ΔN. The patient developed metastatic disease in 2005 and the tumor removed in 2005, was very aggressive involving bone and therefore, considered as metastatic. This patient subsequently received gamma knife radiation treatment in December 2005 and CVD chemotherapy (combination of cyclophosphamide, vincristine and dacarbazine) in August 2006 with no effect and with a further progression of metastatic disease. Patient S22 had the tumor removed in 2002, received CVD chemotherapy in June 2004, October 2004, and Jan 2005 and subsequently died in 2006. No other treatment or radiation has been given to any of the remaining patients after surgery. No patient received any medication that is known to interfere with tumor growth and its metastatic potential after surgery. The above-mentioned treatments were instituted based on clinical judgment (there was no randomized treatment protocol involved).

Specimen characteristics

4. Upon resection the tissue specimen was placed in a sterile container on ice and conveyed to a waiting pathologist. The specimen was immediately examined and tissue not necessary for diagnostic purposes was dissected from the tumor, excluding necrotic

areas and tumor margin or capsule. Further dissection into pieces no bigger than 0.5 x 0.5 x 0.5 cm was performed in a petri dish on ice and frozen directly in liquid nitrogen.

Assay methods

5. The method of RNA extraction of the biospecimens, the quantitative RT-PCR (qRT-PCR) of CPE-ΔN mRNA assay, and the associated reagents used are described in detail in the Methods section of the text. Quality control and reproducibility were assessed by randomly selecting tumor samples and reassaying them on another day using different batches of the PCR kit. Results all fell within a 10% range of the original value. Assays were blinded to the person conducting the tissue extraction and CPE-ΔN mRNA assay to the end-point. Determination of CPE-ΔN mRNA copy numbers in PHEO/PGL is described in detail in the Material and Methods Supplement.

Study design

6. Patients were retrospectively chosen. Patients were chosen based on malignancy criteria that apply to PHEO and PGL, as detailed under #2. All patients meeting these criteria were used: so these patients are not a subsample from a larger population we had available and hence standard statistical issues do not apply. There was no selection based on age or sex or their outcome (e.g. death vs survival). Patients have had follow ups ranging from 2 to 8 years (for either recurrence or, in the case of those metastatic at resection, for further progression of disease). For the 6 patients remaining disease-free at last follow-up, the average follow-up to date has been 3.9 years. Patient follow-up was done with regular monitoring for recurrence and metastasis by serum catecholamine and

metanephrine levels, computerized tomographic (CT) or magnetic resonance scanning; specific PHEO/PGL nuclear medicine imaging including 123/131-I-MIBG scintigraphy and 18F-fluorodopamine PET scanning. The diagnosis of metastatic disease or recurrence was based on typical imaging findings.

7. Clinical endpoints examined: a) the presence or absence of recurrent, malignant (including a significant local progression), or metastatic disease.

8. List all candidate variables: None

9. Rationale for sample size: We obtained tumor samples from as many patients as possible with these diseases for whom necessary surgical and follow-up information was available.

Statistical analysis methods

10. Only one marker was used. As noted above these N=14 patients constitute the entire relevant population – they are not a statistical sample derived from some larger population for which we want to make statistical inferences. There were no other variables, no selection procedure, no model building, nor any model assumptions. There are no statistical analyses – ALL of the relevant data for the 14 patients is enumerated in Table 4. In Table 5 sensitivities and specificities are reported for 3 potential cut-points for mRNA copy numbers merely as illustrative examples – their main purpose is to emphasize the striking copy number data in Table 1 that copy numbers are either very LOW (200K or below) or very HIGH (5 million or above), there is no “middle ground”

in these data; hence, as the table shows, one has VERY wide latitude in defining a cut-point – and for these 14 patients it will not matter! The technical definition of the dichotomous outcome that copy numbers are predicting and that these sensitivity/specificity values apply to is: a poor outcome is for the patient to have either metastatic disease at resection or to have benign or resectable recurrent disease at resection but then to subsequently recur; a good outcome is if neither of these occurs (during the follow-up we have for that patient).

