Paxillin Is a Target for Somatic Mutations in Lung Cancer: Implications for Cell Growth and Invasion

Ramasamy Jagadeeswaran,1 Hanna Surawska,1 Soundararajan Krishnaswamy,1 Varalakshmi Janamanchi,1 A. Craig Mackinnon,1 Tanguy Y. Seiwert,1 Sivakumar Loganathan,1 Rajani Kanteti,1 Trevor Reichman,3 Vidya Nallasura,1 Stuart Schwartz,1 Leonardo Faoro,1 Yi-Ching Wang,2 Luc Girard,5 Maria S. Tretiakova,2 Salman Ahmed,1 Osvaldo Zumba,1 Lioubou Soulli,1 Vytas P. Bindokas,1 Livia L. Szeto,1 Gavin J. Gordon,1 Raphael Bueno,2 David Sugarbaker,3 Mark W. Lingen,6 Martin Sattler,4 Thomas Krausz,2 Wickii Vigneswaran,2 Viswanathan Natarajan,1 John Minna,1 Everett E. Vokes,1 Mark K. Ferguson,3 Aliya N. Husain,2 and Ravi Salgia1

Departments of Medicine,1 Pathology,1 Surgery,1 and Human Genetics, University of Chicago Cancer Research Center, University of Chicago Medical Center, Pritzker School of Medicine, Chicago, Illinois;2 Department of Pharmacology, College of Medicine, National Cheng Kung University, Tainan, Taiwan, R.O.C.;3 Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center, Dallas, Texas; and4 Department of Surgery, Division of Thoracic Surgery, Brigham and Women's Hospital, and 5 Department of Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts

Abstract

Lung cancer is characterized by abnormal cell growth and invasion, and the actin cytoskeleton plays a major role in these processes. The focal adhesion protein paxillin is a target of a number of oncogenes involved in key signal transduction and important in cell motility and migration. In lung cancer tissues, we have found that paxillin was highly expressed (compared with normal lung), amplified (12.1%, 8 of 66) and correlated with increased MET and epidermal growth factor receptor (EGFR) gene copy numbers, or mutated (somatic mutation rate of 9.4%, 18 of 191). Paxillin mutations (19 of 21) were clustered between LD motifs 1 and 2 and the LIM domains. The most frequent point mutation (A127T) enhanced lung cancer cell growth, colony formation, focal adhesion formation, and colocalized with Bcl-2 in vitro. Gene silencing from RNA interference of mutant paxillin led to reduced cell viability. A murine in vivo xenograft model of A127T paxillin showed an increase in tumor growth, cell proliferation, and invasion. These results establish an important role for paxillin in lung cancer. [Cancer Res 2008;68(1):132–42]

Introduction

The cytoskeleton plays an important role in abnormal growth, invasion, and metastasis, which are characteristics of malignant tumors (1, 2). Paxillin is one of the key components within focal adhesions, contributing to form a structural link between the extracellular matrix and the actin cytoskeleton. In addition to its interaction with cytoskeletal proteins, paxillin has previously been shown to interact with several oncoproteins, such as E6, v-Src, and BCR-ABL (3–7). Cellular adhesions between tumor cells and normal cells and between adjacent tumor cells are essential for the progression of cancer. It is thought that these interactions may regulate the function of paxillin itself, which has not been well characterized. Gene disruption of paxillin in mice affects the development of both extraembryonic and embryonic structures in mice. Paxillin null cells have abnormal focal adhesions, an altered cortical cytoskeleton, decreased tyrosine phosphorylation of focal adhesion kinase (FAK) and p130Cas, decreased efficiency of FAK localization to focal adhesions, decreased activation of mitogen-activated protein kinase, decreased rate of spreading, and decreased cell migration (8). As a central protein within the focal adhesion, paxillin acts as a scaffold protein that provides multiple docking sites at the plasma membrane for an array of signaling and structural proteins. Paxillin provides a platform for protein tyrosine kinases, including FAK and Src, which are activated as a result of adhesion or growth factor stimulation (9–11).

We have previously shown that the actin cytoskeleton is important in cell motility and migration of lung cancer cells (12). Lung cancer is characterized by a poor prognosis, early metastasis, and often refractoriness to cytotoxic chemotherapies. A number of oncogenes, including K-ras, Myc, ERBB gene family, and MET can be overexpressed or mutated in lung cancer, as well as a number of tumor suppressor genes, including RB and p53, can be altered or deleted (13). Although these oncogenes and tumor suppressor genes are unique to lung cancer, they may be necessary but not sufficient for oncogenesis, invasion, and metastasis.

