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ORIGINAL ARTICLE

The Gene Expression Status of the PI3K/AKT/mTOR Pathway in Gastric Cancer Tissues and Cell Lines

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Abstract The PI3K/AKT/mTOR pathway plays a crucial role in the regulation of multiple cellular functions including cell growth, proliferation, metabolism and angiogenesis. Emerging evidence has shown that deregulation of this pathway has a role promoting gastric cancer (GC). The aim was to assess the expression of genes involved in this pathway by qPCR in 23 tumor and 23 non-tumor gastric mucosa samples from advanced GC patients, and in AGS, MKN28 and MKN45 gastric cancer cell lines. Results showed a slight overexpression of *PIK3CA*, *PIK3CB*, *AKT1*, *MTOR*, *RPS6KB1*, *EIF4EBP1* and *EIF4E* genes, and a slightly decreased *PTEN* and *TSC1* expression. In AGS, MKN28 and MKN45 cells a significant gene overexpression of *PIK3CA*, *PIK3CB*, *AKT1*, *MTOR*, *RPS6KB1* and *EIF4E*, and a significant repression of *PTEN* gene expression were observed. Immunoblotting showed that PI3K-β, AKT, p-AKT, PTEN, mTOR, p-mTOR, P70S6K1, p-P70S6K1, 4E-BP1, p-4E-BP1, eIF4E and p-eIF4E proteins were present in cell lines at different levels, confirming activation of this pathway in vitro. This is the first time this extensive panel of 9 genes within PI3K/AKT/mTOR pathway has been studied in GC to clarify the biological role of this pathway in GC and develop new strategies for this malignancy.

Keywords PI3K/AKT/mTOR pathway · Gastric cancer · AGS, MKN28 and MKN45 cell lines

Background

Gastric cancer (GC) is a high priority health problem worldwide. Globally, GC is currently the fifth most frequently diagnosed cancer and the third most lethal malignancy [1]. Every year, almost one million new cases of GC are diagnosed and more than 700,000 people die of this disease, thereby representing 10 % of the world's cancer deaths [1]. The most affected populations are those from Eastern Europe, Asia and Central and South America [2]. The main morphological prognostic factors are the level of infiltration of the tumor in the wall of the stomach (T stage) and lymph

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node involvement (N stage) [3, 4]. In 2014, researchers from The Cancer Genome Atlas (TCGA) research network studied 295 stomach tumors and found that 80 % house different grades of mutations in the *PIK3CA* gene and amplifications of receptors of tyrosine kinases (RTKs) genes such as *ERBB3*, *ERBB2* and *EGFR* that trigger increased activity of their proteins in GC [5]. Therefore, class-IA PI3Ks as PI3Kα and PI3Kβ and their downstream pathways have recovered the interest of researcher in GC, especially in the search for inhibitors of these targets. Moreover, advances in signaling have revealed that the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of the rapamycin (mTOR) signaling pathway plays a crucial role in mediating multiple cellular functions including cell growth, proliferation, metabolism, survival and angiogenesis (Fig. 1) [6]. RTKs (ErbB1/EGFR, ErbB2/HER2 and ErbB3) can mediate the activation of class-IA PI3Ks [7]. PI3Kα or PI3Kβ can induce activation of AKT by phosphorylation. Activated AKT (p-AKT) – phosphorylated at either threonine 308 or serine 473 – can activate mTOR complex 1 (mTORC1) mainly at serine 2448 of mTOR. In addition, p-AKT can phosphorylate TSC2 – one of the two members of tuberous sclerosis complex (TSC1/TSC2) – destabilizing this complex and leading to increased mTORC1 activity. The mTORC1 complex activates P70S6K1 by phosphorylation at threonines 229 and 389, and inactivates 4E-BP1 also by phosphorylation. This inactivation of 4E-BP1 leads to a release of the transcription factor eIF4E that triggers transcription of multiple oncogenes such as *CMYC* and *CCND1*, among others [8–10].

In cancer, there is an increase in the activity of PI3K-AKT axis, and TSC1/TSC2 is inhibited by AKT, allowing mTORC1 activation and subsequently activation of P70S6K1 and EIF4e. However, the negative feedback between P70S6K1 and mTOR complex 2 (mTORC2) decreases during carcinogenesis [9]. All these changes result in uncontrolled and increased mitochondrial processes, angiogenesis and ribosome biogenesis for greater protein synthesis, cell growth, proliferation and autophagy [10–12].

