Oncogene (2012) 31, 2640–2652

www.nature.com/onc

ORIGINAL ARTICLE

Somatic LMCD1 mutations promoted cell migration and tumor metastasis in hepatocellular carcinoma

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Common genetic alteration in cancer genomes is implicated for embracing an aberrant cancer gene participated in tumor progression. In this study, we identified a somatic mutated LIM and cysteine-rich domains-1 (LMCD1) as a putative metastatic oncogene in human hepatocellular carcinoma (HCC) using integrated genomic approaches. In addition to revealing genomic amplification and gene upregulation, we identified recurrent E135K (3/48 cases) mutations in HCC tissues and K237R mutation in the PLC/PRF/5 HCC cell line. Expression of mutant LMCD1 E135K or K237R reduced the stress fiber assembly, increased cortical actin accumulation and induced lamellipodial extension. Consistently, these mutations enhanced cell migration and showed activation of the Rac1-signaling pathway. Inhibition of the LMCD1/Rac1 pathway by an *LMCD1* short-hairpin RNA (*shLMCD1*) or the Rac1 inhibitor NSC23766 suppressed the mutation-mediated lamellipodial protrusion and cell migration. In PLC/PRF/5 cells with endogenous K237R mutation, cell migration was enhanced by estrogen-induced LMCD1 expression but reversed by *shLMCD1* treatment. Moreover, overexpression of LMCD1 E135K mutation significantly promoted systemic lung metastasis in a murine tail vein injection model. Together, our results suggest that LMCD1 mutations are potential oncogenic events in HCC metastasis to promote cell migration through the Rac1-signaling pathway.
Oncogene (2012) **31,** 2640–2652; doi:10.1038/onc.2011.440; published online 26 September 2011

Keywords: somatic mutations; *LMCD1*; LIM domain; metastasis; hepatocellular carcinoma

Introduction

Liver cancer is the sixth most common cancer in the world and the third most common cause of cancer mortality. Majority (>80%) of liver cancers occur in Asia and sub-Saharan Africa (Parkin *et al*., 2005). Hepatocellular carcinoma (HCC) is the most common type of liver cancer, accounting for 83% of all primary liver cancers (Farazi and DePinho, 2006). Tumorigenesis of HCC is a slowly progressive and multistep process with several known etiological factors, including hepatitis viral infection (hepatitis B or C virus), consumption of aflatoxin-B1-contaminated foods and alcohol abuse. Continuous insults of hepatocytes with these etiological agents may induce inflammatory cell injury and consequent rounds of necrosis and proliferation. Liver cirrhosis is commonly observed after these deteriorated hepatocytes were replaced with regenerative nodules surrounded by collagen fibrous scarring. The hyperplastic nodules could further evolve into pre-malignant dysplastic nodules and eventually progress to HCC accompanied by increasing genomic instability and accumulated chromosomal alterations.

Recurrent chromosomal alterations in cancers are commonly associated with putative cancer genes. In HCC, many tumor-suppressor genes and oncogenes have been identified based on genetic lesions; for example, loss of *TP53* (17p13) (Wang *et al*., 2001), *RB* and *BRCA2* (13q) (Knuutila *et al*., 1999), as well as amplification of *c-myc* (8q24) (Kusano *et al*., 1999) and *ERBB2* (17q12-q21) (Niketeghad *et al*., 2001). Epigenetic mechanisms also contribute to HCC, such as CpG hypermethylation of *p16(INK4a)* and *COX2* (Matsuda *et al*., 1999; Murata *et al*., 2004), as well as altered expression of miR-122 and miR-21 (Gramantieri *et al*., 2007; Meng *et al*., 2007). Point mutation is another common mechanism to alter functions of cancer genes. In HCC, frequent point mutations of *p53* and β-*catenin* are involved in key pathways of hepatocarcinogenesis (Ozturk, 1991; de La Coste *et al*., 1998; Jou *et al*., 2004). Other studies have reported HCC mutations in *axin-1*, *Ras*, *M6P/IGF2R*, *Smad2/4* and *PTEN* (Challen *et al*., 1992; De Souza *et al*., 1995; Yakicier *et al*., 1999; Yao *et al*., 1999; Satoh *et al*., 2000; Imbeaud *et al*., 2010). However, HCC remains a highly lethal cancer owing to the lack of biomarkers and targets for early diagnosis, categorization and therapeutic interventions. Although an oral multi-kinase inhibitor, sorafenib, has been demonstrated to extend survival in advanced HCC patients for almost 3 months longer than those adminis-

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Received 3 April 2011; revised 2 August 2011; accepted 25 August 2011; published online 26 September 2011

tered placebo (Llovet *et al*., 2008; Cheng *et al*., 2009), identification of novel target genes with high-throughput genomic technologies should be beneficial to increase therapeutic options for HCC patients.

In this study, we applied a genome-wide approach to analyze copy-number alterations and identified an overlapped amplicon at the 3p26-24 region in cancer genomes. After examining aberrant expression and somatic mutations (6.25% of E135K mutations in 3/48 cases) in HCC tissues, we identified mutated *LMCD1* as a putative metastatic oncogene in HCC. LMCD1 (LIM and cysteine-rich domains-1, dyxin) is a member of the LIM protein family containing two C-terminal LIM domains, a central PET (Prickle, Espinas and Testin) domain and an N-terminal cysteine-rich region (Bespalova and Burmeister, 2000). LIM domains contain two contiguous zinc-finger motifs with a defined consensus sequence, $CX_2CX_{16-23}HX_2(C/H)X_2CX_2CX_{16-21}CX_2(C/H/D)$, and are mostly observed in proteins associated with actin cytoskeleton to regulate cell adhesion or migration (Khurana *et al*., 2002). Nevertheless, many of them can be localized to the nucleus and function in modulating gene expression (Kadrmas and Beckerle, 2004). LMCD1 has been described previously as a transcriptional co-repressor of GATA6 (Rath *et al*., 2005), whereas its role in cytoskeletal compartments remains to be elucidated. To further characterize mutant LMCD1 in HCC tumorigenesis, we hypothesized that mutations of LMCD1 could have a role in tumor cell migration *in vitro* and metastasis *in vivo*. Indeed, we demonstrated that E135K mutant LMCD1 induced lamellipodia formation by upregulation of the Rac1 pathway, which contributed to cell migration *in vitro* and enhanced tumor metastasis *in vivo*.

