行政院國家科學委員會專題研究計畫 期末報告

臺灣本土植物臺灣五葉松萃取物對於類風濕性關節炎致病相關酵素’第二及四型胜?精胺酸脫亞氨?’生化及細胞凋亡作用的影響及其調節類風濕性關節炎之探究

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中 文 摘 要 ： 國內胜肽精胺酸脫亞氨酶與類風濕性關節炎間關係在本研究計畫中討論，並整理胜肽精胺酸脫亞氨酶與細胞凋亡作用機制探究，並從臺灣本土植物臺灣五葉松萃取物中尋找特定具備調節細胞生理生化與關節炎之天然物。

中文關鍵詞： 五葉松萃取物, 胜肽精胺酸脫亞氨酶, 關節炎

英 文 摘 要 ：

英文關鍵詞：

ABSTRACT

Peptidylarginine deiminase type 2 (PADI2) deiminates (or citrullinates) arginine residues in protein to citrulline residues in a Ca^{2+} -dependent manner and is found in lymphocytes and macrophages. Vimentin is an intermediate filament protein and a well-known substrate of PADI2. It was shown that citrullinated vimentin was found in ionomycin induced macrophage apoptosis. Citrullinated vimentin is the target of anti-Sa antibodies, which are specific to rheumatoid arthritis and play an important role in the pathogenesis of the disease. For investigating the role of PADI2 in apoptosis, we generated a Jurkat cell line that will overexpress PADI2 transgene from a tetracycline-inducible promoter and used the combination of 12-O-tetradecanoylphorbol-13-acetate and ionomycin to activate Jurkat cells. We found overexpression of PADI2 decreased cell viability of activated Jurkat cells in a dose and time dependent manner. PADI2-overexpressed and activated Jurkat cells presented typical manifestations of apoptosis and exhibited more citrullinated proteins, including citrullinated vimentin. Overexpression of vimentin rescued a part of cells from apoptosis. In conclusion, overexpression of PADI2 induces apoptosis of activated Jurkat cells. Vimentin is involved in PADI2 induced apoptosis. Moreover, PADI2-overexpressed Jurkat cells secreted more vimentin after activation, and then expressed more vimentin on cell surfaces at the time they are going to apoptosis. Through artificially highlighting PADI2 and vimentin, we demonstrate PADI2 and vimentin participate in the apoptotic mechanisms of activated T lymphocytes. Secreted and surface-expressed vimentin are possible pathways of antigen presentation to the immune systems.

Keywords:

peptidylarginine deiminase type 2, apoptosis, vimentin

INTRODUCTION

Peptidylarginine deiminases (PADIs; EC 3.5.3.15) deiminate positively charged arginine residues in protein to neutral citrulline residues in a Ca^{2+}-dependent manner. To date, there are five isotypes of PADIs (PADI type 1-4, and PADI type 6), all of which display extensive mutual sequence homologies. The most difference between them is their tissue-specific expression (reviewed in Vossenaar et al., 2003). PADI type 2 (PADI2) is the most widely expressed type and observed in skeletal muscle, brain, spleen, macrophages and lymphocytes (Asaga et al., 2001; Vossenaar et al., 2004; Foulquier et al., 2007). PADI type 4 (PADI4) is expressed mainly in granulocytes (Asaga et al., 2001; Nakashima et al., 2002; Hagiwara et al., 2002) and monocytes (Nakashima et al., 1999; Vossenaar et al., 2004b). PADI4 has a NLS motif and is found to reside in the cell nucleus (Nakashima et al., 2002). It targets nuclear proteins, including histones and nucleophosmin/B23 (Nakashima et al., 2002; Hagiwara et al., 2002).

This post-translational modification (deimination or citrullination) causes the substrates decreasing in net charge, loss of potential ionic bonds, interference with H bonds and unfolding (Tarcsa et al., 1996). It may have a big impact on the structure and function of proteins. Citrullination can also lead to increasing proteolytic susceptibility. For example, citrullinated myelin basic protein is prone to be digested by aspartyl protease cathepsin D (Pritzker et al., 2000; Nicholas, 2011); citrullinated filaggrin and alpha enolase by calpain-1 (Kamata et al., 2009; Jang et al. 2012); citrullinated vimentin by calpains (reviewed in Zhou and Ménard, 2002).

The activation of PADIs is often observed during terminal differentiation and apoptosis. Citrullination of filaggrin is presumably an apoptotic event in the terminally differentiated keratinocytes (Senshu et al., 1996). In calcium ionophore-induced apoptosis of macrophages, citrullinated vimentin was found localized around the periphery of round-shaped nucleus, which was an early morphological signs of apoptosis (Asaga et al. 1998). Citrullination of nuclear proteins have also been reported during apoptosis (Mizoguchi et al. 1998). In our previous study, we found overexpression of PADI4 induces apoptosis in HL-60 and Jurkat cells (Liu et al, 2006).

Many autoimmune and neurodegenerative diseases are associated with citrullination, such as rheumatoid arthritis (RA) (De Rycke et al., 2005; Foulquier et al., 2007), multiple sclerosis (Nicholas et al., 2004; Moscarello et al. 2007), Alzheimer's disease (Ishigami et al., 2005) and Parkinson's disease (Nicholas, 2011). RA is an autoimmune disease, characterized by chronic symmetric destructive polyarthritis of peripheral joints. Among the autoantibodies found in patients with RA, autoantibodies against citrullinated proteins (anti-citrullinated protein antibodies, ACPAs) are most specific for RA. Citrullinated vimentin is the target of

anti-Sa autoantibody (one of ACPAs) (Vossenaar et al., 2004a) and presents in immune complexes from synovial fluid of RA patients with ACPAs (Van Steendam et al., 2010) and in inflamed synovial tissues (Tilleman et al., 2008). It has an important role in triggering specific autoantibody production and the pathogenesis of RA (Van Steendam et al., 2011).

Vimentin, a well-known substrate of PADI2 (Hojo-Nakashima et al., 2009), is a widely expressed intermediate filament protein in all mesenchymal cells and tissue (Hay, 1989). As other intermediate filaments, the vimentin network spreads from the nucleus to the plasma membrane. It has important roles in maintaining cell and tissue integrity (Lundkvist et al., 2004).