Because of the limited number of studies in GC addressing more than three target genes within the canonical PI3K/AKT/mTOR signaling pathway, we aimed to determine the gene expression levels of a panel of 9 targets within this important pathway (*PIK3CA*,

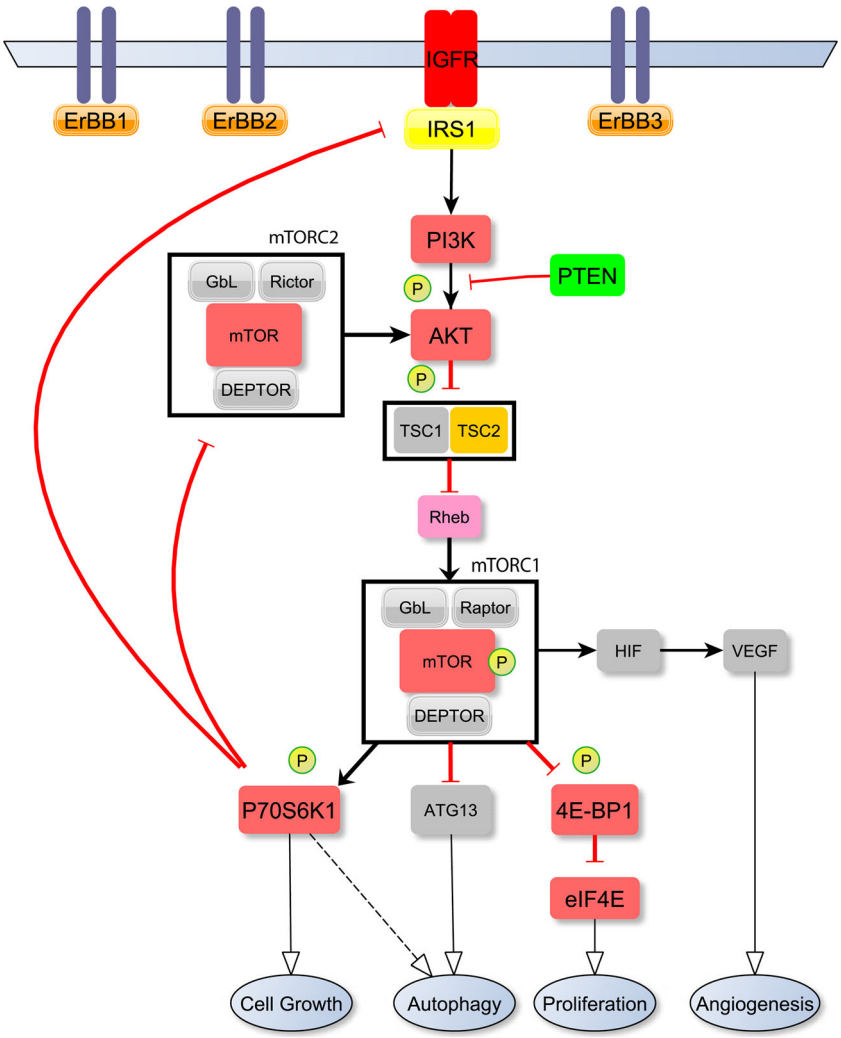

Fig. 1 The canonical PI3K/AKT/mTOR signaling pathway

PIK3CB, *PTEN*, *AKT1*, *TSC1*, *MTOR*, *RPS6KB1*, *EIF4EBP1* and *EIF4E*) by real-time PCR (qPCR) in GC tissues, gastric non-tumor tissues and in AGS, MKN28 and MKN45 cell lines. This approach is useful to ascertain, on a transcriptional level, how the PI3K/AKT/mTOR signaling pathway behaves in order to obtain a complete landscape of potential therapeutic targets within this pathway and propose new models – with activation of components from this pathway – for future analyses in GC.

Materials and Methods

Gastric Tissues and Gastric Cancer Cell Lines

A total of 46 gastric tissues were used in this study corresponding to 23 matched tumor samples from patients with advanced gastric adenocarcinoma and 23 non-tumor gastric samples from the same patients. All gastric samples consisted of frozen tissues collected with RNAlater storage reagent (Lifetechnologies, USA) at the time of surgery through a strict tissue collection protocol approved by the Pontificia Universidad Católica de Chile.

Gene and protein expression were evaluated in AGS, MKN28 and MKN45 cell lines. These three cell lines were grown in RPMI 1640 medium (Thermo Scientific Hyclone, USA) supplemented with 10 % fetal bovine serum (FBS), 10 units/ml penicillin and 10 mg/ml streptomycin (1 % P/S) (Thermo Scientific Hyclone, USA). They were incubated at 37 °C in a humidified atmosphere containing 5 % CO_2, and were subcultured during the logarithmic phase.

RNA Purification and Quantification of mRNAs by Real-Time PCR

Total RNA was extracted from gastric tissues and cell lines using the TRIzol Reagent (Lifetechnologies, USA) according to the manufacturer's protocol. RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). RNA was reverse-transcribed with random primers at 42 °C for 50 min using M-MLV reverse transcriptase 200 U/μl (Promega, USA). The newly synthesized cDNA was subsequently amplified by PCR using the Brilliant II Ultra-Fast SYBR® Green qPCR Master Mix according to the manufacturer's recommendation in a Stratagene Mx-3000P Real-Time PCR System (Agilent Technologies, USA). Relative fold levels were determined using the $2^{-\Delta\Delta CT}$ method, with *GAPDH* and *ACTB* genes being used as normalizer controls. The primer sequences of the PI3K/AKT/mTOR genes used are described in Table 1. Primers were tested to determine their optimal concentrations for PCR analysis and the resulting products were run on 2 % agarose gel to confirm the appropriate size. Efficiency of the real-time PCR reaction was calculated from standard curves (data not shown).

