

# The prolyl isomerase Pin1 is overexpressed in human esophageal cancer

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**Abstract.** Peptidyl-prolyl isomerase Pin1 specifically catalyzes the *cis/trans*-isomerization of proline in the target sequence of phosphorylated Ser/Thr-Pro in over 50 critical regulatory proteins. Pin1 is abnormally overexpressed in a range of human cancers, including lung, breast, colon and prostate cancers. However, few reports of Pin1 overexpression are currently available in clinical samples. Therefore, we examined the expression of Pin1 and p53 in non-pathological human tissues and esophageal cancer tissues. In esophageal cancer tissues, Pin1 and p53 immunoreactivity was detected in cancer cells in 67 and 58% of cases, respectively. Moreover, Pin1 and p53 immunoreactivity was significantly correlated with lymph node-positive disease and more advanced cancer stage. The results demonstrated that high expression levels of Pin1 correlated with high levels of p53. Therefore, Pin1 is suggested to play key roles in the regulation of esophageal cancer.

## Introduction

The human Pin1 (EC 5.2.1.8) was identified in 1996 by yeast two-hybrid screens as a protein that binds and inhibits the toxicity of never-in-mitosis A (NIMA), a fungal mitotic kinase (1). Human Pin1 contains 163 amino acid residues and consists of a substrate-recognition WW domain in the N-terminal and a C-terminal catalytic PPIase domain (2).

Subsequent studies found that Pin1 specifically binds and isomerizes pSer/Thr-Pro motifs in a large and defined subset of phosphoproteins, which are usually the substrates of proline-directed protein kinases. Proline-directed phosphorylation plays an essential role in normal, as well as in malignant, cell proliferation (3-5).

Functionally, the phosphorylation-dependent *cis/trans*-isomerization mediated by Pin1 has a profound impact on cell events. Such a conformational change regulates the activities of its substrates including catalysis, protein-protein interaction, subcellular localization, protein dephosphorylation and stability (5-7). For instance, Pin1 is abnormally overexpressed in a range of human cancers, including lung, breast, colon and prostate cancers, and is considered a biomarker of poor prognosis (8-12).

Extensive studies on Pin1 have identified over 50 proteins as biological substrates of Pin1. These proteins include cell cycle-regulated proteins such as Raf-1, Cdc25, cyclin D1, cyclin E, GTP-binding protein Rab4, anti-apoptotic protein Bcl-2, transcription factors c-Jun,  $\beta$ -catenin, NF- $\kappa$ B, p53, c-Myc, p73, c-fos, and Alzheimer's disease-related proteins APP and Tau (13-18).

Pin1 regulates the cancer suppressor protein p53 via its WW domain by promoting p53's stability in response to DNA damage induced by genotoxic drugs, UV light and ionizing radiation (19,20). Pin1 is capable of interacting with a number of pSer/Thr-Pro motifs in p53, including pSer33, pSer46, pThr81 and pSer315 (21-23). Binding of Pin1 mediates a p53 conformational change, which in turn enhances interaction between p53 and the checkpoint kinase 2 (Chk2) and subsequent p53 phosphorylation at Ser20. Interaction between p53 and Chk2 protects p53 from Mdm2-mediated ubiquitination, nuclear export and degradation. Accumulation of p53 in turn enhances its transcriptional activity towards the cell cycle inhibitor p21. This elevation eventually leads to a cell cycle checkpoint arrest in response to DNA damage (24,25).

Therefore, Pin1 and p53 are considered to be involved in the regulation of cancer progression. In the present study, we conducted a quantitative investigation on 110 esophageal cancer specimens and matching normal esophageal tissue to examine the levels of Pin1 expression in esophageal cancer and normal esophageal specimens by means of immunohistochemistry and reverse transcription polymerase

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chain reaction (RT-PCR) to ascertain the effect of Pin1 on esophageal cancer pathogenesis and development.

## Materials and methods

**Patients and tissues.** Prior informed consent for the following studies was obtained from all patients. A total of 110 cancer specimens and matching normal esophageal tissues were obtained in consultation with the surgeon and the pathologist at the Xiamen Hospital of Traditional Chinese Medicine, Xiamen, China, between September 2004 and November 2008. The tissues were obtained immediately following surgery. The tissues for RNA isolation were snap-frozen and stored at -80°C, and those for immunohistochemistry were fixed with 10% formalin and embedded in paraffin wax. The clinicopathological characteristics of the subjects are shown in Table I.