11. Clarify how marker values were handled: As noted in #10, in Table 5, 3 potential cut-points are used simply for illustrative purposes; no formal cut-points are being posited. These 3 examples however, over a 4-fold range, do illustrate the relative insensitivity of the predictiveness of CPE-ΔN to what cut-point is used; this is evident in Table 2-i that reports all the actual data.

RESULTS

Data

12. Describe the flow of patients through the study: Our patients were chosen retrospectively since PHEO/PGL is a very rare disease and there are few patients in whom all clinical data including frozen tumor tissue are available. Once patients were chosen they were followed until February 2010 if possible to evaluate development of recurrent or metastatic disease. However 1 patient (M15) who had surgery in 2004 was

not followed up due to an address change. In addition, one patient (S22) presented with metastatic disease at the initial visit, had surgery and the tumor obtained, but died 4 years after the operation in 2006. Another patient (M20) presented with metastatic disease at the initial visit in 2005 had the tumor removed in the same year and died in 2008.

13. Report distribution of basic demographic characteristics: Age: 31-66 years; Gender: 7 males and 7 females. There are no prognostic variables for these two diseases to report on. The values of the CPE-ΔN mRNA copy number are reported for each patient in Table 4, along with the patient's initial status, disease status at last follow-up, and the years of follow-up disease-free or until the first recurrence subsequent to the resection date.

Analysis and presentation

14. There are no standard prognostic variables for these tumors.

15. Table 4 shows all data; for illustration purposes Table 5 shows the accuracy of copy numbers in predicting recurrence or metastatic disease using different possible cutoffs, as reproduced here:

Values of CPE-ΔN levels for predicting metastasis/recurrence in PHEO/PGL

Pheo/Pgl copy number cut-off for 95% CI	Sensitivity %	Specificity %	PLR	NLR	DOR
250K	100	100	NA	NA	NA
500K	100	100	NA	NA	NA
1000K	100	100	NA	NA	NA

16. There are no multivariate analyses.

17. Because there are no standard prognostic variables, this item is Not Applicable

18. There are no assumptions to be made. The Table reproduced in #15 above is a simple sensitivity analysis that demonstrates in this group of patients that the accuracy of using CPE-ΔN mRNA copy number to predict outcomes is quite insensitive to what cutoff value one may choose. from the raw copy numbers in Table 4, one can see that the difference between the disease-free patient with the highest copy number (among this subset of n=7) and the recurrent/metastatic patient with the lowest copy number (among this other subset of 7) is roughly a 20-fold difference, so one may have wide latitude in choosing a formal cutoff and still achieve excellent sensitivity and specificity.

DISCUSSION

19 and 20 Results discussed in the main text

Supplemental Figure 1.

