Mst1, RanBP2 and eIF4G are new markers for in vivo PI3K activation in murine and human prostate

Oliver Renner, Jesus Fominaya, Soledad Alonso1, Carmen Blanco-Aparicio, Juan F.M.Leal and Amancio Carnero*

Experimental Therapeutics Programme and 1Molecular Pathology Programme, Spanish National Cancer Centre (CNIO), C/ Melchor Fernandez Almagro 3, 28029 Madrid, Spain

to whom correspondence should be addressed. Tel: +34 91 732 8021; Fax: +34 91 732 8051; Email: acarnero@cnio.es

Phosphatidylinositol 3-kinases (PI3Ks) constitute important regulators of signaling pathways. The PIK3CA gene encoding the p110-alpha catalytic subunit represents one of the highly mutated oncogenes identified in human cancer. Here, we report new markers for in vivo PI3K activation in prostate. To that end, we used a transgenic mouse model, which expresses a constitutively active p110-alpha subunit in the epithelial cells of the prostate. The activity of the PI3K pathway in the prostate was proven by assessing the phosphorylation of the PI3K direct target AKT1 and of the mTOR target eukaryotic translation initiation factor 4G (eIF4G). To establish also transcriptional (late) targets of the PI3K pathway, we tested two genes, Mst1 and RanBP2, which we recently described as transcriptional targets of the growth factor platelet-derived growth factor-beta. We show that the levels of both proteins are elevated in transgenic animals. Additionally, we describe that the phosphorylation of AKT and eIF4G, as well as the elevation of the Mst1 and RanBP2 protein levels, can be inhibited in vivo in transgenic animals by the PI3K inhibitor LY294002. Finally, we performed human tissue microarray experiments with the four markers. Since they define overlapping but not identical subsets of the tested tissue panel, a combination of all four markers might lead to a more accurate diagnosis of the status of the PI3K-signaling cascade in cancer patients.

Introduction

The phosphatidylinositol 3-kinase (PI3K) family plays a central role in transducing a variety of extracellular stimuli into a wide range of cellular processes, including cell-cycle progression, cell growth, survival, cell adhesion and cell motility (1). When activated by cell-surface receptors, PI3K regulates phosphoinositide lipid metabolism and the production of phosphatidylinositol 3,4,5-trisphosphate at the plasma membrane (2). The production of phosphatidylinositol 3,4,5-trisphosphate at the inner leaflet of the plasma membrane facilitates the recruitment of AKT via its pleckstrin homology domain, leading to its phosphorylation at threonine 308 and serine 473 (3). As the primary downstream mediator, AKT transmits the PI3K signal to a large number of the mTOR target eukaryotic translation factor 4G, thereby diversifying the PI3K signal into various functional outcomes. The activation of mTOR, for example, is important for the stimulation of protein synthesis and cell proliferation (4). The main effects of PI3K/AKT activation with regard to cancer biology are the support of cell survival, proliferation and growth (5). Abnormalities in the PI3K-signaling pathway have been found to be common in human tumors. PI3K itself is frequently activated by mutation in a variety of cancer types (6). The tumor suppressor and PI3K antagonist phosphatase and tensin homologue deleted on chromosome 10 has been found to be frequently mutated and epigenetically silenced (7). Of the downstream targets, genomic and epigenetic changes have been described for AKT, p70/S6 kinase and the forkhead family of transcription factors (8). Since PI3K and its downstream effectors are involved in many different tumor types, it constitutes a promising target for cancer therapies. In the study presented here, we describe a novel mouse model for the constitutive activation of the PI3K-signaling cascade in the epithelial compartment of the prostate. Using this model, we established three new markers for PI3K activity, eukaryotic translation factor 4G (eIF4G), Mst1 and RanBP2. We used these markers to characterize human prostate samples in tissue microarray (TMA) experiments.