Table 1 Primer sequences of PI3K/AKT/mTOR pathway genes

Gene	Sequence 5′- 3	Fragment size (pb)
PIK3CA	Fw: GGTTGTCTGTCAATCGGTGACTGT	108
	Rv: GAACTGCAGTGCACCTTTCAAGC	
PIK3CB	Fw: TTGTCTGTCACACTTCTGTAGTT	166
	Rv: AACAGTTCCCATTGGATTCAACA	
PTEN	Fw: GGTTGCCACAAAGTGCCTCGTTTA	129
	Rv: CAGGTAGAAGGCAACTCTGCCAAA	
AKT1	Fw: TTCTGCAGCTATGCGCAATGTG	181
	Rv: TGGCCAGCATACCATAGTGAGGTT	
MTOR	Fw: GCTTGATTTGGTTCCCAGGACAGT	194
	Rv: GTGCTGAGTTTGCTGTACCCATGT	
EIF4EBP1	Fw: GCATCAGCTTTTAGGTGCAAAGGA	188
	Rv: GGCAACTGCCAAAAGTGATTCAGC	
EIF4E	Fw: GAACGAACCCTTCCTTCCGAATGA	143
	Rv: AGGGCGAAGGTGGCTTTTATTTCC	
RPS6KB1	Fw: ACTGTAGTGTTGACTGCCTGACCA	105
	Rv: TAGCCAGCCAATCACAGTGCTCAT	
TSC1	Fw: GCAGCGTGACACTATGGTAACCAA	144
	Rv: AGTTCTATCCGCAGCTCCGCAAT	
GAPDH	Fw: TGCACCACCAACTGCTTAGC	130
	Rv: GGCATGGACTGTGGTCATGAG	
ACTB	Fw: GACAGGATGCAGAAGGAGATTACT	117
	Rv: TGATCCACATCTGCTGGAAGGT	

Rapamycin Treatment

For in vitro assays on AGS cells, rapamycin (LC Laboratories, USA) was dissolved initially in DMSO as a stock solution and stored at −20 °C. Then rapamycin was diluted in culture medium before each in vitro replicate at a 50 nM and 0.1 % DMSO in culture medium was used as a vehicle control. Treatment was performed for 24 h.

Western Blot Analyses

AGS, MKN28 and MKN45 cells were lysed using RIPA buffer (50 mM Tris, pH 7.2; 150 mM NaCl; 1% Triton X-100; and 0.1 % SDS) containing protease (1:100, Roche, USA) and phosphatase (1:100, Sigma-Aldrich, USA) inhibitors. Protein concentrations were determined by a bicinchoninic acid assay (Pierce, Thermo Scientific, USA). Sixty micrograms of proteins were separated by SDS-PAGE and transferred (Bio-Rad, USA) to PVDF membranes (Millipore, USA). Protein expressions were quantified through the use of rabbit polyclonal antibodies against PI3K-β, AKT, p-AKT, PTEN, mTOR, p-mTOR, P70S6K1, p-P70S6K1, 4E-BP1, p-4E-BP1, eIF4E and p-eIF4E were diluted to 1:1000 in TBST-1% BSA solution (Cell Signaling Technologies, Danvers, MA, USA). The expressions of these proteins were standardized to human α-actin using a mouse polyclonal anti-α-actin antibody (1:5000, Millipore, USA). Primary antibodies were detected using goat anti-rabbit or goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000, Santa Cruz Biotechnology, USA). Immunoreactive bands were visualized by Western Lightning Chemiluminescence Reagent Plus (PerkinElmer, USA) according to the manufacturer's instructions.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software Inc., USA). Wilcoxon signed-rank was used for analyzing gene expression from paired tumor and non-tumor gastric tissues. Means of gene expression in GC cell lines were compared using a two-way ANOVA. Each experiment was repeated at least three times and performed in triplicate. A *P*-value of 0.05 was considered statistically significant.

Ethics Statement

The Institutional Review Board of the School of Medicine of Pontificia Universidad Católica approved this study and issued a waiver authorizing the use of archival material without informed consent for samples more than two years old, preserving patient anonymity.

