Abstract

Purpose: hMena, member of the enabled/vasodilator-stimulated phosphoprotein family, is a cytoskeletal protein that is involved in the regulation of cell motility and adhesion. The aim of this study was to determine whether or not the expression of hMena isoforms correlated with sensitivity to EGFR tyrosine kinase inhibitors and could serve as markers with potential clinical use.

Experimental Design: Human pancreatic ductal adenocarcinoma cell lines were characterized for in vitro sensitivity to erlotinib, expression of HER family receptors, markers of epithelial to mesenchymal transition, and expression of hMena and its isoform hMena+11a. The effects of epidermal growth factor (EGF) and erlotinib on hMena expression as well as the effect of hMena knockdown on cell proliferation were also evaluated.

Results: hMena was detected in all of the pancreatic tumor cell lines tested as well as in the majority of the human tumor samples (primary (92%) and metastatic (86%)). Intriguingly, in vitro hMena+11a isoform was specifically associated with an epithelial phenotype, EGFR dependency, and sensitivity to erlotinib. In epithelial BxPC3 cells, epidermal growth factor up-regulated hMena/hMena+11a and erlotinib down-regulated expression. hMena knockdown reduced cell proliferation and mitogen-activated protein kinase and AKT activation in BxPC3 cells, and promoted the growth inhibitory effects of erlotinib.

Conclusions: Collectively, our data indicate that the hMena+11a isoform is associated with an epithelial phenotype and identifies EGFR-dependent cell lines that are sensitive to the EGFR inhibitor erlotinib. The availability of anti-hMena+11a-specific probes may offer a new tool in pancreatic cancer management if these results can be verified prospectively in cancer patients.
In vitro in non–small cell lung cancer, an EMT-like transition is (26–29). Of interest, a recent gene profiling study showed that through a significant reorganization of the actin cytoskeleton lead to a subsequent disassembly of cell adherens junctions and expression and concurrent expression of mesenchymal markers associated cells, is characterized by a loss of E-cadherin.

Furthermore, Buck et al. (30) reported that pancreatic and colorectal tumor cell lines that expressed low levels or mutant forms of the epithelial junctions constituents E-cadherin and γ-catenin and gained expression of mesenchymal proteins such as vimentin, zeb1, and snail were more resistant to EGFR inhibition in vitro. Gain of mesenchymal markers in human pancreatic and colorectal tumor tissues was also associated with a more advanced tumor stage. More recently, Black et al. (31) showed that in a panel of urothelial carcinoma cell lines, loss of E-cadherin expression and enhanced invasive/tumorigenic potential were markers of poor response to the antiproliferative effect of EGFR targeting by the monoclonal antibody cetuximab. Similar results were also reported previously with gefitinib (32).

Enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) are key regulatory molecules controlling cell shape, movement, and actin organization at cadherin adhesion contacts, which are frequently affected after malignant transformation (33). We have shown that human Mena (hMena), a member of the Ena/VASP family, is overexpressed in human breast tumors, and a splice variant termed hMena+11a was recently isolated from a breast cancer cell line with an epithelial phenotype. Of interest, experimental data suggest that hMena couples tyrosine kinase signaling to the actin cytoskeleton (34–36).

The overall goal of our research is to identify molecular markers associated with EGFR dependency that could help clinicians to prospectively select pancreatic cancer patients who are most likely to benefit from erlotinib and other EGFR antagonists. Here, we show that the hMena+11a isoform is a marker of epithelial phenotype and EGFR activation, prerequisite to the antiproliferative effect of EGFR inhibition in human pancreatic cancer cells.