In this study, we found overexpression of PADI2 induces apoptosis in activated Jurkat T cells and investigated the role of vimentin in PADI2-induced apoptosis.

MATERIALS AND METHODS

Cell culture and chemical materials

Cells were grown in 90% RPMI 1640 medium with 10% fetal bovine serum (FBS) obtained from Gibco BRL (Grand Island, NY) at a temperature of 37°C under a humidified and 5% CO2 environment. Ribonuclease A (RNase A), acridine orange and doxycycline (Dox), 12-O-tetradecanoylphorbol-13-acetate (TPA) and ionomycin (Ion) were purchased from Sigma (St. Louis, MO).

Human *padi2* and *vimentin* gene sub-cloning and cell transfection

The human PADI2 and vimentin cDNA was purchased from RZPD Deutsches Ressourcenzentrum für Genomforschung. To amplify PADI2 cDNA, we used a sense primer (PADI2-s: 19-mer) consisting of a 5' *Not*I site (underlined) and a 11-nt sequence (nt 65–75): 5'-GCGGCCGCATGCTGCGCGA-3', an anti-sense primer (PADI2-as: 18-mer) consisting of a 5' *Sal*I site (underlined) and a 12-nt sequence (nt 1,441–1,430): 5'-GTCGACCACCCCAAAAGA-3' and *Taq* DNA polymerase (MDBio, TWN) as previous study (Liu et al, 2006). Another, we amplified vimentin cDNA (encoding 466 amino acids) used a sense primer (VIM-s: 20-mer) consisting of a 5' *Not*I site (underlined) and a 12-nt sequence (nt 292–303): 5'-GCGGCCGCATGTCCACCAGG-3' and an anti-sense primer (VIM-as: 18-mer) consisting of a 5' *Sal*I site (underlined) and a 12-nt sequence (nt 1,678–1,689): 5'-GTCGACTTCAAGGTCATC-3'. The polymerase chain reaction (PCR) product was ligated into T vector and to be transformed into E. *coli* strain JM109. The bacteria was grown overnight at 37℃, eluted plasmids and the amplified plasmid was digested with *NotI-Sal*I and subcloned into the NotI-EcoRI site of pTRE2hyg for Tet-On system (BD Biosciences Clontech, San Jose, CA). pTRE2hyg-PADI2 or-VIM and pTRE2hyg vector only were transfected into JK-Tet-On system cells (BD Biosciences, Clontech) using calcium phosphate-mediated transfection for stable transfection selection. Stably transfected cells were selected with the antibiotic hygromycin (400μg/ml). After approximately 3 weeks, hygromycin-resistant clones were screened for mRNA overexpression, protein expression by immunoblot. In vitro, promoter induction of JK-Tet-On cell system was accomplished by addition of Dox to the growth medium.

Cell viability and acridine orange staining

Living cells were counted by using trypan blue exclusion assay. The quantity of cell viability was calculated by the viable cell numbers from experiment groups divided by those in the control group. To identify apoptotic characters, 5×10^4 cells in 10μl cell suspension were mixed with an equal volume of acridine orange solution (10μg/ml)

in phosphate-buffered saline (PBS) on each slide. Green fluorescence was detected and photographed by a fluorescence microscope as being between 500-525 nm (Olympus America, St Huntington, NY).

DNA fragmentation analysis

In PBS, cells (5×10^6) were harvested and lysed overnight in a digestion buffer (0.5 % sarkosyl, 0.5mg/ml proteinase K, 50mM Tris-HCl, pH 8.0 and 10 mM EDTA) at 55°C. Cells were subsequently treated with 0.5µg/ml RNase A for 2 hours. The genomic DNA was extracted by phenol-chloroform-isoamyl alcohol and was analyzed by gel electrophoresis at 50 volts for 90 minutes using 2% agarose. Approximately 20µg of genomic DNA was loaded in each well, visualized under ultraviolet (UV) light and photographed.

Immunoblot

To purify total proteins, cells were lysed in cold lysis buffer (10% v/v glycerol, 1% v/v Triton X-100, 1mM sodium orthovanadate, 1mM EGTA, 10mM NaF, 1mM sodium pyrophosphate, 20mM Tris, pH 7.9, 100µM β-glycerophosphate, 137mM NaCl, 5mM EDTA, 1mM PMSF, 10µg/ml aprotinin and 10µg/ml leupeptin), then homogenized and centrifuged. The supernatants were boiled in loading buffer and an aliquot corresponding to 100µg of protein separated by SDS-PAGE. After blotting, the membranes were incubated with anti-PADI2 (MDBio), anti-caspase-3 (Cell Signaling), anti-PARP (Cell Signaling), anti-vimentin (Santa Cruz), anti-citrulline (Upstate), and anti-actin antibodies (Santa Cruz) for 6 hours and the second antibody labeled with horseradish-peroxidase was adjacently incubated for 1 hour. The antigen-antibody complexes were visualized by the enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

Immunoprecipitation

Protein extracts (500µg per assay) were pre-absorbed with 1µg of anti-vimentin antibodies at 4°C for 1 hour, and then protein A/G agarose was added at 4°C overnight. After extensive washing, immunoprecipitated proteins were harvested and analyzed by immunoblot using anti-citrulline and anti-vimentin antibodies.

Fluorescence and differential interference contrast microscopy

After stimulation, JK-Tet-On-Vector and JK-Tet-On-PADI2 cells (1×10^6) were fixed in 2% paraformaldehyde at room temperature (RT) for 15 minutes, washed with PBS and cytospun on the coverslips. After blocking with 3% bovine serum albumin at RT for 2 hours, cells were stained with anti-vimentin antibodies (1:50) at 4°C overnight,

washed with PBS, and then incubated with goat anti-mouse IgG conjugated with rhodamine antibodies (Santa Cruz) at 37°C for 2 hours, respectively. Coverslips were mounted onto glass slides and examined by confocal microscopy.

Protein concentration

Following incubation, the conditioned media were collected and concentrated by means of an Amicon Ultra-15 centrifugal filtration device (Millipore) with a molecular weight cutoff at 30kDa.

