An N-terminal truncated carboxypeptidase E splice isoform induces tumor growth and is a biomarker for predicting future metastasis in human cancers

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Metastasis is a major cause of mortality in cancer patients. However, the mechanisms governing the metastatic process remain elusive, and few accurate biomarkers exist for predicting whether metastasis will occur, something that would be invaluable for guiding therapy. We report here that the carboxypeptidase E gene (CPE) is alternatively spliced in human tumors to yield an N-terminal truncated protein (CPE-ΔN) that drives metastasis. mRNA encoding CPE-ΔN was found to be elevated in human metastatic colon, breast, and hepatocellular carcinoma (HCC) cell lines. In HCC cells, cytosolic CPE-ΔN was translocated to the nucleus and interacted with histone deacetylase 1/2 to upregulate expression of the gene encoding neural precursor cell expressed, developmentally downregulated gene 9 (Nedd9) — which has been shown to promote melanoma metastasis. Nedd9 upregulation resulted in enhanced in vitro proliferation and invasion. Quantification of mRNA encoding CPE-AN in HCC patient samples predicted intrahepatic metastasis with high sensitivity and specificity, independent of cancer stage. Similarly, high CPE-ΔN mRNA copy numbers in resected pheochromocytomas/paragangliomas (PHEOs/PGLs), rare neuroendocrine tumors, accurately predicted future metastasis or recurrence. Thus, CPE-ΔN induces tumor metastasis and should be investigated as a potentially powerful biomarker for predicting future metastasis and recurrence in HCC and PHEO/PGL patients.

Introduction
Cancer mortality often results from metastatic disease and is not the direct effect of the primary tumor. With advances in cancer treatment, control of the primary tumor can be managed by multimodal therapy; however, metastatic disease is frequently refractory to the same therapeutic approaches. Thus, identification of biomarkers that could accurately predict metastasis from the state of the primary tumor would be invaluable for guiding therapy.

Currently, determination of the likelihood of developing metastatic disease is based on morphological and histological criteria such as the size of the primary tumor, local invasion, tumor differentiation together with vascular and capsular invasion, and the presence of cancer cells in lymph nodes. Although these criteria put many patients in a very high-risk category for metastatic disease, a significant number of them do not develop metastatic spread. On the other hand, patients with favorable conventional prognostic factors may still develop metastasis. Numerous mechanisms have been described that modulate metastatic potential, including those both endogenous and exogenous to the tumor cells. Such alterations may recruit genes or proteins crucial in the pathogenesis of metastatic spread, recurrence, or significant local invasiveness. Recently, several studies have identified markers of metastatic spread or to predict metastasis. The density of a tumor microenvironment of metastasis, defined as the tripartite arrangement of an invasive breast carcinoma cell, a macrophage, and an endothelial cell, was found to predict the development of systemic, hematogenous metastases in human breast carcinoma (1). Genetic profiling, pointing toward chromosomal aberrations in patients with early-stage colorectal cancer, identified patients who developed metastatic relapse (2). Murine double minute oncogene (Mdm2) and Ki-67 are well established as important predictors of metastasis in some cancers (3). However, identifying a prognostic marker that would be common to various cancers in their ability to predict development of metastasis or recurrence is of utmost importance. Elucidating and studying new molecules that drive tumor metastasis could lead to a better understanding of the mechanisms underlying this complex process (4, 5) and also provide potential prognostic markers and therapeutic targets for clinical use. In the present study we show, for the first time to our knowledge, that a splice isoform of the pro-hormone processing enzyme carboxypeptidase E (CPE) (6, 7), CPE-ΔN, induces tumor growth and metastasis and is a powerful biomarker for predicting future development of extra- and intrahepatic metastasis (recurrence) in patients with hepatocel-
Hence, mice with cholesterol/sphingolipid-rich microdomains at the trans-Golgi of non-endocrine cancers, including colon-rectal, renal, and cervical obesity susceptibility gene, CPE (MW 5.5 and is found primarily in endocrine and neuroendocrine cells (6). Peptide hormones and neuropeptides are synthesized as precursors at the rough endoplasmic reticulum, transported to the Golgi complex, and packaged into secretory granules, where they are processed sequentially, first by prohormone convertases (PC1/3 and PC2), which cleave on the carboxyl side of paired basic residues to yield basic residue-extended peptides (8). Soluble CPE (MW ~53 kDa) then cleaves the basic residues to generate biologically active peptide hormones and neuropeptides (6, 7). An approximately 55-kDa membrane form of CPE anchored to cholesterol/phospholipid-rich microdomains at the trans-Golgi network (TGN) functions as a receptor for sorting prohormones into the regulated secretory pathway granules for secretion (9, 10). Hence, mice with Cpe mutations that lack CPE, and Cpe-knockout mice, have many pathophysiological conditions, such as diabetes, infertility, obesity, low bone mineral density, and deficits in learning and memory (11–14).

CPE of various molecular weights, some different from the 55-kDa WT CPE, have been found in human pulmonary neuroendocrine tumors (15), insulinomas (16), and epithelial-derived hepatomas (17). Moreover, high-throughput microarray studies have correlated elevated CPE mRNA levels with tumorigenesis in a number of non-endocrine cancers, including colon-rectal, renal, and cervical cancers (GEO Profile ID: GDS2609, GDS1780, GDS505, GDS2416; see Bioinformatics section in Methods). However, the role of CPE in tumor progression was undescribed. Alternative splicing of mRNA transcripts can result in proteins that either function differently or are localized to different cellular compartments compared with their primary transcript (18–20). Given a possible new role of this obesity susceptibility gene, Cpe (21), in tumorigenesis, independent of its enzymatic function, we carried out a bioinformatics search (see Methods) for alternatively spliced transcripts of this gene. A splice variant transcript of the Cpe gene, which encodes an isoform of CPE lacking the N-terminus (CPE-ΔN), was identified (Figure 1A). Its expression was associated with an increase in the expression of the neural precursor cell expressed, developmentally downregulated gene 9 (Nedd9) and with cell proliferation in mouse Neuro2a cells, a neuroendocrine cell line (our unpublished data). NEDD9 is expressed transiently during embryonic development, and its expression is downregulated in adult mice (22, 23). It has been implicated in cancer development (24, 25) and tumor cell migration (26), and has been identified as a metastasis-promoting gene in melanomas (27), where it has distinct roles in proliferation and invasion of melanoma cells. NEDD9 enhances the invasion and metastasis of melanocytes by interacting with focal adhesion kinase (FAK), which increases focal contact formation and cell attachment, to the extracellular matrix. The mechanism of action of NEDD9 in migration and invasion via interaction with the FAK-CAS-Crk-DOCK180 complex is well described (27, 28). Hence, we investigated the expression of CPE-AN and its action in inducing Nedd9 gene expression, growth, and metastasis in human cancers. In translational studies, we determined that CPE-AN could serve as a prognostic biomarker for predicting future metastasis and recurrence in patients with cancers of different origin, HCC and PHEO/PGL.