**Immunohistochemistry.** Sections (4 µm) of formalin-fixed, paraffin-embedded tissue samples were cut with a microtome and dried overnight at 37°C on a silanized slide. The protocol for the avidin-biotin-peroxidase complex method was followed for each sample. Samples were deparaffinized in xylene at room temperature for 30 min, rehydrated with graded ethanol and washed in phosphate-buffered saline (PBS). The samples were then laced in 10 mM citrated buffer (pH 6.0) and boiled in a microwave for 10 min for epitope retrieval. Endogenous peroxidase activity was quenched by incubating tissue sections in 3% H<sub>2</sub>O<sub>2</sub> for 10 min. The primary antibodies mouse Pin1 (Abnova Corp., Taipei, Taiwan) and mouse p53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used overnight at 4°C, at dilutions of 1:300 and 1:200, respectively. The slides were washed and biotinylated goat anti-mouse secondary antibody (Santa Cruz Biotechnology) was applied for 1 h. Following rinsing in PBS, the slides were treated using the peroxidase-labeled Vectastain Elite ABC kit, and the peroxidase was subsequently developed with a diaminobenzidine kit and counterstained with hematoxylin. Goat serum was used for the negative controls instead of the primary antibody for Pin1 and p53.

Specimens of immunohistochemical staining for Pin1 and p53 were evaluated in a semi-quantitative manner, which considers the intensity of the staining and the percentage of cells stained at each intensity. In each case, the intensity (weak, moderate or strong) and pattern (incomplete or complete) of nuclear and cytoplasmic staining, and the percentage of neoplastic immunoreactive cells (cut-off of 10%) were evaluated. Tumors were scored as: score 0, no appreciable staining or staining in <5% of neoplastic cells; score 1+, tumors with faint/barely appreciable incomplete nuclear and cytoplasmic staining in 5-25% of neoplastic cells; score 2+, tumors with weak to moderate complete nuclear and cytoplasmic staining or containing 25-50% of neoplastic cells with moderate incomplete basolateral nuclear and cytoplasmic immunostaining; score 3+, strong immunoreactivity of the entire nucleus and cytoplasm in >50% of neoplastic cells or containing >50% neoplastic cells with strong basolateral incomplete nuclear and cytoplasmic immunoreactivity. Tumors were classified as 0 or 1+, 'negative' and 2+ or 3+, 'positive'.

**Primers.** Primer sequences were designed for the qRT assay using Primer Premier Analysis Software, version 5.0. To avoid

Table I. Clinicopathological characteristics of 110 esophageal cancer patients.

| Parameters         | No. of patients (N) |
|--------------------|---------------------|
| Patients           | 110                 |
| Lymph node         |                     |
| Positive           | 65                  |
| Negative           | 45                  |
| Differentiation    |                     |
| Low                | 30                  |
| Middle             | 69                  |
| High               | 11                  |
| Stage              |                     |
| I                  | 31                  |
| II                 | 40                  |
| III+IV             | 39                  |
| TNM classification |                     |
| T1                 | 8                   |
| T2                 | 67                  |
| T3                 | 11                  |
| T4                 | 24                  |
| T1+T2              | 75                  |
| T3+T4              | 35                  |
| Gender             |                     |
| Female             | 18                  |
| Male               | 92                  |
| Age                |                     |
| <49                | 17                  |
| 50-59              | 18                  |
| 60-69              | 46                  |
| >70                | 29                  |

possible amplification of contaminating genomic DNA, primers were designed so that each PCR product covered at least one intron. The primer sequences utilized were: Pin1 forward, 5'-TGATCAACGGCTACATCCAG-3'; reverse, 5'-CAAACGAGGCGTCTTCAAAT-3'; and GAPDH forward, 5'-CATGACA ACTTTGGTATCGTG-3'; reverse, 5'-GTGTCGCTGTTGAAGTCAGA-3'. GAPDH was amplified as an internal reference housekeeping gene for assessing the status of the mRNA sample.