Materials and methods

Cloning of the p110-alpha transgene into a vector containing a myristoylation signal

First, a ‘myristoylated’ version of the vector pMB is been generated. By polymerase chain reaction (PCR), the human myristoylation sequence has been introduced in the Nhel and XhoI sites, thereby creating a SpeI restriction site 3’ of the myristoylation sequence (forward primer: 5’-GCTAGGCCGGCTACATGGGAGGACAGCAAGGACAAAGCCAAGGCCCCAGACCCGCGGGGGAACATGTCGGCGCTCGAC-3’ and reverse primer: 5’-CTCGAGGCGC GACCTTGGTCTCGGCTAGGACTTTCGCCGGCGCGCTCGAC-3’). The cDNA for murine p110-alpha was amplified by PCR, thereby introducing a SpeI restriction site at the 5’-end and a MluI restriction site at the 3’-end of the cDNA. After digestion with SpeI and MluI, the cDNA was inserted into the myristoylated version of the vector pMB.

Generation, handling and analysis of transgenic mice

All animal experiments were done under the experimental protocol approved by the Institutional Committee for Care and Use of Animals of the Spanish National Cancer Centre that complies with European legislation on the care and use of animals, National Institutes of Health guidelines for the use of laboratory animals and related codes of practice. Transgenic mice were generated by microinjecting the 3.2 kb fragment of pMIMTV-MYRp110-alpha digested with AatII and SalI into the pronucleus of single-cell embryos isolated from super-ovulated B6CBA mice. The embryos were implanted into pseudo-pregnant females and allowed to develop to term. The genotype of transgenic mice was assessed by PCR (forward primer: 5’-TGAATTTGCGCGCTCGGGAATC-3’ and reverse primer: 5’-CAGGCGGATGCCAATAAC-3’). Formalin-fixed prostate samples were stained with antibodies against p110-alpha (Upstate Biotechnology, Lake Placid, NY), phospho-S473AKT1, phospho-ser1108 eIF4G and Mst1 (New England Biolab, Beverly, MA) and RanBP2 (ABR, Golden, CO).

Western blot analysis

Total protein was extracted from snap-frozen tissue by homogenization using a Dounce homogenizer in 1 ml extraction buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 15 mM ethylenediaminetetraacetic acid, 10 mM NaF, 2 mM p-touleuensulfonlfy-argnine methyl ester, 5 mM benzamide, 10 mg/ml aprotinin, 40 mg/ml bestatin, 10 mg/ml E64, 300 mg/ml phosphoramidon, 50 mg/ml antipain, 2.5 mM Na2P04, 15 mM p-nitrophenyl phosphate, 1 mM dihydrothiolo, 60 mM beta-glycerophosphate, 10 mg/ml leupeptin, 0.14 mg/ml chymostatin, 0.7 mg/ml pepstatin, 1 mg/ml pefabloc and complete protease inhibitor cocktail tablet (Roche Molecular Biochemicals, Barcelona, Spain)). Following antibodies were used: phospho-S473AKT1 and anti-AKT1 (New England Biolab) and alpha-tubulin (Sigma, St Louis, MO).

Marine tissue immunohistochemistry

Tissue slices of 3 μm from paraffin blocks were applied to special immunohistochemistry coated slides (DAKO, Glosstrup, Denmark). These slides were baked overnight in a 56°C oven, deparaffinized in xylene for 20 min, rehydrated through a graded ethanol series and washed with phosphate-buffered saline. A heat-induced epitope retrieval step was performed in a solution of sodium citrate buffer, pH 6.5. The slides were then heated for 2 min in a conventional pressure cooker and rinsed in cool running water for 5 min. Endogenous

Abbreviations: eIF4G, eukaryotic translation factor 4G; MMTV, mammary tumor virus; PCR, polymerase chain reaction; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol 3-kinase; PIN, prostatic intra-epithelial neoplasia; PSA, prostate-specific antigen; TMA, tissue microarray.