### Materials and Methods

#### Reagents.
Erlotinib was kindly provided by Roche. A stock solution was prepared in DMSO and stored at -20°C. Recombinant human EGF was purchased from Promega. The antibodies used for Western blot analyses were from the following sources: rabbit anti–total AKT, rabbit anti-p-AKT (Ser473), rabbit anti–total mitogen-activated protein kinase (MAPK; p42/44), and mouse anti-pMAPK (Thr202-Tyr204) were from Cell Signaling Technology; rabbit anti-pEGFR (Ty1068) was from Biosource; rabbit anti–total EGFR, rabbit anti-HER2, mouse anti-HER3, and rabbit anti-HER4 were from Santa Cruz; mouse anti–E-Cadherin from BD Biosciences; mouse anti–N-Cadherin and mouse anti–vimentin from DakoCytomation, and mouse anti–α-actin was from Sigma-Aldrich. We previously developed the anti-Mena rabbit polyclonal antibody (C6KL1) against the human 11a exon (RDSPRKNQVFDNRSYDSLH) sequence (Covance Research Products). The human 11a peptide with a terminal cysteine residue was covalently bound to an Iodoacetyl-based resin (SulfoLink Coupling Gel, Pierce), and the antibodies were affinity purified following manufacturer’s instruction. The specificity of the antibody was evaluated on Ena/VASP-deficient cells stably expressing enhanced green fluorescent protein (EGFP)-Mena or EGFP-Mena+11a.

#### Cell lines and culture conditions.
The following cell lines were purchased from the American Type Culture Collection: BxPC3, Panc1, MiaPaCa-2, Hs766T, and HEK 293 Phoenix (human embryonic kidney cells). The L3.6pl human pancreatic cancer cell line was kindly provided by Dr I.J. Fidler (The University of Texas M. D. Anderson Cancer Center, Houston, TX). The T3M4, PACA44, and PT45 cell lines were kindly provided by Dr. F. Velotti (Tuscia University, Viterbo, Italy). All cell lines were maintained in RPMI 1640 (Life Technologies, Inc.). The medium was supplemented with 10% fetal bovine serum (Life Technologies), l-glutamine (Bio Whittaker), and antibiotics (penicillin/streptomycin; Bio Whittaker). Adherent monolayer cultures were incubated at 37°C in a mixture of 5% CO2 and 95% air. MV379, Ena/VASP-deficient mouse embryonic fibroblastic cells, were isolated as described in Bear et al. (37) and cultured at 32°C in Immorto medium [high-glucose Dulbecco’s modified Eagle’s with 15% FCS, penicillin/streptomycin, l-glutamine, and 50 U/ml recombinant mouse IFN-γ (Invitrogen)].
Retroviral packaging, infection, and fluorescence-activated cell sorting. EGFP-Mena and EGFP-Mena\(^{1-11a}\) were subcloned into pMSCV retroviral plasmid by using standard techniques. Retroplasmids were transiently transfected into 293 Phoenix cells, and supernatant was collected after 48 h. MV\(^{39}\) cells were exposed to infectious supernatant for 24 h in the presence of 4 \(\mu\)g/mL polybrene and cultured to 90% confluence, trypsinized, and fluorescence-activated cell sorting was collected after 48 h. MV D7 cells were exposed to infectious supernatant for 24 h in the presence of 4 \(\mu\)g/mL polybrene and cultured to 90% confluence, trypsinized, and fluorescence-activated cell sorting was collected after 48 h.

Cell treatments. Cells were grown in 6-well plates to confluence in RPMI supplemented with 10% fetal bovine serum. After 18 h in serum-free medium, the cells were treated with different amounts of rhEGF for 24 h. Erlotinib (10 \(\mu\)mol/L) was added 2 h before EGF treatment.

Western blot analyses. Cells were lysed as reported (34). Lysates (30 or 100 \(\mu\)g) were resolved on 10% polyacrylamide gel and transferred to nitrocellulose membranes (GE Healthcare). Membranes were blocked in 5% nonfat milk in TBS containing 0.1% Tween 20 for 1 h at room temperature, incubated overnight with relevant antibodies, washed, and probed with species-specific secondary antibodies coupled to horseradish peroxidase, and detected by enhanced chemiluminescence (Amersham Biosciences).

Proliferation assays. Cell proliferation was determined by measurement of cellular ATP levels with a high sensitivity cell proliferation/cytotoxicity kit (Vialight Plus; Cambrex Bio Sci Rockland, Inc.). Briefly, cells were plated in 96-well plates at a density of 1 \(\times\) 10\(^4\) per well and exposed 24 h later to various concentrations of erlotinib (0.1-10 \(\mu\)mol/L) in serum-free medium. After 24 h, nucleotide-releasing agent (reagent) (100 \(\mu\)l) was added to each well and the plate was incubated for 10 min at room temperature. Cell lysate (180 \(\mu\)l) was transferred to a luminescence compatible plate. The 96-well plates were read using a Perkin-Elmer LS 50B luminometer. ATP levels in cells were normalized to levels in untreated control cultures.

