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中文摘要

免疫細胞創造一個複雜的細胞素環境促進癌細胞存活、血管新生、侵襲及轉移。然而要生長在這樣的環境，癌細胞表現的特定抗原必須進化成可以躲過免疫細胞的偵測及毀滅。因此，免疫逃脫是癌症發展重要的特徵，但是關於它分子基因機制的探討仍是缺乏。免疫逃脫跟侵襲及轉移等癌症惡化特徵間的相互作用依然是含糊不清的。侵犯及轉移性的癌細胞可以被具有相當活性的免疫系統給消滅，證明如果可以克服免疫逃脫，也許可以提供一個良好癌症治療的方法，但是免疫逃脫的機制必須先去了解。

IDO 是 kynurenine 途徑中分解色氨基酸的初始及速率決定步驟。老鼠胚胎中 IDO 活性對於防止同種異體胎的排斥作用扮演一個重要角色。藉由除去色氨基酸，IDO 可以防止 T 細胞淋巴球的增生。T 細胞淋巴球對色氨基酸的缺乏相當的敏感，會促使 T 細胞淋巴球停留在 G1 細胞週期。這些觀察指出 IDO 活性的表現可以藉由抑制 T 細胞淋巴球的增生來抑制免疫反應。IDO 活性的表現同時也可以在暴露於干擾素的細胞及活化巨細胞及樹突細胞中被觀察到，這些觀察可以證實 IDO 確實調控免疫反應。

上皮細胞生長因子接受器(HER, ErbB)家族(包含 HER1, HER2, HER3, and HER4)不正常活化會引起癌細胞的生長及惡化。在這四個 HER 家族中，HER2 是最強力的致癌蛋白。在 30%乳癌病人中，可以發現有 HER2 放大及過度表現的情形，臨床的治療效果會很差，此外也與癌細胞的轉移有正相關。除此之外，HER2 增加老鼠及人類癌細胞轉移的能力，並且在轉殖老鼠模式中發現 HER2 可以誘發乳癌轉移到肺。免役逃脫是癌症重要的特徵，但是相對於其他癌症特徵，如: 抵抗細胞死亡、血管新生、侵犯及轉移，關於免疫逃脫的分子機制仍然所知有限。最近的研究指出 IDO 對於免疫反應的調控扮演一個重要角色。HER2 過度表現提升癌症轉移的能力並且與較差的治療效果有關。在本計劃當中，我們初步的數據指出 HER2 會誘發 IDO 蛋白表現。因此，我們能釐清 HER2 跟 IDO 間的關係。本計劃的中心假說是希望能找出是否 HER2 在癌症轉移的地方誘發 IDO 表現，引起 T 細胞活性下降，近一步在那裡建立轉移的癌細胞。本計劃長遠的目標是希望藉由改善癌症所引起的免疫力下降來治療乳癌。

Abstract

Immune cells create a complex cytokine environment that promotes cancer cell survival, angiogenesis, invasion and metastasis. To survive in the environment, however, cancer cells expressing recognizable tumor antigens must evolve strategies to thwart immune detection and destruction. Immune escape is thus a hallmark of cancer progression, but its underlying molecular genetic basis remains poorly understood. The interplay between immune escape and other hallmarks of malignant conversion, such as invasion and metastasis, is similarly obscure.

IDO is an enzyme catalyzing the initial and rate-limiting step in the catabolism of tryptophan along the kynurenine pathway. The IDO activity of mouse placenta has an essential role in preventing rejection of allogeneic fetuses. By depleting tryptophan locally, IDO seems to block the proliferation of alloreactive T lymphocytes. T

lymphocytes are extremely sensitive to tryptophan shortage, which causes their arrest in the G1 phase of the cell cycle. These observations introduced the concept that IDO expression could suppress immune responses by blocking T-lymphocyte proliferation locally.

The aberrant activation of the human epithelial growth factor receptor (HER, ErbB) family of receptor tyrosine kinases (RTKs), which includes HER1 (ErbB1, epithelial growth factor [EGFR]), HER2 (ErbB2, neu), HER3, and HER4, has been implicated in tumor growth and progression. Of the four HER family members, HER2 is the most potent oncoprotein. HER2 is amplified or overexpressed in about 30% of breast cancers and is associated with a poor clinical outcome, including a positive correlation with metastasis. In addition, HER2 increases the metastatic potential in murine and human cancer cell lines and induces mammary tumors and lung metastases in transgenic animal models.

Immune escape is a central hallmark of cancer, but compared to other recognized hallmarks of cancer—apoptotic resistance, angiogenesis, invasion and metastasis—much less is known about the genetics of immune escape. Recently, IDO was found to play a very important role in the regulation of immune responses. Overexpression of HER2 enhances the metastatic potential and correlates with poor prognosis. Here, our data show that HER2 upregulates IDO expression.