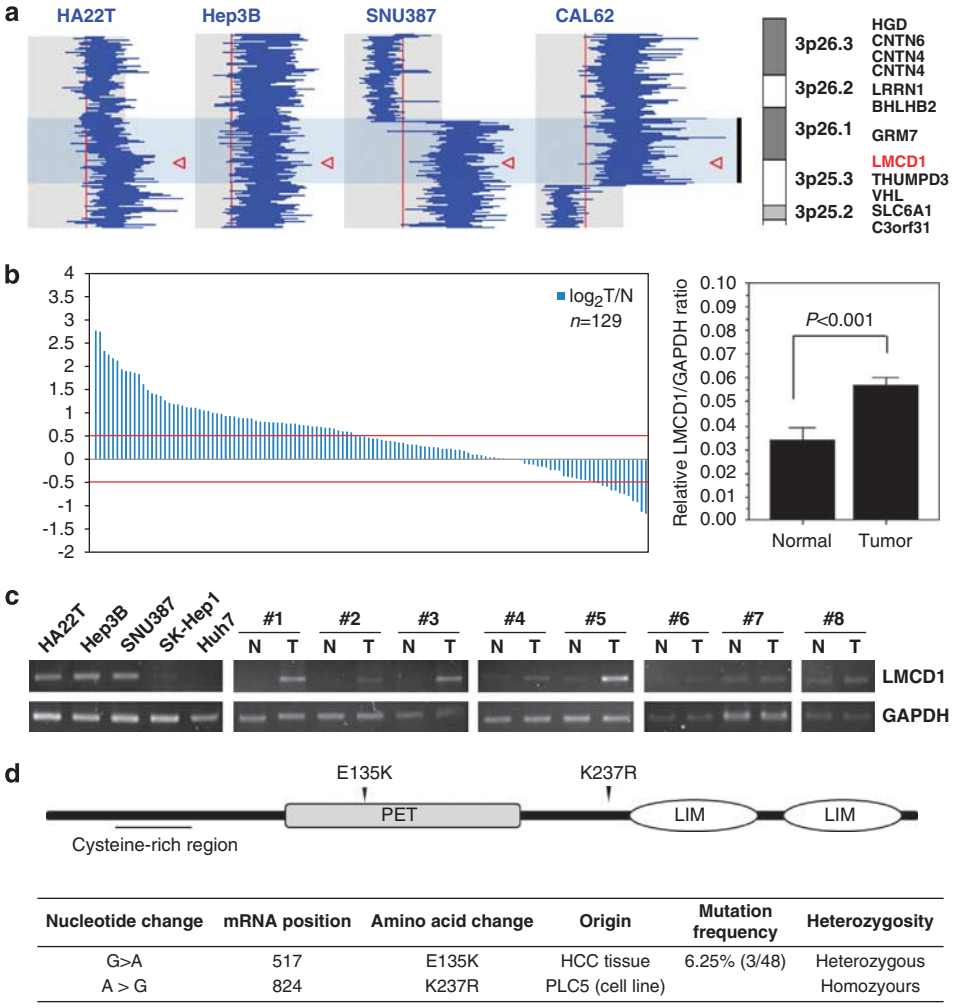
Results

Amplification, upregulation and point mutations of LMCD1 *in HCC*

To search for putative cancer-related genes in a high-resolution genomic approach, we performed a genome-wide DNA copy-number alteration study in multiple human cancer cell lines using Affymetrix 500K SNP GeneChip Arrays and used non-matched normal reference genomes as controls (Chen *et al*., 2010). The median smoothing method was used with a window size of five continuous single-nucleotide polymorphisms (SNPs) to minimize data variation and obtain the inferred copy number (ICN) of each SNP. An overlapped amplified region on chromosome-3p was detected in three HCC cell lines, HA22T (ICN = 2.71), Hep3B (ICN = 2.76) and SNU387 (ICN = 3.32). Interestingly, by incorporating a 3p amplified region from the thyroid carcinoma CAL62 cell line (ICN = 3.46), we narrowed down and defined a 2.53-Mb amplicon at 3p26.1-25.3 (Figure 1a). Among 8 known and 6 predicted genes residing in this region, *LMCD1* was chosen as a candidate cancer gene based on the following three reasons: (1) *LMCD1* is located at the amplification peak of overlapped amplicons in SNU387 and Hep3B cells; (2) several LIM domain-containing proteins have been shown to be relevant to tumor progression (Bagheri-Yarmand *et al*., 2006; Jagadeeswaran *et al*., 2008) and (3) *LMCD1* is commonly overexpressed in HCC tissues based on *in silico* analysis of HCC data sets from the iCOD database (61 of 129, $\log_2 T/N > 0.5$), with a significantly higher average *LMCD1*/*GAPDH* level in HCCs than in normal references ($P < 0.001$ by two-sample *t*-test) (Figure 1b). The upregulated *LMCD1* was further validated in these three HCC cell lines, HA22T, Hep3B and SNU387, and eight pairs of HCC tissues by semi-quantitative reverse transcription–PCR assay (Figure 1c). Furthermore, we also identified a novel recurrent point mutation, G517A (E135K), in the PET domain that occurred in 3 of 48 HCC cases (6.25%) after sequencing analysis of all exons of *LMCD1*. In the PLC/PRF/5 HCC cell lines, another A824G (K237R) mutation just before the first LIM domain was also identified (Figure 1d and Supplementary Figure S1). Both mutations were not found in the existing SNPs in dbSNP database and the expression of mutant alleles were further validated by DNA sequencing of cDNAs converted from mRNAs of HCC tissues and PLC/PRF/5 cell lines. These results suggest that *LMCD1* might be a hotspot of genetic alterations in HCC.

LMCD1 mutations E135K and K237R induce lamellipodia formation

We established SK-Hep1 transfectants stably expressing wild-type (wt) and mutant LMCD1, wtLMCD1, LMCD1-E135K and LMCD1-K237R, with confirmation of protein expression by western blot analysis (Figure 2a). In contrast to most wtLMCD1 cells displaying classical spindle-shaped epithelial morphology similar to that of mock cells, a significant high proportion of cells expressing LMCD1-E135K and LMCD1-K237R protruded apparent lamellar extensions (77.43 and 65.56% of E135K and K237R cells, respectively, in compared with 28.71% of mock cells and 31.24% of wtLMCD1 cells) (Figures 2b and c). By staining with lamellipodia marker Arp3, the significant enrichment of Arp3 staining indicated the increased formation of lamellipodia in the leading edge of LMCD1-E135K and LMCD1-K237R cells (Figure 2c). We further examined the subcellular localization of LMCD1 by immunofluorescence staining with anti-Flag antibody, together with rhodamine-phalloidin, for F-actin visualization. Although wtLMCD1 widely expressed in nucleus and cytoplasm, a portion of cytoplasmic wtLMCD1 was shown to co-localize with stress fibers (Figure 2d). LMCD1-E135K and LMCD1-K237R expression somewhat reduced the stress fiber assembly but predominantly augmented and co-localized cortical actin accumulation, which was consistent with their capability to induce lamellipodial extension. These results indicate that LMCD1 may play a role in the regulation of actin cytoskeleton organization to modulate cell morphology and mutants LMCD1 redistributes the F-actin pool from stress fiber to lamellipodia.



Nucleotide change	mRNA position	Amino acid change	Origin	Mutation frequency	Heterozygosity
G>A	517	E135K	HCC tissue	6.25% (3/48)	Heterozygous
A > G	824	K237R	PLC5 (cell line)		Homozyours

Figure 1 Somatic alterations of *LMCD1* in HCC. (**a**) A common amplicon is amplified in HA22T, Hep3B and SNU387 HCC cells. The shaded region across the amplicons indicates the overlapped amplicon 3p26.1-25.3 narrowed down by incorporating a thyroid carcinoma CAL62 cell line. The red triangles indicate the location of *LMCD1*. (**b**) *LMCD1* is commonly upregulated in 61 of 129 (47.29%) HCC tissues from iCOD data sets with a criterion of $\log_2$T/N over 0.5. Relative expression ratios of *LMCD1*/*GAPDH* in normal and HCC tissues are shown on the far right. The data represent means ± s.e.m. (**c**) Expression of LMCD1 in HCC cell lines and tumor pairs by semi-quantitative reverse transcription–PCR assay. (**d**) Top: Schematic illustration of the mutant residues of LMCD1. LMCD1 contains a cysteine-rich region in the N-terminal region, a PET domain in the central part and two LIM domains in the C-terminal end. Bottom: *LMCD1* mutations of 48 HCC tumor tissues and 13 HCC cell lines.

