Tissue Transglutaminase Regulates Focal Adhesion Kinase/AKT Activation by Modulating PTEN Expression in Pancreatic Cancer Cells

Amit Verma,1 Sushovan Guha,2 Huamin Wang,3 Jansina Y. Fok,1 Dimpy Koul,4 James Abbruzzese,5 and Kapil Mehta1,6

Abstract

Purpose: Pancreatic ductal adenocarcinoma (PDAC) progresses rapidly and exhibits profound resistance to treatment. We recently reported that a great majority of PDAC tumors and tumor cell lines express elevated levels of tissue transglutaminase (TG2). Here, we provide first evidence that TG2 expression in PDAC cells results in constitutive activation of focal adhesion kinase/AKT by modulating the expression of the tumor suppressor phosphatase PTEN.

Experimental Design: Using PDAC cell lines, we determined the effect of TG2 overexpression on PTEN stability and functions. We confirmed the correlation between TG2 expression and PTEN levels in a few (n = 51) PDAC tumor samples.

Results: We observed that expression of TG2 is inversely correlated with PTEN expression in PDAC cells. Ectopic expression of TG2 inhibited PTEN phosphorylation and promoted its degradation by ubiquitin-proteasomal pathway. Conversely, down-regulation of TG2 by small interfering RNA up-regulated PTEN expression. Clinical relevance of these results was evident in an athymic nude mouse model where down-regulation of endogenous TG2 caused a significant retardation in PDAC xenograft growth. Importantly, the analysis of 51 tumor samples from patients with stage II PDAC revealed that overexpression of TG2 was associated with loss of PTEN expression (P = 0.023; odds ratio, 4.1). In multivariate analysis, TG2-mediated loss of PTEN was a prognostic factor for overall survival in patients with stage II pancreatic ductal carcinoma independent of tumor stage/lymph node status and tumor differentiation (P = 0.01).

Conclusion: TG2 expression in PDAC promotes degradation of PTEN by ubiquitin-proteasomal pathway and results in constitutive activation of focal adhesion kinase/AKT cell survival signaling.

Approximately 33,000 new cases of pancreatic ductal adenocarcinoma (PDAC) are diagnosed each year in the United States and nearly same number of patients die from the disease (1). At the time of diagnosis, most patients have disseminated disease and exhibit marked resistance to radiation and chemotherapy, leaving surgical resection the only hope for cure. Unfortunately, the disease is so aggressive that only 10% to 20% patients with the disease are surgically resectable. Therefore, understanding the molecular factors that contribute to the development of intrinsic resistance of PDAC to chemotherapy could yield better treatment options.

The observation of increased tissue transglutaminase (TG2) expression in drug-resistant and metastatic cancer cells (2) has kindled great interest in understanding the contributions of this protein in carcinogenesis. TG2 is a unique multifunctional protein that, in addition to catalyzing calcium-dependent post-translational modification of proteins, can also catalyze calcium-independent protein disulfide isomerase, GTP/ATP hydrolase, and serine/threonine kinase functions (3–8). Recently, we reported that overexpression of TG2 in PDAC is associated with resistance to gemcitabine (9). Increased expression of TG2 in breast and pancreatic cancer cells led to constitutive activation of the nuclear factor-κB and conferred resistance to drugs (10, 11). Accordingly, inhibition of TG2 activity in glioblastoma tumors sensitized them to N-nitrosourea chemotherapy (12). We also observed that expression of TG2 in PDAC cells results in constitutive activation of focal adhesion kinase (FAK) and its downstream phosphatidylinositol 3-kinase (PI3K)/AKT pathway (9). However, the link between PI3K/AKT activation and TG2 expression was not elucidated. Because the tumor suppressor PTEN (phosphatase and tensin homologue deleted on chromosome 10, also called MMAC1 or TEP1) is a negative regulator of FAK and PI3K/AKT signaling pathways (13, 14), we reasoned that TG2 might affect FAK/AKT activation by regulating PTEN expression or function.
The results reported here show a novel role for TG2 in regulating PTEN expression. TG2 associates with PTEN and inhibits its phosphorylation at Ser380, the site that is known to affect the stability of PTEN. TG2 promoted the ubiquitination of PTEN and its degradation by proteasomal pathway. Down-regulation of TG2 significantly decreased PDAC tumor growth and tumor volume in nude mice. Similarly, TG2 expression in PDAC tumor samples was associated with down-regulation of PTEN expression and poor survival of patients. To our knowledge, this is the first report documenting the role of TG2 in posttranslational modification of PTEN. TG2 promotes the degradation of PTEN via ubiquitination and results in the activation of FAK/akt signaling pathway, the events that strongly affect the ability of pancreatic cancer cells to invade and respond to anticancer therapies.