計劃緣由與目的

The HER2 gene, also known as neu or erbB2, encodes a 185 kDa transmembrane receptor tyrosine kinase belonging to the epidermal growth factor receptor (EGFR) family. Overexpression of HER2 is found in about 30% of human breast cancers and in many other cancer types. The HER2 phosphorylates downstream substrates and activates a variety of signaling cascades, including the phosphatidylinositol-3 kinase (PI3K)/Akt and Ras/MAPK pathways. These regulatory signal cascades promote cell survival and tumor growth and metastasis. It has already been used as a target for cancer therapies such as trastuzumab (Herceptin), an anti-HER2 antibody that has shown a good clinical benefit in patients with HER2-driven metastatic breast cancer. Although the functionality of HER2 in breast cancer has been extensively studied. its role in tumor progression is still poorly completely understood, especially its role in tumor immune escape.

IDO, an enzyme that specifically catabolizes tryptophan, is an amino acid essential for T cell viability and proliferation. IDO is found under basal conditions in the epididymis, thymus, gut, lung, placenta, and some subsets of dendritic cells. IDO was originally discovered in 1967 in the rabbit intestine and is the object of renewed attention by immunologists in view of its capacity to act as an inducible negative regulator of T cell viability, proliferation, and activation during inflammation. In addition to potential in direct effects by IDO on APC function, IDO has been proposed to suppress T cells by degrading tryptophan and increasing the level of tryptophan degradation products (kynureneria and quinolinate). Both of these activities suppress T cell response by inducing T cell apoptosis. Recently, IDO has been found in human cancers of variable origin and is implicated in tumor evasion in several murine models. Due to its ability to suppress immune response endogenous tumor Ags, IDO represents an ideal target for immunomodulatory drugs.

Our preliminary study suggested a link between HER2 over-expression and upregulation of IDO. IDO expression was also shown to predict tumor immune escape. We were interested in addressing the question of whether HER2 could enhance IDO transcription and identifying the downstream molecules of HER2 that are involved in this process. In the present study, the mechanism of the IDO expression induced by HER2 mediated signaling was investigated and HER2-overexpressing cancer cells drive immune escape and provide crucial evidence of a function between the HER2 and IDO.

方法與結果

- **Increased expression of IDO in HER2-overexpressing cancer cell lines**

It is well known that HER2 enhances cancer progression and metastasis, and the Immunomodulatory protein IDO is involved in the metastasis and immune escape of cancer. We hypothesized that IDO plays a role in HER2-mediated tumor metastasis and immune escape. To test this hypothesis, we examined IDO expression in different breast and ovarian cancer cell lines by western blotting (WB) analysis. We found IDO was highly expressed in HER2 over-expression cell lines.(Fig.1A, B) By using FACS, the expression of IDO of HER2 over-expression breast cancer cells, MDA-MB-453, was higher than HER2 basal level cells, MDA-MB-231.(fig.1C) The results show that increased expression of IDO in HER2-overexpressing breast and ovarian cancer cell lines.

- **HER2 the enhances Immunomodulatory protein IDO expression**

It has been reported that the level of HER2 on tumor cells affected the production of proteolytic enzymes. Aberrant HER2 activation has also been demonstrated to increase immune escape and metastasis by stimulating the expression of several key molecules including MMP-1 and MMP-9. To investigate whether HER2 over-expression associates with IDO, HER2 basal level breast cancer cell lines, MCF-7 and MDA-MB-435 were transfected with the plasmid pcDNA3.1/HER2. The stable clones over-expressing HER2 were selected by G418 and the increased level of HER2 expression confirmed by Western blot. The western blotting data show that IDO expression was higher in the HER2 transfectants of MDA-MB-435 breast cancer cells (435/HER2) and MCF-7/HER2 than that in the vector control cells (435/neo) and parental cell, MCF-7 (fig.2 A. B). In addition, this phenomenon was supported in two independent HER2 stable transfectants of MDA-MB-435 and MCF-7 breast cancer cells by Western blot and also observed in the NIH 3T3 cell and its HER2 stable transfectant (Fig2 C).

The increase in IDO expression by HER2 was further supported by fluorescence confocal microscopy. The 435/HER2 and MCF-7HER2 cells expressed much higher levels of IDO than did the vector control cells (435/neo) and parental cell (MCF-7) (red) (Fig3 A.B). And we determined IDO expression by fluorescence-activated cell sorting (FACS) analysis and found that IDO expression was higher in the HER2 transfectants of MDA-MB-435 breast cancer cells (435/HER2) than that in the vector control cells (435/neo) (Fig 3C).

- **HER2 knocked-down reduced IDO level in HER2-overexpressing cells**

The HER2 status was confirmed using an HER2-shRNA plasmid. To further

examine whether HER2 is required for the enhanced IDO expression in HER2-overexpressing cancer cells, we used a HER2-shRNA, that is known to target HER2 mRNA and knock-down the HER2 expression. By using FACS and western blotting, we found that HER2-shRNA decreases the IDO expression in HER2 stable transfectants MDA-MB-435/HER2 cells which is compared to untransfected cell, tansfection reagent only(mock) and luciferase-shRNA transfected. Similar results were also observed by using FACS to demonstrate the IDO expression was down-regulated in HER2-shRNA transfected MDA-MB-435/HER2 cells (Fig.4 A.B). Down-regulating IDO by HER2-shRNA was also observed in endogenous HER2 overexpressing SKOV3 cells western blotting (Fig.4C). Therefore, these results indicate that HER2 is able to increase the expression of the immuosuppression protein IDO.