**Real-time RT-PCR assay.** Total cellular RNA from tissue specimens was isolated using a TRIzol reagent (Gibco BRL Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. The total RNA (3 µg) was reversed transcribed using Moloney murine leukemia virus reverse transcriptase and random primers (Promega, Madison, WI, USA).

Real-time PCR was carried out with an Opticon 2 Real-Time Thermocycler (two colors) detection system (MJ Research, Canada). The PCR reaction solution (25 µl) contained cDNA from 250 ng of total RNA, 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP, 0.6 µM of each primer, 1 unit Taq polymerase (Takara, Otsu, Shiga, Japan), 0.625 µl 1X SYBR-GreenI (Molecular Probes,

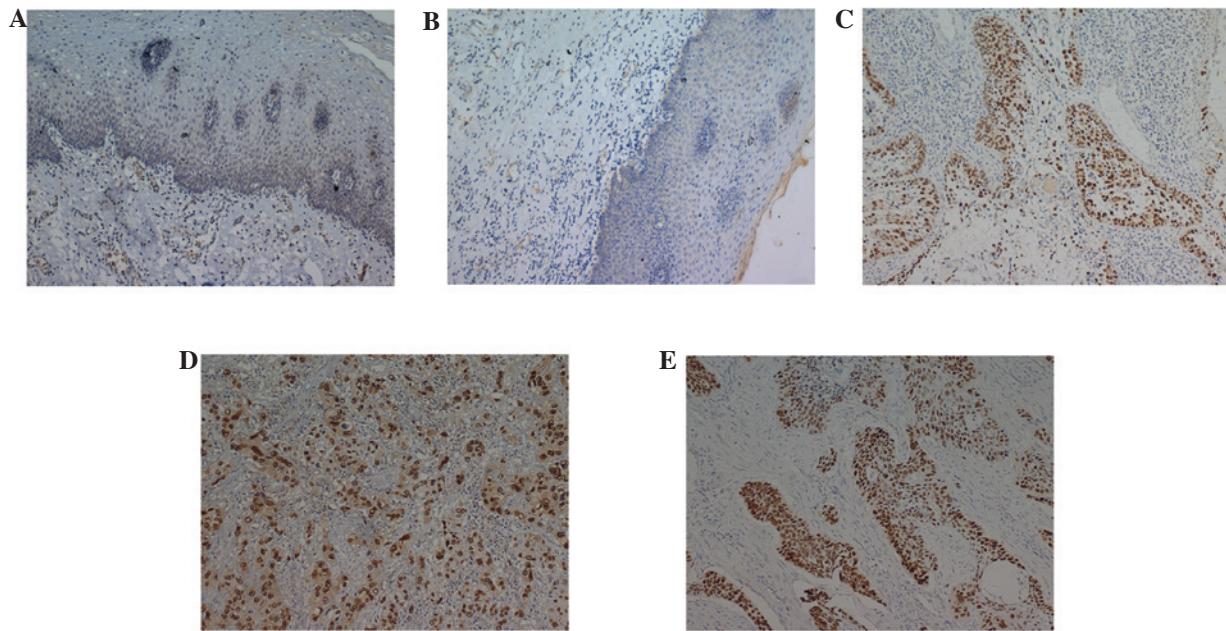


Figure 1. Representative immunohistochemical staining for Pin1 and p53. Sections from paraffin-embedded tissues were subjected to antigen retrieval treatment, followed by immunostaining with anti-Pin1 or anti-p53 antibodies. (A) Normal esophageal tissue with Pin1 immunostaining; (B) high differentiation of tumor tissue with Pin1 immunostaining; (C) middle differentiation of tumor tissue with p53 immunostaining; (D) low differentiation of tumor tissue with Pin1 immunostaining; and (E) tumor tissue with p53 immunostaining. Magnification,  $\times 100$ . The specimens were evaluated independently by two pathologists in a blinded manner without previous knowledge of the qRT results on Pin1 mRNA expression. The immunohistochemistry results for Pin1 were arbitrarily classified into four scores dependent on the intensity of immunoreactivity as described in Materials and methods.

Eugene, OR, USA; dilution 1:1000) and  $2.5\ \mu\text{l}$  10X AmpliTaq buffer for the final volume. PCR was performed under the following conditions: samples were initially denatured by heating at  $94^{\circ}\text{C}$  for 5 min, followed by 40 cycles of denaturation at  $94^{\circ}\text{C}$  for 30 sec and annealing at  $58^{\circ}\text{C}$  for 30 sec for the two genes, and extension at  $72^{\circ}\text{C}$  for 1 min. Each assay was performed at least three times to verify the results.