E135K and K237R mutations promote cell migration

To evaluate the functions of wild-type and mutant LMCD1 in HCC tumorigenesis, we examined SK-Hep1 transfectants for cell proliferation by Alamar Blue assay and transformation by anchorage-independent growth assay in soft agar. To our surprise, capabilities of cell proliferation and *in vitro* cellular transformation were unchanged in comparison between mock control and all transfectants (Figures 3a and b). As a lot of LIM-domain-containing proteins were characterized to function in cytoskeletal organization or cell migration (Arber *et al*., 1998; Pratt *et al*., 2005; Barrientos *et al*., 2007), and as both mutants induced lamellipodia formation in our results, we speculated that LMCD1 mutants might potentially have a role in cell migration regulation. To test this hypothesis, we performed three different cell migration assays for reflecting alteration of directed migration, random migration and chemotaxis. Directed migration of these SK-Hep1 transfectants was inspected by wound-healing assay. In accordance with previous results, lamellipodia formation of LMCD1-E135K and LMCD1-K237R cells at wound edge was conspicuous and could be detected as early as 2 h after wounding (Supplementary Figure S2). Accelerated wound closure was observed for LMCD1-E135K and LMCD1-K237R cells as expected (Figure 3c). To further monitor the morphology of wound-edge migrating cells, live-cell imaging was performed (Supplementary Videos 1–4). The LMCD1-E135K and LMCD1-K237R cells moved significantly faster than mock or wtLMCD1 cells, with more dynamically prominent lamellipodia formation. Tracking of randomly selected cells revealed advanced directed migration velocity of 1.65- and 1.75-fold for LMCD1-E135K and LMCD1-K237R cells, respectively, in comparison with mock or wtLMCD1 cells (Figure 3d). Similar results were

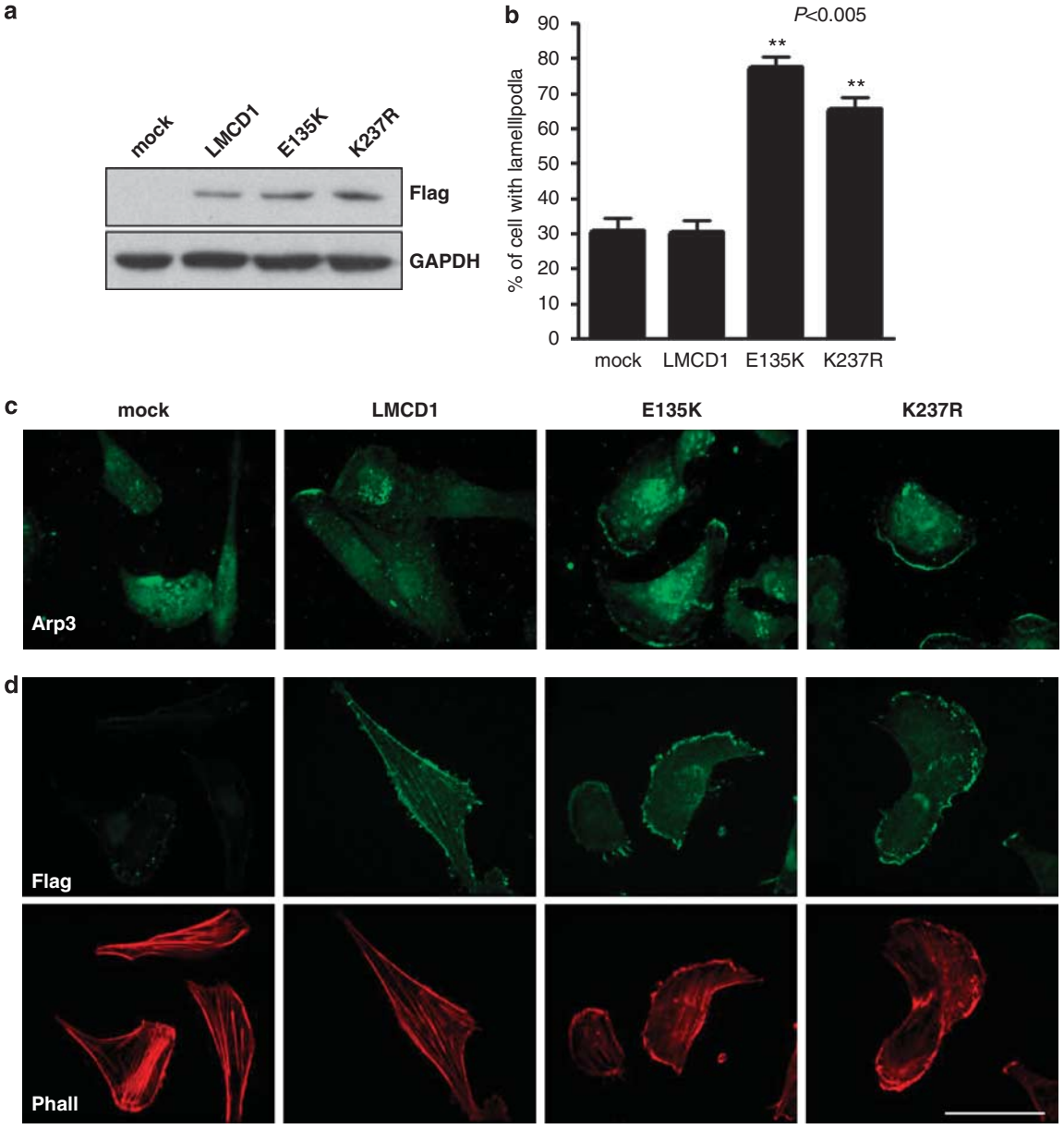


Figure 2 Mutant LMCD1 induces lamellipodia formation. (**a**) Western blot analysis of LMCD1 transfectants in SK-Hep1 cells. (**b**) Percentage of cells with lamellipodia formation calculated by counting 100 randomly selected cells in each group. The data represent means ± s.e.m. (**c**) Immunofluorescence staining using an anti-Arp3 antibody indicates induced lamellipodia formation in LMCD1-E135K- and LMCD1-K237R-expressing cells but not wtLMCD1 cells. (**d**) Double staining with LMCD1 (anti-Flag antibody) and F-actin (rhodamine–phalloidin) shows that wtLMCD1 can colocalize with stress fibers whereas the LMCD1-E135K and LMCD1-K237R mutants reduce stress fiber assembly and colocalize with F-actin in the cell leading edge. Bar: 50 μm.

observed in random migration assay, in which average migration velocity for LMCD1-E135K and LMCD1-K237R cells increased to about 1.47- and 1.8-fold in comparison with mock or wtLMCD1 cells (Supplementary Figure S3). Consistent with the aforementioned two migration experiments, expression of LMCD1-E135K and LMCD1-K237R resulted in 1.62- and 2.78-fold increase in cell chemotaxis mobility in transwell migration assay (Figure 3e).