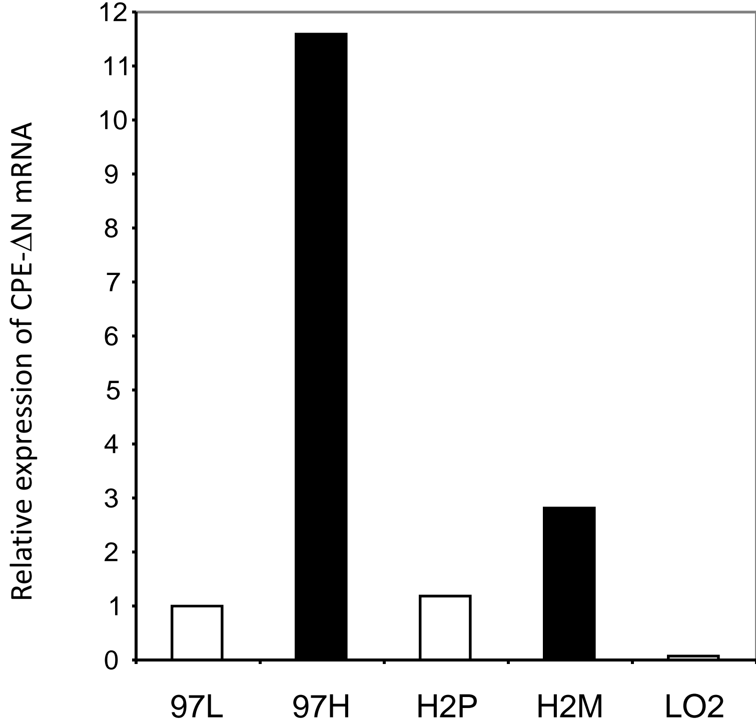


Figure 1. Levels of CPE-ΔN mRNA in HCC cells and a primary hepatocyte cell line
qRT-PCR quantification of hCPE-ΔN mRNA demonstrate negligible levels of CPE-ΔN mRNA in a primary hepatocyte cell line (LO2), whereas highly metastatic cell lines MHCC97H(97H) and H2M show increased levels in hCPE-ΔN mRNA compared to low metastatic cell lines MHCC97L (97L) and H2P respectively.

Supplemental Figure 2.

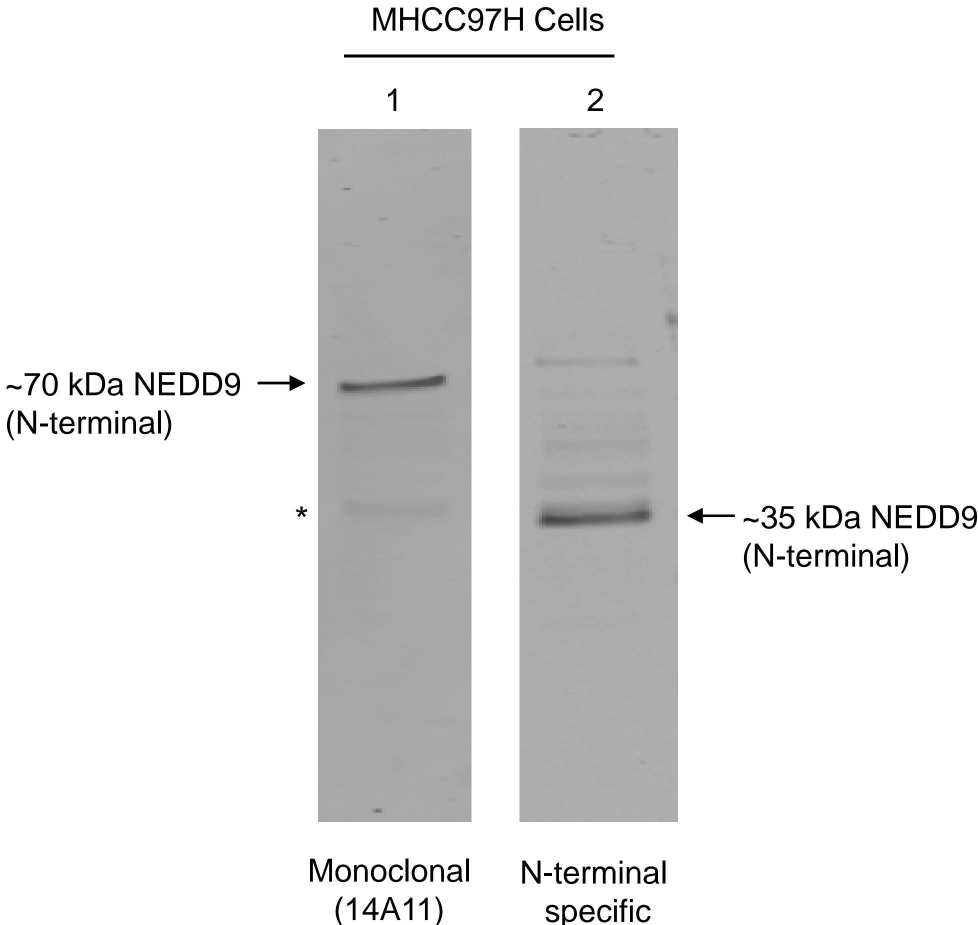


Figure 2. Western blot showing the 70 kD NEDD9 band as the processed product and the 35 kD NEDD9 band as the N-terminal cleavage product derived from cleavage of the 105 kD NEDD9 in MHCC97H cells. MHCC97H cells were probed with NEDD9 N-terminal mouse monoclonal antibody clone 14A11 which immunostained the 70 kD form of NEDD9 (lanes 1), or an extreme N-terminal polyclonal antibody (lanes 2) derived from antigenic peptide sequence: CEYPSRYQKDVYDIPPSH, a gift from Prof. Morimoto, Tokyo University, Japan, which immunostained the 35 kD product. * indicates a non-specific band.