Results

CPE-ΔN is highly expressed in human metastatic tumor cell lines, and repression in xenograft tumors decreased metastatic lesions in mice. To investigate the role of CPE-AN in tumor metastasis, we used human HCC cells as a model system. Semiquantitative RT-PCR using specific primers showed that highly metastatic MHCC97H cells had elevated levels of CPE-AN mRNA compared with cells with low metastatic potential (MHCC97L) derived from the same parental cell line (Figure 1B). Northern blotting confirmed a CPE transcript of approximately 2.4 kb, consistent with the predicted size of CPE-AN mRNA, different from the WT CPE transcript, which is 2.5 kb in size (ref. 29 and Figure 1C).

Moreover, WT CPE mRNA (see Methods) and protein (our unpublished observations) were not detected in these epithelial-derived MHCC97 cells. qRT-PCR showed that the CPE-AN mRNA level was 8.5-fold higher in MHCC97H versus MHCC97L cells (Figure 2A). However, a normal hepatocyte cell line, LO2, showed essentially no expression of CPE-AN mRNA compared with HCC cell lines (Supplemental Figure 1; supplemental material available

Figure 1

Schematic and characterization of ΔN-splice variant of CPE in HCC cells. (A) Diagram showing human WT and human ΔN-splice variant CPE-ΔN mRNA and protein derived from bioinformatic analysis (see Methods). (B) Semiquantitative PCR showing the 148-bp predicted product of CPE-ΔN and 18S RNA in high (H) and low (L) metastatic MHCC97 cells after 31 cycles of co-amplification. (C) Northern blot showing the CPE-ΔN transcript of approximately 2.4 kb in MHCC97H cells. (D) Western blot showing the endogenous ΔN-splice variant CPE-ΔN product in MHCC97H cells. The identity of the approximately 40-kDa band as hCPE-ΔN isoform was verified by immunostaining with both a mouse monoclonal CPE antibody and a rabbit polyclonal antibody (no. 6135) generated against a CPE peptide (mouse pre-pro-CPE amino acids 362–379) in our laboratory. Furthermore, absorption control with the antigenic peptide abolished this approximately 40-kDa band. The left lane was run on a separate blot; the right two lanes were run on the same blot but were noncontiguous.
Elevated expression of CPE-ΔN and NEDD9 in metastatic tumor cell lines. (A) qRT-PCR quantification of hCPE-ΔN mRNA in HCC tumor cell lines. Graphs show fold difference in expression of CPE-ΔN mRNA in tumor cell lines relative to primary tumor cells with lowest hCPE-ΔN mRNA expression (first blue bar) made equal to 1. Red bars, highly metastatic cell lines; blue bars, low metastatic cell lines. Note higher expression of hCPE-ΔN mRNA in the highly metastatic cell lines compared with low metastatic cells. Asterisks indicate known highly metastatic or aggressive cell lines. Assays were done in quadruplicate. (B) Top: Western blots showing approximately 40 kDa hCPE-ΔN and 70 kDa NEDD9 in HCC tumor cell lines. Bottom: The intensities of the bands from the Western blots were quantified by densitometry. Graphs show expression levels of hCPE-ΔN (blue) and NEDD9 (red) for each cell line, corrected for actin levels and expressed as mean ± SEM in arbitrary units (n = 3 experiments). (C) Western blot showing approximately 40 kDa CPE-ΔN and 70 kDa NEDD9 in highly metastatic HCC cells (MHCCCLM3). Cells were infected with si-Scr or CPE-ΔN siRNA (sequences 1, 2, 3) to downregulate CPE-ΔN mRNA expression. All 3 clones showed coordinated downregulation of CPE-ΔN and NEDD9 protein.

To demonstrate that CPE-ΔN induces growth and cell invasion, highly metastatic human liver MHCCCLM3 cells were downregulated in CPE-ΔN expression by siRNA. Suppression of CPE-ΔN expression in highly metastatic MHCCCLM3 cells led to inhibition of growth and invasion (Supplemental Figure 3). Furthermore, in each of the 3 clones stably transduced with 3 different CPE-ΔN siRNA sequences, a decrease in NEDD9 was also observed (Figure 2C). To complement these observations in vivo, we used two animal models. In one, nude mice were subcutaneously injected in the right flank with luciferase-expressing MHCCCLM3 cells transduced with either si-CPE-ΔN (sequence 2) or si-Scr (Figure 3A and ref. 31). Thirty days after cell inoculation, bioluminescence imaging showed that control mice bearing the si-Scr MHCCCLM3 cells had tumors 16.2-fold greater in intensity than mice injected with si-CPE-ΔN cells. In another model, a metastatic orthotopic mouse model (Figure 3B and ref. 32), the tumors from mice described in the first model above were removed, cut into 1- to 2-mm cubes, and implanted into the liver of nude mice (32). Thirty-five days after implantation, bioluminescence imaging showed that the si-Scr MHCCCLM3–derived tumors in the mice were 13.9-fold greater in intensity compared with those from si-CPE-ΔN–treated cells. They also developed intrahepatic metastasis and extrahepatic metastasis to the lung (Figure 3B and Supplemental Figure 4) as previously described (33). Mice inoculated with si-CPE-ΔN MHCCCLM3–derived tumors had smaller tumors and showed no metastasis. These in vitro and in vivo results demonstrate that CPE-ΔN promotes HCC growth and metastasis.

Similar results were observed in other human tumor cell lines. Highly metastatic breast, colon, and head and neck cancer cells had elevated expression of CPE-ΔN mRNA compared with matched tumor cell lines with low metastatic potential (Figure 4). Western blot analysis of these cell lines (MDA-MB-231, HT-29, and MDA-1986) showed expression of only CPE-ΔN but not WT CPE (data not shown). Suppression of CPE-ΔN by the stably transfected siRNA in these cell lines (Supplemental Figure 3A) led to 56%–85% inhibition of growth (Supplemental Figure 3B) and 70%–85% inhibition of Matrigel invasion (Supplemental Figure 3C).