© The Author 2007. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oxfordjournals.org
peroxide activity was quenched with 1.5% hydrogen peroxide (DAKO) in methanol for 10 min and incubation with the primary antibodies phospho-S473-AKT1 (polyclonal, 1:50; New England Biolab), Mst1 (polyclonal, 1:25; Cell Signaling, Danvers, MA), RanBP2 (polyclonal, 1:50; ABR) and phospho-S1108-eIF4G (polyclonal, 1:10; Cell Signaling) were performed (40 min). After incubation, immunodetection was carried out with the peroxidase-based Peroxidase-anti peroxidase system (DAKO) using diaminobenzidine chromogen as substrate, according to the manufacturer’s instructions. Slides were cover slipped in Pertex (HistoLab, Gothenburg, Sweden). The immunostaining was performed in a TechMate 500 automatic immunostaining device (DAKO). Incubations omitting the specific antibody were used as a control of the technique.

TMA construction
To corroborate the in vitro results, immunohistochemical studies were performed in human prostate specimens included in a TMA that contains representative samples of prostatic carcinoma (10 cases), prostatic intra-epithelial neoplasia (PIN) (8 cases) and benign prostate (16 cases). In addition, normal tissues such as breast and tonsil were included to serve as internal controls. This TMA was constructed by a standard method (9) using cases kindly provided by the Spanish National Cancer Tumour Bank Network. Briefly, for each sample, two 1 mm diameter cylinders of tissue were obtained from representative areas of each archived paraffin block and arrayed into a new recipient paraffin block with a custom-built precision instrument (Beecher Instruments, Silver Spring, MD, USA). The two cores from each sample were arranged in opposite sites of the array, in order to control for uneven distribution of the antibody and staining solutions on the array. The original tissues had been fixed in 4% buffered formalin and paraffin embedded according to routine procedures. New hematoxylin and eosin-stained slides from each case were performed for review and selection of the most representative areas.

After construction, initial sections of the TMA were stained for hematoxylin and eosin and reviewed by clinical pathologists to verify the histopathological findings. Moreover, p63 and high-weight cytokeratin (34BE12) were performed for staining of basal cells in order to help in difficult cases or small lesions to distinguish carcinoma from PIN lesions.

TMA immunohistochemistry
Tissue slices of 3 μm from the TMA block were applied to special immunohistochemistry coated slides (DAKO). These slides were baked overnight in a 56°C oven, deparaffinized in xylene for 20 min, rehydrated through a graded ethanol series and washed with phosphate-buffered saline. A heat-induced epitope retrieval step was performed in a solution of sodium citrate buffer, pH 6.5. The slides were then heated for 2 min in a conventional pressure cooker and rinsed in cool running water for 5 min. Endogenous peroxidase activity was quenched with 1.5% hydrogen peroxide (DAKO) in methanol for 10 min and incubation with the primary antibodies phospho-S473-AKT1 (polyclonal, 1:25; New England Biolab), Mst1 (polyclonal, 1:10; Cell Signaling), RanBP2 (polyclonal, 1:50; ABR), phospho-S1108-eIF4G (polyclonal, 1:50; Cell Signaling) and p63 (monoclonal, 1:50; DAKO) were performed (40 min). After incubation, immunodetection was carried out with Envision (DAKO) as visualization system using diaminobenzidine chromogen as substrate, according to the manufacturer’s instructions. Slides were cover slipped in Permount® (Fisher Scientific, Cayce, PR). The immunostaining was performed in a TechMate 500 automatic immunostaining device (DAKO). Incubations omitting the specific antibody were used as a control of the technique.

Scoring system
Immunostaining intensity was evaluated by S.A. and O.R. and scored using uniform and clear cut-off criteria to maintain the reproducibility of the method. Briefly, the result of the immunostaining was recorded as positive or negative and low versus high expression taking into account the expression of the protein in tumoral cells. As a general criterion, cut-offs were selected to facilitate reproducibility, considering negative (0): 0–5% staining; low positivity (1): 5–50% and high positivity (2): >50% of positive tumoral cells (see Table 1). Selected sample replicates were highly reproducible (95%), as other authors had found (10,11). Mouse epithelial-specific signal was quantified using the NIH ImageJ software package. Of each immunohistochemical image, five selected sample replicates were highly reproducible (95%), as other authors