Results

Gene Expression of PI3K/AKT/mTOR Pathway in Gastric Tissues

Gene expression analysis of key regulator genes within the PI3K/AKT/mTOR pathway (*PIK3CA*, *PIK3CB*, *PTEN*, *AKT1*, *TSC1*, *MTOR*, *RPS6KB1*, *EIF4EBP1* and *EIF4E*) showed that there is a slight tendency to overexpression for most of the analyzed genes, with the exception of *PTEN* and *TSC1*, which are repressed in most of the study tissues. However, the difference in gene expression relative to the corresponding normal tissues was very slight in all genes. None of them reached statistical difference (*P* = NS for all) and fold change values of tumor tissues were

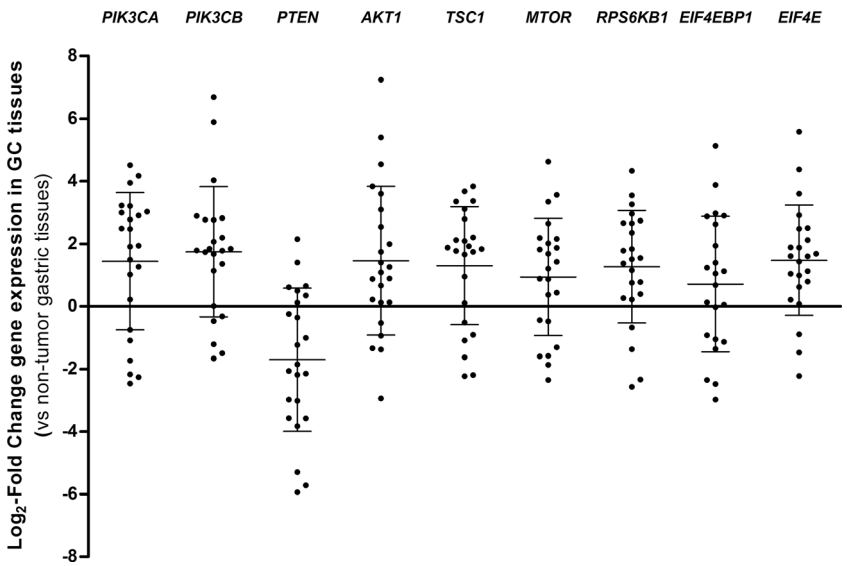

Fig. 2 Gene expression of the PI3K/AKT/mTOR pathway in tumor samples. Each gastric tumor sample was calibrated against its corresponding non-tumor gastric tissue and was processed in triplicate. The 0 represents the gene expression of non-tumor gastric tissues. Bars represent the mean ± SD of all cases. The Wilcoxon signed-rank test was used. (**P* < 0.05; ***P* < 0.01; ****P* < 0.001)

solely between −1 and 1 times of expression relative to non-tumor tissues (Fig. 2).

The gene expression of the PI3K/AKT/mTOR pathway in this study was compared to the protein expression of this signaling pathway observed in a different cohort of formalin-fixed paraffin-embedded tissue samples described in a previous study conducted by our group [13]. The immunohistochemical analyses performed on tissue microarrays (TMAs) of 71 gastric adenocarcinoma samples and 71 paired non-tumor gastric mucosa samples from patients with advanced GC showed statistically significant differences in 10 of 12 proteins investigated (Fig 3). High protein expression levels were found in tumor tissue versus non-tumor tissue for PI3K ($p = 0.001$), AKT ($p = 0.01$), p-AKT ($p = 0.03$), p-mTOR ($p < 0.001$), P70S6K1 ($p = 0.002$), p-P70S6K1 ($p = 0.01$), p-4E-BP1 ($p < 0.001$), eIF4E ($p < 0.001$), and p-eIF4E ($p < 0.001$). Conversely, PTEN was found to be down-regulated in tumor tissues ($p < 0.001$). Neither mTOR nor 4E-BP1 showed statistically significant differences. The comparison between the two different cohorts of gastric tissue samples is important to show the consistency of data about the activation of PI3K/AKT/mTOR pathway in GC given the very similar pattern of expression either in mRNA or protein.

Gene Expression of PI3K/AKT/mTOR Pathway in AGS, MKN28 and MKN45 Cell Lines

The gene expression analysis of the PI3K/AKT/mTOR pathway showed that *PIK3CA*, *PIK3CB*, *AKT1*, *MTOR*, *RPS6KB1* and *EIF4E* were significantly overexpressed in all three cell lines (Fig. 4). Conversely, the tumor suppressor gene *PTEN* exhibited significantly suppressed expression in the three cell lines. For this analysis, the expression values of the cell lines were normalized using expression values obtained from a pool of total RNA from non-tumor gastric tissues. Moreover, the *TSC1* gene only showed significant overexpression in the MKN28 cell line ($P = 0.025$), and the *EIF4EBP1* gene