CPE-ΔN drives tumor invasion by interacting with HDAC1/2 to upregulate Nedd9 metastasis gene expression. To determine whether CPE-ΔN promotes tumor growth and invasion through activation of Nedd9 gene expression, we transfected low metastatic MHCC97L cells with CPE-ΔN cDNA. Concomitant with an increase in Nedd9 mRNA upon transfection (2.99 ± 0.76-fold; P < 0.001, n = 3), the transfected cells exhibited elevated levels of both CPE-ΔN and NEDD9 protein (Figure 5A). Consequently, significant increases in proliferation and invasion compared with cells transfected with an empty vector were seen (Figure 5B). This enhanced invasion was suppressed when these MHCC97L cells stably expressing CPE-ΔN were infected with lentivirus carrying Nedd9 siRNA.
to downregulate NEDD9 expression (Figure 5, C and D). This result provides direct evidence that CPE-ΔN promoted cell invasion by driving up Nedd9 gene expression.

Thus, CPE-ΔN, which lacks the signal peptide, can be translocated into the nucleus to modulate gene expression. This is consistent with a recent high-throughput protein-protein interaction study that showed a direct interaction of CPE with a chromodomain-helicase-DNA-binding protein (CHD3)/histone deacetylase (HDAC1 and -2) complex (34); however, the authors did not relate a physiological significance to this interaction. Our bioinformatic studies (see Methods) identified a domain homologous to HDAC-interacting proteins in CPE-ΔN (human CPE amino acids 111–196, with a consensus of 60%) (35). Hence, we carried out co-immunoprecipitation studies. Our results indicate that CPE-ΔN interacts with HDAC1/2.
Complementing this result, when the 99 patients were divided into those who showed recurrence at any time during follow-up versus those that did not, a dramatic difference in mean T/N values of 2 or less, whereas 92.3% (24 of 26) of the patients carrying NEDD9 siRNA (Si) or scrambled (Scr) siRNA. (D) The increased invasion of MHCC97L cells overexpressing CPE-ΔN was prevented by treatment of cells with NEDD9 siRNA (Scr: 4.6 ± 0.1–fold, mean ± SEM, n = 3, versus Si: 1.5 ± 0.3–fold, mean ± SEM, n = 3, **P = 0.002).

CPE-ΔN expression. Thus, CPE-ΔN upregulates Nedd9 gene expression, through its interaction with HDAC1/2, to induce tumor cell proliferation and migration.

CPE-ΔN is a powerful prognostic biomarker for predicting future metastasis in HCC patients. We evaluated CPE-ΔN as a potential prognostic biomarker for predicting future extrahepatic or intrahepatic metastasis (also referred to as recurrence) in HCC patients using two retrospective cohorts of patients. In one group of 99 patients, qRT-PCR was used to measure CPE-ΔN mRNA in the primary tumor (T) and surrounding non-tumor tissue (N); in a separate group of 80 patients, Western blotting was used to measure protein levels in the tumor and non-tumor tissues. RT-PCR and Western blotting confirmed that only CPE-ΔN mRNA and no WT CPE mRNA or protein was expressed in primary HCC tumors (see Methods). CPE-ΔN mRNA tumor versus non-tumor was expressed as a ratio (T/N). The cutoff value for T/N of greater than 2 for prediction of future recurrence was first established using a subset of 37 of the 99 patients (the training set; see Reporting Recommendations for Tumor Marker Prognostic Studies [REMARK] guidelines, ref. 38; compliance in Supplemental Data). Then 62 additional HCC patients were studied as a test or confirmatory set to independently assess the usefulness of this cutoff value. Of patients evaluable 2 years after surgery for disease-free status (no metastasis/recurrence), 75.8% (25 of 33) of those who were disease-free had CPE-ΔN mRNA ratios of 2 or less, whereas 92.3% (24 of 26) of the patients with recurrence had a T/N ratio greater than 2 (Tables 1–3).

Complementing this result, the 99 patients were divided into those who showed recurrence at any time during follow-up versus those that did not, a dramatic difference in mean T/N values between the two groups was observed (Figure 7A). Thus, high levels of CPE-ΔN mRNA correlate with metastasis/recurrence. A Kaplan-Meier plot of disease-free survival of the 62 confirmatory HCC patients (Figure 7B) showed shorter disease-free survival times (P < 0.0001) when CPE-ΔN mRNA T/N ratios were greater than 2 (high) in the primary tumor compared with patients with T/N values of 2 or less (low).

Western blots of resected tumors from an additional cohort of 80 HCC patients also showed significantly higher CPE-ΔN (Figure 8A) levels in patients with recurrence compared with the disease-free group. Quantitative analysis (Figure 8B) of the T/N ratios of CPE-ΔN protein from such Western blots from these 80 patients revealed that 82.4% (28 of 34) of the patients with primary HCC who were disease-free 6 months after surgery had tumor CPE-ΔN T/N levels of 2 or less, whereas 76% (35 of 46) of the patients who developed intra- or extrahepatic metastasis within 6 months had primary tumor CPE-ΔN T/N levels of greater than 2. Analysis of NEDD9 expression in resected HCC tumors of these patients showed significantly elevated levels in the recurrent group versus the non-recurrent group (Supplemental Figure 6).

Immunohistochemistry (IHC) of CPE-ΔN in HCC tumors from 37 patients revealed immunostaining primarily in the nuclei of tumor cells in patients who subsequently developed recurrence that was absent in cell nuclei of patients who remained disease-free (Figure 8, C–F). The intensity of immunostaining determined by image analysis (0.402 ± 0.032 vs. 0.279 ± 0.036, mean ± SEM) was statistically different (P < 0.02) between the groups. CPE-ΔN was further evaluated as a biomarker for HCC, following REMARK guidelines (see Supplemental Data).

As noted above, statistical analyses revealed that CPE-ΔN mRNA T/N ratios with a cutoff of greater than 2 predicted future recurrence within 2 years after surgery, with a sensitivity value of 92.3% and a specificity value of 75.8% (Table 2); similar values, 90% and 78%, respectively, were obtained for patients with recurrence 3 years after surgery (data not shown). Very importantly, correlation of a high CPE-ΔN mRNA T/N ratio (>2), with recurrence did not appear to be dependent on the clinicopathological stage of the cancer (Table 3) – despite the inherent imprecision arising from small sample sizes within stages, the odds ratios (ORs) for the impact of a CPE-ΔN ratio greater than 2 in all 3 higher stages were consistent, ranging from 13.3 to 62.1. There were HCC
patients with early, stage 2 tumors who had a CPE-ΔN mRNA T/N greater than 2 and experienced recurrence, while some patients with late, stage 3 and 4 tumors who had CPE-ΔN mRNA T/N less than 2 showed no recurrence 2 or 3 years after surgery. In a logistic regression model for predicting recurrence within 2 years, CPE-ΔN added very significantly to cancer stage as a predictor (OR, 37.6; 95% CI, 6.7–210.6 with associated P = 1.8 × 10^−7).