Small interfering RNA treatment. Cells in exponential growth phase were transfected with 100 nmol/L hMena-specific pooled small interfering RNA (siRNA) duplexes (siRNA SMART pool) or control nonspecific siRNA (Dharmacon) using Lipofectamine 2000 reagent (Invitrogen). After culturing for 48 h, cells were serum deprived for 18 h and then differently treated for Western blot analysis or proliferation assays.

Two-dimensional electrophoresis. Cells were washed, lyophilized, and proteins were solubilized with two-dimensional electrophoresis buffer (9 mol/L urea, 10 mmol/L Tris, 4% CHAPS, 65 mmol/L DTT, 2% IPG buffer ampholine (pH 3-10), protease inhibitor cocktail). Protein samples (250 \(\mu\)g) were applied to 7-cm IPG strips (pH 3-10; nonlinear; Amersham Biosciences), and isoelectric focusing was done with an IPGphor system (Amersham Biosciences) following a protocol described as (38). Strips were equilibrated in 100 mmol/L Tris-HCl buffer (pH 8.8) containing 6 mol/L urea, 30% glycerol, 2% SDS, and 2% DTT, followed by an incubation in the same buffer replacing DTT with 2.5% iodoacetamide. The strips were loaded on top of 10% acrylamide SDS-PAGE gels for the second dimension separation. Proteins were electrotransfered onto nitrocellulose membranes, and Western blot was done as described above. Images were acquired at high resolution, and two-dimensional immunoreactivity patterns were analyzed using Progenesis PG240 v2005 software (Nonlinear Dynamics). Relative molecular mass (M\(_r\)) was estimated by comparison with M\(_r\) reference markers (Precision; Bio-Rad) and isoelectric point (pI) values assigned to detected spots by calibration as described in the Amersham Biosciences guidelines.

Patients and tissue specimens. A series of 26 patients (median age, 62 y; range 39-78 y) who underwent pancreatic resection or biopsy at the Regina Elena National Cancer Institute between 2002 and 2005 with a diagnosis of pancreatic adenocarcinoma were retrospectively collected for immunohistochemical studies. This series included 12 primary (9 stage II, 1 stage III, and 2 stage IV) and 14 metastatic carcinomas (11 liver metastasis and 3 other abdominal sites). Tumors were staged according to the Union Internationale Contre le Cancer TNM System 2002 and collected according to the Internal Ethic Committee guidelines.

Immunohistochemistry. Pancreatic cancer specimens were fixed for 18 to 24 h in buffered formaldehyde and then processed through to paraffin wax. hMena expression was evaluated by immunohistochemistry using the monoclonal antibody clone 21 (BD Transduction; 2.5 \(\mu\)g/mL) that recognizes all the hMena isoforms and does not crossreact with other members of Ena/VASP family proteins (34). Dewaxing, antigen retrieval, incubation with the primary antibody, chromogenic reaction with 3,3'-diaminobenzidine, and counterstaining with Mayer Haematoxylin were done with an automatic autostainer (Vysion Biosystems Bond; Menarini). Sections were mounted in aqueous mounting medium (Glycergel; Dako-Cytomation). The intensity of hMena staining, detected in the cytoplasm, was scored from 0 to 3+ according to the following criteria: no staining, score 0; weak cytoplasmatic staining of neoplastic cells, score 1+; moderate cytoplasmatic staining, score 2+; and strong cytoplasmatic staining, score 3+. Evaluation of the immunohistochemical data was done independently and in blinded manner by two investigators.

Statistical analysis. All experiments were repeated at least twice. Statistical significance was determined by Student's t test (two tailed) comparison between two groups of data. Asterisks indicate significant differences of experimental groups compared with the corresponding control condition (\(*, P < 0.05; **, P < 0.01\). Statistical analysis was done using GraphPad Prism 4, V4.03 software (GraphPad, Inc.). Change in the phosphorylation status was evaluated, using Progenesis v.2004 software (Nonlinear Dynamics), by absorbance indicated as normalized spot volume. Normalization was done by multiplying the total spot volume by the constant factor 100, which produces spot percentage volume. Densitometric quantitation of hMena immunoreactivity was determined by ImageJ and normalized in comparison with the actin immunoreactivity.