Supplemental Figure 3.

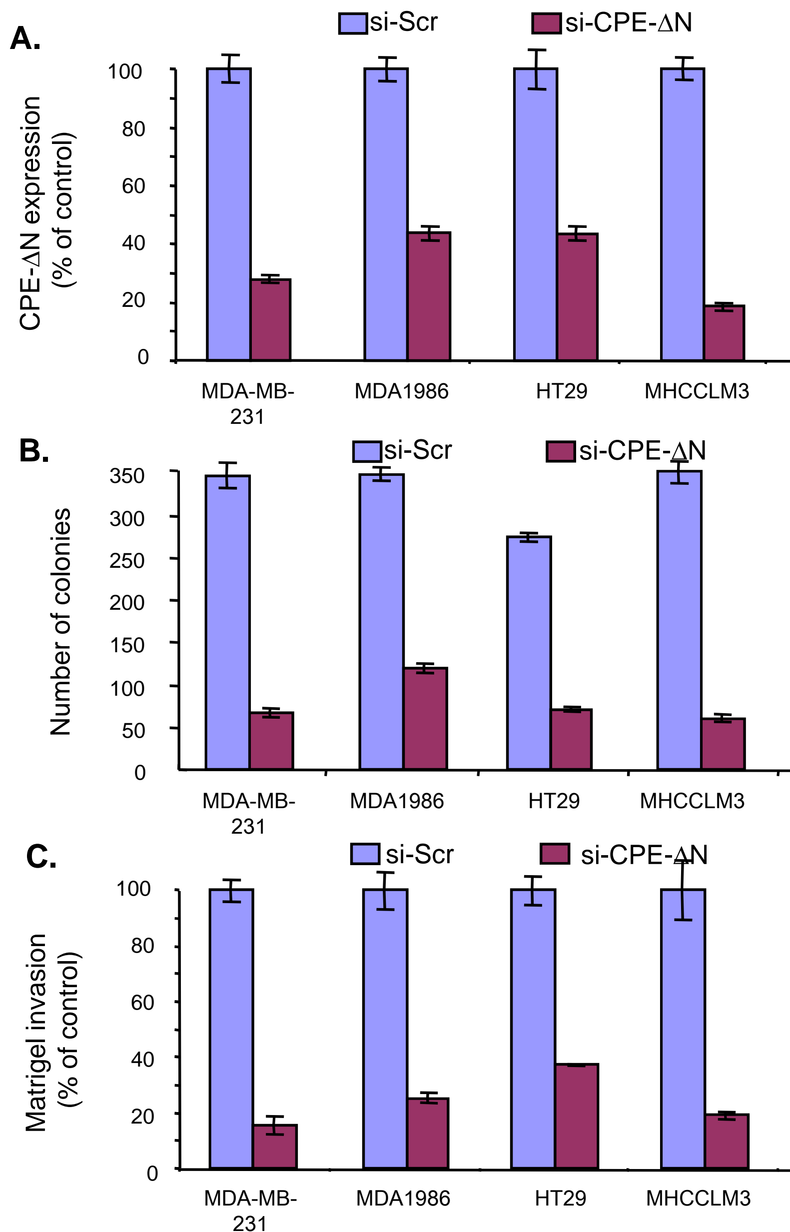


Figure 3. Down-regulation of CPE-ΔN inhibits tumor growth and metastasis. **(A)** Bar graphs show % of ~40 kD CPE-ΔN in various si-CPE-ΔN treated cancer cells (red) relative to si-scr treated cells (control, made equal to 100%, blue). Mean values ± SEM (n=3) are shown. **(B)** Growth analysis: bar graphs show the number of colonies with >50 cells in the si-scr treated cells (blue) and si-CPE-ΔN treated cells (red). Mean values ± SEM (n=3) are shown. **(C)** Invasion assay: bar graphs show the % invasion of si-CPE-ΔN treated cells (red) relative to the si-scr treated cells (made equal to 100%, blue). Mean values ± SEM (n=3) are shown.

Supplementary Figure 4.