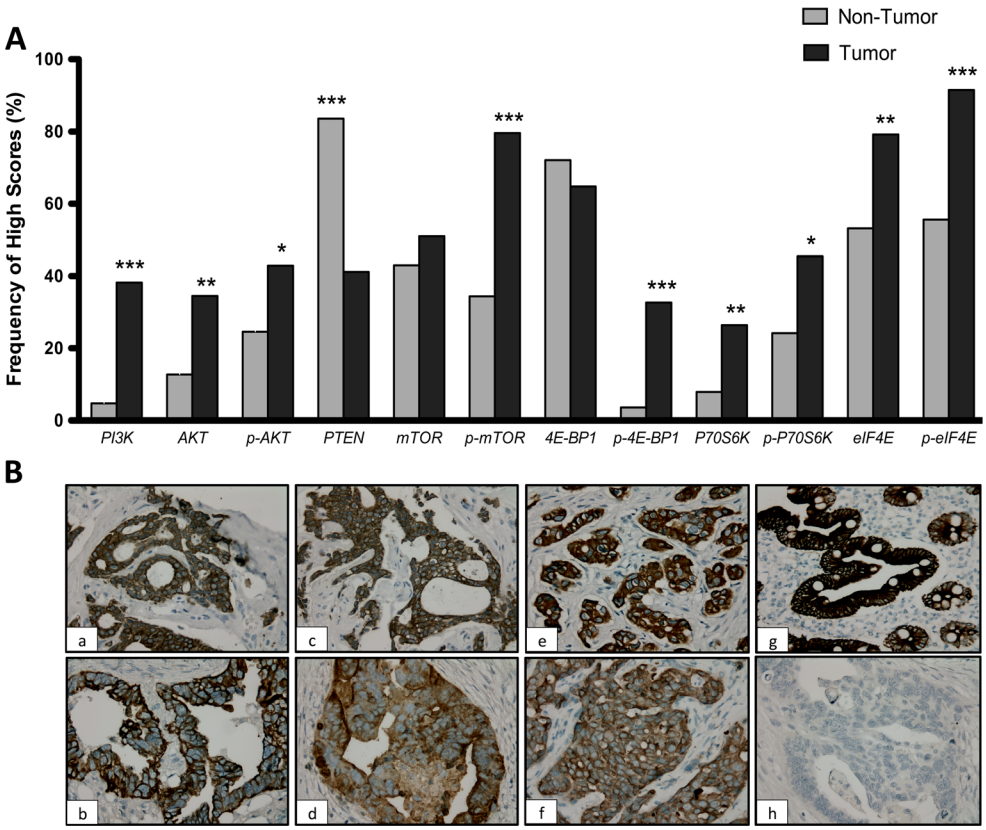

Fig. 3 Immunohistochemical expression of the PI3K/AKT/mTOR pathway in gastric tumor and non-tumor samples. **a** Frequency of cases with high staining scores of proteins of the PI3K/AKT/mTOR pathway in non-tumor and tumor tissues. Each bar indicates the percentage of high scoring cases per tissue type according to criteria. Immunohistochemistry was evaluated using a semiquantitative scoring system: staining intensity was scored as 1 (negative), 2 (weak), 3 (moderate), and 4 (intense). The percentage of positive cells was quantified as 0 (none), 1 (1–25%), 2 (26–50%), 3 (51–75%), and 4 (76–100%). For statistical analysis, the sum score of intensity and extent of staining was grouped into low (final score, 0 to 4) or high (final score, 5 to 7). The TMA slides were examined independently by two pathologists (OT and JCR) who were blinded to both the clinical and pathological data. The associations between immunohistochemical expression and tumor/non-tumor condition were examined using chi-square test and Fisher's exact test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). **b** Examples of immunohistochemical expression of proteins of the PI3K/AKT/mTOR pathway in gastric cancer. *a* PI3K in tumor, *b* p-P70S6K1 in tumor, *c* p-eIF4E in tumor, *d* p-mTOR in tumor, *e* p-4E-BP1 in tumor, *f* AKT in tumor, *g* PTEN in non-tumor tissue, *h* PTEN in tumor (×40)