Results

hMena and hMena\(^{1-11a}\) isoform expression in pancreatic cancer–derived cell lines. To acquire insights into the expression, modulation, and function of the hMena and its isoform in pancreatic cancer, we first characterized the hMena and hMena\(^{1-11a}\) expression in a panel of eight pancreatic cancer cell lines by Western blot analysis. Using an anti-hMena antibody recognizing all isoforms (pan-hMena), we observed (Fig. 1A) that hMena was consistently expressed at different level in all the tumor cell lines tested. Because hMena and hMena\(^{1-11a}\) isoforms are not distinguishable by Western blot because they comigrate (88-90 kDa), we used an anti-hMena\(^{1-11a}\) antibody that specifically recognize this isoform. The specificity of this antibody was tested on cell lysates from Ena/VASP-deficient cells, stably expressing EGFP-Mena and EGFP-Mena\(^{1-11a}\) (Fig. 1B). hMena\(^{1-11a}\) was selectively expressed in different level in all the tumor cell lines tested. Because hMena and hMena\(^{1-11a}\) isoforms express in pancreatic cancer–derived cell lines.
was revealed by pan-hMena (Fig. 1C). These two set of spots correspond to the two different isoforms, hMena and hMena+11a, as previously reported in breast cancer (36). A different pattern was observed in Panc1 cells, which such as BxPC3 cells, expressed the 5.4 to 6.0 set of spots (hMena). However, the set of spots corresponding to hMena+11a were absent, and a new set of protein spots displaying a lower molecular weight and more basic pI (range, 5.9-6.7) was present (we are currently characterizing this isoform in more detail). Because expression of hMena+11a seems to be restricted to cells with an epithelial phenotype, we evaluated markers of EMT in our panel of pancreatic cancer cell lines by Western blot analysis. As shown in Fig. 1D, E-cadherin was highly expressed in all of the hMena+11a–positive cell lines (L3.6pl, BxPC3, T3M4, and PACA44) and was absent in the hMena+11a–negative cell lines. Conversely, we detected expression of the mesenchymal marker vimentin in PT45, Panc1, and MiaPaCa-2 and N-cadherin in MiaPaCa-2 and Hs766T, suggesting that hMena+11a is a marker of an epithelial phenotype in pancreatic cancer cell lines.

hMena+11a isoform expression correlates with sensitivity to EGFR inhibition in pancreatic cancer cell lines. Recently, we have shown that in breast cancer, hMena may couple tyrosine kinase signaling to the actin cytoskeleton (36). In view of the role of EGFR as a relevant therapeutic target in the treatment of pancreatic cancer patients, we evaluated the growth inhibitory effect of the EGFR tyrosine-kinase inhibitor erlotinib in our panel of pancreatic cancer cell lines by exposing them to increasing concentrations (0-10 μmol/L) of the drug. As shown in Fig. 2, we observed a significant heterogeneity in drug responsiveness. Considering the average steady-state plasma concentrations in erlotinib-treated patients, we divided our panel in sensitive (L3.6pl, BxPC3, T3M4, and PACA44), displaying at least a 50% inhibition of proliferation at concentrations of erlotinib ≤1 μmol/L, and resistant (PT45, Panc1, MiaPaCa-2, and Hs766T) cell lines, in which the growth rate...
was not significantly affected even with an erlotinib concentration of 10 μmol/L (39, 40). Of interest, all the erlotinib-sensitive cell lines expressed hMena+11a, indicating that this isoform identifies a specific cell phenotype in which EGFR-tyrosine kinase inhibition significantly affects cell proliferation. No hMena+11a expression was in fact observed in the erlotinib-resistant cell lines. To evaluate whether the expression of other EGFR family members might affect the responsiveness of tumor cells to EGFR kinase inhibitors, we analyzed the levels of EGFR family members in our panel of pancreatic cancer cell lines by Western blot (Fig. 3). The expression of HER family members was not correlated with hMena and hMena+11a and no correlation was found with erlotinib sensitivity, confirming previous results (41–43). However, a constitutive EGFR phosphorylation was seen exclusively in the sensitive pancreatic cancer cell lines hMena+11a positive, with PACA44 expressing the lowest level of phosphorylation being the least sensitive among them. This observation is consistent with our previous findings that EGFR-mediated signaling networks are “on” in the erlotinib-sensitive cells, driven by availability of the autocrine ligand production (44).