To avoid selection bias of transfected clones to enhance cell migration, another set of independent clones were examined with the same migration experiments and produced similar results (Supplementary Figure S4). In addition, we also established LMCD1-expressing cells in Huh7 cells to clarify whether the altered functions of E135K and K237R mutations are universal to other HCC cell lines. Two distinct clones of Huh7 transfectants for each construct with different protein expression levels were selected to test in transwell migration assay. Similar to the results in SK-Hep1 cells, expression of LMCD1-E135K and LMCD1-K237R, but not of wtLMCD1, in Huh7 transfectants increased cell migration (Supplementary Figure S5). Taken together, these analyses suggest that LMCD1-E135K

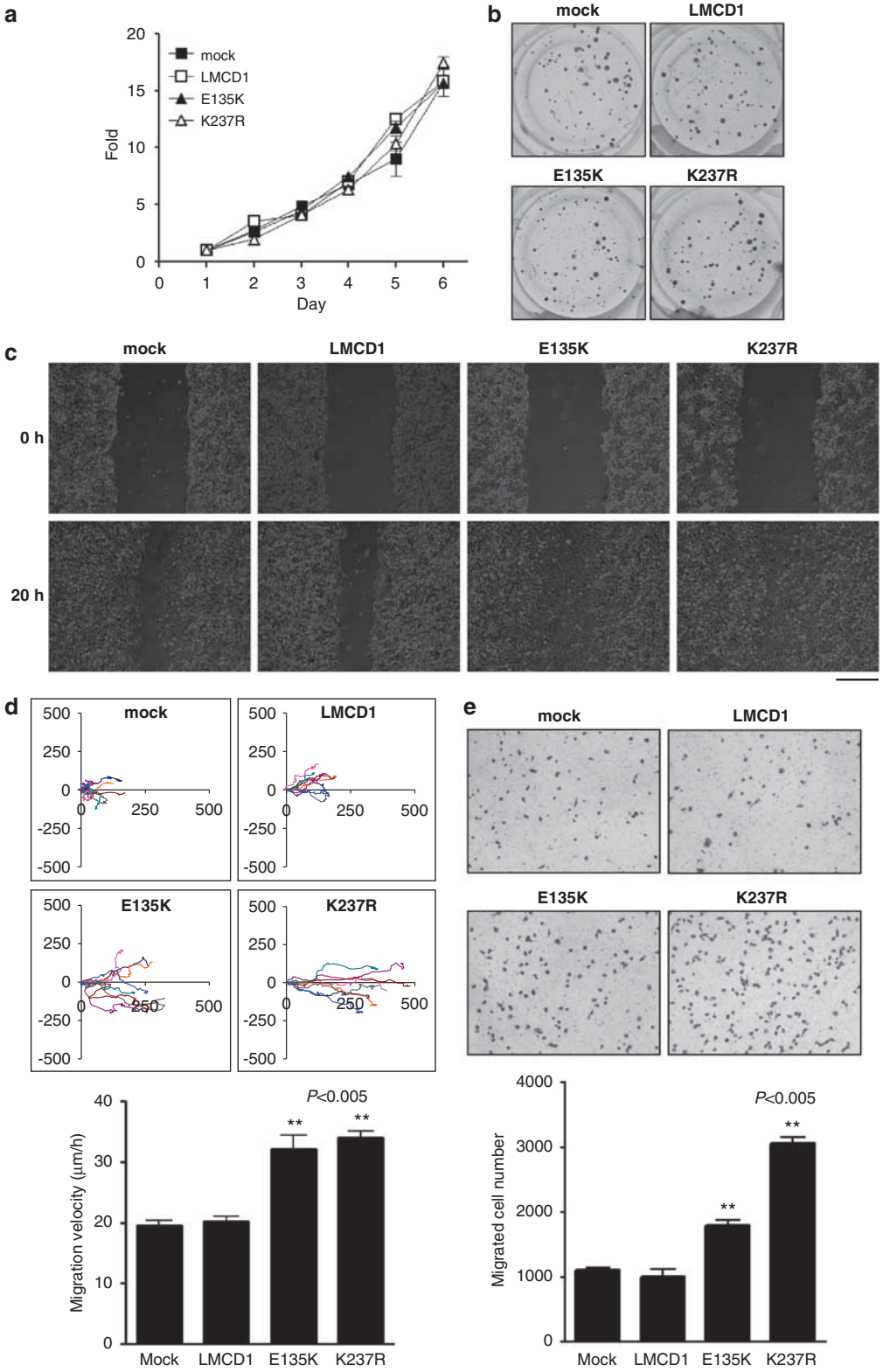


Figure 3 Expression of LMCD1 mutants specifically enhances cell migration. (**a**) Proliferation assay of mock and LMCD1 transfectants in SK-hep1 cells. (**b**) Representative images of anchorage-independent growth assay of LMCD1 transfectants. (**c**) LMCD1-E135K and LMCD1-K237R cells showed increased mobility as examined by wound-healing assays photographed at 0 and 20 h after wounding. Bar: 500 μm. Images are representative of at least three independent experiments. (**d**) Plotted tracks of representative migrating cells during wound healing according to Supplementary Videos 1–4. The starting point of each cell was transposed to the origin of the *x*–*y* coordinates. Quantification of migration velocity is shown at the bottom. The data represent means ± s.e.m. (**e**) Transwell migration assay of LMCD1 transfectants (1×10^4 cells). Images are representative of three independent experiments performed in triplicate. Quantification of migrated cell number is shown at the bottom. The data represent means ± s.e.m.

and LMCD1-K237R do not alter cell proliferation and transformation capabilities, but conduce to the advantages of cell migration in multiple HCC cells.

RNA interference knockdown of LMCD1 *can reverse mutation-induced lamellipodia formation and cell migration*

In order to validate the LMCD1-E135K- and LMCD1-K237R-enhanced cell migration, *LMCD1* expression was knocked down by *shLMCD1* lentiviral infection (Figure 4a). *LMCD1* knockdown did not change the cell morphology of mock and wtLMCD1 cells. However, the enriched Arp3 in the lamellipodia and periphery extension of LMCD1-E135K and LMCD1-K237R cells were no longer observed after introduction of *shLMCD1* (Figure 4b). In agreement with their decreased lamellipodia, *shLMCD1* also diminished the wound closure rate of LMCD1-E135K and LMCD1-K237R cells to a level similar to that of mock or wtLMCD1 cells (Figure 4c). These results confirm that induced lamellipodia formation and cell migration indeed arise from mutant LMCD1 expression.

To demonstrate whether endogenous LMCD1-K237R mutation modulates cell mobility in PLC/PRF/5 cells, we induced *LMCD1* expression by estrogen treatment as reported in breast cancer cells (Harvell *et al*., 2006; Dudek and Picard, 2008) and examined the migration alteration of PLC/PRF/5 cells. Our results demonstrated that addition of estrogen to PLC/PRF/5 cells could indeed upregulate the expression of *LMCD1-K237R* and cell migration. Further treatment with *shLMCD1* abrogated endogenous *LMCD1-K237R* expression and impaired cell mobility concomitantly with or without estrogen induction (Figures 4d and e). These data provide evidence that LMCD1-K237R mutation at an endogenous level is necessary for PLC/PRF/5 cell mobility regardless of estrogen induction.