Statistical analysis

Statistical analysis for significant differences between the control and experimental groups were evaluated using Student's t test.

RESULTS

Overexpression of PADI2 induces apoptosis of activated Jurkat cells

We generated a cell line that will overexpress PADI2 transgene from a tetracycline-inducible promoter in an otherwise isogenic background (Gossen et al., 1995). Briefly, we constructed *padi2* and *vector* genes into Tet-On expression plasmid (pTRE2hyg), respectively, and then transfected them to parental JK-Tet-On cells, namely "JK-Tet-On-PADI2" and "JK-Tet-On-Vector" cells. We used the combination of 12-O-tetradecanoylphorbol-13-acetate (TPA) 15ng/ml and ionomycin (Ion) 1μM (TPA/Ion) to induce the activation of Jurkat cells. After being treated with doxycycline (Dox) 50μM for 6 hours, and then activated by TPA/Ion for another 6 hours, JK-Tet-On-PADI2 cells expressed more PADI2 protein than JK-Tet-On-Vector cells did (Fig. 1A).

We observed the effects of PADI2 overexpression on cell viability of JK-Tet-On-PADI2 cells and their counterparts after being treated with Dox 10 or 50μM for 6 hours and then activated by TPA/Ion. The data revealed that the cells kept on proliferating in all the three groups of JK-Tet-On-Vector cells (+50μM Dox; +10μM Dox+TPA/Ion; +50μM Dox+TPA/Ion) and the group of JK-Tet-On-PADI2 cells treated only with 50μM Dox (Figure 1B). There were no statistical differences between these four groups. That is, PADI2 overexpression only does not cause cell death, even not influence cell proliferation. The same is Jurkat cell activation or different doses of Dox. However, when Jurkat cells with overexpressed PADI2 were activated (JK-Tet-On-PADI2+10μM Dox+TPA/Ion and JK-Tet-On-PADI2+50μM Dox+TPA/Ion), cell viability was decreased significantly in a time and dose dependent manner within 24 hours (Figure 1B). In short, overexpression of PADI2 decreases cell viability of activated Jurkat cells.

The morphologic changes of PADI2-overexpressed and activated Jurkat cells included chromatin condensation, membrane blebbing and shrinkage, and apoptotic bodies (Figure 2A). Jurkat cells without PADI2 overexpression (JK-Tet-On-Vector+Dox+TPA/Ion) or without activation (JK-Tet-On-PADI2+Dox+Control) showed the normal appearances of living cells (Figure 2A). DNA fragmentation (Fig. 2B), Caspase 3 activation and cleavage of PARP (Fig. 2C) presented in the PADI2-overexpressed and activated Jurkat cells (JK-Tet-On-PADI2+Dox+TPA/Ion), but not in others. All these evidences indicated that the cause of decreasing the cell viability of activated Jurkat cells with PADI2 overexpression is apoptosis.

Enhanced by cell activation, overexpression of PADI2 increases citrullinated proteins, including citrullinated vimentin

To explore the function of overexpressed PADI2 on JK-Tet-On-PADI2 cells, we detected citrullinated proteins and vimentin. Intracellular citrullnated proteins were detected by immunoblot with using anti-citrulline antibodies. Non-activated Jurkat cells with PADI2 overexpression (JK-Tet-On-PADI2+Dox) had more citrullinated proteins than their non-activated counterparts (JK-Tet-On-Vector+Dox). When Jurkat cells with PADI2 overexpression were activated (JK-Tet-On-PADI2+Dox+TPA/Ion), they had more citrullinated proteins than non-activated Jurkat cells with overexpressed PADI2 (JK-Tet-On-PADI2+Dox) (Figure 3A). We immunoprecipitated vimentin, and immunoblotted the precipitant with anti-citrulline antibodies. Again, citrullinated vimentin was increased 1.2 times in non-activated, PADI2-overexpressed cells (JK-Tet-On-PADI2+Dox), and 2 times in activated and PADI2 overexpressed cells (JK-Tet-On-PADI2+Dox+TPA/Ion), compared with the control (Figure 3B). These evidences revealed that PADI2 protein, overexpressed by transfected gene, is functional and will deiminate intracellular substrates, including vimentin. The enzyme activity is further enhanced by Jurkat cell activation.

Vimentin is involved in PADI2 induced apoptosis of activated Jurkat cells

To investigate the role of vimentin in PADI2-induced apoptosis, We transiently transfected vimentin into JK-Tet-On PADI2 and JK-Tet-On Vector cells for 24 hours to force express more amount of vimentin in cells (Fig. 4A). Overexpression of vimentin did not change the cell viability or proliferation of activated Jurkat cells without PADI2 overexpression (Fig. 4B). In activated Jurkat cells with PADI2 overexpression, the cell viability was significantly increased in cells with force-expressed vimentin than in those without. Overexpresison of vimentin rescued a part of Jurkat cells from apoptosis, caused by PADI2 overexpression (Fig. 4B). That is, vimentin is involved in PADI2 induced apoptosis in activated Jurkat cells.

Vimentin is expressed on cell surfaces and secreted out by activated Jurkat cells with overexpressed PADI2

It has been reported that vimentin is secreted out by activated macrophages (Mor-Vaknin et al., 2003) and is partially exposed on the surface of apoptotic T cells (Boilard et al., 2003). We wanted to know whether vimentin is expressed on cell surfaces and secreted during the course of PADI2 induced apoptosis. After JK-Tet-On-Vector and JK-Tet-On-PADI2 cells were treated with Dox 50μM for 6 hours, then with TPA/Ion, the supernatants were collected at 12 and 24 hours, and all the cells were harvested at 24 hours.

PADI2-overexpressed and activated Jurkat cells (JK-Tet-On-PADI2+Dox+TPA/Ion) were immunostained stronger than their counterparts by anti-vimentin antibody,

combined with rhodamine-conjugated anti-mouse secondary antibody (Fig. 5). We merged with the photographs of differential interference contrast microscopy (DIC) and fluorescence microscopy (VIM) and found that the binding sites of anti-vimentin antibody were on the cell surfaces. These evidences indicate that vimentin is expressed on cell surfaces of PADI2-overexpressed and activated Jurkat cells at the time they are going apoptosis.