**Materials and Methods**

**Materials.** Rabbit polyclonal antibodies to phosphorylated PTEN (pSer380), phosphorylated Akt (pSer473), total PTEN, and total Akt were purchased from Cell Signaling Technology. Mouse monoclonal antibodies to phosphorylated FAK (pThr397) and total FAK were from BD Biosciences Pharmingen. Anti-TG2 monoclonal antibody CUB7401 was purchased from NeoMarkers. Anti-ubiquitiniquinominonal antibody (P4D1) was from Santa Cruz Biotechnology, Inc. Anti–β-actin antibody was from Sigma Chemical Co. and horseradish peroxidase–conjugated goat anti-rabbit and sheep anti-mouse were purchased from Amersham Biosciences. Trublot anti-mouse and anti-rabbit Ig immunoprecipitation beads were from eBiosciences. DMEM/F12, RPMI 1640, fetal bovine serum, and Normocin antibiotic were all purchased from InvivoGen.

**Cell lines.** The PDAC cell lines were either purchased from the American Type Culture Collection or provided by Dr. Shrikant Reddy (The University of Texas M. D. Anderson Cancer Center, Houston, TX). All cell lines were maintained in the log phase of cell growth by culturing in RPMI 1640 or DMEM/F12 medium supplemented with FCS (10%), v/v). Normocin (0.1 mg/mL), l-glutamine (2 mM/L), and HEPES (10 mM/L; U.S. Biochemical) at 37°C in a CO2 incubator.

**TG2 short hairpin RNA stable clones.** TG2 short hairpin RNA (shRNA) expression plasmid was constructed using the psiSTRIKE neomycin vector, which contains a human U6 promoter and two Pslr sites (Promega). For the construction of TG2 hairpin-type small interfering RNA (siRNA) expression plasmids, we constructed oligonucleotides with the hairpin sequence TG2si1382 (GGGCGAACCACCTTG2 in posttranslational modification of PTEN. TG2 promotes the degradation of PTEN via ubiquitination and results in the activation of FAK/akt signaling pathway, the events that strongly affect the ability of pancreatic cancer cells to invade and respond to anticancer therapies.

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Results

TG2 expression inversely correlates with PTEN. We recently reported that aberrant expression of TG2 in PDAC cells promotes activation of FAK and its downstream PI3K/AKT pathway (9). However, the mechanism by which TG2 affects these functions was not delineated. We hypothesized that TG2 might affect FAK/PI3K/AKT pathway by modulating the expression of PTEN, a lipid/protein phosphatase that is known to inhibit FAK and PI3K/AKT pathways. To test this hypothesis, first, we tested several PDAC cell lines for TG2 and PTEN expression. Using Western blot analysis, we observed that the cell lines that expressed high basal level of TG2 protein contained low levels of PTEN (Fig. 1A). To our knowledge, none of the cell lines that we used harbors PTEN mutation.

We further confirmed the inverse correlation between TG2 and PTEN expression in two stably TG2 shRNA-transfected Panc-28 sublines (Panc-28/cl.10 and Panc-28/cl.12). The two Panc-28 clones showed 70% to 80% decrease in TG2 expression compared with the parental Panc-28 cells (Fig. 1B). Importantly, the decrease in TG2 expression in both the clones was associated with a parallel increase in total PTEN expression (Fig. 1B). This inverse correlation between TG2 and PTEN expression in PDAC cell lines suggested that TG2 might be involved in the regulation of PTEN expression.

TG2 regulates PTEN expression. We next determined the effect of TG2 overexpression on PTEN levels. The BxPC-3 cells (expressing low basal levels of TG2) were infected with an adenovirus construct containing TG2 cDNA. TG2-infected cells
were then chased for PTEN expression over a period of 24 h. Results shown in Fig. 2A revealed that overexpression of TG2 protein (Fig. 2A, top) results in ~60% decrease in PTEN expression after 24 h of incubation (Fig. 2A, middle). In a subsequent experiment, we used 48 h of infection with adenovirus alone (LacZ), which resulted in 5- to 6-fold increase in TG2 expression and >80% decrease in PTEN expression (Fig. 2B). The infection of cells with adenovirus alone (LacZ) had no effect on TG2 or PTEN expression (Fig. 2B). We next investigated the status of PTEN phosphorylation at Ser^{380}, the site known to affect its stability (17, 18). Ectopic expression of TG2 in BxPC-3 cells resulted in hypophosphorylated (Ser 380) PTEN (Fig. 2B). Interestingly, like wild-type TG2, forced expression of the mutant TG2, which lacks transamidation activity due to a point mutation in the active site (C277S; ref. 3), also induced a similar decrease in PTEN phosphorylation and expression (Fig. 2B). These results suggest that TG2-mediated regulation of PTEN expression is independent of its catalytic/transamidation function.