Lung metastasis

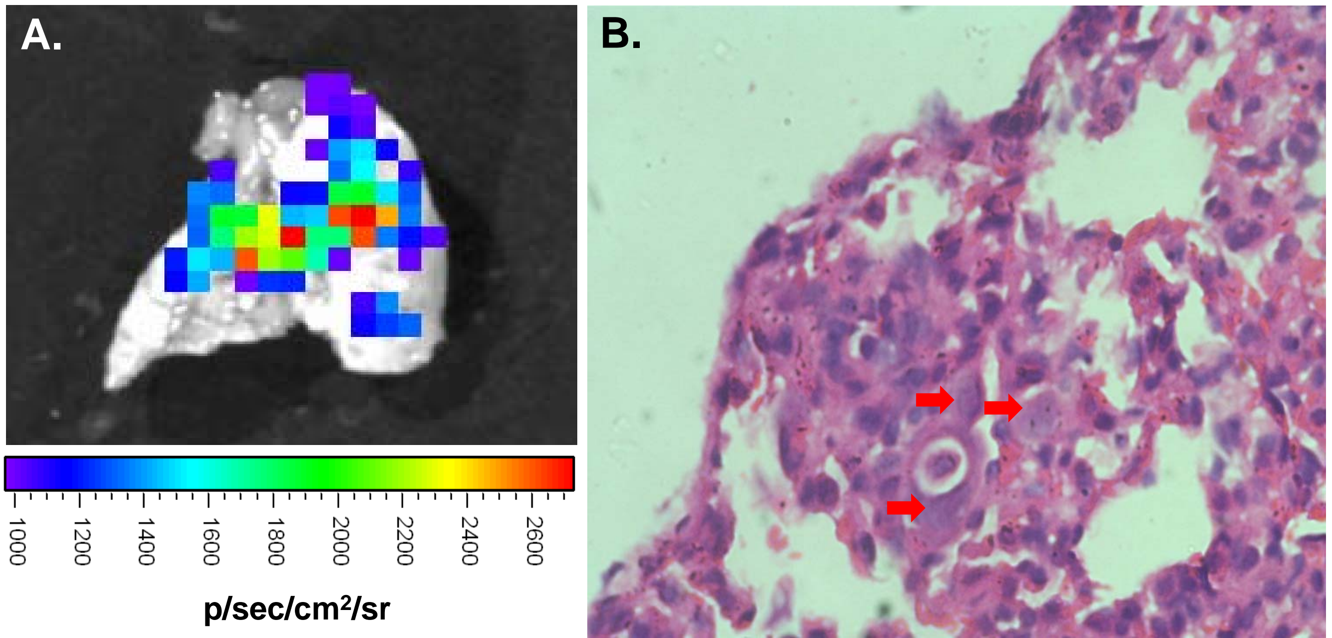


Figure 4. Detection of lung metastasis by ex vivo whole lung imaging. **(A)** Pseudoimage of emitted light in photons/s/cm^2/steradian, superimposed over the gray-scale image of the lungs from a nude mouse implanted with MHCCLM3 cells treated with si-scr RNA as described in Fig. 3B. **(B)** Histological staining of the left lung from panel A (H & E stain, x 400). Red arrows represent micrometastasis of the cells from the orthopically implanted MHHCML3 tumor in the liver.

Supplementary Figure 5.