The supernatants were concentrated by using a centrifugal filtration device (Millipore) with a molecular weight cutoff at 30kDa, and then analyzed by immunoblot with anti-vimentin antibodies. After activating JK-Tet-On vector cells, there were more vimentin in the supernatant collected at 24 hours, than in that collected at 12 hours (Fig. 6). At 12 hours, the supernatant of PADI2-overexpressed and activated Jurkat cells had more vimentin than the supernatant of their counterparts (Fig. 6). That is, cell activation and PADI2 overexpression promote the secretion of vimentin. However, at 24 hours, the supernatant of PADI2-overexpressed and activated Jurkat cells had less vimentin.

DISCUSSION

Triggering of the antigen-specific T-cell receptor (TcR)/CD3 complex initiates a cascade of signal transductions across the cell membrane, resulting in activation and proliferation. One signal transduction pathway involves a phospholipase C species that hydrolyses phosphatidylinositol 4,5-bisphosphate and generates two second messengers, diacylglycerol and inositol 1,4,5-trisphosphate (reviewed in Altman et al., 1990). Diacylglycerol activates protein kinase C (PKC) (Isakov et al. 1987; Nishizuka, 1988). Inositol 1, 4, 5-trisphosphate releases Ca^{2+} from the endoplasmic reticulum, leading to increase the intracellular concentration of free Ca^{2+} (Imboden and Stobo, 1985). Therefore, TcR/CD3-induced cellular response includes a rise in cytosolic calcium concentration and protein kinase C activation. The phorbol ester TPA, structurally related to diacylglycerol, directly binds to, and activates PKC (Castagna et al., 1982). The calcium ionophore, ionomycin, stimulates Ca^{2+} influx (Liu and Hermann, 1978). Thus, the combination of TPA and ionomycin mimics antigen-induced signal at the onset of lymphocyte activation (Truneh et al., 1985; Altman et al., 1992).

PADI2 and PADI4 are the only two isotypes expressed in the synovial tissue of patients with RA and those with other arthritides. Inflammatory cells, including lymphocytes and monocytes, are infiltrating in the synovial tissue and are the major source of PADI2 and PADI4. The expression levels of PADI2 and PADI4 are correlated with the intensity of inflammation. (Foulquier et al., 2007) In our study, Jurkat T lymphocytes have endogenous PADI2 protein. Six hours after using the combination of TPA and ionomycin to active Jurkat cells, we found that activated Jurkat cells expressed more PADI2 and has higher enzyme activity of PADIs (Data not shown). The expression of endogenous PADI2 may be transcriptionally regulated by TPA, because there are four plausible Sp1 binding sites in a PADI2 promoter region (Dong et al., 2005; Kim et al., 2004). Our PADI2-Tet-On system worked successfully to express exogenous PADI2 in activated Jurkat cells (Fig. 1A). And overexpressed PADI2 had enzyme activity, indirectly indicated by causing more citrulinated proteins shown in figure 3.

PADIs rely strongly on the presence of Ca^{2+} for activity. The usual cytosolic Ca^{2+} concentration is approximately 10^{-7} M, which is too low for activity of PADI2 (Asaga et al., 1998; Vossenaar et al., 2004b). The minimum Ca^{2+} concentration required for PADI2 activity is approximately 100-fold higher than the normal cytosolic Ca^{2+} (Takahara et al., 1986). Although non-activated Jurkat cells expressed endogenous and exogenous PADI2, most of the enzymes were inactivated because of the low cytosolic Ca^{2+} concentration. That is the reason that overexpression of PADI2 did not cause cell death of non-activated Jurkat cells. When Jurkat cells were activated,

raising the cytosolic Ca^{2+} concentration will activate PADI2 to deiminate intracellular substrates. In the meanwhile, we observed the activated cells with overexpressed PADI2 presented the typical manifestations of apoptosis. Here, we first provide the direct evidence that overexpression of PADI2 induces apoptosis of activated Jurkat cells.

Citrullinated proteins were detected in the inflammatory synovial fluid, not only from patients with RA, but also from patients with spondyloarthritis (Kinloch et al., 2008). However, the presentation of intracellular citrullinated proteins in the synovium is specific for RA (Baeten et al., 2001) and is co-localized with PADI2 (De Rycke, L., 2005). There are many well-known or suspected intracellular substrates of PADI2 or PADI4, such as vimentin (Asaga et al., 1998; Vossenaar et al., 2004b), histones (Nakashima et al., 2002), nucleophosmin/B23 (Hagiwara et al., 2002), F-actin capping protein alpha 1 subunit, cathepsin D, beta-actin (Matsuo et al., 2006), aldolase, alpha-enolase, Phosphoglycerate kinase 1, calreticulin, 60 kDa heat shock protein, the far upstream element-binding proteins 1 and 2 (Goëb et al., 2009), glucose-6-phosphate isomerase (Umeda et al., 2013) and IKKγ(Lee et al., 2010). Among these, vimentin is the major citrullinated protein in human monocytic leukemia THP-1 cells during macrophage differentiation (Hojo-Nakashima et al., 2009).

In our study, vimentin was one of target proteins of PADI2 in Jurkat cells during activation and/or apoptosis, as reported in mouse peritoneal macrophages and human ex vivo macrophages (Vossenaar et al., 2004b; Asaga et al., 1998) and human monocytic leukemia THP-1 cells (Hojo-Nakashima et al., 2009). Besides its mechanical and structural properties, vimentin plays important roles in adhesion, migration, survival and cell signaling (reviewed in Hendrix et al., 1996; Wang and Stamenovic, 2002). Not fully elucidated, the exact mechanisms of vimentin function have been attributed to the dynamic disassembly/assembly and spatial reorganization, regulated by phosphorylation (Inagaki et al., 1989; Ivaska et al., 2007). PADI2 deiminates predominantly the non-alpha-helical N-terminal head domain of vimentin, which is rich in arginine, serine and threonine, and prone to phosphorylation (Inagaki et al., 1989; Ivaska et al., 2007). Citrullination of vimentin results in the disassembly of the vimentin filaments, incompetence of the assembly (Inagaki et al., 1989), loss of functions and transformation of the fine network across the whole cell into amorphous clusters located around the nucleus (Asaga et al., 1998; Hojo-Nakashima et al., 2009).