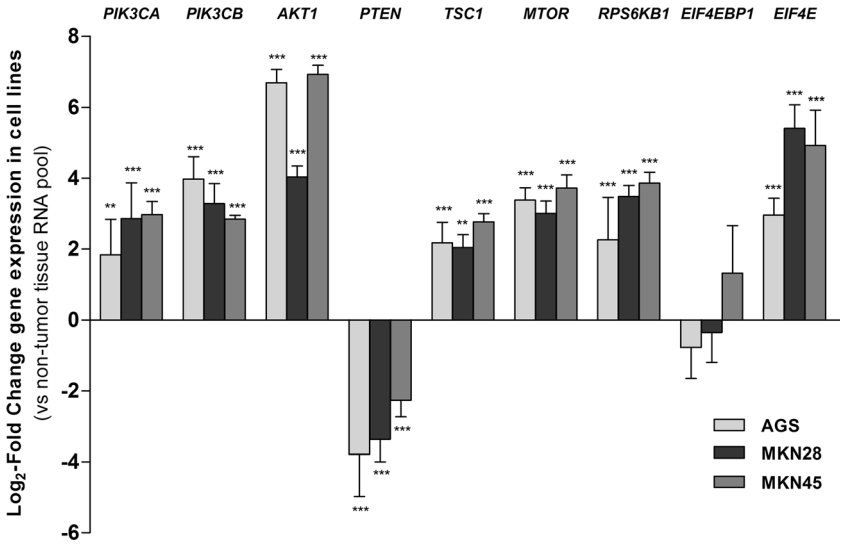

Fig. 4 Gene expression of the PI3K/AKT/mTOR pathway in AGS, MKN28 and MKN45 gastric cancer cell lines. Each cell line was processed in biological and technical triplicate. Calibration was done with a pool of non-tumor gastric tissues. The 0 represents the gene expression of non-tumor gastric tissues. The bars represent the mean ± SD of gene expression. Two-way ANOVA was used. (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$)

showed no statistically significant expression values in any cell line (P = NS, for all three cell lines).

Protein Expression of PI3K/AKT/mTOR Pathway in AGS, MKN28 and MKN45 Cell Lines

Protein expression analysis in AGS, MKN28 and MKN45 cell lines performed by western blot technique showed that all target proteins were expressed in the three studied cell lines (Fig. 5). This expression pattern is consistent with what was found in gene expression of PI3K/AKT/mTOR in these GC cell lines. As protein expression was more intense in AGS cells, this cell line was treated with 50 nM rapamycin for 24 h and western blots were performed for total and phosphorylated proteins of mTOR, P70S6K1, 4E-BP1 and eIF4E. Results showed that rapamycin treatment did not induce a significant change in the protein expression of total mTOR, P70S6K1, 4E-BP1 and eIF4E. However, rapamycin induced a significant decrease in the phosphorylation of both mTOR and its downstream effectors, which coincides with a decrease in activity attributed to mTORC1 over their targets P70S6K1 and 4E-BP1. Finally, a decreased phosphorylation 4E-BP1 causes more retention and, therefore, less activation of eIF4E (Fig. 6).

Discussion

Multiple signaling pathways have been linked to the development of gastric cancer. Among them, the PI3K/AKT/mTOR pathway is critical in the regulation of many cellular functions, mainly the translation of those proteins that lead to cell growth and proliferation, and to the decrease of negative regulator translation of cycle cell progression [14–17]. When cells are in a carcinogenic state, an increase in PI3K-AKT signaling, mTORC1 activation and a decreased feedback activity between P70S6K1 and mTORC2 are observed. All these events induce uncontrolled processes of increased metabolism, increased angiogenesis and increased ribosome biogenesis for an altered protein synthesis which leads to cell growth, proliferation, autophagy, among others, that help trigger cancer formation [8, 18–20].

Previous studies involving the main targets of the PI3K/AKT/mTOR pathway protein expression levels measured by immunohistochemistry (IHC) in both gastric cancer tissues and non-tumor gastric tissues have shown that levels of PI3K [21, 22], AKT [22], p-AKT [23], mTOR [18], p-mTOR [24–26], P70S6K1 [13], p-P70S6K1 [18], 4E-BP1 [27], p-4E-BP1 [26, 27], eIF4E [28–30] and p-EIF4e [28, 29] are in general elevated in tumor samples compared to non-tumor samples. Conversely, PTEN protein levels have been found to be lower in gastric cancer [13, 31, 32]. These results indicate that the PI3K/AKT/mTOR pathway is activated in this malignancy. This activation has been associated with clinical pathological features such as metastasis (PI3K) [21], lower survival (p-AKT) [23], pathogenesis and progression (mTOR and p-P70S6K1) [18, 24], among others. Moreover, our previous immunohistochemical study performed in a different cohort consisting in paired gastric tissues (tumor and non-tumor) from 71 patients with advanced GC supports the idea of a strong activation of canonical PI3K/AKT/mTOR pathway in GC at a protein level. The importance of the present study is that provides the first evidence of the activation in the complete canonical PI3K/AKT/mTOR pathway in GC at a transcriptional level.

Results of this work show a slight overexpression of *PIK3CA*, *PIK3CB*, *AKT1*, *MTOR*, *RPS6KB1*, *EIF4EBP1* and *EIF4E* genes, and a slightly decreased expression of *PTEN* and *TSC1*. These results are consistent with previously mentioned studies; however, unlike what was found in most immunohistochemistry studies, differences in gene expression between tumor and non-tumor tissue were not statistically