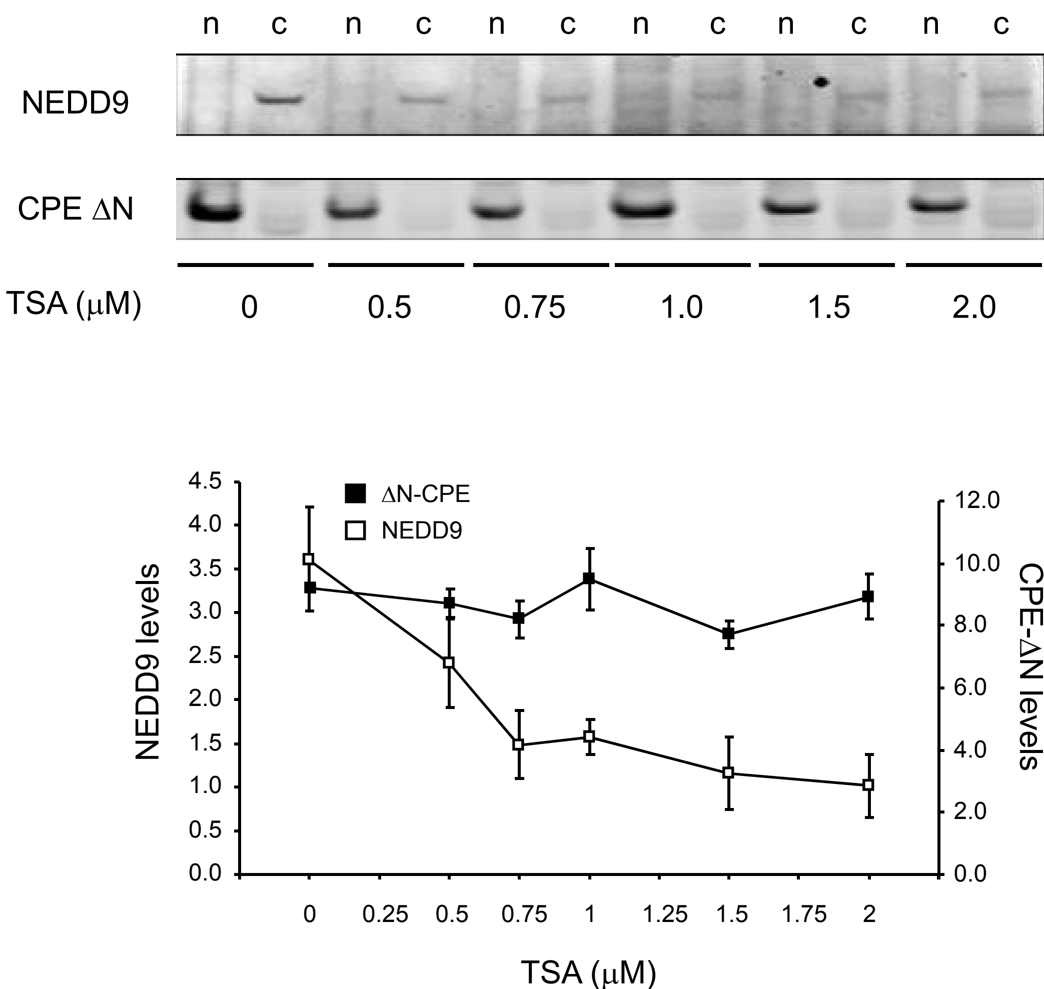


Figure 5. Effect of HDAC inhibitor, TSA, on NEDD9 expression in MHCC97L cells stably expressing CPE-ΔN. Cells were treated with TSA at various concentrations (0–2 μM) for 24h. Cells were harvested and fractionated into cytosolic and nuclear fractions and analyzed by Western blot. A representative Western blot shows expression of NEDD9 relative to CPE-ΔN at different concentrations of TSA as indicated. (c) and (n) represent cytosol and nuclear fractions respectively. The graph shows the quantification of NEDD9 and CPE-ΔN bands from Western blots of 3 different experiments. Values are the mean ± SEM.

Supplemental Figure 6.