Here, we show novel somatic mutations of paxillin that are unique to lung cancers compared with other tumors. Most frequently, an A127T substitution in paxillin was found and correlated with F-actin stress fiber formation, association with Bcl-2, and reduced requirement for serum-dependent cell growth. Forced expression of wild-type paxillin in paxillin null non–small cell lung cancer (NSCLC) H522 cells promoted a nodular tumor phenotype with increased cell proliferation and microvessel density. Of special interest here is that the expression of A127T paxillin promoted nodular, invasive, and proliferative tumor growth without increasing microvessel density or stroma formation. Overall, these results implicate paxillin as a central mediator of a number of biological processes in lung cancer.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Ravi Salgia, Department of Medicine, Division of Hematology/Oncology, University of Chicago, M255, 5841 South Maryland Avenue, Chicago, IL 60637. E-mail: rsalgia@medicine.bsd.uchicago.edu.

Materials and Methods

Cell lines and tissue specimens. H441, SK-LU-1, H1993, A549, H522, H358, H661, SW1573, H2170, SW-900, normal human bronchial epithelial (NHBE), and BEAS-2B cell lines were obtained from the American Type Culture Collection and cultured as described.9 Genomic DNA (total, 483) was isolated from 191 lung cancer tissues from Caucasians and African-Americans, 70 lung cancer tissues from Asians, 151 non–lung tumor tissues, and 71 lung cancer cell lines by standard procedures (14). The Institutional Review Board at the University of Chicago and the respective institutions approved use of human materials in this study.

DNA sequencing and mutational analysis. Eleven pairs of PCR primers to cover 11 exons spanning the entire coding region of paxillin were used (Supplementary Table S1) and sequenced (4). The adjacent “normal” tissues of the surgically resected tumor specimens was identified histopathologically, and sequenced as well. The numbering of the nucleotide positions was relative to the first base of the translational initiation codon according to full-length human α paxillin cDNA. All mutations were confirmed by sequencing in both directions.

Constructs of wild-type and mutant paxillin plasmids. Paxillin was subcloned into pcDNA3.1 DEST47 through Gateway cloning (Invitrogen). Briefly, primers 5′CCACATGACCAGCTTCGAGCCCGG 3′ and 5′GACAGAGA-GCTTTAGAGACGCTTGCAG 3′ were used to PCR amplify the paxillin coding region, which was subsequently TOPO cloned into a PENTR/SD/D-TOPO entry vector using LR clonase II. The paxillin insert was transferred into the destination vector pcDNA3.1 DEST47 by a recombination reaction. The A127T (379G→A) mutation was independently introduced into paxillin in DEST47 using Quick-Change site-directed mutagenesis (Stratagene) using the primers 5′CCCCCAAGCAAGAATCAACTGAGCCTTCACCCACCG 3′, 5′CGGTTGCTGGAAGGATAGTGTGTTCTGTCTGTTGGGG 3′. The insert alteration was confirmed by direct sequencing.

Immunoblotting and antibodies. Cell lysate preparation, SDS-PAGE, and immunoblot analysis was performed as previously described (5). Polyclonal phosphorylation site-specific paxillin antibodies were obtained from Biosource International. Paxillin monoclonal antibody was obtained from Neomarkers, Lab Vision Corp. (clone 5H11). Phosphorylated AKT (Ser473) was obtained from Cell Signaling Technology. c-Met (c-12), Bcl-2, and CD31 antibodies were obtained from Santa Cruz Biotechnology, and β-actin monoclonal antibody and all other chemicals were purchased from Sigma.

Confocal microscopy and immunohistochemistry. Cells were grown on glass coverslips, and immunofluorescence was procedure performed as previously described (15). Images were captured using a confocal microscope and saved as digital images. For immunohistochemistry, we analyzed paraffin-embedded, formalin-fixed tissue slides and tissue microarrays with institutional review board–approved protocols. Expression of paxillin was quantified manually in each core at 400× magnification by pathologists and with automated cellular imaging system (ACIS). Two independent pathologists reviewed all of the staining, and immunoscoring was confirmed.

Fluorescence in situ hybridization. Fluorescence in situ hybridization (FISH) analysis was done using three different BAC probes: RP11-144B2, localized to 12q24.23, contains the full-length paxillin gene; RP11-433C10, localized to 7p11.2, contains the full-length epidermal growth factor receptor (EGFR) gene; and RP11-163C9, localized to 7q31.2, contains the c-Met gene. Two color FISH was done for NSCLC cell lines, using RP11-144B2 (labeled in red) together with RP11-163C9 (labeled in green) and RP11-433C10 together with RP11-163C9. Probes were labeled, and FISH was done as previously described (16). At least 10 metaphase cells were analyzed for each cell line.