The standard curve for quantifying mRNA copy number was established by amplifying six aliquots of templates with known copy numbers ( $2.1 \times 10^3$ – $2.1 \times 10^8$  copies). cDNA was synthesized as follows: RT-PCR on the sample RNA was performed, electrophoresis was run on a 2% agarose gel, the Pin1 cDNA and GAPDH cDNA were ligated separately into pCR II-TOPO cloning vectors (Invitrogen, San Diego, CA, USA), the cDNA clones were transformed into *Escherichia coli* DH5- $\alpha$  cells and the cultures were expanded as previously described (9). Plasmids containing the target gene were purified and quantified for use in the qRT setup. To confirm that the inserted PCR product size was correct, plasmids were digested with specific restriction enzymes, and the cDNA clone PCR products were then run on gel electrophoresis. Finally, the two plasmids were sequenced by another biology company (Invitrogen) to confirm the sequencing results.

**Statistical analysis.** Statistical analysis was carried out using the Kappa analysis. Results were considered significant when  $P < 0.05$ .

## Results

This study set out to examine the protein expression level of Pin1 and p53. A total of 110 esophageal cancer specimens

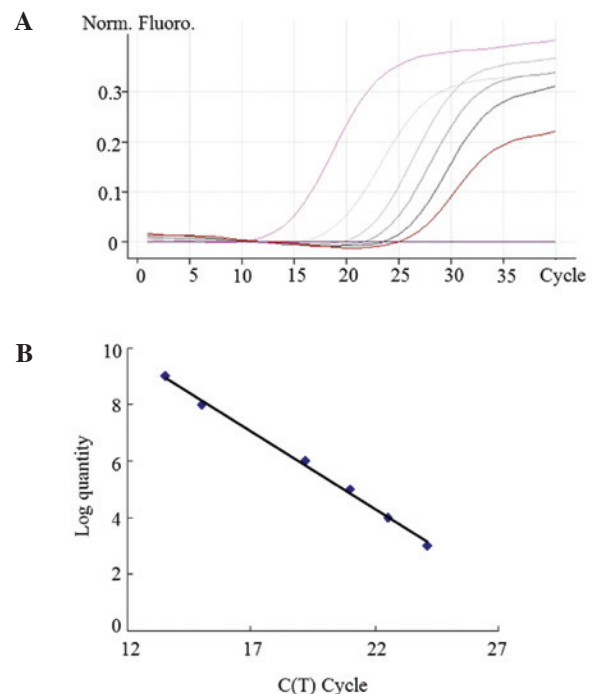


Figure 2. Representative q-RT analysis for Pin1 mRNA copy levels. (A) Serially diluted plasmids containing Pin1 cDNA ( $2 \times 10^3$ – $2 \times 10^9$ ) were analyzed for controls. (B) Standard curve (correlation coefficient, 0.9969). Norm. Fluoro; normal fluorescence.

and matching normal esophageal tissues were analyzed by immunohistochemistry. Fig. 1 shows representative cancer tissue sections stained for the Pin1 and p53 proteins. A number of clinicopathological categories were used to evaluate the

Table II. The correlation between Pin1 and p53 protein expression and clinicopathological characteristics.