The role of vimentin in apoptosis has been demonstrated in Withaferin-A (WFA) induced apoptosis (Lahat et al., 2010). WFA, a naturally derived bioactive compound, binds to vimentin by covalently modifying its cysteine residue, which is present in the

highly conserved alpha-helical coiled coil 2B domain (Bargagna-Mohan et al., 2007). It transforms vimentin filaments to punctate cytoplasmic aggregates that co-localize vimentin and F-actin (Bargagna-Mohan et al., 2007). Vimentin is degraded rapidly by casapses upon different pro-apoptotic stimuli, including ionizing radiation, Fas, TRAIL, tumour necrosis factor α (TNFα) and tamoxifen administration (Prasad et al., 1998; Morishima, 1999; Hashimoto et al., M., 1998). Inhibition of vimentin degradation (by caspase inhibitors or overexpress of caspase-resistant vimentin) abrogates WFA-induced apoptosis (Lahat et al., 2010). If vimentin is degraded by activated caspases, potential pro-apoptotic fragments will be released and markedly enhance apoptosis (Byun et al., 2001). A positive feedback loop is formed, whereby activated caspases degrade vimentin and these degraded fragments in turn activate caspases to amplify apoptosis.

Back to our topic, we found overexpression of vimentin rescued at least in part of overexpressed PADI2 and activated Jurkat cells. The cells with more vimentin exhibit more ability to stand the impact of citrullination and prevent the apoptosis. That is, vimentin is involved in the mechanism of PADI2-induced apoptosis. Overexpression of PADI2 increased citrullinated proteins, including citrullinated vimentin. Citrullinated vimentin lost its normal functions, and was probably unfolded and degraded by calpains. Taking together, the inferential mechanism of PADI2-induced apoptosis may be contributed to initiating the apoptotic signals by loss of normal vimentin functions, by the unfolded protein response, or by pro-apoptotic vimentin fragments.

Although vimentin is an intracellular protein, it is found to be expressed on cell membranes in some special conditions, such as by platelets and macrophages during activation (Podor et al., 2002; Mor-Vaknin et al., 2003), or by lymphocytes and neutrophils during apoptosis (Boilard et al., 2003; Moisan and Girard, 2006). Vimentin is partially exposed on the surface of apoptotic T cells and binds human group IIA phospholipases A2 (PLA2) via its rod domain in a calcium-independent manner (Boilard et al., 2003). The binding of these two proteins enhanced the activity of PLA2, suggesting that vimentin may play a role in PLA2-mediated cellular arachidonic acid release. Vimentin is expressed on the surface of apoptotic neutrophil (Moisan and Girard, 2006) and is cleaved by the neutrophil specific protease membrane-type 6 matrix metalloproteinase (Starr et al., 2012). Cleaved vimentin on the cell surface potently promotes phagocytosis and functions as an “eat-me” signal to macrophages. It shows another role of vimentin in increasing phagocytic removal of neutrophils to resolve inflammation (Starr et al., 2012). Compatible with previous studies, we found vimentin was expressed on the cell surface of PADI2-overexpressed and activated Jurkat cells at the time they were

going to apoptosis. However, our data cannot answer whether PADI2 contribute this cell-surface expression or expressed vimentin is citrullinated. More in-depth investigations are needed to resolve these questions.

In addition, vimentin can be secreted out by activated macrophages (Mor-Vaknin et al., 2003). Secretion of vimentin is blocked by anti-inflammatory cytokine interleukin-10, but triggered by pro-inflammatory cytokine TNFα. Extracellular vimentin is involved in bacterial killing and the generation of oxidative metabolites (Mor-Vaknin et al., 2003) and is a chemo-attractant for monocytes (Starr et al., 2012). We found vimentin was secreted by activated Jurkat cells, further enhanced by PADI2 overexpression in initial 12 hours of activation. However the amount of vimentin in the supernatant of PADI2 overexpressed and activated Jurkat cells was decreased 24 hours after activation. Citrullinated vimentin was found in all the supernatants (data not shown). But, we failed to demonstrate an explainable and constant result. The possible reason is there are many proteases in the intracellular and extracellular spaces, such as caspases, calpains and matrix metalloproteinases, which are activated during cell activation or apoptosis and degrade vimentin.

T lymphocytes are central to the specific immune response, and following their activation they are eliminated by a Fas-mediated cell death program (Savill, 1997). The presence of activated T cells in the inflamed synovium of patients with RA is a hallmark of the disease, and apoptosis is an important mechanism for their elimination and the eventual resolution of inflammation (Savill, 1997; Savill et al. 1989). It is generally believed that the apoptotic immune cells can become secondary necrosis and release their contents in the extracellular space when they are too much or can't be normally cleared. In extracellular space with the high Ca^{2+} concentration, PADI2 and PADI4 will citrullinate extracellular proteins which are then exposed to the immune system and elicit the formation of ACPAs (van Venrooij et al., 2011). Through artificially highlighting PADI2 and vimentin, we demonstrate PADI2 and vimentin participate in the apoptotic mechanisms of active T lymphocytes. Even in the normal course of T cell apoptosis, secreted and surface-expressed vimentin are possible pathways of antigen presentation to the immune systems.

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Figure legends

Figure 1. Overexpression of PADI2 decreases cell viability of activated Jurkat cells.
(A) JK-Tet-On PADI2 and JK-Tet-On Vector cells were treated with doxycycline (Dox) 50μM for 6h, then treated with 12-O-tetradecanoylphorbol-13-acetate (TPA) 15ng/ml and ionomycin (Ion) 1μM (TPA/Ion) for 6h. PADI2 and actin were detected by immunoblot with anti-PADI2 antibodies (PADI2) and anti-actin antibodies (Actin).
(B) JK-Tet-On PADI2 and JK-Tet-On Vector cells were treated with Dox 10 or 50μM for 6h, and then treated with or without TPA/Ion. Cell viability was determined at indicated time points by trypan blue exclusion assay. * $P < 0.05$ and ** $P < 0.01$.