Results
Generation of a transgenic mouse line expressing an epithelial-specific, constitutively active p110-alpha from the Y chromosome
The purpose of the study is the investigation of PI3K activity in the epithelial tissue compartment. We constructed a transgenic mouse line that expresses a constitutively active p110-alpha under the control of the epithelial-specific mouse mammary tumor virus (MMTV) promoter. To ensure the localization to the membrane and thereby the constitutive enzymatic activity, we fused the myristoylation sequence of the src kinase to the N-terminus of p110-alpha. We identified a mouse line, which transmitted the transgene only to male progeny. Among the F1 generation (19 litters, 72 females and 80 males) and the F2 generation (15 litters, 53 females and 52 males) obtained by crossing the founder male with C57BL/6 wild-type female mice, none of the female progeny was positively genotyped for the transgene, raising the question if the transgene might have been integrated into the Y chromosome. To clarify this question, we cloned the insertion site by the PCR-based Genome Walker method (CLONTECH, BD Bioscience, Palo Alto, CA). The sequence analysis of the fragments revealed a genomic region of the strain C57BL/6 mouse genome that occurs at two sites of the Y chromosome. The features flanking the amplified region include a sequence similar to Sycp3-like Y-linked isoform 1, Spindlin-like protein-2, Y-linked testis-specific protein isoform 2, Y-linked zinc finger protein-2 and sex-determining region Y.

Expression of active MMTV-MYRP110-alpha in male reproductive organs
The activity of the constitutively active p110-alpha transgene was examined in the following organs of transgenic males: breast and fat tissue including lymph node, salivary gland, skin, testis, epididymis, seminal vesicle, prostate, urinary bladder, pancreas, spleen, liver, lung, thymus, heart and brain. The phosphorylation of AKT at serine 473 as end-point read-out of PI3K activation was assessed first by western blot experiments. For the comparison of equivalent protein amounts, the levels of total AKT and alpha-tubulin were determined. Second, we performed immunohistochemistry with the antibody against phosphorylated AKT mentioned above. The immunohistochemical analysis of the organs listed above was conducted to pick up transgene expression in subpopulations of cells that might not be sufficient in number to be detected by western blot. Elevated PI3K activity could be demonstrated by western blot and immunohistochemistry in testis, seminal vesicles, epididymis and prostate (Figure 1A and B). By the immunohistochemical analysis, we additionally detected slightly elevated PI3K activity in the brain (data not shown). The increased expression of the p110-alpha protein was confirmed by immunohistochemical analysis using an antibody against p110-alpha (Figure 1B). PI3K activity is commonly detectable in different cell types all over the prostate. The higher expression of p110-alpha and the highly elevated AKT phosphorylation in transgenic prostates were distributed uniformly all over the prostate, as well. But in contrast to the wild-type situation, the higher signals in transgenic prostates could only be observed in the epithelial compartments of the prostates. This observation was expected since the expression of the transgene was driven by the epithelial-specific MMTV promoter. The quantification of this epithelial-specific signal is shown in Figure 1C. The differences between the transgenic and respective wild-type tissues were found to be significant.

Because of the high clinical relevance of prostate pathologies, we decided to perform the following analyses with the prostate as the model organ. To examine if the phosphorylation of AKT could be used to test for the pharmacological inhibition of PI3K activity, we injected intra-peritoneally either the PI3K-specific inhibitor LY294002 or the solvent agent (50% dimethyl sulfoxide in phosphate-buffered saline) without the drug into transgenic littermates. As displayed in Figure 2A, the levels of phosphorylated AKT are lower in the prostates of transgenic mice injected with LY294002 than in the control mice and the protein levels of p110-alpha remain unchanged. The inset of each panel shows in higher magnification that the strong signal of the untreated transgenic prostate tissue is located to the epithelium. We quantified this epithelial-specific signal, and values of the immunohistochemical images of LY294002-treated prostates were compared with the values of the respective control images (transgenic prostates treated with the solvent only). As displayed in the graph of Figure 2A, 1419
the phosphorylation of AKT was decreased to 48% of the control level ($P = 0.004$).