High CPE-ΔN mRNA copy numbers in resected tumors predicted metastasis and recurrence in PHEO/PGL patients. To evaluate CPE-ΔN as a prognostic biomarker for another type of cancer, we carried out a prospective study on patients with pheochromocytomas/paragangliomas (PHEO/PGLs), rare tumors of neuroendocrine origin. These tumors are often fatal due to catecholamine excess or development of metastatic disease (39). Currently, there are no reliable markers that would predict malignant behavior of these tumors. Among familial syndromes in PHEO/PGL, those related to succinate dehydrogenase subunit B gene mutations (SDHB) are known to have a high frequency of metastatic disease (40). Here we analyzed tumors from 8 patients with SDHx gene mutations (6 with SDHB and 2 with subunit D [SDHD] mutations); 5 patients with RET gene mutation, which leads to multiple endocrine neoplasia type 2 (MEN2); and 1 patient who had SDHB gene polymorphism (patient S18) of unclear clinical significance, but with metastatic disease at an initial diagnosis. CPE-ΔN mRNA copy numbers in the resected tumors were determined, since surrounding normal tissue for comparison was unobtainable. Patients in the SDHB/D category diagnosed with metastatic tumors at time of surgery had tumor CPE-ΔN mRNA copy numbers of 5–11 million, compared with 150–200 thousand in 4 patients with primary solitary or multiple benign tumors (based on their location and behavior); these 4 patients were disease free with regular follow-up from 2 to 4 years after resection (Table 4). However, a fifth patient (S31), with a benign tumor at resection, had a CPE-ΔN mRNA copy number similar to that in metastatic tumors (Table 4) and hence was predicted to develop recurrent or metastatic disease. At 2 years of follow-up, this patient was documented to have a local recurrence.
with metastasis to bone. In the MEN2 category, 4 patients had resected benign primary PHEO and 1 patient (M06) had recurrent adrenal PHEO (Table 4). Of these 5 patients, 2 (M05 and M06) had high tumor CPE-ΔN mRNA copy numbers (6.8–14.8 million) compared with the other 3 who had tumor CPE-ΔN mRNA copy numbers in the 170–200 thousand range, similar to those found in SDHx/B patients with non-metastatic tumors. In follow-up studies, 2 of these low-copy-number patients were disease free for 6.5 and 6 years, respectively; the third was not available for subsequent follow-up. However, both patients, M05 and M06, who were predicted to develop metastasis/recurrence based on their high CPE-ΔN mRNA copy numbers, were found to have recurrence of PHEO in the surgical bed at 8 and 4 years, respectively. CPE-ΔN was further evaluated as a biomarker for PHEO/PGL, following REMARK guidelines (see Supplemental Data). With poor outcome in these patients defined as either having metastatic disease (SDHx) at the time of resection or development of a post-resection recurrence (MEN2), high CPE-ΔN mRNA copy numbers (e.g., >1 million) correlated with poor outcome, while low copy numbers correlated with disease-free survival, with sensitivity and specificity values of 100% (Table 5; cut-points for copy numbers over a wide range gave identical results). These results indicate that CPE-ΔN is a powerful prognostic biomarker for metastatic SDHB or recurrent MEN2 PHEO/PGL.

Discussion

Initially, in pilot studies we found, in cDNA microarray analysis comparing gene expression profiles of highly metastatic MHCC97H and low metastatic MHCC97L cells derived from the same parental cell line, that one of the genes expressed at highest levels in MHCC97H cells was CPE. Western blotting and immunostaining of MHCC97H cells (Figure 6A) revealed an approximately 40-kDa CPE protein localized in the nucleus. The decreased size and nuclear localization of immunoreactive CPE in these cells, which differ from those of WT CPE, prompted us to link the smaller splice variant form of CPE lacking the N-terminal signal peptide we obtained from a bioinformatic search to cancer metastasis. In this study, we have characterized the alternatively spliced CPE gene transcript encoding CPE-ΔN, a CPE isoform, and show that it plays a pivotal role in driving tumor metastasis, invasion, and recurrence through what we believe to be a novel mechanistic pathway. This CPE isoform, lacking an amino-terminal domain, has a distinct cellular localization and phenotype. The function of CPE-ΔN is in stark contrast to that of full-length WT CPE, the product of the primary transcript of the CPE gene, the role of which in the maturation of neuropeptides and peptide hormones in (neuro)endocrine cells is well established. Our data provide compelling evidence that this variant of CPE, CPE-ΔN, increases cell proliferation, alters cell motility, and plays a role in inducing metastasis. Interestingly, we found that CPE-ΔN is expressed transiently in mouse embryos, suggesting a possible physiological role in embryonic development (our unpublished observations).

Our siRNA suppression studies showed that CPE-ΔN promotes growth and invasion ex vivo in several types of human tumor cell lines and metastasis of human HCC cells in vivo in nude mice. Specifically, we demonstrated in low metastatic HCC cells transfected with CPE-ΔN that the mechanism of induction of growth and invasion involves upregulating the expression of the Nedd9 metastatic gene. The function and mechanism of action of the NEDD9 protein in tumor cell proliferation and metastasis are well documented (27).

Bioinformatic studies have identified a domain, homologous to HDAC-interacting proteins, in the CPE sequence (human CPE amino acids 111–196, with a consensus of 60%) (35). Analysis of the molecular structure of CPE (41) revealed that part of this HDAC-binding domain comprises a loop structure that is exposed on the surface of the molecule, while some of it remains shallowly embedded but masked by the amino-terminal domain. Expression of a CPE protein without this amino-terminus, as in the case of CPE-ΔN, would result in an HDAC-binding domain that would be more accessible, thus allowing for an increase in the potential to interact with HDAC. Our studies showing co-immunoprecipitation of CPE-ΔN with HDAC1 and HDAC2 in HCC cells demonstrate CPE-ΔN interaction with HDAC1/2. Perhaps CPE-ΔN may also interact with other members of the CHD3 complex, as suggested by a previous yeast two-hybrid high-throughput protein-protein interaction study (34). Furthermore, using HDAC inhibitors, we showed that upregulation of Nedd9 gene expression was dependent on a mechanism requiring histone deacetylase activity, which could lead to chromatin remodeling. Indeed, this corroborates clinical studies showing a direct correlation between high levels of HDAC1 and tumor aggressiveness and shorter survival in patients with HCC (42).