Quantitative real-time PCR. Quantitative real-time PCR for gene copy number measurement for DNA samples obtained from tumors of patients with NSCLC was done as described before (17) using the Stratagene Mx3000P system and the iQ SYBR green PCR kit (Bio-Rad Laboratories). Relative gene copy number was calculated from the real-time PCR efficiencies, which were determined for each individual run, and the crossing point deviations of the target and reference genes in a test sample versus a control. Long interspersed element-1 (LINE-1) served as reference gene, which is a repetitive element for which copy numbers per diploid genome are similar in healthy or malignant human cells (18). Primer sets were used for PAX, MET, EGFR, and LINE-1 were listed in Supplementary Table S1. Reactions were done in triplicate under standard thermocycling conditions (one cycle of 95°C for 12 min, followed by 45 cycles of 95°C for 20 s and 58°C for 1 min), and the mean threshold cycle number was used.

Transfection and serum starvation assays. For paxillin transient transfection, H522 cells were plated the day before in six-well plates and transfected in duplicate with 2 μg total plasmid DNA and 5 μL of Lipofectamine PLUS (Invitrogen) according to manufacturer’s instructions. Cells were serum starved and harvested 24 and 48 h later in lysis buffer. Paxillin levels were assessed by immunoblotting. Cell survival was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described (4). For colony survival (clonogenic) studies as described previously (4), transfected H522 cells were reseeded at 0.1 × 10³ cells per well in six-well plates. After incubation for 4 weeks, the colonies were fixed and stained with methylene blue in 50% ethanol for 20 min and then rinsed with water and air-dried. Colonies were counted with a Gel Doc-XR Imager (Bio-Rad Laboratories) using the colony counting program.

Gene silencing assays. Small interfering RNA (siRNA) gene silencing studies were done using methods as previously described (15). siRNA oligonucleotides targeting paxillin mRNA were obtained from Dharmaco, Inc. and used according to the instructions of the manufacturer.

Xenograft assays in nude mice. We investigated the in vivo oncogenic properties of the mutated paxillin by studying the ability of A127T paxillin and wild-type paxillin–stably expressing H522 cells to induce tumor formation in nude mice. Cells expressing wild-type paxillin (H522-Wt paxillin) or A127T paxillin (H522-A127T paxillin) and control (H522 control vector) were injected s.c. into nude mouse flank region (5 × 10⁶ cells per flank). Animal experiments were done according to institutional approved protocols (IACUC). The animals were monitored for tumor formation every week and sacrificed 8 to 12 weeks later. Tumor growth was measured every week over a 12-week period.

Tumor morphology, invasion, and immunohistochemistry were studied at the end of the experiment. Tumor tissues were fixed in 10% formalin and embedded in paraffin. Representative tumor sections were obtained from paraffin-embedded tumor tissue and stained with H&E, Masson’s trichrome, and specific antibodies. Areas (n = 10) of immunohistochemistry staining with Ki67, Masson’s trichrome, and CD31 were analyzed. Expression levels were quantified using the automated cellular imaging system (ACIS).

Statistical analyses. Data are expressed as the mean ± SE. Statistical significance was tested using statistics software 15.0. For comparison between means of two groups, Student’s t test or χ² test was used. For comparing means between >2 groups, one-way ANOVA was used. For evaluation of correlations, Spearman’s test was used. Unless otherwise stated, representative figures reflect the findings in a minimum of n = 3 evaluations, and mean values reflect data obtained in a minimum of n = 6 mice.

Results

Level of Paxillin Expression Correlated with Higher Stage and Metastasis in Lung Cancer

To understand the role of paxillin in lung cancer, we have systematically examined paxillin expression using immunohistochemical studies. Paxillin expression was correlated with histology, stage, and metastasis. In samples of primary lung tumors versus lymph node or brain metastases, the level of paxillin expression was significantly (P < 0.001) higher in metastatic samples. The expression of paxillin in adjacent normal lung of the corresponding tumors was

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negative (Fig. 1A). In NSCLC, large cell carcinomas had high expression (score, 2+ to 3+) of paxillin (51%) compared with adenocarcinomas (33%) and squamous cell carcinomas (23%; Fig. 1B). The paxillin expression level increased with increasing stage (Fig. 1C). This did not reach statistical significance ($P = 0.11$; Fig. 1C). In the samples of primary versus lymph node or brain metastasis, the level of paxillin expression was increased in the metastatic setting.

**Expression of Paxillin in Lung Cancer Cell Lines**

Expression of *paxillin*, *MET*, and *EGFR* were analyzed by immunoblotting in a panel of lung cancer cell lines and two normal lung epithelial cell lines. There was a high expression of paxillin and coexpression of MET but not EGFR in NSCLC cell lines. Low level expression of paxillin was observed in NHBE and BEAS-2B cells when compared with A549 (NSCLC) cells (Fig. 2A).