**Phosphorylation of eIF4G as a surrogate marker for PI3K activity**

It would be desirable to have additional markers to follow the transmission of the PI3K signal further downstream. We examined if the phosphorylation of eIF4G is dependent on PI3K activity. Western blot and immunohistochemical analyses with an antibody against the phosphorylated serine 1108 revealed a strong elevation of phosphorylation in the prostates of transgenic animals compared with age-matched wild-type mice (Figure 3A and C, respectively). The quantification of this epithelial-specific signal is shown in Figure 3B. The difference between the transgenic and respective wild-type prostate was found to be significant.

As shown in Figure 4, the phosphorylation level of eIF4G in the epithelial compartment of the prostate was significantly diminished 24 h after intra-peritoneal injection of LY294002. These results confirm that phosphorylation of eIF4G is dependent on PI3K activity.
Expression of Mst1 and RanBP2 is activated by PI3K

In a recent study, we had identified novel target genes of the platelet-derived growth factor (PDGF) receptor-beta pathway (12). The activation of the PDGF receptor-beta leads to the activation of the PI3K pathway (reviewed in ref. 13). We used the list of PDGF targets to look for genes activated by the PI3K-signaling cascade. We wanted to select proteins with different activities. With its kinase activity, Mst1 may be a candidate member of the PI3K phosphorylation cascade. RanBP2 has been described to be implicated in protein sumoylation and nuclear transport, e.g. in the transport of the growth factor receptor ErbB2 into the nucleus. By choosing these two factors with very different activities, we hope to identify markers of different...
levels/branches of the PI3K-signaling cascade. We assessed the expression of Mst1 and RanBP2 in total cell lysates of three whole prostates each of transgenic mice and age-matched wild-type control mice by western blot experiments (Figure 3A). The measurement of alpha-tubulin expression was used to control the comparable loading of each lane, as well as to correct the quantified signals for AKT-P, eIF4G-P, Mst1 and RanBP2. For each marker, the signals obtained in the wild-type and in the transgenic prostate lysates were compared. The significance of each comparison was calculated by the Mann–Whitney test. The mean value of all four markers was higher in the transgenic situation than in the wild-type control; with a P-value of 0.05 for all four markers, this difference was significant. The immunohistochemical analysis with specific antibodies against Mst1 and RanBP2 confirmed the increase of the levels of both proteins in the epithelium of the prostates of transgenic mice in comparison with age-matched wild-type mice (Figure 3C).

These elevated protein levels could be lowered in vivo by intra-peritoneal injection of LY294002 (Figure 4). The insert of each panel of Figure 4A shows in higher magnification that the strong signal of the untreated transgenic prostate tissue is located to the epithelium. The values of the immunohistochemical images of LY294002-treated prostates were compared with the values of the respective control images (transgenic prostates treated with the solvent only). As displayed in the graph of Figure 4B, the phosphorylation of eIF4G and the expression of Mst1 and RanBP2 were decreased to 33, 88 and 71%, respectively, of the control levels (P = 0.004 in all three cases).

**Fig. 4.** The intra-peritoneal injection of the PI3K-specific inhibitor LY294002 reduces the elevated levels of P-eIF4G, Mst1 and RanBP2 in the prostates of transgenic animals after 24 h. (A) Representative photomicrographs are shown. Original magnification, ×100. The inserts display the staining of the epithelium in higher magnification (×400); the lumen of the gland is located in the right lower corner of each insert. (B) The staining in the cytoplasm of epithelial cells was quantified using the NIH ImageJ software package. The difference between LY294002-treated and control tissue was found to be significant for phosphorylated eIF4G, Mst1 and RanBP2 (P-value = 0.004 in all cases, Mann–Whitney test). Transgenic and wild-type mice were 9 weeks old.