Translating our findings to clinical studies, we demonstrated that quantification of the CPE-ΔN mRNA levels in primary tumors from HCC and PHEO/PGL patients by qRT-PCR can be used as a powerful tool for predicting future development of recurrence and metastatic disease (Tables 1–5 and REMARK in Supplemental Data). CPE-ΔN is the first highly reliable single-molecule prognostic biomarker for HCC; other single markers such as α-fetoprotein are only useful for diagnosis and have no prognostic value (43, 44). Alteration in CPE-ΔN mRNA is easily detected, with a clear threshold of 2-fold over expression in the primary tumor associated with

### Table 1
Correlation of CPE-ΔN mRNA expression with recurrence in HCC patients

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<th>Clinicopathological variables</th>
<th>CPE-ΔN expression</th>
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<tr>
<td>Recurrence at 2 years</td>
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<tr>
<td>Yes</td>
<td>23</td>
</tr>
<tr>
<td>No</td>
<td>33</td>
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OR, 37.5; 95% CI, 6.44–363.96.

### Table 2
Sensitivity and specificity of CPE-ΔN as a biomarker for recurrence at 2 years

<table>
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<th>Value</th>
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<tr>
<td>Sensitivity (%)</td>
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<tr>
<td>Negative likelihood ratio</td>
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<tr>
<td>Diagnostic OR</td>
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prediction of future intrahepatic metastasis (recurrence) within 2 years, with sensitivity and specificity values of 92% and 76%, respectively. Moreover, prediction of recurrence was highly correlated with T/N ratio (>2) of CPE-ΔN mRNA, independent of cancer stage, demonstrating that it is superior to staging, currently used as a prognostic tool for HCC patients.

Measurement of the CPE-ΔN mRNA copy number in resected PHEO/PGL primary tumors established numbers in the millions for metastatic tumors versus hundreds of thousands for most benign tumors. More importantly, patients with tumors with CPE-ΔN mRNA copy numbers in the millions, but characterized as benign or non-metastatic at the time of surgery, were predicted to develop recurrent or metastatic disease, which subsequent follow-up studies indeed confirmed. Thus, CPE-ΔN is the only biomarker to date that could determine future metastasis and recurrence for PHEO/PGL patients diagnosed with benign tumors (Tables 4 and 5 and REMARK guidelines in Supplemental Data). With a poor outcome defined as one when either the patient had metastatic disease at resection (SHDB) or developed recurrence after resection (MEN2), this biomarker achieved sensitivity and specificity values of 100% in this group of 14 patients (Tables 4 and 5).

In summary, the importance of this CPE-ΔN biomarker is that it is predictive of future development of recurrence as well as the metastatic potential and invasiveness of the tumor, based on CPE-ΔN mRNA present in the primary tumor, independent of tumor stage. Additionally, CPE-ΔN can also serve as a diagnostic biomarker to confirm metastasis diagnosed by other parameters. Furthermore, it is an advantage that this one biomarker can be used for predicting metastasis and recurrence in cancers with different origin, such as epithelial (HCC) and neuroendocrine cancers (PHEO/PGL). We showed that CPE-ΔN mRNA is also found to be elevated in metastatic breast, colon, and head and neck cancer cell lines (Figure 4). Additionally, others have found in high-throughput microarray studies that the elevation of CPE mRNA is correlated with tumorigenesis and metastasis in different types of human cancers, including cervical, colorectal, and renal cancers, Ewing sarcomas, and various types of astrocytomas and oligodendrogliomas (GEO Profile ID: GDS 2416, GDS 2609, GDS505, GDS1813, GDS971; see Bioinformatics section in Methods). However, the form of the CPE transcript expressed in these tumors has not been investigated, and it may well be CPE-ΔN. Indeed, we recently carried out IHC studies and found immunopositive staining for CPE in the nucleus of colon cancer cells that had metastasized to the liver in humans. This is consistent with the form of CPE in colon cancer being CPE-ΔN, especially since highly metastatic human colon rectal cancer cell lines express CPE-ΔN mRNA (Figure 4) but no WT CPE (our unpublished observations). Moreover, we also found that high levels of CPE-ΔN mRNA expression in the primary resected tumor is correlated with lymph node invasion and distant metastasis in patients with colon-rectal cancer (Supplemental Figure 7). Thus, CPE-ΔN could also potentially be a prognostic/diagnostic biomarker for those cancers shown in high-throughput studies to express CPE mRNA. While CPE-ΔN appears to be a general cancer marker, its function as a prognostic marker for predicting future metastasis may be limited to specific cancers.

In conclusion, we have identified CPE-ΔN as an inducer of metastasis, and it acts by translocation into the nucleus where it interacts with HDAC1/2 to upregulate NEDD9 metastatic gene expression, leading to enhanced proliferation, migration, and invasion of tumor cells. Its assay provides a major breakthrough in biomarker discovery with invaluable utility in cancer prognosis for predicting future metastasis and recurrence in patients with HCC and PHEO/PGL, and potentially for other types of cancer as well. Moreover, CPE-ΔN could also be a useful target for therapeutic intervention (45).

**Methods**

*Cell lines.* Three lines of human oral squamous cell carcinoma (Tu167, Tu159, and MDA1986) (46), established from freshly resected human tumors, were obtained from Gary Clayman, University of Texas M.D. Anderson Cancer Center, Houston, Texas, USA. Human HCC cell lines

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**Table 3**

<table>
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<th>Stage</th>
<th>Non-recurrence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Recurrence&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>≤2</td>
<td>≤2</td>
<td>2/2</td>
<td>0/2</td>
</tr>
<tr>
<td>2 (n = 18)</td>
<td>&gt;2</td>
<td>&gt;2</td>
<td>10/13</td>
<td>3/13</td>
</tr>
<tr>
<td>3 (n = 24)</td>
<td>&gt;2</td>
<td>&gt;2</td>
<td>7/10</td>
<td>3/10</td>
</tr>
<tr>
<td>4 (n = 12)</td>
<td>&gt;2</td>
<td>&gt;2</td>
<td>4/5</td>
<td>1/5</td>
</tr>
</tbody>
</table>

<sup>a</sup>Patients with 2-fold or less, or greater than 2-fold CPE-ΔN/total number of patients.