**Gene Copy Numbers of PXN and Correlation with MET and EGFR in Lung Cancer Cell Lines and Tumor Tissues**

Gene copy numbers of *PXN*, *MET*, and *EGFR* were analyzed by FISH analysis in a panel of lung cancer cell lines. There were increased gene copy numbers of the PXN in H1993, H1838, H358,
H661, and SW1573 NSCLC cell lines. A total genomic gain of PXN in lung cancer cell lines was 62.5% (five of eight cell lines tested). H1993 cells had increased gene copy numbers of PXN (>4 copies) as well as clustered gene amplification (>10 copies). H1838 cells had clustered gene amplification (>10 copies) of PXN and MET (Fig. 2B and C). There was association between paxillin and MET in terms of expression and copy number alterations in most of the NSCLC cell lines tested. We further quantitatively analyzed gene copy numbers of NSCLC tumor tissues from 66 patients for PXN, MET, and EGFR using quantitative real-time PCR method. Histologic cell types of these NSCLC tissues included adenocarcinoma (n = 26), large cell carcinoma (n = 24), and squamous-cell

Figure 2. Paxillin expression and amplification in lung cancer. A, immunoblots for protein expression from lysates of the NSCLC and bronchial epithelial (NHBE, BEAS-2B) cell lines using antibodies against paxillin, c-Met, EGFR, and β-actin (loading control). The upper 170-kDa protein band of c-Met represents the glycosylated precursor c-Met and the lower 145-kDa band is the biologically active transmembrane β-subunit of c-Met. B, NSCLC cell lines with FISH-positive increased copy numbers (>4), clustered amplification (>10) or normal (diploid) PXN, MET, and EGFR genes. C, representative FISH analysis image of metaphase and interphase H1838 cells. Red arrows, tightly clustered gene amplification of PXN; green arrows, tightly clustered gene amplification of MET in H1838 cells. Boxed regions, higher magnification, 2×. D, distribution of PXN, MET, and EGFR copy numbers (>4) for each NSCLC subtype by real-time PCR analysis. Change in PXN copy numbers was highly correlated with MET copy numbers (Spearman r = 0.40, P = 0.001).
carcinoma (n = 16). Seventeen percent of large cell carcinoma exhibited increase in gene copy numbers of PXN, and those of positive samples also showed increase gene copy numbers of MET (>4 copies) and high copy numbers of EGFR (>10 copies). Eight percent of adenocarcinoma exhibited increase in gene copy numbers of PXN and those positive samples showed only high gene copy numbers of MET (>10) but not EGFR. Thirteen percent of squamous cell carcinoma exhibited increase gene copy numbers of PXN and MET. Thirty-three percent of large cell carcinoma exhibited increase gene copy numbers of MET and EGFR (Fig. 2D).

**PXN Mutations in Lung Cancer**

We further examined the mutations of the PXN gene in lung cancer. The paxillin coding region (11 exons) was sequenced in cancer DNA specimens, cancer cell lines, and corresponding normal DNA. DNA samples obtained from corresponding normal tissues identified pathologically as distant normal tissues or adjacent normal tissues through laser capture microdissection (Supplementary Fig. S1) were sequenced for the paxillin gene. A total of 21 unique paxillin mutations were identified in lung cancer tissue specimens and cell lines (Fig. 3A). The normal tissues showed a wild-type paxillin sequence, confirming that the mutations were somatic in origin.

Some of the mutations were found as homozygous in the SK-LU-1 (A127T paxillin) and H820 (E423K paxillin) cell lines; in addition, one large cell carcinoma tumor tissue (sample T11) contained two homozygous mutations, P233L, and D399N. All other mutations identified were heterozygous.

The overall rate of paxillin mutations in lung cancer was 9.4% (18 of 191). The frequency of paxillin mutations was high in NSCLC, which included large cell carcinomas (18.4% or 7 of 38), adenocarcinomas (8.6% or 8 of 93), and squamous cell carcinomas (6% or 3 of 51). There were no paxillin mutations in the SCLC primary tissue samples examined (0 of 9). The A127T paxillin mutation, the most frequent mutation detected in NSCLC specimens, was also identified in H69 and H249 SCLC cell lines (2 of 36 cell lines tested). Overall, the paxillin mutation rate in the 71 lung cancer cell lines tested was 5.6% (4 of 71; Supplementary Table S2).
PXN Mutations in Malignancies Other than Lung Cancer

In contrast to lung cancer, paxillin mutations were infrequent in many other cancers, including mesothelioma (rate of 2 of 73), head and neck cancer (1 of 10), breast cancer (0 of 16), melanoma (0 of 4), cervical cancer (0 of 6), myelodysplastic syndrome (0 of 20), and chronic myelogenous leukemia (0 of 22). The overall mutational rate for these malignancies, not including lung cancer, was 2% (3 of 151; Supplementary Table S2).