**Discussion**

Regulatory changes in the PI3K/AKT pathway are common in cancer. PI3K itself, its antagonist PTEN, and the target AKT are frequently changed genetically or epigenetically (14). Abnormalities commonly occur in related molecules and pathways, too. Growth factors and their receptors—e.g. PDGF-beta (c-sis)—that use the PI3K pathway have been found to be involved in tumor development. Because of the prominent role of the PI3K pathway in cancer, drugs against different members of this signaling cascade have been developed (14). Whether one of these drugs succeeds or fails in a particular patient depends not least in the correct assessment of the molecular constitution of the tumor to be treated. Therefore, assays and marker molecules are needed to determine which pathway is altered in a particular tumor and/or to follow the effects of a therapeutic treatment. Because of
the variety of downstream effectors and interconnected pathways regulated by PI3K, markers that indicate at which level the complex network is activated would be helpful. The purpose of the study presented here is the investigation of the PI3K pathway in the epithelial compartment of specific tissues. We choose the MMTV promoter to target the expression of p110-alpha, the catalytic subunit of PI3K, to epithelial cells. To ensure the localization to the membrane, we fused the myristoylation sequence of the src kinase to the N-terminus of p110-alpha. It has been shown that targeting the catalytic subunit of PI3K to the cell membrane by fusing a myristoylation sequence to its N-terminus is sufficient to ensure its constitutive enzymatic activity (15). We identified a mouse line in which the transgene has been integrated into the Y chromosome. While no tumor had been observed up to now due to the young age of these mice, immunohistochemical analysis confirmed the elevated expression of the p110-alpha protein and the increased phosphorylation of the PI3K target AKT in various organs, mainly of the reproductive system. The highest AKT phosphorylation could be detected in the testis and the prostate. We also could show that this activity—but not the higher expression of p110-alpha—can be reversed in vivo by the PI3K-specific inhibitor LY294002. Therefore, we used this model to investigate the consequences of the constitutive activation of the PI3K pathway and to search for downstream markers for its accurate assessment. PI3K stimulates protein synthesis via an mTOR-dependent mechanism. The translation factor eIF4G is involved in the recruitment of the translational machinery to the 5'-end of mRNA, a key regulatory step in protein synthesis. The C-terminal region of eIF4G contains serum-stimulated phosphorylation sites, including Ser1108, Ser1148 and

Fig. 5. Human prostate microarray experiments. Exemplary photomicrographs of the immunohistochemical analysis of human prostate biopsies of different disease stages are shown. For phosphorylated AKT and eIF4G, stainings of two non-tumoral control tissues are depicted, representing the maximal variations occurring with these markers. An immunostaining for p63 was performed to check for the presence of basal cells in non-tumoral tissues and PIN lesions, and for their absence in adenocarcinomas. Original magnification, \( \times 100 \).
Table I. Immunohistochemical analysis of a human prostate TMA

<table>
<thead>
<tr>
<th>Patient</th>
<th>Pathology</th>
<th>P-AKT</th>
<th>P-eIF4G</th>
<th>Mst1</th>
<th>RanBP2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Non-tumoral tissue</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>Non-tumoral tissue</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Non-tumoral tissue</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Non-tumoral tissue</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>Non-tumoral tissue</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>Nr</td>
</tr>
<tr>
<td>5</td>
<td>Non-tumoral tissue</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>Non-tumoral tissue</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>Non-tumoral tissue</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>Non-tumoral tissue</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>Non-tumoral tissue</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>Non-tumoral tissue</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>Non-tumoral tissue</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>Non-tumoral tissue</td>
<td>2</td>
<td>1</td>
<td>Nr</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
<td>Non-tumoral tissue</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>Non-tumoral tissue</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>Non-tumoral tissue</td>
<td>2</td>
<td>1</td>
<td>Nr</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>Non-tumoral tissue</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Non-tumoral tissue versus PIN</td>
<td>0.119</td>
<td>0.381</td>
<td>0.017</td>
<td>0.113</td>
<td></td>
</tr>
<tr>
<td>Non-tumoral tissue versus adenocarcinoma</td>
<td>0.081</td>
<td>0.024</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>PIN versus adenocarcinoma</td>
<td>—</td>
<td>0.225</td>
<td>0.304</td>
<td>0.069</td>
<td></td>
</tr>
</tbody>
</table>

0, No cytoplasmatic staining (0–5% of positive cells). 1, low cytoplasmatic staining (5–50% of positive cells). 2, high cytoplasmatic staining (>50% of positive cells). Nr, tissue section is not representative for the respective lesion. In the lower part of the table (last three rows), the unilateral exact significance (exact statistics of Fisher and Chi-square tests) is indicated for each marker in all tissue combinations. For this statistical evaluation, a staining (values 1 and 2) has been set 'yes' and no staining (value 0), 'no'.