---

**Figure 7**

Elevated CPE-ΔN mRNA expression in HCC tumors correlates with poor prognosis. (A) qRT-PCR quantification of hCPE-ΔN mRNA in tumor versus surrounding non-tumor tissue in HCC clinical samples from <i>n</i> = 99 patients that were followed for recurrence (intrahepatic or extrahepatic metastases) for a median of 25.4 months after surgical resection (range, 0–110 months). Bar graphs show mean ± SEM (**<i>P</i> < 0.01) of T/N in the disease-free (blue) versus recurrence groups (red). (B) Kaplan-Meier plot of disease-free survival of <i>n</i> = 62 HCC patients (not used in determining expression level cut-point) stratified by CPE-ΔN expression levels. Patients with CPE-ΔN mRNA T/N levels greater than 2 (high) were compared with those with T/N of 2 or less (low). For CPE-ΔN (T/N ≤ 2), the median survival time was more than 90 months. For CPE-ΔN (T/N > 2), the median survival time was 8.6 months. Log-rank test showed significant differences between the 2 groups (<i>P</i> < 0.0001) (see Statistics for case-control sampling scheme).
MHCC97L and MHCC97H, derived from the same parental cell line, and MHCCCLM3 (47) were obtained from Liver Cancer Institute, Fudan University (Shanghai, China); H2P and H2M derived from the same parental cell line (12) were obtained from X.Y. Guan, Department of Clinical Oncology, University of Hong Kong, Hong Kong, China. Human colon cancer cell lines (HCT-116, HT-29, and SW480) and human breast cancer cell lines (MDA-MB-231, T47D, and MCF-7) were obtained from ATCC.

Bioinformatic: A non-redundant nucleotide sequence database search was carried out with human and mouse CPE nucleotide sequence as queries (NM_001873.2 and NM_013494.3, respectively). Potential spliced variants (Genbank AK090962 and BY270449) were screened based on differences in nucleotide sequence between the query and the subject sequences. Specific primers at the splice junctions were designed to amplify these variants by PCR in MHCC97 cells. CPE microarray data were mined from GEO Profile data (http://www.ncbi.nlm.nih.gov/geo/).

Semi-quantitative PCR of WT CPE and CPE-ΔN transcripts in HCC cells. RNA was extracted from MHCC97L and MHCC97H using the RNeasy Mini Kit (QIAGEN), and first-strand cDNA was synthesized with 1 μg of total RNA from these cells using Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). Semi-quantitative PCR was performed to quantify CPE-ΔN transcripts using GoTaq Green Master Mix (Promega). 18S RNA was used for normalization. Primer sequences specific for the human ΔN-splice variant CPE-ΔN RNA were fwd: 5'-ATG GCC GGG GCATG AGG GCC GC-3', rev: 5'-GCTGCCGCCCCACCGTGA AA-3'. Primer sequences for amplifying 18S RNA were fwd: 5'-CTCTTAGCAGTGTCCTGGCGC-3', rev: 5'-CTGATGCTTCTGAACTCC-3'. cDNA (0.2 μg) from MHCC97L and MHCC97H cells was used for every reaction. PCR cycling was at 94°C for 15 seconds, annealing at 65°C for 30 seconds, extension at 72°C for 15 seconds, annealing at 65°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 10 minutes. Eighteen microliters of each sample was removed every 3 cycles from 24 to 35 cycles in each reaction to amplify CPE-ΔN and 18S fragments. Amplified PCR products were separated on 1.5% agarose gels with Tris-borate EDTA buffer and stained with ethidium bromide. Gels were captured as digital images and band intensity in such blots for ΔN transcripts using fast SYBR Green Master Mix PCR kit (Applied Biosystems); cycling conditions were 95°C for 5 minutes, followed by 40 cycles of 95°C for 15 seconds, 62°C for 60 seconds. Reactions were performed using an ABI PRISM 7900 Sequence Detector (Applied Biosystems). Fluorescence signals were analyzed using SDS 1.9.1 software (Applied Biosystems). 18S rRNA was used as an endogenous normalization control. Primer sequences for CPE-ΔN RNA and 18S rRNA were as outlined above. All qRT-PCRs were performed in duplicate and averaged to obtain the data point for each specimen. The relative amount of CPE-ΔN mRNA was normalized to an internal control, 18S

Table 4
CPE-ΔN mRNA copy number in SDHB/D and MEN2 PHEO/PGL tumors and prediction of metastasis and recurrence

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Diagnosis4</th>
<th>Genotype</th>
<th>Copy no.</th>
<th>Follow-up status5</th>
</tr>
</thead>
<tbody>
<tr>
<td>S55</td>
<td>Benignc</td>
<td>SDHD</td>
<td>167,550</td>
<td>Disease free (4 yr)</td>
</tr>
<tr>
<td>S65</td>
<td>Benign</td>
<td>SDHB</td>
<td>187,809</td>
<td>Disease free (2 yr)</td>
</tr>
<tr>
<td>S73</td>
<td>Benign</td>
<td>SDHB</td>
<td>200,000</td>
<td>Disease free (2.5 yr)</td>
</tr>
<tr>
<td>S82</td>
<td>Benign</td>
<td>SDHB</td>
<td>200,714</td>
<td>Disease free (2.4 yr)</td>
</tr>
<tr>
<td>S31</td>
<td>Benign</td>
<td>SDHB</td>
<td>11,894,562</td>
<td>Metastatic (2 yr)</td>
</tr>
<tr>
<td>S18</td>
<td>Metastatic</td>
<td>SDHB</td>
<td>5,583,686</td>
<td>Metastatic</td>
</tr>
<tr>
<td>S22</td>
<td>Metastatic</td>
<td>SDHB</td>
<td>10,937,462</td>
<td>Metastatic</td>
</tr>
<tr>
<td>S65-A-1</td>
<td>Metastatic</td>
<td>SDHB</td>
<td>6,181,873</td>
<td>Metastatic</td>
</tr>
<tr>
<td>M20</td>
<td>Metastatic</td>
<td>SDHB</td>
<td>11,057,100</td>
<td>Metastatic</td>
</tr>
<tr>
<td>M12</td>
<td>Benignc</td>
<td>MEN2</td>
<td>194,801</td>
<td>Disease free (6.5 yr)</td>
</tr>
<tr>
<td>M13</td>
<td>Benignc</td>
<td>MEN2</td>
<td>190,139</td>
<td>Disease free (6 yr)</td>
</tr>
<tr>
<td>M15</td>
<td>Benignc</td>
<td>MEN2</td>
<td>168,984</td>
<td>No follow-up information</td>
</tr>
<tr>
<td>M06</td>
<td>Recurrentf</td>
<td>MEN2</td>
<td>6,750,151</td>
<td>Recurrent (4 yr)</td>
</tr>
<tr>
<td>M05</td>
<td>Benignc</td>
<td>MEN2</td>
<td>14,825,680</td>
<td>Recurrent (8 yr)</td>
</tr>
</tbody>
</table>

4Diagnosis at time of surgery. 5Years of follow-up or time to recurrence/metastasis. 6Benign adrenal: No clinical symptoms or signs of disease, with negative imaging and biochemical data where applicable. 7Benign extra-adrenal. 8Recurrent in contralateral adrenal surgical bed. Benign: no metastatic lesions; recurrent: reappearance in surgical bed; metastatic: presence of tumor at sites where chromaffin cells not normally present. Patients S22 and M20 died of metastatic disease at 4 and 3 years, respectively, after surgery; S18 and S95 show progressive metastatic disease since surgery.
Table 5

<table>
<thead>
<tr>
<th>CC cut-point (×10⁸)</th>
<th>SN</th>
<th>SP</th>
<th>PLR</th>
<th>NLR</th>
<th>DOR</th>
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</thead>
<tbody>
<tr>
<td>2.5</td>
<td>100</td>
<td>100</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>100</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>100</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

CC, CPE-ΔN mRNA copy number; SN, sensitivity; SP, specificity; PLR, positive likelihood ratio; NLR, negative likelihood ratio; DOR, diagnostic OR.