Ethnic Variations and Nature of PXN Mutations

Most of the identified paxillin mutations (19 of 21) clustered in the region between LD motifs 1 and 2 (amino acid residues Pro30 to Gly139) and the region spanning the LIM domains (Supplementary Figs. S2–S5). In lung cancer tissues examined, the paxillin mutations were identified in samples from Caucasians and African-Americans, each with a unique mutational spectrum. However, there were no mutations for paxillin identified in Taiwanese samples (0 of 70). Figure 3B shows the differences between ethnic groups in paxillin mutations. The mutational spectrum of paxillin was characterized by a high proportion of GC to AT (97%) transitions (Fig. 3C). We have observed 3% of AT to GC, 97% of GC to AT transition, and 0% of AT to TA changes. The paxillin mutations identified as shown in Supplementary Table 2 are new and unique, and none of the mutations identified have been reported previously or present in the National Center for Biotechnology Information–single-nucleotide polymorphism (SNP) database. In addition, we have identified synonymous SNPs apart from previously known synonymous SNPs and nonsynonymous SNPs, such as S73G, for paxillin and differences noticed in the various ethnic groups (Supplementary Tables S3 and S4).

A127T Paxillin Mutant Function In vitro for Lung Cancer

Increased cell growth and colony formation. To investigate the role of paxillin and its mutant form of paxillin (A127T) in lung cancer, we stably expressed wild-type and mutant enhanced green fluorescence protein (EGFP) fusion paxillin in the paxillin negative HS22 cell line. Exogenous higher molecular weight (95 kDa) EGFP fusion paxillin was confirmed by immunoblotting. The viability of HS22 cells expressing wild-type paxillin was comparable with that of control vector cells when subjected to starvation. HS22 cells expressing A127T paxillin grew more rapidly (2-fold faster) than cells expressing wild-type paxillin or the control vector (Fig. 4A). This was corroborated by the significantly higher number of colonies formed by H522 cells expressing A127T paxillin compared with cells expressing wild-type paxillin or vector control (Fig. 4B).

Decreased cell viability with PXN siRNA. Paxillin knockdown was effectively achieved in both the BEAS-2B (normal bronchial epithelial cells) and SK-LU-1 cell lines by 70% to 90% after 72 h of paxillin-specific siRNA transfection compared with control siRNA transfection. A significant reduction (40 ± 1.3%) of cell viability was observed in paxillin siRNA-transfected SK-LU-1 cells compared with BEAS-2B cells (6.5 ± 1.5%; Fig. 4C).

Redistribution of mutant PXN and association with Bel-2.

We examined paxillin localization using confocal microscopy. HS22 cell line was chosen as a representative of NSCLC, with minimal to no paxillin expression. In H522 cells, localization of exogenous wild-type paxillin was diffused throughout the intracellular compartments in the cell. In contrast, A127T paxillin expression was localized toward the cell periphery and had the characteristic

Figure 4. Effects of mutant A127T paxillin on lung cancer growth, transformation, and inhibition with siRNA. A, cell growth in HS22 cells transfected with EGFP vector, EGFP fusion wild-type paxillin, and EGFP fusion A127T paxillin shown for the indicated starvation time. *, P < 0.001. The traces of endogenous 68-kDa of paxillin in H522 cells and significant exogenous expression of EGFP fusion paxillin (95 kDa) was observed by immunoblot with anti-paxillin (SH11) antibody. B, anchorage-independent cell growth and colony formation of transfected H522 cells with vector, wild-type paxillin, and A127T mutant paxillin. Colonies stained and counted. Representative of colony formation assay of three independent experiments. Columns, number of colonies obtained in control, wild-type paxillin, and A127T paxillin; bars, SE; #, P = 0.0005; *, P < 0.0001. C, SK-LU-1 and BEAS-2B cells were either transfected with control siRNA (scrambled sequences) or with paxillin-specific siRNA duplexes (100 pmol/mL). Cell viability was measured in triplicate by MTT assay method after 72 h siRNA transfection. Percentage cell viability inhibition by paxillin targeting siRNA was shown with control siRNA in BEAS-2B and SK-LU-1 cells. Columns, mean percentage of cell growth; bars, SE (n = 3). Immunoblot analysis was used to confirm siRNA-directed knockdown of the paxillin expression in BEAS-2B and SK-LU-1 cells. The same membrane was then stripped and probed with anti–β-actin antibody for protein loading control.
punctate appearance of focal adhesion points (Fig. 5A). We further studied paxillin localization using nonimmortalized primary NHBE cells and compared with localization of A127T paxillin mutant SK-LU-1 cells. A well-defined actin cytoskeleton was observed in control NHBE cells and was found to be disrupted with reduced paxillin focal adhesion and F-actin fibers after starvation. In contrast, massive stress fiber formation with redistribution of paxillin to the ends of stress fibers occurred in SK-LU-1 cells (harboring the A127T paxillin mutant). Redistribution of paxillin and enhancement of the cortical actin ring were noticed