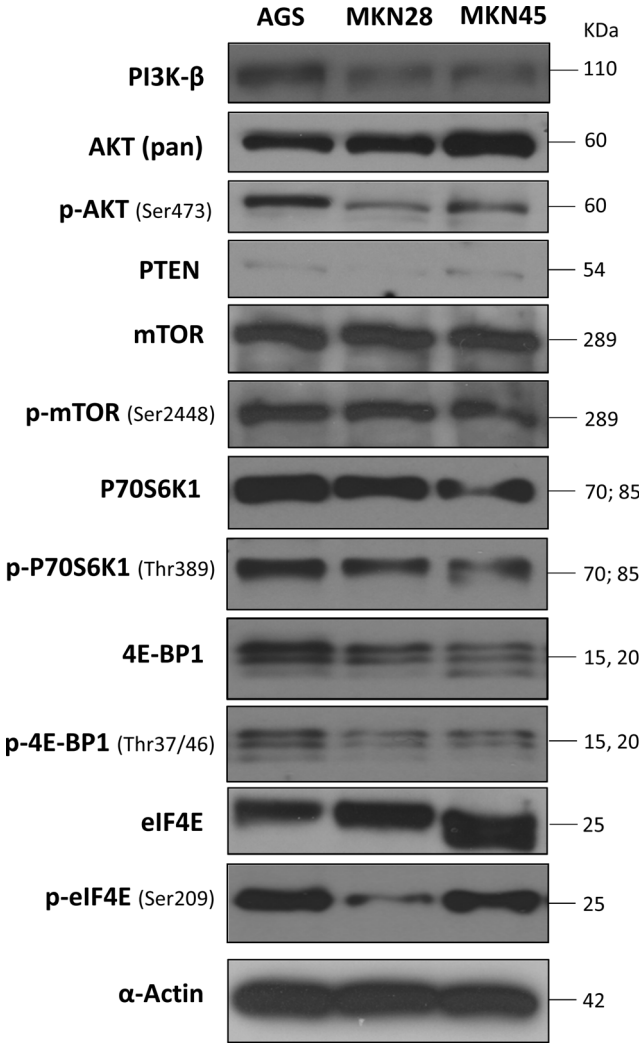

Fig. 5 Protein expression of the PI3K/AKT/mTOR pathway in AGS, MKN28 and MKN45 gastric cancer lines. Total protein (60 μg) of each cell line was processed by immunoblotting using antibodies against PI3K-β, AKT, p-AKT, mTOR, p-mTOR, P70S6K1, p-P70S6K1, 4E-BP1, p-4E-BP1, eIF4E and p-eIF4E. Protein loading was normalized with α-actin

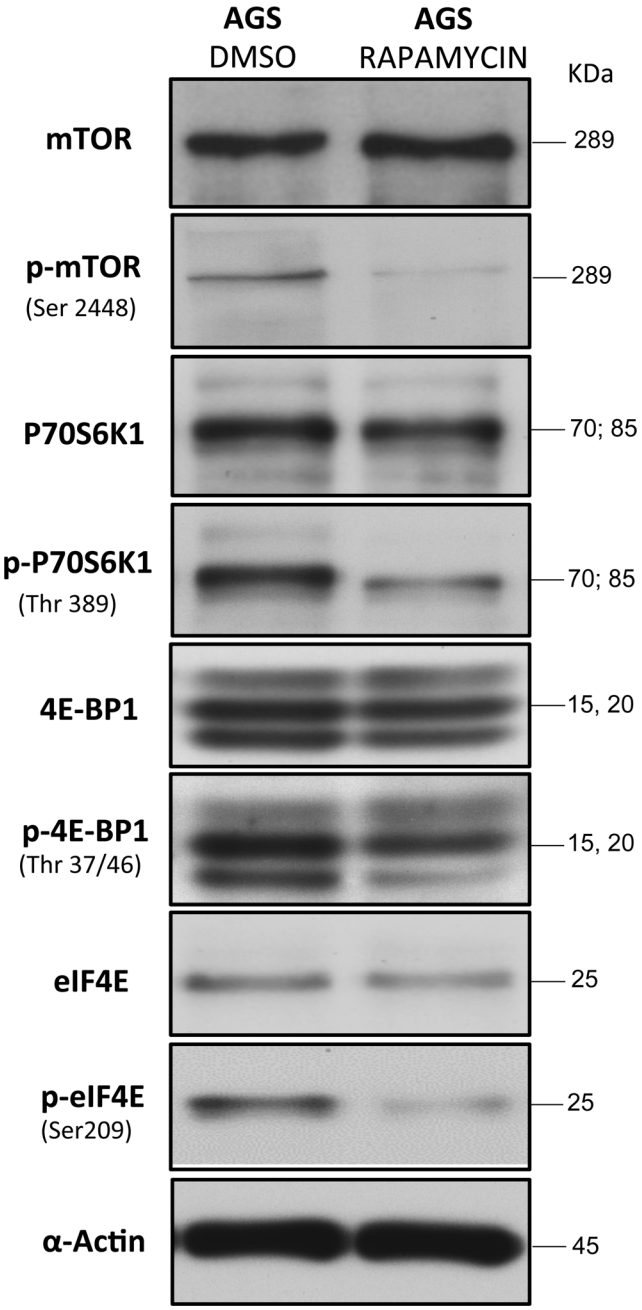

Fig. 6 Protein expression of total and phosphorylated proteins of mTORC1 downstream regulators in AGS cells with and without rapamycin treatment (50 nM). Total protein (60 μg) of AGS lysates was processed by immunoblotting using antibodies against mTOR, p-mTOR, P70S6K1, p-P70S6K1, 4E-BP1, p-4E-BP1, eIF4E and p-eIF4E. Protein loading was normalized with α-actin