RNA, and to a calibrator, given by Livak and Schmittgen (48): \(2^{-\Delta\Delta C_T}\), where \(\Delta C_T = (C_T[^{\text{CPE}}]) - (C_T[^{\text{WT}}])\). A threshold value \((C_T[^{\text{WT}}])\) was defined as the fractional cycle number at which the amount of amplified target reached a fixed threshold. The C-value correlates with the input target mRNA levels, and a lower \(C_T[^{\text{WT}}])\) value indicated a higher starting copy number. One of the samples was designated as the calibrator to compare the relative amount of target in different samples and used to adjust for plate-to-plate variation in amplification efficiency. The relative expression level of CPE of each patient was evaluated as the relative fold change in log2 scale.

**Determination of CPE-ΔN mRNA copy numbers in PHEO/PGL:** Total RNA was extracted from frozen PHEO/PGL tumor pieces after homogenization in TRIzol reagent (Invitrogen) followed by RNeasy Mini kit (QIAGEN) according to the manufacturer’s recommendations. Total RNA (0.2 μg) was then converted to cDNA using the Roche First Strand cDNA Synthesis Kit. CPE-ΔN mRNA copy numbers were determined by setting up a standard curve using known concentrations of hCPE cDNA. A complete clone of hCPE cDNA was excised from its plasmid and purified, and its concentration was determined spectrophotometrically. The conversion of microgram value to picomoles was performed using the following formula:

\[ \text{pmol of dsDNA} = \frac{\text{μg of dsDNA}}{106 \text{ pg/μg} \times 1 \text{ pmol/660 pg}} \times 1/\text{Nbp} \]

(where Nbp is length of the amplicon in bp). Serial dilutions of samples were used as templates for qRT-PCR to generate a standard curve. The qRT-PCR was carried out in triplicate for 8 different conditions using primers specific for hCPE (fwd: 5’-CTCATCCTCCGT-GGAAGGATAA-3’ and rev: 5’-CCTGGAGCTGAGGCTTGAAG-3’). The crossing point was determined from the qRT-PCR program and averaged for each sample. The mean ± SEM of AU from 3 separate experiments. The expression of CPE and NEDD9 levels of each cell line was corrected for their actin level and expressed as the mean ± SEM of AU from 3 separate experiments.

**Lentivirus-based suppression of CPE-ΔN and NEDD9 in tumor cell lines.** Lentiviral particles (Dana Farber Cancer Institute–Broad Institute RNAi Consortium) expressing shRNAs against human CPE and human NEDD9 were used to downregulate their mRNA. Transduced cells were selected with 2 μg/ml puromycin. For CPE, initially 3 shRNAs were used: CPE-sh1-CCGGCCGGAATCATGATCCTGACATCTCGAGATTCACG-GAATACCTCCGAGATTTTTG; CPE-sh2-CCGGTTCCGAGGCTATCTCGAGATTCACG-GAATACCTCCGAGATTTTTG; and CPE-sh3-CCGGGATAGGATAGTGTACGTGAATCTCGAGATTCACG-GAATACCTCCGAGATTTTTG. Subsequently, lentivirus CPE-sh2 was used in the tumor cell lines described in Figure 2. For NEDD9, the shRNA used was Nedd9-sh1-CCGGCCGGAATCATGATCCTGACATCTCGAGATTCACG-GAATACCTCCGAGATTTTTG. A scrambled shRNA lentivirus was used as the negative control.

**Immunofluorescence of CPE-ΔN in HCC tumor cells.** MHCCCL3 cells transfected with either si-scrambled or si-CPE, which downregulates CPE-ΔN mRNA expression, were cultured on chambered slides, permeabilized with 0.1% Triton X-100, and fixed with 4% paraformaldehyde in PBS. The cells were incubated with monoclonal antibodies against carboxypeptidase E (1:100) (R&D Systems). The secondary antibody was TRITC-conjugated goat anti-mouse IgG (Molecular Probes, Invitrogen). The slide was subse-
A set of 99 patients. Due to limited follow-up in a few patients, only 92 of one patient did not have usable normal tissue available, all analyses used the end of the follow-up period was September 2009, with a median follow-up of 1 year. The 99 were analyzable for the primary end point of 2-year recurrence. The expression of ΔN in HCC cells; thus, the immunostaining reflects ΔN mRNA from resected primary tumor and surrounding normal tissue from a subset of 37 of the 99 HCC patients (the “pilot” or “training” set) was used. A cut-point T/N value of 2 was established — ratios above this cut-point indicated likely tumor recurrence within 2 years. This cut-point provided a near optimal balance between sensitivity and specificity in this subset of patients. The remaining 62 of the 99 patients were used to predict, using the cutoff of 2 for the T/N ratio, which patients would be disease-free and which would experience recurrence within 2 years. Similarly, the 80 patients with protein levels established by Western blot, were evaluated for recurrence within 6 months after surgery, comparing protein T/N ratios between those with recurrence and those without. Although not prospectively evaluated at this cut-point, a protein T/N ratio greater than 2 again appeared to be highly correlated with recurrence during this time period.

HCC patient follow-up and survival analysis. All patients were followed monthly in the first year and thereafter quarterly, with regular monitoring for recurrence by serum α-fetoprotein level and ultrasonography or CT scan 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 the last follow-up. Actuarial survival was measured from the date of hepatic resection to the date of death or the last follow-up.

Tumor samples and clinical pathology of PHEO/PGL patients. For a prospective blinded study, all PHEO and PGL patients were enrolled and patient samples collected under IRB-approved protocol 00-CH-0093 at the NIH, Bethesda, Maryland, USA. Results for all n = 14 patients are included in this report (no selection of cases). All patients provided written informed consent, and all but one were followed for a minimum of 2 years after resection of their primary tumors. Upon resection, the tissue specimen was placed in a sterile container on ice and transported for immediate examination by a pathologist, and tissue that was not necessary for diagnostic purposes was dissected from the tumor, excluding necrotic areas and tumor margin or capsule. Further dissection into pieces no larger than 0.5 x 0.5 x 0.5 cm was performed on ice, and samples were frozen directly in liquid nitrogen.