Figure 5. A127T paxillin localized to the focal adhesion and, during starvation, colocalized with Bcl-2. A, confocal images of H522 cells transfected with EGFP vector (control), EGFP fusion wild-type paxillin, and EGFP fusion A127T paxillin. N, nuclear location. Boxed regions shown zoomed twice. B, cells were stained for F-actin and paxillin, and projections of optical sections corresponding to the plane of adhesion are shown. Confocal images of F-actin (red) and paxillin (green) were shown in NHBE and SK-LU-1 cells. Nuclei were labeled in blue using 4',6-diamidino-2-phenylindole (DAPI). Boxed regions are shown zoomed thrice and contrast enhanced. Bar, 20 μmol/L. C, confocal laser scanned images of control and serum-starved SK-LU-1 cells double-stained using specific primary and fluorescence-conjugated secondary antibodies for paxillin (green), Bcl-2 (red), and Mitotracker (red). Nuclei were labeled in blue using DAPI. Note the cytoplasmic, mitochondrial pattern of paxillin and colocalization with Bcl-2 or Mitotracker after starvation in SK-LU-1 cells. Boxed regions shown zoomed thrice. Bar, 10 μmol/L.
Paxillin or A127T Paxillin Increases Tumor Growth and Invasion In vivo

In vivo properties of A127T paxillin and wild-type paxillin-expressing cells in nude mice were determined. H522 cells expressing wild-type paxillin or A127T paxillin and control vector group of xenografts were generated. Tumor growth was measured every week up to 12 weeks. At 12 weeks, tumor growth in the A127T mutant paxillin group markedly exceeded (P = 0.0019) that in the control vector group or wild-type paxillin (Fig. 6A). Paxillin-negative H522 cells grew in nude mice as a solid mass without any invasion, whereas either wild-type paxillin or A127T paxillin mutant expressing H522 cells grew as nodular tumors. In addition to nodularity, A127T paxillin-expressing H522 xenograft tumors were highly invasive into the adjacent muscle tissue. Upon gross examination, the A127T paxillin tumors had larger nodules compared with the wild-type paxillin tumors (Fig. 6B). Immunohistochemical analysis of tumor sections from mice in various groups were examined using antibodies specific for nuclear antigen Ki-67 (a marker for active cell division) and CD31 (measuring microvessel density). Immunohistochemistry of xenografts revealed that compared with control vector H522, there was enhanced cell proliferation, increased stroma, and increased microvessel density in wild-type paxillin H522 xenografts. In the A127T paxillin H522 xenografts, compared with paxillin-negative or paxillin-positive cells, there was enhanced cell proliferation with less stroma and microvessel density (Fig. 6C and D).

Discussion

Lung cancer can involve a number of abnormalities of oncogenes and tumor suppressor genes (13). Biologically, lung cancers are highly invasive and metastatic tumors (19). We have determined that the cytoskeletal protein paxillin had elevated levels of paxillin (compared with normal adjacent lung) in NSCLC, especially with higher stages, implicating it in invasion and metastasis. The gene amplification for \(\text{PAXN}\) was also high in large cell carcinoma (17%) compared with other histologies. Interestingly, \(\text{MET}\) was highly amplified with or without \(\text{PAXN}\). Because there is precedence in the literature that there are somatic mutations, such as \(\text{K-ras}\) and EGFR (20, 21), and germ-line mutations, such as c-Met (4, 14), for a number of genes, we also investigated whether the paxillin gene could be mutated in lung cancer. Somatic paxillin mutations were identified in lung cancer tumor tissue samples. The mutations were particularly clustered between the LD domains, as well as in the LIM domains. Our current studies focused only on frequent A127T paxillin mutation, and in the future, it would be interesting to determine the role of other mutants in the progression of lung cancer. A127T paxillin showed increased cell growth, focal adhesion formation, colocalization with Bcl-2, and proliferation within a mouse model.