Rac1 has an important role in the mutant LMCD1-mediated increase of migration

As Rac1 is a key regulator of lamellipodia formation, we hypothesized that Rac1 is involved in the signaling pathway of mutant LMCD1-enhanced cell mobility and lamellipodia formation. To test this possibility, a short-hairpin (shRNA) and a specific inhibitor of Rac1, *shRac1* and NSC23766, respectively, were applied to LMCD1 transfectants (Gao *et al*., 2004). Our results indicated that treatment with *shRac1* or NSC23766 inhibited the lamellipodia extension of LMCD1-E135K and LMCD1-K237R cells (Figure 4b). Furthermore, Rac1 activity was assayed during wound-healing migration. As expected, LMCD1-E135K and LMCD1-K237R cells showed an increased Rac1 activation level, whereas wtLMCD1 overexpression did not influence endogenous Rac1 activity (Figure 5a). To further confirm that mutant LMCD1-mediated cell migration enhancement was Rac1-dependent, we performed western blot analysis and wound-healing assays in the presence of NSC23766. Addition of NSC23766 decreased Rac1 activity and cell mobility in a dose-dependent manner (Figures 5b and c). More importantly, treatment with 25 μM NSC23766 caused 31 ~ 32.3% migration inhibition for LMCD1-E135K and LMCD1-K237R mutant transfectants in comparison with only 12.1 ~ 16.4% inhibition in mock and wtLMCD1 cells (Figure 5c). Treatment with *shRac1* had a similar inhibitory effect on mutant LMCD1-mediated cell migration (Figures 5d and e). These results indicate that the LMCD1-E135K and LMCD1-K237R transfectants addicted to the mutation-mediated increase of cell migration are more sensitive than the wtLMCD1 transfectant to Rac1 inhibitor or shRNA.

LMCD1 E135K but not K237R mutation promoted systemic tumor metastasis in a nude mice model

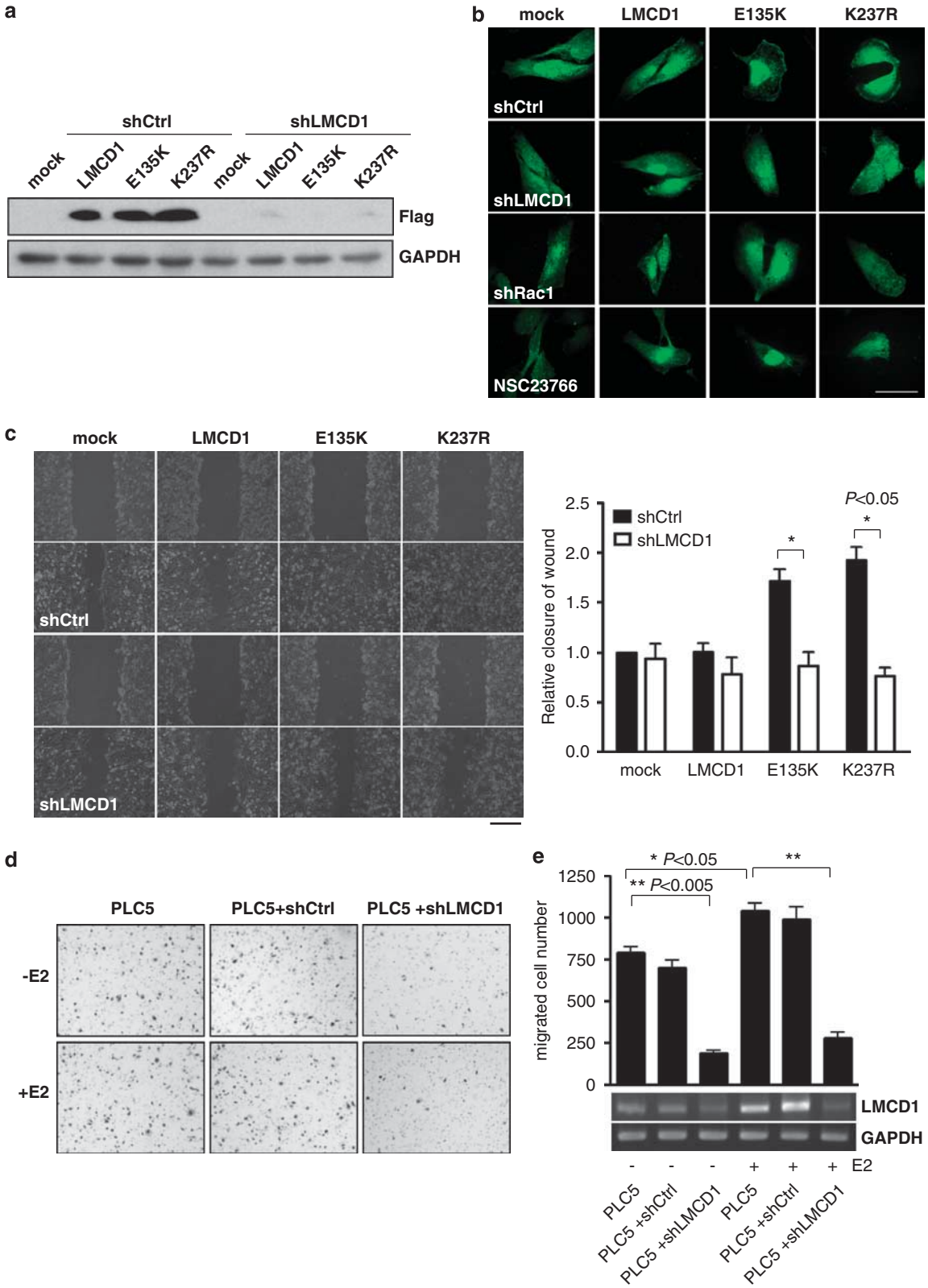
To further investigate the influence of LMCD1 mutations on tumor metastasis *in vivo*, we established red fluorescent protein (RFP)-labeled LMCD1 transfectants of SK-Hep1 cells for tail vein injection in a nude mice metastasis model. After 8 weeks of cell injection, lung tissues were isolated and RFP-labeled metastatic cells were quantified by using the photon-counting technique of the Living Image software. Our results showed that the mock and wtLMCD1 transfectant showed weak intrinsic metastatic ability whereas the LMCD1-E135K transfectant (mutations identified in HCC tissues) significantly promoted lung metastasis as predicted (Figure 6). However, it is to our surprise that there is no increase in tumor metastases formed by the LMCD1-K237R (mutation identified in PLC/PRF/5 cells) transfectant. Nevertheless, the capability of LMCD1-E135K to promote systemic metastasis and its clinical occurrence in HCC patients make LMCD1-E135K of greater importance in HCC progression.

Discussion

In this study, we adopted a comprehensive approach, including cancer genome aberration study, gene expression analysis and mutation screening in HCC cells, to search for potential HCC target genes. We found that *LMCD1*, residing in the 3p26.1-25.3 amplicon, was a hotspot of somatic alterations, with recurrent mutation at E135K in HCC tissues and K237R in PLC/PRF/5 HCC cells. Expression of mutant LMCD1-E135K and LMCD1-K237R facilitated the switch of stress fiber assembly to lamellipodia formation and promoted cell migration through upregulation of Rac1 activation. Our *in vivo* study further demonstrated the capability of LMCD1-E135K to increase systemic tumor metastasis in mouse models.

LIM proteins are characterized by the containment of an LIM domain, which is generally considered to function as a mediator for protein interaction but lacking a specific binding motif on targeted proteins (Khurana *et al*., 2002). At least 64 human LIM proteins were identified and classified to 17 subfamilies based on other comprising domains or motifs, which may influence the distribution of the protein to different

cellular compartments and their involvement in distinct biological functions (Zheng and Zhao, 2007). Except for the members of the LHX (LIM homeodomain) and LMO (LIM-only) families located only in the nucleus, most LIM proteins shuttled between the nucleus and the cytoplasm, and associated with the actin cytoskeletal or cell adhesion apparatus. Despite being categorized in various LIM subfamilies, many LIM proteins are



aberrantly expressed and serve as positive or negative regulators in tumorigenesis and metastasis in various cancers, such as methylation silencing of LMX1a; overexpression of LMO3, LMO4, LASP1, PCD1 and LIMK1; activation of LMO1 and LMO2 by chromosomal translocation (Boehm *et al*., 1991; Kang *et al*., 2000; Visvader *et al*., 2001; Aoyama *et al*., 2005; Bagheri-Yarmand *et al*., 2006; Grunewald *et al*., 2007; Liu *et al*., 2009) and oncogenic somatic mutation at A127T of paxillin (Jagadeeswaran *et al*., 2008).