| Parameters         | N   | Pin1 staining (IHC) |          |         | p53 staining (IHC) |          |         |
|--------------------|-----|---------------------|----------|---------|--------------------|----------|---------|
|                    |     | Positive            | Negative | P-value | Positive           | Negative | P-value |
|                    | 110 | 74                  | 36       |         | 63                 |          |         |
| Lymph node         |     |                     |          |         |                    |          |         |
| Positive           | 65  | 54                  | 11       |         | 40                 | 25       |         |
| Negative           | 45  | 20                  | 25       | 0.000   | 23                 | 22       | 0.277   |
| Differentiation    |     |                     |          |         |                    |          |         |
| Low                | 30  | 24                  | 6        |         | 22                 | 8        |         |
| Middle             | 69  | 45                  | 24       |         | 36                 | 33       |         |
| High               | 11  | 5                   | 6        | 0.095   | 5                  | 6        | 0.104   |
| Stage              |     |                     |          |         |                    |          |         |
| I                  | 31  | 18                  | 13       |         | 20                 | 11       |         |
| II                 | 40  | 34                  | 6        |         | 22                 | 18       |         |
| III+IV             | 39  | 29                  | 10       | 0.038   | 21                 | 18       | 0.626   |
| TNM classification |     |                     |          |         |                    |          |         |
| T1                 | 8   | 7                   | 1        |         | 7                  | 1        |         |
| T2                 | 67  | 55                  | 12       |         | 35                 | 32       |         |
| T3                 | 11  | 8                   | 3        |         | 7                  | 4        |         |
| T4                 | 24  | 19                  | 5        | 0.847   | 14                 | 10       | 0.275   |
| T1+T2              | 75  | 62                  | 13       |         | 42                 | 33       |         |
| T3+T4              | 35  | 27                  | 8        | 0.492   | 21                 | 14       | 0.693   |
| Gender             |     |                     |          |         |                    |          |         |
| Female             | 18  | 12                  | 6        |         | 12                 | 6        |         |
| Male               | 92  | 70                  | 22       | 0.484   | 50                 | 42       | 0.260   |
| Age                |     |                     |          |         |                    |          |         |
| <49                | 17  | 12                  | 5        |         | 11                 | 6        |         |
| 50-59              | 18  | 12                  | 6        |         | 11                 | 7        |         |
| 60-69              | 46  | 28                  | 18       |         | 26                 | 20       |         |
| >70                | 29  | 18                  | 11       | 0.868   | 15                 | 14       | 0.833   |

In each case, the intensity (weak, moderate or strong) and pattern (incomplete or complete) of nucleus and cytoplasm staining, and the percentage of neoplastic immunoreactive cells (cut-off of 10%) were evaluated. Tumors were scored as follows: score 0, no appreciable staining or staining in <5% neoplastic cells; score 1+, tumors with faint/barely appreciable incomplete nucleus and cytoplasm staining in 5-25% neoplastic cells; score 2+, tumors with weak to moderate complete nucleus and cytoplasm staining or containing 25-50% neoplastic cells with moderate incomplete nucleus and cytoplasm immunostaining; and score 3+, strong immunoreactivity of the entire nucleus and cytoplasm >50% neoplastic cells or containing >50% neoplastic cells with strong nucleus and cytoplasm immunoreactivity. Tumors classified as 0 or 1+ were considered 'negative' and those scored as 2+ or 3+ were classified as 'positive'.

expression of the targets and their potential correlation with the status of the disease. As shown in Table II, the protein level of Pin1 was significantly higher in cancer tissue than in normal tissue. A favorable correlation was found between Pin1 expression and the stage of the disease. However, there significant correlation was observed between Pin1 expression and histological type or the level of differentiation of the cancer. On the other hand, the results indicated that p53 was expressed more often in cancer tissue than in normal tissue.

To further examine the expression pattern of Pin1 in esophageal cancer tissues, we investigated the mRNA expression of Pin1 by quantitative real-time-PCR (q-RT).

Total cellular RNA from tissue specimens was extracted for RT-PCR analysis. Among the total RNA extractions from 110 esophageal cancer specimens, only the RNA extractions from 40 specimens qualified for further RT-PCR analysis. We generated standard curves using known quantities of Pin1 prepared and confirmed by sequencing in our laboratory. A representative graph of the curves obtained from serial dilutions of Pin1 is shown in Fig. 2. The Pin1 mRNA was identified as 'overexpressed' if, when calibrated against GAPDH, the tumor had a  $\geq 1.8$  expression ratio compared to normal tissue. The level of mRNA was then compared to various clinicopathological characteristics. In accordance



Table III. Correlation between Pin1 mRNA expression and clinicopathological characteristics.