Paxillin was initially discovered as a substrate for the nonreceptor tyrosine kinase oncogene pp 60v-src in Rous sarcoma virus–transformed fibroblasts (22). The human paxillin gene was originally cloned via antibody expression cloning from a BCR/ABL-containing cell line (7). With its unique ability to act as an adaptor protein, paxillin has been shown to bind to a number of proto-oncogenes, including BCR/ABL, \(\gamma\)-Src, and E6 (23–25). We have identified that there can be amplification or increased copy numbers of paxillin gene in lung cancers, and it is therefore possible that the overexpressed amount of paxillin is available to bind further to oncogenes for transformation. It was also shown that a number of tumors could have combined \(\text{PAXN/MET}\) gene amplification. This would be a therapeutic target approach, because MET inhibition has come to clinical fruition (26). We now have identified mutations between the LD motifs of paxillin. Because the LD motifs uniquely bind to various oncogenes, the mutations in this region of paxillin may alter the association with different proteins. The LIM domains of paxillin (especially LIM3) have been shown to be important for actin-localization (27). Mutations of the LIM domain may therefore be important in localization of paxillin, as well as signal transduction. Since the cloning of \(\alpha\)-paxillin, a number of isoforms have been identified as \(\beta\)-paxillin, \(\gamma\)-paxillin, and \(\delta\)-paxillin (28, 29). There are also homologous proteins with LIM domains, including Hic-5 and leupaxin (30, 31). Our studies focused only on \(\alpha\)-paxillin, and in the future, it would be interesting to determine the role of these other proteins in the etiology and progression of lung cancer. In addition, it will be important to determine the frequency of paxillin mutations in a larger cohort, with verification from cDNA and genomic DNA sequencing, as well as analysis of germ-line DNA.

The pattern of paxillin mutations may provide insights into the development of lung cancer. The mutational spectrum of paxillin was characterized by a high proportion of GC to AT (97%) transitions. Recent mouse lung tumor studies showed that the distribution of nucleotide changes (AT to GC, GC to AT, and AT to TA) leading to a tobacco-specific carcinogen \([4\text{-}(\text{methylnitrosa-mino})\text{-1-(3-pyridyl})\text{-1-butaneone (NNK)}]–\text{induced K-ras mutation (1%, 96%, and <1%, respectively)}\) was uniquely specific compared with that of spontaneous mutations (24%, 29%, and 20%), as well as other carcinogens, such as ethyl carbamate (31%, 3%, and 64%; ref. 32). It is possible that a single carcinogen, such as NNK, could be the causative mutagen.

The mutations identified were of different frequencies in the various histologies for lung cancer. As has been shown recently, there are molecular differences between small cell cancer, large cell cancer, squamous cell cancer, and adenocarcinomas of the lung (33, 34). As an example, \(\text{EGFR}\) mutations are more common in adenocarcinomas than the other histologies of lung cancer (40% versus 3%; ref. 21). \(\text{EGFR}\) mutations and amplification in NSCLC can serve as a prognosticator as well as a predictive marker to therapeutic inhibition (35). Paxillin can also potentially serve as a molecular marker, with a large number of mutations occurring within large cell lung cancer. Currently, we are investigating if there are differences in expression, invasion, and metastases of large cell lung cancers with and without paxillin mutations. Interestingly, there is a subtype of large cell lung cancer with neuroendocrine features (36), and it is not known how paxillin is affected in these tumors. Ultimately, it would be useful to determine the role of paxillin in prognosis for NSCLC as well as predictive marker for therapeutic response.

Not only was there a difference in histology for paxillin mutations, but there was also a difference between various ethnic populations. Unlike the \(\text{EGFR}\) gene, which can frequently be mutated in Asians with lung cancer (21), paxillin was not mutated in Taiwanese lung cancer samples. That is, the frequency of paxillin predominantly in starved SK-LU-1 cells (Fig. 5B). Because mutant paxillin staining in the cytoplasm resembled the staining seen with mitochondria, we determined whether paxillin localized to the mitochondria and colocalized with Bcl-2 using Mitotracker red dye and antibody specific to Bcl-2, respectively, In SK-LU-1 cells, A127T mutant colocalized with Bcl-2 in the mitochondria and not in NHBE after starvation (Fig. 5C).

Paxillin, a Target for Somatic Mutations in Lung Cancer

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mutations was higher in Caucasians and African-Americans and nonexistent in Asians. This could certainly be due to the intrinsic genetic differences, but could also be a result from environmental effects (such as the pattern or type of smoke) or a combination of both. As we have determined the relative rates of SNPs and mutations of paxillin in the Taiwanese, African-American, and Caucasian patients in our sample set, it would be important to determine the relative frequency of these SNPs/mutations in other populations. In terms of tumorigenesis, it is possible that, in the non-Asian population, there is more reliance on paxillin mutations to enhance tumorigenesis, especially because the frequency of EGFR mutations is quite low compared with the Asian population.