In a converse experiment, we studied the effect of TG2 knockdown on PTEN phosphorylation (Ser^{380}) and expression. We used the siRNA approach using two different siRNA sequences, which resulted in 80% to 90% down-regulation of endogenous TG2 expression in Panc-28 cells (Fig. 2C). Down-regulation of endogenous TG2 was associated with an increase in PTEN phosphorylation (Ser^{380}) and PTEN expression. siRNA-induced effect on PTEN phosphorylation and expression was related to a decrease in TG2 expression, as under similar conditions transfection of Panc-28 cells with control siRNA did not alter TG2 or PTEN protein expression (Fig. 2C).

To determine the mechanism by which TG2 affects PTEN expression, we next induced TG2 expression in BxPC-3 cells by adenoviral infection. Immediately following the infection, cells were treated with cycloheximide in the presence or absence of the proteasome inhibitor MG132 (20 μmol/L). Cells were harvested at 0, 6, and 12 h after infection and processed to determine the PTEN levels by immunoprecipitation, as described in Materials and Methods. Whole-cell lysate and immunoprecipitates were then probed with anti-phosphorylated PTEN (Ser^{380}) and anti-total PTEN antibodies. Bottom, Western blot showing ubiquitination and polyubiquitination of PTEN in samples shown in top panel after the membrane was stripped and reprobed with ubiquitin antibody. E, Western blot analysis showing PTEN expression in Panc-28 cells after infection with adenovirus containing wild-type PTEN construct, as described in Materials and Methods. Cells infected with adenovirus alone (LacZ) served as control. The membranes were stripped and reprobed with anti-TG2 and anti-PTEN antibody. Finally, the membranes were probed with β-actin antibody to ensure even loading of proteins in each lane. Results shown are from a representative experiment repeated twice with similar results.
inhibits PTEN phosphorylation and promotes its ubiquitination and subsequent degradation via proteasomal pathway. An alternate possibility of TG2 regulation by PTEN was also investigated. PTEN was overexpressed in Panc-28 cells by infection with adeno virus containing PTEN construct. Results shown in Fig. 2E revealed that overexpression of PTEN does not affect TG2 levels or cell viability for at least 48 h after infection of Panc-28 cells.

**TG2 regulates PTEN-mediated functions.** PTEN expression is known to affect the invasiveness of cancer cells (13). Therefore, we next determined the effect of TG2 down-regulation on invasive functions of Panc-28 cells. For this purpose, we used two subclones (Panc-28/cl.10 and Panc-28/cl.12) that were derived by stable transfection of Panc-28 cells with TG2 shRNA and expressed 70% to 80% lower TG2 with a parallel increase in PTEN expression (Fig. 1B). Results shown in Fig. 3A showed that inhibition of endogenous TG2 in Panc-28 cells was associated with decreased invasiveness through the Matrigel. Furthermore, PTEN is known to negatively regulate FAK and PIP3 activation, resulting in the inhibition of FAK/PI3K/AKT pathway (13, 14). PTEN can directly associate with FAK and inactivate/dephosphorylate it (13). Therefore, we next determined the association of FAK and PTEN in Panc-28 and BxPC-3. In a pull-down experiment with anti-FAK antibody, the immunoprecipitates from Panc-28 and BxPC-3 cell extracts revealed the presence of PTEN (Fig. 3B). The association between FAK and PTEN was further confirmed by confocal microscopy (data not shown). We found no appreciable difference in total FAK levels in Panc-28 and BxPC-3 cells. Panc-28 cells, which expressed high basal levels of TG2, showed low association of FAK with PTEN and expressed more phosphorylated FAK (pThr397; Fig. 3B). On the other hand, low TG2-expressing BxPC-3 cells showed high association of FAK with PTEN and contained low level of phosphorylated FAK (Thr397; Fig. 3B). Furthermore, Western blot analysis of untreated BxPC-3 cells or cells infected with wild-type or mutant TG2 revealed the presence of a major phosphorylated FAK band in TG2-infected cells (Fig. 3C). A similar experiment revealed an increase in phosphorylated AKT (Ser473) in response to overexpression of TG2 in BxPC-3 cells (Fig. 3C). These results suggested that TG2-mediated down-regulation of PTEN results in constitutive activation of the FAK/PI3K/AKT pathway.