Immunostaining and quantification of CPE-ΔN in human tissue sections. HCC tumor tissue and surrounding non-tumor tissue were formalin fixed and paraffin embedded. Four-micrometer sections were cut, dewaxed in xylene and graded alcohols, hydrated, and washed in PBS. After pretreatment in a microwave oven (12 minutes in sodium citrate buffer [pH 6.1]), the endogenous peroxidase was inhibited by 0.3% H2O2 for 30 minutes, and the sections were incubated with 10% normal goat serum for 30 minutes. Mouse monoclonal anti-carcinoembryonic antigen (1:100) (R&D Systems) was applied overnight in a humidity chamber at 4°C. (While the antibody used detects both CPE-ΔN and WT CPE, the latter is not expressed in HCC; thus, the immunostaining reflects CPE-ΔN.) A standard avidin-biotin peroxidase technique (Dako) was applied. Briefly, biotinylated goat anti-mouse immunoglobulin and avidin-biotin peroxidase complex were applied for 30 minutes each, with 15-minute washes in PBS. The reaction was finally developed with the Dako Liquid DAB+ Substrate Chromogen System (Dako). Slides were imaged on an Aperio Scanscope CS imager, generating 0.43-μm/pixel whole slide images. These images were compiled and analyzed using the Aperio Spectrum software with a pixel count algorithm (49). Quantified expression of tumor tissue minus adjacent normal tissue was compared for patients with and without recurrence.

Statistics. The primary statistics used for non-patient samples were means and SEMs for descriptive statistics, and standard unpaired t tests for comparing independent observations (paired versions for comparing paired
observations). The statistical methods used for the PHEO/PGL section are descriptive, with no formal analyses being performed due to the small number of patients and their variable follow-up. Table 4 shows all pertinent data for the 14 patients. Although Table 5 shows several sensitivity/specificity values, they are only illustrative, emphasizing what is apparent from the patient-by-patient data listing: copy numbers divide into two very separate groupings; and, using current patient follow-ups, these copy numbers perfectly separate those having a bad outcome — defined as either being metastatic at resection or developing recurrent or metastatic disease during follow-up — from those having a good outcome.

For HCC patients, standard t tests (and means and SEMs) are reported for comparing — either in the group of 99 patients evaluated for mRNA by qRT-PCR or the group of 80 patients evaluated by Western blot for protein — the levels of these products in those with recurrence and those without. The primary end point for HCC patients was 2-year recurrence in the 99 patients with mRNA T/N ratios. A training subset of 37 patients from this group was used to evaluate potential T/N cut-points; using these results, a cut-point of 2 was established, based on its excellent sensitivity and specificity. This cut-point was then evaluated on the remaining 62 patients — the test subset. The training and test sets were very similar with respect to recurrence rates and other patient characteristics: in the training set, 45.5% were 53.5 years and 55.1 years, respectively; and the proportions of males were 78.4% and 85.5%, respectively.

Using 2-year (very similar results were obtained using 3 years) recurrence as the primary end point was dictated by the initial case-control selection of 50 recurrences and 50 non-recurrences from the full population; because this was not a random selection from the population, there is the potential that survival-based analyses could lead both to biased estimates of survival curves and to statistical tests giving erroneous \( P \) values (see ref. 50 for a study, based on actual data, of the possible associated problems). Using a dichotomous outcome eliminates this problem. Basing analyses on ORs or Fisher’s exact test insures the validity of the results (see ref. 51 regarding the applicability of OR-based methods, including logistic regression and Fisher’s test, for case-control designs).

With 2-year recurrence as the end point, 92 of the 99 had sufficient follow-up for analysis: 33 of 37 in the training set and 59 of 62 in the test set. Since 2-year follow-up captured 41 of all 50 recurrences (82%), only a small amount of information was lost (analyses using 3-year recurrence captured 47 of the 50 recurrences). Any small loss of power was irrelevant, since the primary \( P \) values were around \( 10^{-7} \). Besides standard estimates of ORs, their exact CIs, and exact \( P \) values based on Fisher’s test (StatXact, Cytel Software Corp.), logistic regression was used to assess whether CPE-AN added significantly to a model with cancer stage (the only standard prognostic variable for this disease) as a predictor (Stata). This was the only multivariate analysis, and the only one involving any modeling assumptions.

To complement these analyses of 2- and 3-year recurrence rates, we present a Kaplan-Meier plot and log-rank \( P \) value for time to recurrence, although case-control sampling may introduce some bias (and we did not have the data that could “adjust” the nominal log-rank \( P \) value to account for the bias). Our 3-year recurrence rate was 52.5%, very similar to the 48.2% seen in the full population of \( n = 317 \); hence, the bias in the Kaplan-Meier estimates should be only a few percent (similar to ref. 50), which is swamped by the enormous differences between the two survival curves in Figure 7B. The nominal log-rank \( P \) value (\(< 10^{-7}\)) is likely too small, due to case-control sampling bias and the \( \chi^2 \) approximation; however, the valid OR \( P \) values at 2 or 3 years were 1.7 \( \times \) 10\(^{-7}\) and 2.2 \( \times \) 10\(^{-8}\), so a valid log-rank \( P \) value should be in a similar range; to be conservative, we simply report it as less than 0.0001.

Other methods. Colony formation assay, Matrigel invasion assay, animal studies, and Northern blotting are presented in Supplemental Data.

Acknowledgments

We thank Tamara Prodanov and Alicia Woronowicz (NICHHD) for helpful discussions, Rebecca McGirr (Lawson Health Research Institute) for technical assistance, David Carter (London Regional Genomics Centre) for microarray analysis, Jana Wo and Simon Yau (University of Hong Kong) for animal work and qRT-PCR of the clinical tumor samples, respectively, and Chikao Morimoto (Tokyo University) for NEDD9 N-terminal antibody. We thank Steven Coon (NICHHD) for assisting in Northern blots. This research was supported by the Intramural Research Program of the NICHHD, NIH; a Natural Sciences and Engineering Research Council of Canada Discovery Grant to S. Dhanvantari; and University of Hong Kong research grants to R.T. Poon and I.O. Ng. I.O. Ng is Loke Yew Professor in Pathology.

Received for publication August 13, 2010, and accepted in revised form December 1, 2010.

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