Ser1192 (16). We investigated whether an antibody specific for the phosphorylation at serine 1108 can be used as a marker for PI3K activity. We could prove that the phosphorylation of eIF4G is elevated in the prostates of transgenic mice, and that this phosphorylation can be inhibited by LY294002. To our knowledge, this is the first time that the correlation of PI3K activation and phosphorylation of eIF4G could be demonstrated in vivo. To identify markers that are located more downstream, we looked for transcriptional targets of PI3K activation. Recently, we had identified a panel of 147 genes that are transcriptionally activated by the PDGF-beta (12). Since the binding of PDGF-beta to its receptor leads to activation of the PI3K pathway (13), we checked two of those proteins, Mst1 and RanBP2, for their expression in transgenic mouse prostates. We demonstrated an elevated expression, which could be inhibited in vivo by LY294002. To our knowledge, both proteins have not been shown before to be regulated by the PI3K pathway. Mst1 is a 487 amino acid Group II GC (protein ser/thr) kinase. It has been shown to be activated by severe stress in cell culture and in chick embryo fibroblasts transformed by v-Src (17). RanBP2, also known as Nup358, is the largest nucleoporin characterized so far (18). Beside its function as a docking factor in nuclear transport, RanBP2 serves as an E3 ligase for sumoylation (19). The discovery that RanBP2 has SUMO E3 ligase activity suggests that SUMO modification may occur at nuclear pore complexes as proteins are transported between nucleus and cytoplasm. Sumoylation can have important functional consequences, particularly in regulating gene activity. Interestingly, RanBP2 has been found to be involved in the transport of the cell membrane receptor ErbB2 into the nucleus (20). Since ErbB2 activates the PI3K-signaling pathway, our finding of the transcriptional activation of RanBP2 by PI3K suggests that ErbB2 might trigger its own localization to the nucleus by a PI3K-dependent mechanism. The experiments in the mouse model suggested that the phosphorylation of AKT and eIF4G and the expression of Mst1 and RanBP2 could be used as markers for PI3K activity and the diagnosis of neoplastic and tumoral tissue. To explore this further, we performed immunohistochemistry on a TMA of human prostate biopsies. The tissue sample subsets recognized by the markers overlap but are not identical, suggesting that these markers might be useful to define more accurate the specific pathway activated in each sample. For more than a decade, prostate-specific antigen (PSA) has been the most reliable marker for prostate cancer and is used as a marker to assess the response to cancer therapies. However, in androgen-independent prostate cancer, regulation of PSA is complicated, i.e. PSA levels do not often correlate with the grade of the disease (21). Therefore, additional markers are needed. The proposal that molecules of the PI3K pathway are useful for this purpose are supported by recent data showing that the PI3K/AKT pathway plays a crucial role in the negative regulation of PSA in prostate cancer cells (22). Since anti-apoptotic survival signals induced by several receptors are mediated by PI3K, this pathway may decisively contribute to drug resistance of tumor cells. Therefore, this pathway has become an attractive target for the development of novel drugs supporting conventional chemotherapy. The markers we describe here may serve not only for the diagnosis of tumor samples but additionally for the identification and characterization of potential drug candidates.
Acknowledgements

We acknowledge the Tumor Bank of the Spanish National Cancer Centre (CNIO) Tumour Bank for providing us the prostate tumor samples. This work has been supported by grants from Spanish Ministry of Health (FIS-02/0126), Fundación Mutua Madrileña and the Spanish Ministry of Education and Science (SAF2005-00944).

Conflict of Interest Statement: None declared.

References


Received December 15, 2006; revised March 6, 2007; accepted March 9, 2007