Effect of EGF and erlotinib treatment on hMena expression in pancreatic cancer cell lines. To further test our hypothesis that hMena isoforms are along the EGFR-signaling pathway, we explored the effects of EGF and erlotinib treatment on hMena expression in BxPC3, erlotinib-sensitive, and Panc1, erlotinib-resistant cell lines (Fig. 4). Twenty-four hours treatment with two different EGF concentrations (50 and 100 ng/mL) clearly increased hMena and hMena+11a protein level as detected by Western blot analysis in both cell lines. Furthermore, the addition of erlotinib to the EGF-treated cell lines down-regulated hMena expression only in the hMena+11a positive BxPC3 cell line.

hMena knockdown reduces proliferation, AKT, and MAPK activation in the erlotinib-sensitive pancreatic cancer cell lines. In view of the role of hMena+11a on the proliferative activity in breast cancer cells, we transiently knocked down, with high efficiency, hMena+11a–positive, erlotinib-sensitive BxPC3, and in hMena+11a–negative and erlotinib-resistant Panc1 cell lines via RNA interference (Fig. 5A). In parallel, we analyzed constitutive AKT and MAPK phosphorylation levels and found that they were high in both the cell lines, consistent with the general relevance of these pathways in driving proliferation and survival in pancreatic cancer cells. However, whereas AKT and MAPK phosphorylation were strongly reduced in hMena-silenced BxPC3 cells, hMena knockdown did not or slightly affect constitutive AKT and MAPK phosphorylation in Panc1 cells. The effects of hMena knockdown on these pathways were associated with a significant reduction of the baseline growth rate in BxPC3 (45% versus 100%; \( P = 0.002 \)) compared with the growth rate of untransfected cells and cells transfected with a control-nonspecific siRNA. In the Panc1 cell line, hMena knockdown also reduced proliferation rates, but the effects were much less dramatic (78% versus 100%; \( P = 0.01 \); Fig. 5B). Notably, combined exposure to the hMena siRNA construct (48 hours)
plus erlotinib (100 nmol/L; 24 hours) resulted in an additive decrease in proliferation in the BxPC3 cells but not in the hMena−/−negative Panc1 cells compared with untransfected cells (Fig. 5B).

**Discussion**

The feeble and heterogeneous clinical responses to EGFR-inhibitors therapies in patients with locally advanced or metastatic carcinomas highlight the importance of identifying the molecular determinants of patients’ responsiveness, which may be translatable to the clinical setting (5). We have previously shown that a subset of gefitinib-sensitive pancreatic cancer cells is dependant on autocrine transforming growth factor-α–mediated activation of the EGFR for cell proliferation and display constitutive EGFR phosphorylation in vitro and in vivo, whereas insensitive cell lines do not exhibit transforming growth factor-α

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Table 1. hMena protein expression in invasive and metastatic pancreatic carcinoma by immunohistochemistry

<table>
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<tr>
<th>No. of cases</th>
<th>hMena score</th>
<th>0/1+ (%)</th>
<th>2+/3+ (%)</th>
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<tbody>
<tr>
<td>Primary tumors: 12</td>
<td>1 (8.4) 11 (91.6)</td>
<td></td>
<td></td>
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<tr>
<td>Metastatic tumors: 14</td>
<td>2 (14.2) 12 (85.8)</td>
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expression and EGFR constitutive activation (44). To contribute in the understanding of the mechanisms leading to EGFR-inhibitors sensitivity and in light of accumulating data implicating the EGFR in the modulation of the actin cytoskeleton, in the present study, we evaluated whether hMena and hMena+11a actin-binding proteins that are able to couple tyrosine kinase signaling to the actin cytoskeleton in breast cancer, may represent key mediators of EGFR activity in pancreatic cancer cells (36, 45, 46).