| Parameters         | N  | Pin1 mRNA (q-RT)   |                     | P-value |
|--------------------|----|--------------------|---------------------|---------|
|                    |    | Overexpression (%) | Underexpression (%) |         |
|                    | 40 | 16 (40)            | 24 (60)             |         |
| Diff               |    |                    |                     |         |
| Low                | 13 | 5                  | 8                   | 0.959   |
| Middle             | 18 | 7                  | 11                  |         |
| High               | 9  | 3                  | 6                   |         |
| Stage              |    |                    |                     |         |
| I                  | 9  | 2                  | 7                   | 0.164   |
| II                 | 12 | 7                  | 5                   |         |
| III+IV             | 19 | 7                  | 12                  |         |
| TNM classification |    |                    |                     |         |
| T1                 | 3  | 0                  | 3                   | 0.099   |
| T2                 | 26 | 8                  | 18                  |         |
| T3                 | 5  | 4                  | 1                   |         |
| T4                 | 6  | 2                  | 4                   | 0.110   |
| T1+T2              | 29 | 8                  | 21                  |         |
| T3+T4              | 11 | 6                  | 5                   |         |
| Gender             |    |                    |                     |         |
| Female             | 6  | 3                  | 3                   | 0.403   |
| Male               | 34 | 11                 | 23                  |         |
| Age                |    |                    |                     |         |
| <49                | 6  | 2                  | 4                   | 0.985   |
| 50-59              | 12 | 5                  | 7                   |         |
| 60-69              | 15 | 6                  | 9                   |         |
| >70                | 7  | 3                  | 4                   |         |

Diff, differentiation.

with the earlier comparison with the expression protein levels, high-level Pin1 mRNA was significantly correlated with the presence of lymph node metastasis and with the stage of disease. No significant correlation was found between Pin1 mRNA expression and the histological or differentiation types of the tumors, the size of the tumor, or between Pin1 expression and the age or gender of the patient. These results are shown in Table III. As shown in Table IV, no significant correlation between the level of Pin1 protein expression and Pin1 mRNA expression.

## Discussion

Pin1 has been shown to play a significant role in numerous steps of oncogenic signaling pathways (24,25). For example, Pin1 collaborates with Ras signaling to increase the transcriptional activity of c-Jun towards cyclin D1 (24,25). Furthermore, Pin1 is involved in the DNA damage response through modulation

Table IV. Correlation between Pin1 protein expression (IHC) and mRNA expression (q-RT).

|                            |    | Pin1 protein (IHC) |          |         |
|----------------------------|----|--------------------|----------|---------|
|                            | N  | Positive           | Negative | P-value |
| Pin1 mRNA                  |    |                    |          |         |
| (q-RT)                     |    |                    |          |         |
| Positive                   | 24 | 19                 | 5        | 0.757   |
| Negative                   | 16 | 12                 | 4        |         |
| IHC, immunohistochemistry. |    |                    |          |         |

of p53 function upon genotoxic stress (24,25). Pin1 is overexpressed in human breast and oral cancers, and a high Pin1 expression is correlated with cancer development and poor prognosis in patients with prostate cancer (24,25). However, whether or not there is any correlation between Pin1 expression and the clinical outcome of cancer patients remains to be determined. To address this question, we determined Pin1 expression in 110 specimens using immunohistochemistry, followed by quantitative real-time-PCR.

This is the first large-scale study of Pin1 expression in clinical samples of esophageal cancer. We have shown that Pin1 is overexpressed in esophageal cancer, and that its presence correlates with lymph node involvement and late stage disease. We also examined tumors for a correlation between Pin1 expression and the expression of p53. In tumors with high levels of Pin1 expression, there was also a strong likelihood for the tumors to exhibit high levels of p53 expression. Since Pin1 is overexpressed in tumors, and since high levels of expression correlate with a poorer prognosis for patients, Pin1 may have the potential to be an excellent tumor marker.

Furthermore, an elevated Pin1 expression correlates with clinical stage. The Pin1 expression level may prove useful during biopsies, perhaps indicating a need for further treatment for patients with high Pin1 tumors. The strong correlation between the Pin1 level and clinical outcome of esophageal cancer suggests the involvement of Pin1 in the progression of the disease. We have previously found that Pin1 is activated by oncogenic pathways via the transcriptional factor E2F, and that Pin1 overexpression activates multiple steps in oncogenic signaling pathways. No significant correlation was observed between the level of Pin1 protein expression and Pin1 mRNA expression. Therefore, this result would have suggested post-transcriptional regulation. Thus, our study establishes the role of Pin1 as a prognostic maker for biochemical recurrence in esophageal cancer and suggests that Pin1 is a novel target for treating patients with esophageal cancer.

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