Based on the comprising LIM and central PET domains, LMCD1 is classified into the Testin subfamily. Testin is a focal adhesion protein that regulates stress fiber organization and cell spreading through modulation of the RhoA pathway (Coutts *et al*., 2003; Griffith *et al*., 2005). Meanwhile, Testin is suggested to function

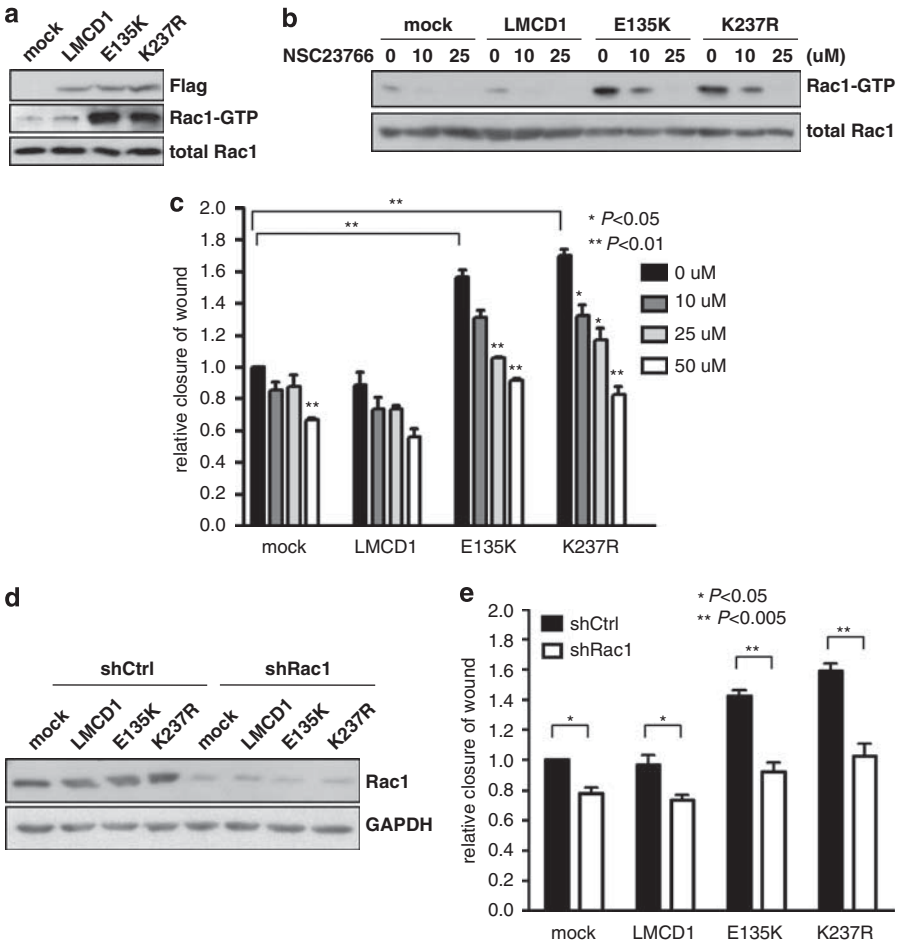


Figure 5 Expression of LMCD1 mutants increases Rac1 activity to promote cell migration. (**a**) Western blot analysis of total and GTP-bound Rac1 expression in LMCD1 transfectants after repeated scratching of cells and GTP-bound Rac1 pull down by GST-PAK1 PBD. (**b**) The dosage effects of the Rac1 inhibitor NSC23766 on Rac1 activity in LMCD1 transfectants using western blot analysis. (**c**) The inhibitory effects of the Rac1 inhibitor NSC23766 on the wound closure of LMCD1 transfectants in a dose-dependent manner. The data represent means ± s.e.m. (**d**) Knockdown efficiency of shRac1 on Rac1 expression in LMCD1 transfectants using western blot analysis. (**e**) The inhibitory effects of *shRac1* on the wound closure of LMCD1 transfectants. The degrees of wound closure were normalized to that of mock cells. The data represent means ± s.e.m.

Figure 4 Knockdown of LMCD1 mutants eliminates lamellipodia formation and cell migration. (**a**) Western blot analysis of LMCD1 knockdown efficiency in multiple stable clones infected with lentiviral *shLMCD1* or luciferase shRNA (shCtrl) as control. (**b**) Treatment with *shLMCD1*, *shRac1* and the Rac1 inhibitor NSC23766 reduced the lamellipodia formation induced by LMCD1-E135K and LMCD1-K237R. Bar: 50 μm. (**c**) LMCD1 knockdown further reversed the increase of cell mobility in the wound-healing assay. Bar: 500 μm. Images are representative of at least three independent experiments. Quantification of migration results normalized with mock cells is shown on the far right. The data represent means ± s.e.m. (**d**) Transwell migration assays of endogenous *LMCD1-K237R* expression in PLC/PRF/5 cells (1×10^5 cells) induced by 10 nM 17β-estradiol and knocked down by *shLMCD1*. Images are representative of three independent experiments performed in triplicate. (**e**) Quantification of migration results in panel **d**. The data represents means ± s.e.m. The endogenous *LMCD1-K237R* expression induced by 10 nM 17β-estradiol and knocked down by *shLMCD1* in PLC/PRF/5 cells was confirmed by reverse transcription–PCR using LMCD1-specific primer pairs. GAPDH was used as an internal control.