Figure 2. Overexpression of PADI2 induces apoptosis of activated Jurkat cells.
JK-Tet-On-Vector and JK-Tet-On-PADI2 cells were treated with Dox 50μM for 6h, then treated with or without TPA/Ion for 24h.
(A) Cells were stained with acridine orange and analyzed by light microscope and fluorescent microscope.
(B) DNA fragmentation was detected by DNA gel electrophoresis.
(C) Total proteins were extracted and analyzed by immunoblot with anti-caspase 3 (Caspase 3) and anti-PARP (PARP) antibodies.

Figure 3. Jurkat cells with overexpressed PADI2 increase citrullinated proteins and citrullinated vimentin, and more after cell activation.
The amounts of citrullinated proteins and vimentin in Jurkat-Tet-On Vector and PADI2 cells were analyzed.
(A) JK-Tet-On-Vector and JK-Tet-On-PADI2 cells were treated with or without Dox 50μM for 6h, and then treated with or without TPA/Ion for 6h. Cells were harvested and total proteins were extracted for immunoblot with anti-citrulline antibodies.
(B) The cells were lysed, and the lysates were immunoprecipitated with anti-vimentin antibodies (VIM). The immunoprecipitates were analyzed by immunoblot with anti-vimentin (VIM) and anti-citrulline (Cit) antibodies. Data are quantified with a density meter, compared with the control group.

Figure 4. Overexpression of vimentin attenuates the apoptotic effect of PADI2 on activated Jurkat cells.
(A) JK-Tet-On-Vector and JK-Tet-On-PADI2 cells were transfected transiently with vimentin for 24h. Cells were harvested and total proteins were extracted for immunoblotting with anti-vimentin (Vimentin) and anti-actin (Actin) antibodies.
(B) Transfected and non-transfected cells were treated with Dox 50μM for 6h, and

then treated with TPA/Ion for 24h. Cell viability was determined by trypan blue exclusion assay. * $P < 0.05$.

Figure 5. Vimentin is expressed on cell surfaces of activated Jurkat cells with overexpressed PADI2.

JK-Tet-On-Vector and JK-Tet-On-PADI2 cells were treated with Dox 50μM for 6h, and then treated with TPA/Ion for 24h. Stained with anti-vimentin antibody, then rhodamine-conjugated anti-mouse secondary antibody (red), cells were observed and photographed by differential interference contrast microscopy (DIC) and fluorescence microscopy (VIM). Confocal merge showed the location of vimentin.

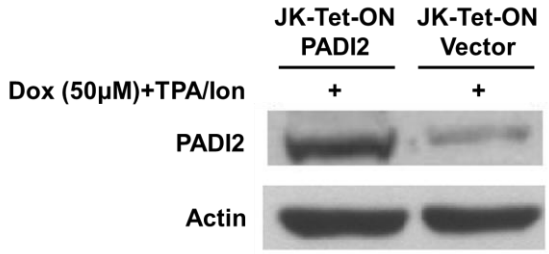
Figure 6. Vimentin presents in the supernatants of activated Jurkat cells.

JK-Tet-On-Vector and JK-Tet-On-PADI2 cells were treated with Dox 50μM for 6h, and then treated with TPA/Ion. The supernatants were collected at 12h and 24h, concentrated and then analyzed by immunoblot with anti-vimentin antibody (Vimentin).

Figures

Figure 1.

(A)



(B)

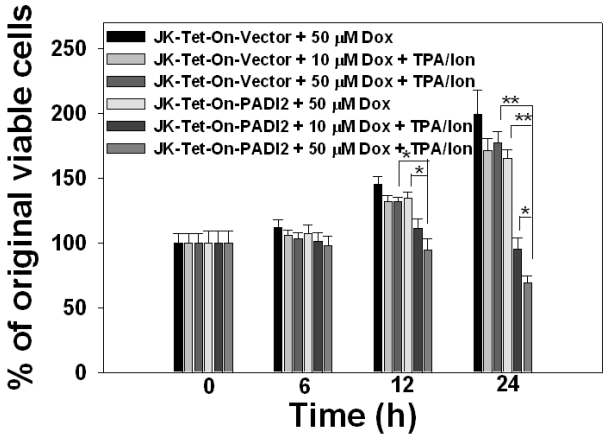
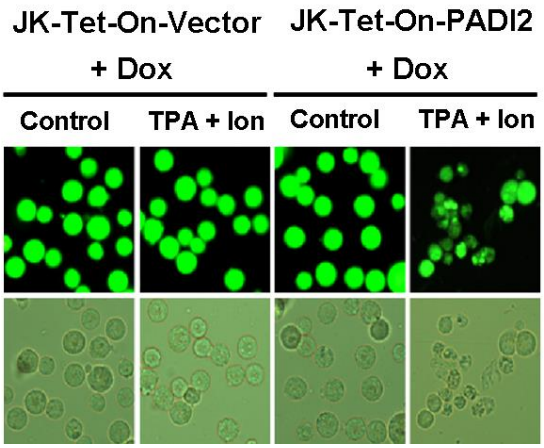


Figure 2

(A)



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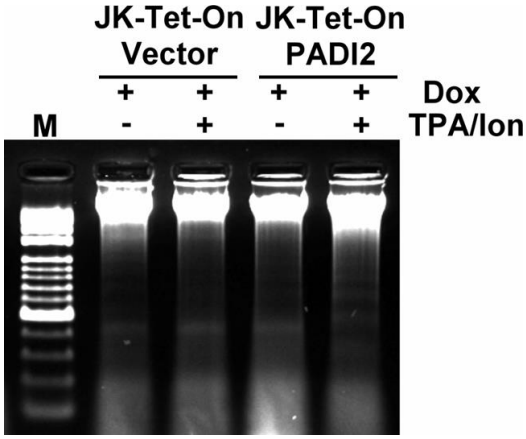
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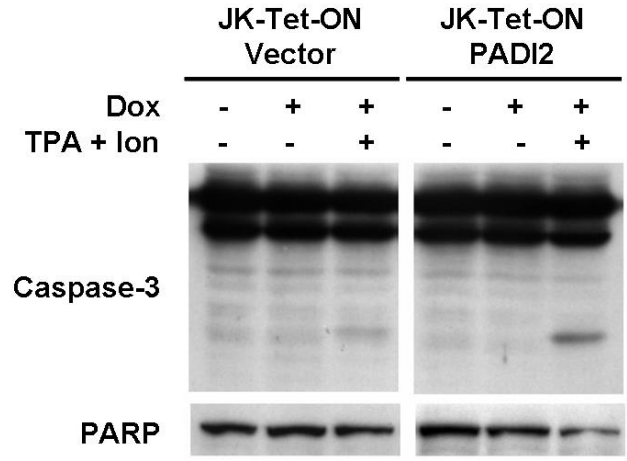
+ + + + Dox
M - + - + TPA/Ion