Down-regulation of endogenous TG2 by siRNA resulted in massive accumulation of cytoplasmic vacuoles after 48 h of transfection (data not shown). Recently, a role for PTEN in promoting autophagic cell death in cancer cells has been documented (19). In line with these observations, we recently showed that down-regulation of TG2 in Panc-28 cells resulted in the accumulation of autophagosome and cell death via autophagy (20).

**TG2 is associated with PTEN.** Interestingly, in a pull-down experiment using an anti-TG2 antibody, the immunoprecipitates from Panc-28 and BxPC-3 cell extracts revealed the presence of PTEN when the blots were probed with anti-PTEN antibody (Fig. 4A). The association of TG2 with PTEN was also evident in a reverse experiment where immunoprecipitates with anti-PTEN antibody showed the presence of TG2 protein.
ascertain that TG2 expression remained suppressed when compared with Panc-28/empty vector controls (Fig. 5A). Representative PDAC tumor sections, showing inverse correlation between TG2 and PTEN expression, are shown in Fig. 6B. No staining appeared in sections treated with the isotypic control antibody (data not shown). Eleven of the 51 tumor samples (21%) showed both the TG2 and PTEN expression, suggesting that other unknown factors could also contribute in the regulation of PTEN expression. Although the expression of TG2 did not correlate with the patients’ survival (P = 0.68), the TG2-mediated loss of PTEN correlated significantly with the overall survival (P = 0.004). In multivariate analysis, TG2-mediated loss of PTEN was a prognostic factor for overall survival in patients with stage II pancreatic ductal carcinoma independent of tumor stage/lymph node status and tumor differentiation (P = 0.01). Thus, overall survival in TG2+/PTEN− patients was significantly poor (20.7 months) than in TG2+/PTEN+ patients (68.6 months).

**Discussion**

The data presented here show a novel role for TG2 in regulating PTEN expression at posttranslational level. PDAC cell lines and tumor samples showed inverse correlation between TG2 expression and PTEN levels. Our results suggest that aberrant expression of TG2 can down-regulate PTEN expression. Thus, ectopic expression of the catalytically active or inactive (C277S) TG2 suppressed PTEN expression, suggesting that irrespective of its transamidation activity TG2 plays a role in PTEN regulation. Conversely, down-regulation of endogenous TG2 by siRNA resulted in increased expression of PTEN. TG2 inhibited PTEN phosphorylation at Ser380 and promoted PTEN ubiquitination and subsequent degradation via proteasomal pathway. Importantly, TG2 overexpression in conjunction with a loss in PTEN expression was associated with slow tumor growth in vivo in nude mice and predicted poor disease outcome in PDAC patients. Thus, the present study provides the first evidence on the regulation of PTEN by TG2 at posttranslational level, which can affect the invasiveness of pancreatic cancer cells and their response to anticancer therapies by modulating PI3K/AKT signaling pathway.

Previously, we found that TG2 is involved in constitutive activation of FAK and its downstream PI3K/AKT cell survival pathway. Down-regulation of TG2 inhibited pancreatic tumor growth. Of the two TG2 shRNA stable clones, we used Panc-28/cl.10 cells for in vivo studies as this clone showed more decrease in endogenous TG2 levels when compared with wild-type Panc-28 cells (Fig. 1B). Xenografts were grown s.c. in athymic nude mice using Panc-28/cl.10 or Panc-28/empty vector (control) cells. Panc-28/cl.10 xenografts showed significantly retarded growth when compared with Panc-28/empty vector controls (Fig. 5A). The tumor growth was followed for 5 weeks, and mice were sacrificed during the 6th week. The Panc-28/cl.10 cells consistently produced xenografts with 60% to 80% lower tumor volumes (Fig. 5B) than the Panc-28/empty vector xenografts (P = 0.017). The tumors were analyzed for TG2 expression to ascertain that TG2 expression remained suppressed in vivo in Panc-28/cl.10 tumors. Indeed, TG2 expression in Panc-28/cl.10 xenografts was significantly lower and PTEN expression was higher than in control tumors, as determined by Western blotting (Fig. 5C) and immunohistochemistry (data not shown).

Taken altogether, these results suggest that TG2 expression plays an important role in switching on the activation of FAK/PI3K/AKT cell survival signaling pathways. Moreover, TG2 regulates the activation of FAK/PI3K/AKT pathway by regulating the expression of the tumor suppressor protein PTEN.