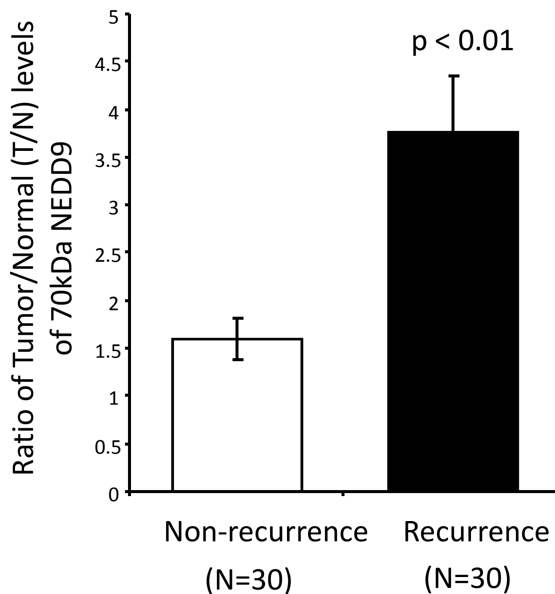
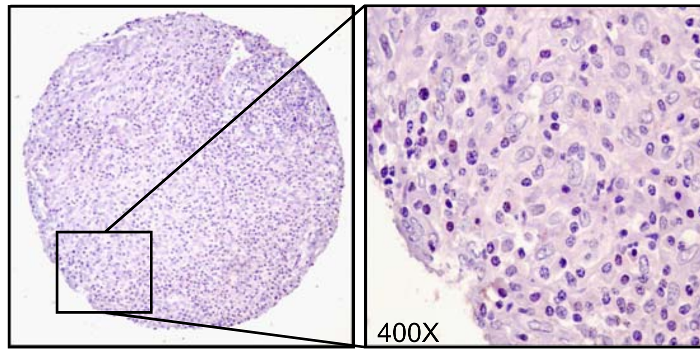


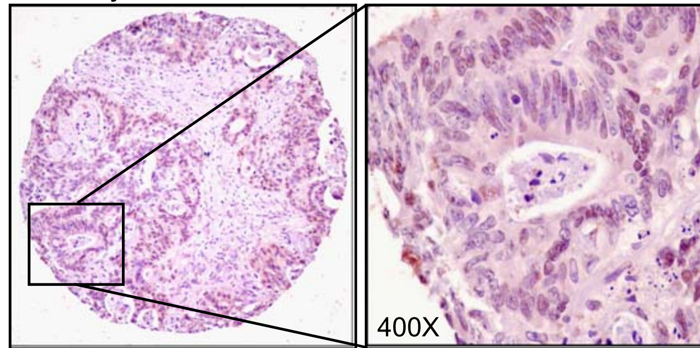
Figure 6. Levels of 70kDa NEDD9 in HCC patients with recurrent and non-recurrent cancers. Bar graphs show expression levels of 70 kDa NEDD9 protein in 60 HCC patients as a tumor/normal (T/N) ratio in Western blots analyses quantified by densitometry.

Supplemental Figure 7.

A



Primary colon cancer



Metastatic colon cancer

B

Clinicopathological variables	*n*	CPE- ΔN expression T/N≤2	CPE- ΔN expression T/N>2	*P* value
Lymph node and Distant metastases				
No	31	26	5	<0.001*
Yes	37	11	26	

*Statistical significance

Figure 7. Expression of CPE-ΔN in colon cancer cells and correlation with metastasis.
A) To determine if CPE-ΔN is expressed in colon cancer cells and can serve as a biomarker for metastasis, we performed tissue microarray analysis (TMA) on matched pairs of primary and metastatic colon cancer samples from 31 patients with colonic cancer who subsequently developed extra-colon metastases to the liver. The figure shows a representative TMA of CPE-ΔN immunostaining with CPE mouse monoclonal antibody in a resected primary colon cancer (top panel) and in the matched sample from the metastatic colon to liver cancer cells from the same patient (bottom panel). Note the presence of few immunostained cells in the primary cancer compared to the large increase in the number and intensity of CPE-ΔN immunopositive cells in the metastatic cancer. Immunostaining was found in the nucleus of these cells, similar to HCC clinical specimens (Fig. 8C-F). While the antibody used detects both CPE-ΔN and WT-CPE, the latter is not expressed in colon cancer cells, thus the immunostaining reflects CPE-ΔN.
B) To determine if CPE-ΔN could be a diagnostic biomarker for metastasis, colon cancer primary tumors from 68 patients were analysed for CPE-ΔN mRNA using specific primers in qRT-PCR. The table shows that 26 of 31 (83.9%) patients that did not have metastatic disease had a CPE-ΔN mRNA T/N ratio of ≤2, whereas 26 of 37 (70.2%) patients that had lymph node or distant metastasis within a year after surgery had a CPE-ΔN mRNA T/N ratio of >2. This data shows a good correlation between the level of CPE-ΔN mRNA and the metastasis, indicating that CPE-ΔN mRNA is a good diagnostic biomarker for metastasis for colon rectal cancer.