(C)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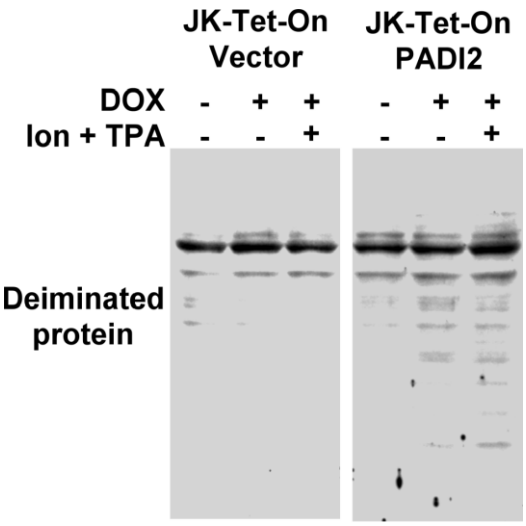
JK-Tet-ON PADI2
Dox - + + - + +
TPA + Ion - - + - - +
Caspase-3
PARP

Figure 3

(A)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JK-Tet-On PADI2
DOX - + + - + +
Ion + TPA - - + - - +
Deiminated protein

(B)

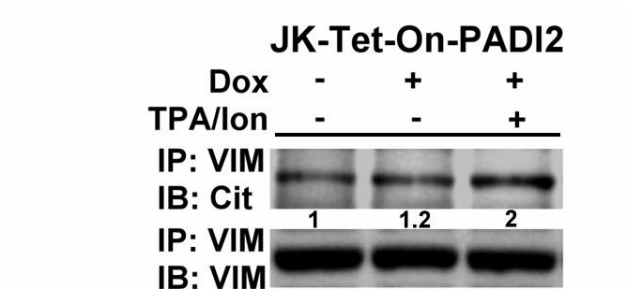
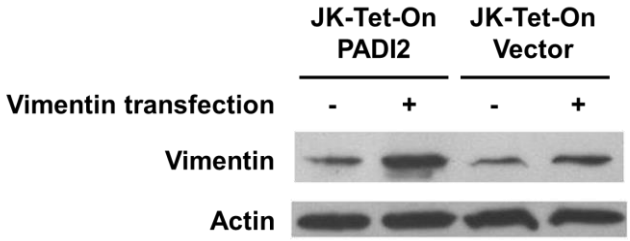


Figure 4

(A)



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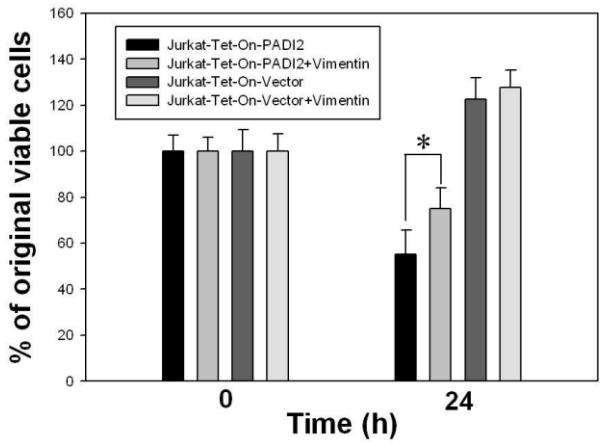


Figure 5

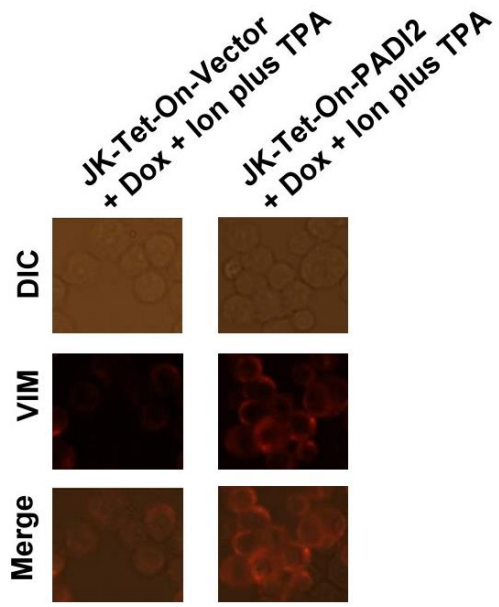
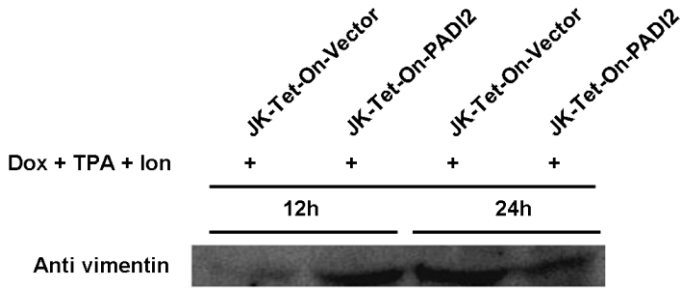

JK-Tet-On-Vector
+ Dox + Ion plus TPA
JK-Tet-On-PADI2
+ Dox + Ion plus TPA
DIC
VIM
Merge

Figure 6


JK-Tet-On-Vector
JK-Tet-On-PADI2
JK-Tet-On-Vector
JK-Tet-On-PADI2
Dox + TPA + Ion
+
+
+
+
12h
24h
Anti vimentin

Tables

Table 1 Demographical and clinical characteristics of study subjects

Variables	RA Patients (*n* = 103)	Controls (*n* = 113)	P value
Age	52.8±12.6	49.6±13.8	0.074†
Gender			0.780§
Male	15 (14.6)	18 (15.9)	
Female	88 (85.4)	95 (84.1)	
Anti-CCP3			< 0.001§
Positive	51 (49.5)	1 (0.9)	
Negative	52 (50.5)	112 (99.1)	

Note: § indicates using chi-square test, † indicates using unpaired t-test.