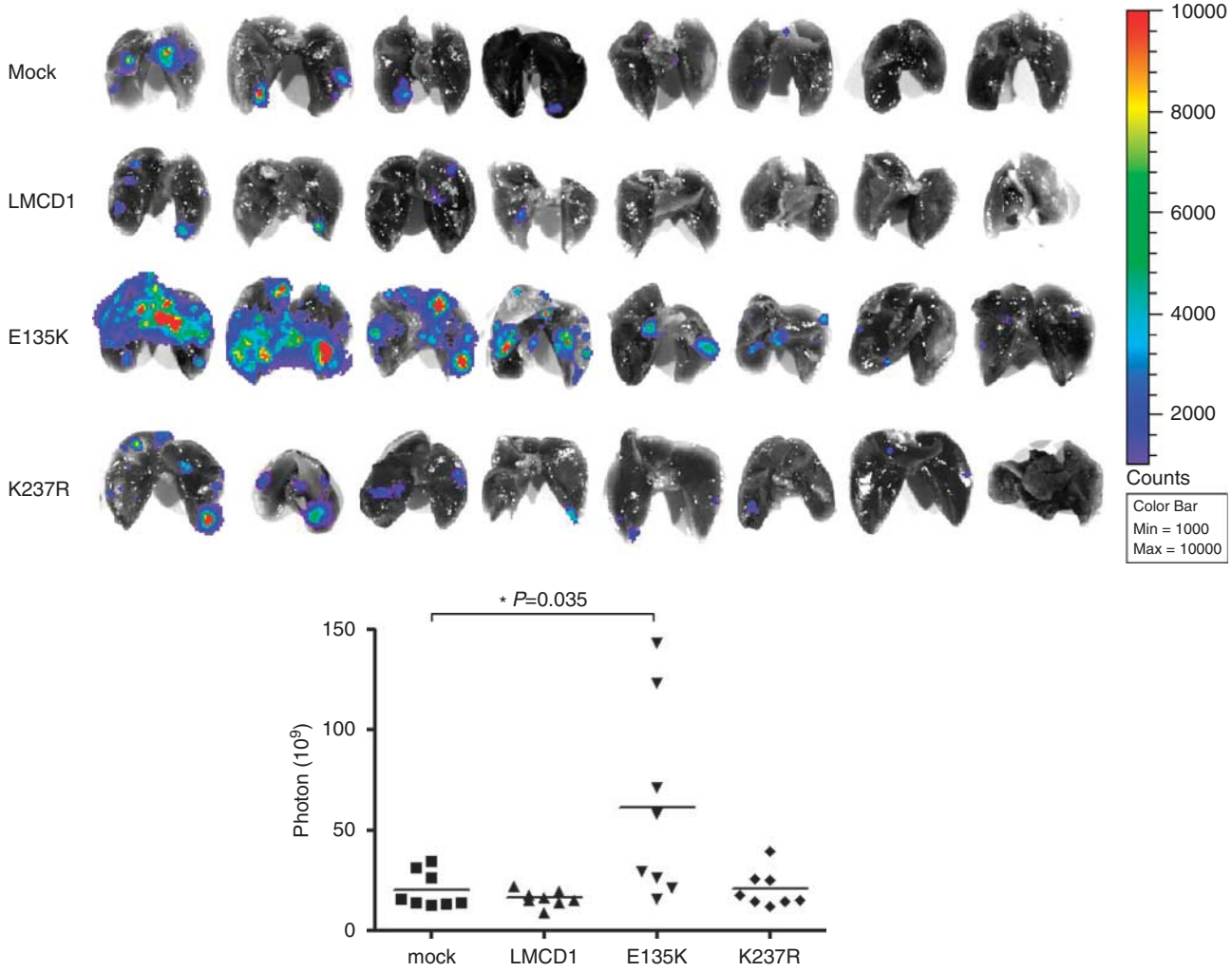


Figure 6 E135K but not K237R LMCD1 mutation promoted systemic tumor metastasis *in vivo*. Images of lung metastasis from nude mice injected with RFP-labeled LMCD1 transfectants in SK-Hep1 cells. Metastatic fluorescence was detected by using the IVIS system. Quantification of photon counts representing efficiency of metastasis is shown at the bottom.

as a tumor-suppressor gene by downregulation through hypermethylation and repression of cell growth in several cancer cell lines and tumor types, including ovarian carcinomas, glioblastoma and acute lymphoblastic leukemia (Tobias *et al*., 2001; Mueller *et al*., 2007). Besides, a *Testin* frameshift mutation resulting in a premature truncation was observed in the breast cancer cell line ZR-75 (Tobias *et al*., 2001). In contrast to Testin, we found that *LMCD1* is commonly upregulated in 47.29% of the HCC tissues from the iCOD HCC data sets; gene-amplified in three HCC cell lines and in 6.25% (3/48) of somatic mutations in HCC tissues. Although the biological function of wtLMCD1 remains elusive, LMCD1 mutants identified in HCC tissues could colocalize with the F-actin cytoskeleton to modulate actin architecture dynamics and function as a metastatic oncogene in HCC.

In this study, we demonstrated a novel pathway connecting LMCD1 mutation to upregulated Rac1 signaling, enhanced lamellipodia extension and cell mobility *in vitro* and *in vivo*. Nevertheless, in-depth studies for at least three issues are needed to clarify the aberrant roles of LMCD1 in HCC tumor progression. First of all, the biological meaning of upregulated LMCD1 in HCC cells and tissues remained unclear. We performed *shLMCD1* knockdown assay in HCC cells HA22T, SNU387 and Hep3B carrying an LMCD1 amplicon for examining the role of up-regulated wtLMCD1 in cell migration. The decrease of cell migration in transwell and wound-healing assays suggested that upregulation of LMCD1 through amplification is a necessary factor for cell migration in HCC cells (Supplementary Figure S6). To further validate LMCD1 upregulation in HCC tissues, we performed additional analysis of *LMCD1* expression in 14 downloaded HCC data sets from public domains and revealed 377 *LMCD1*-overexpressing cases from 949 HCC patients (one-sample *t*-test, $P<0.001$) (Supplementary Figure S7). Interestingly, when analyzing *LMCD1* upregulation using iCOD HCC data sets with some clinicopathological features, *LMCD1* upregulation ($\log_2$T/N over 0.5) was observed in 48 of the 107

patients (44.86%) with an expansive tumor growth pattern and 12 of 16 (75%) with an infiltrative growth pattern ($P=0.0319$, Fisher's exact test). The positive correlation of upregulated *LMCD1* with infiltrative tumor growth pattern, which is frequently observed in HCC specimens with intra-hepatic metastasis (Kanai *et al*., 1987), infers its possible involvement in tumor cell invasiveness. However, we were unable to demonstrate enhancement of cell proliferation, transformation or migration by overexpressing wtLMCD1 in several cell models, including the human HCC cell lines SK-Hep1, HA22T, SNU387 and Hep3B, and rat liver epithelial cell WB (data not shown). One possible explanation is that upregulated wtLMCD1 might contribute to HCC tumorigenesis or metastasis through other mechanism not yet detectable in our experimental models.

Second, despite similar capabilities of E135K and K237R LMCD1 mutations in enhancing Rac1-activation, lamellipodia extension and *in vitro* cell migration, we failed to demonstrate K237R-mediated systemic tumor metastasis in nude mice model. The insufficiency of the LMCD1-K237R mutation to enhance systemic metastasis could be due to the requirement of additional lesions in comparison with LMCD1-E135K to unleash the constraint in the multistep process and the microenvironment of the *in vivo* metastasis model. One possibility is that different LMCD1 mutations might alter their capabilities in modulating cell adhesion and/or metastasis. Our preliminary results suggested that no significant difference was detected between wild-type and mutant LMCD1 transfectants after surface coating with the extracellular matrix components collagen-I, laminin or fibronectin (Supplementary Figure S8). Future experiments on other extracellular matrix components and concerning other cellular functions, such as adhesion complex (dis)assembly or extracellular matrix digestion, would be needed to dissect this issue.

Finally, development of inhibitors against LMCD1 mutations or downstream Rac1 activity based on protein structure to better understand mutation-mediated metastatic effects may be of great significance in HCC therapy. Although a high-resolution LMCD1 protein structure is not available, we infer that E135K might change the LMCD1 protein structure or function owing to amino-acid charge alteration from Glu to Lys and its location inside the PET domain. The PET domain has been suggested to have a role in protein–protein interactions with proteins involved in planar polarity signaling or organization of the cytoskeleton. By contrast, K237R is neither located in the PET or LIM domains, nor results in any significant charge difference. However, Lys can be modulated by several post-translational modifications, including acetylation, methylation, ubiquitination, sumoylation and biotinylation. We therefore cannot rule out the possibility that K237R mutation might alter the accessibility of protein modification. Nevertheless, the high concordance of mutation-mediated metastasis based on functional and structural interpretations warrants efforts for further identification of LMCD1 mutations to serve as a therapeutic biomarker or target in cancers.