significant (P = NS for all). These non-significant differences in the fold change gene expression between gastric cancer tissues and non-tumor tissues – compared to p-values obtained in immunohistochemical analyses – might be explained, in part, by the quantitative and semi-quantitative nature of the technologies used in these studies and by the presence of post-transcriptional or post-translational regulators that could induce less of a difference in expression between these two types of tissue. Also, it is known that mRNA levels do not always explain the abundance of their respective proteins [33]. Moreover, gastric tumor cells can generally exhibit significant genomic alterations such as loss of heterogeneity (LOH), aneuploidy or other genomic instability, which mask the gene expression values. Nevertheless, we attribute these results mainly to the way in which tumors are processed. When a mass of advanced gastric tumors is processed, not all of the processed cells belong to stomach-epithelium-derived cells, but immune and stromal cells comprising such tumors were also included, causing an overlapping effect in gene expression differences with other cells. Although differences in gene expression between tumor and non-tumor tissues were not significant, these data do not rule out that transcriptionally

there may be a real mRNA overexpression. This conclusion is reinforced with the total protein expression data in publications where IHC was used. Therefore, to solve this issue, it would be very useful to use a laser microdissection system to remove stromal tissue and immune cells in these cases.

Supporting this last idea, the results obtained in AGS, MKN28 and MKN45 cell lines showed significantly high levels of gene expression of *PIK3CA*, *PIK3CB*, *AKT1*, *MTOR*, *RPS6KB1* and *EIF4E*, and significantly low levels of *PTEN*. Interestingly, these results in cell lines are also consistent with results previously found in IHC studies [13] and with western blot results performed in the present study on the same cell lines.

Western blot with AGS, MKN28 and MKN45 cell lysates showed that the study proteins were present in the cell lines, but at different levels. The lowest PTEN levels (the weakest band in blots) and the presence of the phosphorylated portions of mTOR, P70S6K1, 4E-BP1 and eIF4E can confirm that this signaling pathway is also activated on these in vitro models in a manner similar to tumor tissues. Therefore, these in vitro models are easy to obtain because there are no special requirements and they are useful for testing new chemical agents in order to block not only PI3K, AKT and mTOR proteins, but also to test agents against P70S6K1 and eIF4E. Moreover, these are interesting models to test shRNA or miRNA mimics in order to repress the expression of a specific gene within this pathway as was confirmed through the rapamycin treatment. Rapamycin not only blocked the phosphorylation of mTOR but also decreased the phosphorylation levels of downstream effectors as P70S6K1, 4E-BP1 and eIF4E.

In 2014, researchers from TCGA network examined 295 stomach tumors and identified subtypes using complex statistical analyses of molecular data obtained from six molecular analysis platforms. As a result, they were able to describe a new molecular characterization that defines four major genomic subtypes of GC: positive for Epstein Barr virus (EBV), microsatellite instability (MSI), chromosomally instability (CIN) and genomically stable (GS). From these subtypes, at least three of them that cover 80 % of the GC cases studied (EBV-positive, MSI and CIN subgroups) house different grades of mutations in the *PIK3CA* gene and amplifications of RTK genes such as *ERBB3*, *ERBB2* and *EGFR* [5, 34]. Therefore, *PIK3CA* and the downstream pathways involving PI3Kα or another class IA PI3K – activated through RTKs – have recovered the interest of researchers in GC, especially in the search for inhibitors of these targets. Moreover, these alterations have been associated with relevant clinical features such as etiology, gender, age at diagnosis and anatomical location. Taken together, these findings highlight the importance of clarifying the different carcinogenic processes that lead to each subtype, as well as the relevant genes and pathways that may be susceptible to therapeutic targeting.

Results with this panel of targets within the PI3K/AKT/mTOR pathway – 9 genes and 12 proteins, evaluated simultaneously in gastric tumor tissues, in gastric non-tumor tissues and in three GC cell lines – have not been previously published in the literature for this disease. Moreover, the bibliography about gene expression of the PI3K/AKT/mTOR pathway in gastric cancer is scarce, particularly in relation to downstream effectors. This approach can help clarify the biological role of this pathway in GC in order to develop new strategies for this malignancy.

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Compliance with Ethical Standards

Conflict of Interest and Funding Sources Authors report no conflicts of interest.

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