**Fig. 4.** TG2 is associated with PTEN. A and B, coimmunoprecipitation, showing the association of TG2 with PTEN protein. Total cell lysates were prepared from Panc-28 and BxPC-3 cells and immunoprecipitated using an anti-TG2 (A) or anti-PTEN (B) antibody. The immunoprecipitates were subjected to SDS-PAGE and Western blotting using anti-PTEN and anti-TG2 antibodies. C, confocal microscopy images of Panc-28 cells showing colocalization of PTEN protein (red fluorescence) with TG2 (green fluorescence), as evidenced by the yellow fluorescence in the merged image. Representative experiment done twice on different occasions with similar results.
signaling pathway (9). Because the tumor suppressor protein PTEN is known to regulate cell growth, invasion, migration, and focal adhesion functions by negatively regulating PI3K/AKT pathway (13), we hypothesized that TG2 might affect FAK/PI3K/AKT pathway by modulating PTEN expression or function. Indeed, the evidence presented here strongly supports this contention and suggests that TG2 expression decreases PTEN expression, which in turn leads to the activation of FAK/PI3K/AKT pathway. However, at this stage, we do not know whether the observed effect of TG2 overexpression on FAK/PI3K/AKT activation is exclusively related to PTEN down-regulation or other upstream pathways, such as src kinase and phosphoinositide-dependent kinases, could also attribute to this event.

Gene deletion and mutation of PTEN are observed frequently in various human cancers. These genetic changes, however, are not necessarily the only way to inactivate PTEN during tumorigenesis but subtle decrease in expression of PTEN, mediated by posttranslational modification, may affect its stability and degradation and may have profound effect on tumorigenesis. Indeed, a recent report by Wang et al. (21) suggested that the oncogenic protein NEDD4.1 serves as an E3 ubiquitin ligase for PTEN and results in its ubiquitination and degradation. Little is known about the regulation and stability of PTEN, except that its phosphorylation can affect the stability in certain cell types (17, 22, 23). The COOH-terminal domain of the PTEN protein is rich in putative phosphorylation sites that are embedded within two PEST motifs, which is a signature in many short-lived proteins, degraded by the ubiquitin pathway (24). The deletion or dephosphorylation of PTEN COOH terminus has been reported to affect PTEN stability without affecting its phosphatase activity (17, 18, 22, 23). Recently, protein kinase 2 (CK2) was shown to phosphorylate PTEN 

in vitro and in vivo, in a constitutive manner, on a cluster of COOH-terminal serine/threonine residues, including Ser370, Ser380, Thr382, Thr383, and Ser385 (18). The phosphorylation-defective mutants of PTEN showed decreased stability and were
Our results suggest that association of TG2 with PTEN can negatively regulate PTEN activity (17). Phosphorylation of separate studies, it was shown that phosphorylation of PTEN diminished the PTEN protein levels. These results suggest that TG2 expression promotes PTEN degradation and increases phosphorylated FAK levels. By inhibiting the phosphorylation of PTEN at site Ser380, TG2 can destabilize PTEN by promoting PTEN ubiquitination and proteasomal degradation. Thus, TG2-mediated suppression of PTEN results in the activation of AKT, which in turn can phosphorylate various substrates leading to the activation of AKT and nuclear factor-κB (NFκB) and inhibition of PDK1 and BAD contributing to increased cell survival and chemoresistance.

Fig. 7. Schematic representation of TG2-induced activation of FAK and downstream PI3K/AKT pathway. TG2 affects PTEN function and expression, resulting in activation of FAK and downstream PI3K/AKT survival pathway. TG2 expression promotes PTEN degradation and increases phosphorylated FAK levels. Inhibiting the phosphorylation of PTEN at site Ser380, TG2 can destabilize PTEN by promoting PTEN ubiquitination and proteasomal degradation. Thus, TG2-mediated suppression of PTEN results in the activation of AKT, which in turn can phosphorylate various substrates leading to the activation of AKT and nuclear factor-κB (NFκB) and inhibition of PDK1 and BAD contributing to increased cell survival and chemoresistance.

In conclusion, we provide the first evidence that increased expression of TG2 in PDAC cells is strongly associated with deregulation of PTEN-mediated cell survival FAK/PI3K/AKT signaling pathway, as depicted schematically in Fig. 7. The TG2-mediated loss of PTEN is associated with poor survival in stage II PDAC patients. The association between TG2, PTEN, and FAK/AKT pathways deserves further analysis in prospective clinical studies.

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References

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