Together, our results provide first evidence that somatic alterations of LMCD1 have important roles in HCC tumor progression especially tumor cell migration and metastasis. Mutant LMCD1 is a putative HCC proto-oncogene involved in dysregulated Rac1 signaling, which results in actin cytoskeleton re-organization and facilitates cell migration and metastasis. Further investigations are to elucidate the aberrant roles of LMCD1 in HCC tumor progression, including the frequency of LMCD1 mutations in larger HCC cohorts, the LMCD1 protein functions and its detailed interactions with HCC pathological pathways. Moreover, as emerging studies have demonstrated that Rac1 has an important role in HCC metastasis (Grise *et al*., 2009), additional studies will be required to link LMCD1 mutations with the Rac1-signaling pathway to serve as a potential target of anti-motility cancer therapy in HCC.

Materials and methods

Antibodies, reagents and cell culture

Monoclonal anti-Flag M2 antibody was purchased from Sigma (St Louis, MO, USA). Monoclonal anti-Rac1 antibody (clone 23A8) and polyclonal anti-Arp3 antibody were purchased from Millipore (Bedford, MA, USA). Polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was purchased from Lab Frontier (Seoul, Korea). Alexa Fluor-488 goat anti-mouse IgG, Alexa Fluor-488 goat anti-rabbit IgG and rhodamine–phalloidin were purchased from Molecular Probes (Eugene, OR, USA). The Rac1 inhibitor NSC23766 was purchased from Calbiochem (San Diego, CA, USA). 17β-Estradiol was purchased from Sigma.

Details on plasmid constructions, routing culturing conditions, cell proliferation and anchorage-independent growth are provided under Supplementary Materials and methods.

Analysis of copy-number alterations, gene expression and somatic mutation

We performed copy-number alteration analysis using genomic data sets conducted using Affymetrix GeneChip Human Mapping 500K SNP Arrays and the dChip software as described previously (Chen *et al*., 2010). The *LMCD1* expression level in clinical HCC tissues was analyzed by using multiple data sets downloaded from the iCOD database (Yoshida *et al*., 2010). Pooled normal references were obtained from the public repository Gene Expression Omnibus (GEO) database. These microarray data sets were imported into the dChip software for expression analysis. *LMCD1* expression level is computed as follows: $\log_2$ (signal of the clinical case/mean signal of references). Other HCC microarray data sets were downloaded from the GEO database and analyzed as described in the Supplementary information.

All of the six exons of *LMCD1* were sequenced in genomic DNA samples from 48 HCC patients and 13 human HCC cell lines for screening of somatic mutation. To confirm nucleotide alterations, forward and reverse sequencing reactions were performed. Both mutations were further confirmed by sequencing cDNAs converted from mRNA samples to ensure expression of mutant LMCD1 in HCC tissues and cell lines.

Small interfering RNA and lentiviral infection

The shRNAs for LMCD1 and Rac1 obtained from the TRC library were TRCN0000134556 and TRCN0000004870,

respectively. Details on the protocol for lentiviral preparation are provided under Supplementary Materials and methods.

Immunofluorescence microscopy
Cells were replated on glass coverslips the day before staining. For observation of cell morphology at wound edge, cells were scratched with a p200 pipette tip 2 h before fixation. The primary antibodies and the corresponding Alexa Fluor-488-conjugated secondary antibodies were applied for 1 h each. Rhodamine–phalloidin for F-actin staining was incubated with cells for 10 min. Coverslips were mounted on slides using the VECTASHIELD mounting medium with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA). Images were obtained with an Olympus BX51 fluorescence microscope or a PerkinElmer Ultra View confocal microscope (PerkinElmer Life Sciences, Boston, MA, USA).

Cell migration assays
For the wound-healing assay, cells were seeded at a confluent density and cultured overnight to grow into a monolayer. The culture was scratched with a p200 pipette tip and the wound was allowed to close for 20 h. The average reduction of distance between two wound edges was calculated to evaluate the rate of wound closure. To determine the inhibitory effect of the Rac1 inhibitor on wound closure, cells were pretreated with the indicated amount of NSC23766 for 6 h. For live video recording of wound healing and random migration, cells were placed in a CO_2- and temperature-controlled chamber on a microscope (Axiovert 200 M; Carl Zeiss MicroImaging Inc., Thornwood, NY, USA). Cell migration was recorded under a $\times 20$ objective lens with a cooled charge-coupled device (CCD) video camera (CollSNAP fx; Roper Scientific, Tucson, AZ, USA). Time-lapse images were captured at 15-min intervals over 18 h and used to assemble the Supplementary Movies by using the Metamorph image analysis software. The migration path of individual cells was manually tracked for each frame and expressed as graphs by using the Excel program (Microsoft).

For transwell assay, cells resuspended in serum-free medium were seeded into the upper chamber of the insert by using an 8.0-μm-pore polycarbonate membrane (BD Biosciences, San Jose, CA, USA) and then placed into the bottom chamber containing 10% fetal bovine serum as a chemoattractant. Cells were allowed to migrate for 24 h, followed by methanol fixation and Giemsa staining (MERCK, Darmstadt, Germany). Un-migrated cells on the membrane apical side were removed. Migrated cells were photographed with a phase-contrast microscope and the number was counted by using the Image-Pro Plus software. All experiments were performed in triplicates.

Rac1 activity assay
Rac1 activity measurement was performed by affinity pull-down assay using a glutathione-*S*-transferase (GST) fusion protein conjugated with the p21-binding domain derived from the Rac1 effector, PAK1 (GST-PAK1 PBD). Cells were lysed in lysis buffer containing 25 mM HEPES (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, $1\times$ protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA), 1 mM sodium vanadate and 10 mM NaF. Cell lysates were normalized by Bradford protein assay for equal amount of protein and incubated with GST-PAK1 PBD-coupled glutathione–Sepharose-4B beads for 1 h at 4 °C. The beads were washed with lysis buffer for five times then boiled in sodium dodecyl sulfate–PAGE loading buffer. Samples were analyzed by sodium dodecyl sulfate–PAGE and western blotting to detect the level of Rac1-GTP using an anti-Rac1 antibody.

In vivo *tumor metastasis*
LMCD1 transfectants were infected with an *RFP* lentivirus and sorted for RFP-positive cells. Expanded cells (1×10^6 per mouse) were intravenously injected into athymic BALB/c nude (nu/nu) mice at 4 weeks age. The mice were killed 8 weeks after injection. Lung metastatic signals were detected by using the IVIS system (Xenogen Corp., Alameda, CA, USA), with the excitation and emission wavelength at 570 and 620 nm, respectively. Photons emitted from lung metastases were quantified by the Living Image software (Xenogen Corp.). All animal procedures were performed in accordance with the guidelines of the Institutional Animal Care and approved by the Animal Committee of Academia Sinica.

Statistical analysis
Statistical significances of the variances and the expression difference of *LMCD1* in microarray samples were calculated by Student's *t*-test.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We thank Dr Pei-Jer Chen and Dr Ding-Shinn Chen at School of Medicine, National Taiwan University, for helpful discussions and advice on the work. We also thank the core facilities from National Research Program for Genomic Medicine, National Science Council, Taiwan, including the National Genotyping Center, for their service in SNP genotyping and the National RNAi Core facility for providing shRNAs. *Financial Support:* This work was funded by the National Research Program for Genomic Medicine of the National Science Council, Taiwan, through grant numbers NSC98-3112-B-001-004, NSC98-3112-B-001-031 and NSC99-3112-B-001-019.

Author contributions: C-YC, S-CL and C-MH performed the experiments; C-YC, W-HS, C-MH and Y-SJ performed data analysis and interpretation; and C-YC and Y-SJ drafted the manuscript and supervised the study.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)