The expression of hMena and hMena+11a was first characterized in a panel of human pancreatic cancer cell lines showing heterogeneity in responsiveness to the tyrosine kinase inhibitor erlotinib. Whereas in other normal tissues hMena expression has been reported at low or no detectable levels, hMena was detected in all the pancreatic tumor cell lines tested, in a human pancreatic ductal epithelial cell line, as well as in pancreatic tissue, primary, and metastatic tumors (34–36). Intriguingly, the expression of hMena+11a, an isoform specific to cell lines that display an epithelial phenotype, was restricted to the non-neoplastic human pancreatic duct epithelial cell line and to the four cancer cell lines that were E-cadherin positive and negative for expression of vimentin and N-cadherin. Notably, these four cell lines also displayed constitutive phosphorylation of the EGFR pathway and significant sensitivity to erlotinib, suggesting, in agreement with recent published data, that the efficacy of EGFR inhibitors is tightly related to the activation of the targeted pathway (47). Collectively, these data lead us to hypothesize that hMena+11a is not only a marker of an epithelial phenotype, but its expression is also able to identify cancer cells that are using the EGFR to drive proliferation, rendering them sensitive to EGFR-specific tyrosine kinase inhibitors. On the contrary, the hMena+11a isoform was not expressed in mesenchymal, erlotinib-resistant cancer cell lines.

Our results are consistent with previous studies that have linked EMT to erlotinib resistance in non–small cell lung cancer cells in vivo and in vitro (22). Furthermore, in a small subset of patients enrolled in a randomized, placebo-controlled non–small cell lung cancer clinical trial, in which erlotinib in combination with chemotherapy failed to show clinical activity, a positive E-cadherin immunostaining was predictor of a better outcome in all measures of clinical benefit in patients treated with erlotinib (48, 49). In pancreatic and colorectal tumor cell lines, the loss of E-cadherin and often the gain of proteins associated with an EMT phenotype correlates with resistance to EGFR inhibition. Furthermore, when the expression of E-cadherin and vimentin was measured in pancreatic and colorectal tissue microarrays containing tumors of varying stages, decreased E-cadherin expression and parallel increased vimentin expression is observed with advanced tumor stages (30). The extent to which the mesenchymal proteins are cellular biomarkers rather than functional participants in producing insensitivity to EGFR inhibitors is unclear at the present time. Recent observations suggest that during EMT, cells acquire abnormal EGFR-independent survival signals in part from the activation of either or both the phosphatidyl inositol 3-kinase or Ras-Raf-Mek-Erk pathways (50).

In view of the above, we analyzed the effect of hMena knockdown in BxPC3 and Panc1 cell lines endowed with differential sensitivity to erlotinib. In BxPC3 cells, displaying the on EGFR pathway, hMena/hMena+11a knockdown significantly impaired cell proliferation, whereas this effect was barely evident in hMena+11a-negative Panc1 cells, possessing an “off” EGFR pathway. This observation was confirmed at the protein level by a significant reduction of the constitutive AKT and MAPK phosphorylation, known to be via EGFR-mediated routes in BxPC3 cell line (44). Conversely, the lack of effect on AKT and MAPK phosphorylation by hMena silencing in Panc1 cells, in which the EGFR pathway is off, again support the hypothesis that hMena is downstream to the EGFR activity. As expected, when hMena knockdown cells were treated with very low concentration of erlotinib, a dramatic decrease on cell proliferation was observed only in BxPC3.

In conclusion, we have shown that hMena acts as a mediator of the EGFR signaling pathway and significantly modulates the growth of pancreatic cancer cell lines dependent on EGFR signaling. These cell lines, which display an epithelial phenotype, are erlotinib sensitive and are selectively characterized by the presence of hMena+11a isoform. As a whole, the results of the present study identify the expression of hMena/hMena+11a as predictive of in vitro response to EGFR inhibitors, thus strongly supporting prospective studies to assess whether this molecular signature may be associated with an improved clinical response to EGFR targeted therapy in pancreatic cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Human Mena$^{11}$a Isoform Serves as a Marker of Epithelial Phenotype and Sensitivity to Epidermal Growth Factor Receptor Inhibition in Human Pancreatic Cancer Cell Lines

Maria S. Pino, Michele Balsamo, Francesca Di Modugno, et al.


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