Figure 6. Phenotypic changes and cell growth effect of wild-type paxillin and A127T paxillin in mouse xenograft models. A, tumorigenicity of H522-control vector (blue), H522-wild-type paxillin (green), and H522-A127T paxillin (red) transfected H522 cells in nude mice. Points, mean value for six tumors per group. Tumor volume was plotted over time. *, P = 0.0016 (compared with control vector). Data analyzed by a two-tailed Student’s t test. B, representative photomicrograph. H&E, Ki67, trichrome, and CD31 staining images of tumor tissue obtained from mice injected with control vector/wild-type paxillin/A127T paxillin stably transfected H522 xenograft. Note the clear-cut border between the tumor (T) and muscle tissue (M) in control vector. A127T paxillin–expressing H522 xenograft showed tumor cell invasion into adjacent mouse muscle tissue. Boxed regions shown with higher magnification. Black arrow, necrotic cells; white arrow, invading tumor cells. C, semiquantitative analysis of tumor tissues stained for Ki67, trichrome, and CD31. Bars, SE.
There is also considerable evidence that \textit{EGFR} mutations are mutually exclusive of \(K\)-ras mutations in lung cancer (21, 37). It is not known at this time how the paxillin mutations correlate with other mutations in lung cancer, such as \(p53\), \(K\)-ras, \textit{EGFR}, and \textit{MET}. Ultimately, in lung cancer, one can envision having molecular profiling of individual tumors and thereafter therapeutic decision making.

Paxillin was shown to be essential in actin filament assembly, as well as in the focal adhesion formation, migration, association with extracellular signal-regulated kinase in DNA synthesis (38), survival, motility (39), and in morphogenesis (40). As has been shown recently, paxillin is phosphorylated in response to reactive oxygen species and c-Met activation (41). Therefore, it is reasonable to consider that paxillin plays an essential role in the increased growth and invasion in lung cancer. We have shown that forced expression of A127T paxillin mutant enhanced \textit{in vitro} cell growth, oncogenic transformation (colony formation), tumor growth, and invasion in a nude mouse model.

A127T paxillin also colocalized with the antiapoptotic Bcl-2 protein. Because it is known that paxillin interacts with Bcl-2 and promotes the survival and morphogenesis of kidney cells (42, 43), the colocalization between A127T paxillin and Bcl-2 is very likely to promote lung cancer cell survival. Here, we discovered the enhanced survival of cells harboring A127T mutant paxillin specifically after serum starvation. Our confocal microscopy results further show that there was increased expression and colocalization of Bcl-2 and paxillin (A127T) in the SK-LU-1 cells. It would now be very interesting to pursue this further, and this would become a study in itself. Expression and starvation experiments further showed the ability of A127T paxillin to support the growth and cell survival of lung cancer cells, particularly in the absence of growth factor. Although we have determined the functionality of one of the frequently occurring mutations, it would be useful to test out the relative biological and biochemical significance of other somatic mutations identified for paxillin.

\textit{In vivo} modeling of wild-type paxillin and A127T paxillin lead to a number of interesting results. We identified that there was increased tumor growth over time, enhanced nodularity, increased invasion into the muscle, and decrease in stroma for mutant paxillin (compared with both control and wild-type paxillin). There was some nodularity of wild-type paxillin when compared with the control vector, but less so than the mutant paxillin. This would implicate the mutant paxillin in the aggressive growth of some lung cancers and could potentially be a mechanism of early metastasis. Also, in quantification of mean-vessel density in the center of the tumor, there were fewer vessels in mutant paxillin compared with wild-type paxillin. We have observed that, because the mutant paxillin tumors grow more rapidly, there was more necrosis in the center of tumor, thus fewer vessels. However, at the edge of the tumor for the mutant paxillin, there were much more vessels compared with wild-type paxillin or control vector.

Overexpression of paxillin in xenografts correlated with angiogenesis. The wild-type paxillin lead to increased nodularity, as well as increased blood vessel formation. It is currently not known if this is an early event in the pathogenesis of lung cancer (especially occurring in metaplasia and dysplasia). Because several direct and indirect treatment strategies are under clinical investigation for treatment of solid tumors, inhibition of tumor angiogenesis through targeting paxillin could also be a promising indirect strategy. As a direct strategy, we have been able to show that small interference targeting of A127T lead to decreased cell viability. It would now be useful to determine the role of inhibition of paxillin in lung cancer and other tumors.

In conclusion, our results indicate that paxillin is a key molecule in tumor growth and metastasis. These findings are particularly interesting in light of recent evidence implicating paxillin in cancer aggressiveness (44), and it will be important to assess the relevance of these alterations in metastasis and understand the types of mutations involved in tobacco-related carcinogenesis. Further experiments will be required to establish the relative structure-function relevance of altered forms of paxillin and potential development of new targeted therapeutics for lung and other cancers showing such alterations.

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Ramasamy Jagadeeswaran, Hanna Surawska, Soundararajan Krishnaswamy, et al.


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