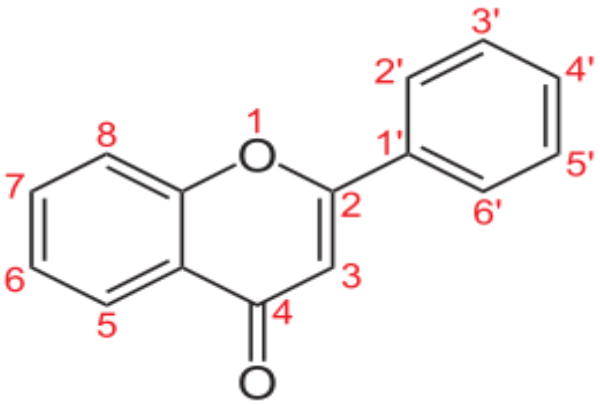
Table 2 The univariate analysis of association between RA susceptible *PADI4* SNPs gene types and RA

Variables	RA Patients	Controls	P value§
padi_89-A/G			0.852
A/A	30 (31.9)	34 (35.8)	
G/G	15 (16.0)	14 (14.7)	
A/G	49 (52.1)	47 (49.5)	
padi_90-C/T			0.485
C/C	30 (32.6)	40 (40.4)	
T/T	18 (19.6)	15 (15.2)	
C/T	44 (47.8)	44 (44.4)	
padi_92-C/G			0.643
C/C	31 (33.0)	38 (38.8)	
G/G	19 (20.2)	16 (16.3)	
C/G	44 (46.8)	44 (44.9)	

Note: § indicates using chi-square test.

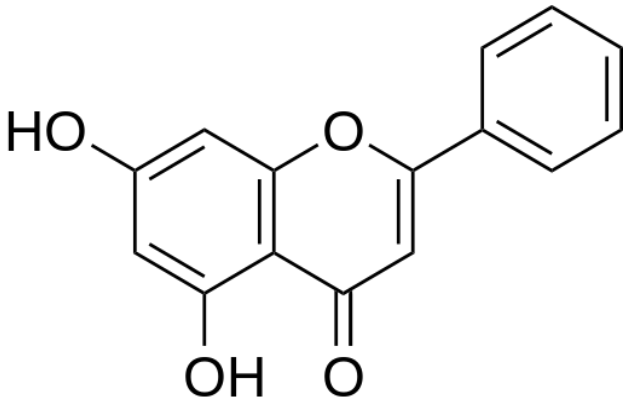
Analysis of the pinus extract

Flavones



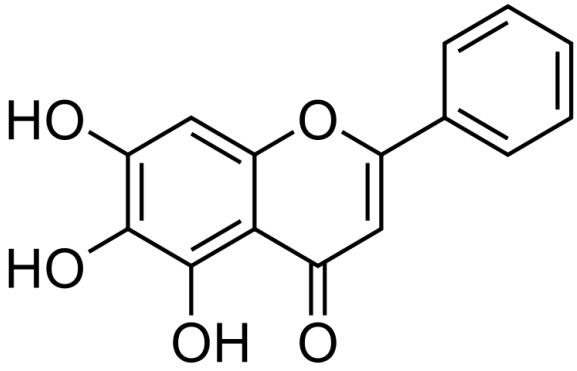
Flavone C

IC50<40 uM



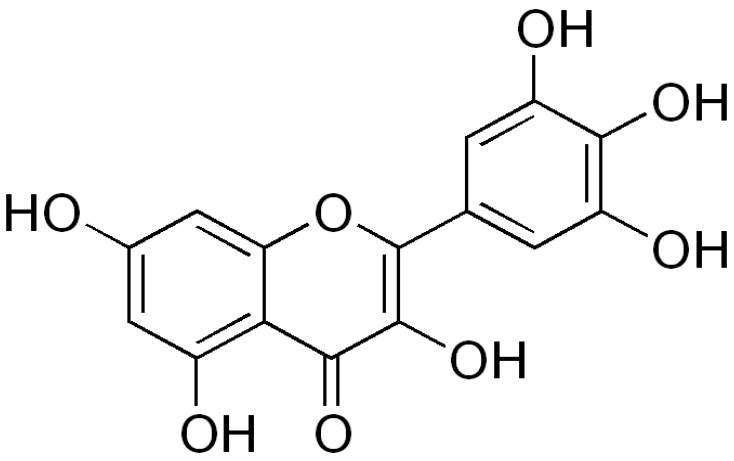
Flavone B

IC50<25 uM



Flavone M

IC50<37 uM



國科會補助計畫衍生研發成果推廣資料表

日期:2013/10/29

國科會補助計畫	計畫名稱: 臺灣本土植物臺灣五葉松萃取物對於類風濕性關節炎致病相關酵素“第二及四型胜?精胺酸脫亞氨?”生化及細胞凋亡作用的影響及其調節類風濕性關節炎之探究
	計畫主持人: 劉光耀
	計畫編號: 101-2320-B-040-017- 學門領域: 保健營養

無研發成果推廣資料

101 年度專題研究計畫研究成果彙整表

計畫主持人：劉光耀	計畫編號：101-2320-B-040-017-

計畫名稱：臺灣本土植物臺灣五葉松萃取物對於類風濕性關節炎致病相關酵素’第二及四型胜?精胺酸脫亞氨?’生化及細胞凋亡作用的影響及其調節類風濕性關節炎之探究

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	1	1	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	1	1	100%	人次	
		博士生	1	1	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	1	1	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	研究成果已進行國際論文投稿

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：□已發表 ■未發表之文稿 □撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：(以 100 字為限)

台灣臨床分析 PADIs, CCP, CCP 抗體間相關

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）(以 500 字為限）

與大陸,歐美分析,可供國內臨床參考