- **HER2/neu induced the expression of IDO at transcriptional level**

To investigate whether HER2 over-expression associates with the transcription of IDO, MCF-7 cells were stable transfected with the plasmid pcDNA3.1/HER2. The stable clones overexpressing HER2 were selected by G418 and the increased level of HER2 expression. Semiquantitative RT-PCR analysis showed that IDO mRNA expression at transcriptional level was very low in MCF-7 cells. The result shows that mRNA expression was significantly increased in MCF-7/HER2 cells (Fig.5A). To determine whether over-expression of HER2 affects IDO promoter activity, the plasmid containing a luciferase reporter gene driven by IDO promoter (pIDO-Luc) was constructed and MCF-7 and MCF-7/HER2 cells were transfected with pIDO-Luc. Parental MCF-7 cells ransfected with pLuc1267 was used as a control. The effect of HER2 on the IDO promoter activity was assessed by luciferase assay. Luciferase activities from MCF-7/HER2 cells showed an almost thirteen fold increase compared with the control cells. And MCF-7/HER2 co-transfected HER2-shRNA and pIDO-Luc were shows down-regulation of IDO promoter activity (Fig. 5B). It’s suggesting that HER2 over-expression induced IDO promoter activity.

- **Effect of HER2-triggered Ras pathway on IDO expression**

HER2 phosphorylates downstream substrates and activates a variety of signaling cascades, including the phosphatidylinositol-3 kinase (PI3K)/Akt and Ras/MAPK pathways and enhance protein synthesis. We next examined whether the enhancement of IDO protein synthesis by HER2 might occur through the PI-3K/Akt or Ras pathway. If PI3K or Ras is involved in the HER2-enhanced IDO systhesis, blockage of the HER2 downstream signals such as PI-3 kinase or mTOR or Erk or JNK should inhibit HER2-induced IDO expression. Treatment of different inhibitors, including the PI3K/Akt signaling inhibitors for PI-3 kinase (LY294002) and and mTOR (rapamycin) or Ras signaling inhibitors for MEK1 inhibitor (PD98059) and JNK inhibitor (SP600125) indeed inhibited CXCR4 expression in MCF-7/HER2 cells. We then determined whether the activity of the IDO promoter and IDO mRNA could be inhibited by inhibitors. Aftertransient transfection with pIDO-Luc into MCF-7/HER2 cells and then treated inhibitors, the IDO promoter activity was measured. As shown in Fig. 6A, that the expression of the MEK1 inhibitor (PD98059) and JNK inhibitor (SP600125) significantly reduced HER2-induced IDO promoter activity. Further confirmed by using RT-PCR, we detected IDO mRNA after treated signaling inhibitors. The result shows that IDO mRNA

were reduced after the treatments of MEK1 and JNK inhibitor (Fig. 6B).

討論

The RTK HER2 and the immunomodulatory protein HER2 are two different type protein, but the our study demonstrates that HER2 enhances IDO expression and that IDO is required for HER2-mediated tumor immune escape, therefore resolving a longstanding puzzle of how HER2 overexpression guides cancer cells metastasis and tumor-derived immunosuppression. IDO expression was recently found to be correlated with tumor immune escape. In view of the fact that HER2 over-expression is a known marker of poor prognosis in breast cancer, our observations add strong support for the identified mechanism, namely HER2 enhancement of IDO expression.

Immunosuppression and evasion can be achieved through a variety of mechanisms, many of which are well-characterized, experimentally supported, and at the disposal of cancer cells. These include secretion of Th2-associated cytokines such as IL-10 or TGF-ß leading to Th2 polarization, over-expression of Fas ligand/TRAIL, overexpression of complement inhibitors (DAF and CD55), and overexpression of HLA-G protecting against NK-induced lysis. A novel mechanism of tumor-derived immunosuppression through IDO has gained attention recently and has stimulated research in cancer immunology. Our results demonstrate HER2 could directly stimulate IDO gene expression in breast cancer cell lines and HER2 increased IDO transcripts in human cancer cells. However, the mechanisms of the transcription regulation of IDO gene are largely unknown.

It has been known that HER2 over-expression is related to structurally related proteases, MMP-2, 7 and MMP-9 expression which is associated with tumor metastasis. In our study, increased mRNA and protein expressions of IDO were observed in MCF-7 stable transfected with HER2. The promoter activity of IDO gene was markedly upregulated in MCF-7/HER2 cells. When we knocked-down HER2 expression of cancer cell by using RNA interference, an decreased promoter activity and protein expression of IDO was observed in both parental SKOV3 and MCF-7/HER2 cells. In depth, we found that IDO was regulated with HER2 through RAS signaling pathway.

計劃成果自評

根據本研究結果證明，不論是在蛋白質層次、基因轉錄層次或 mRNA 表現等方面都清楚得知 HER2 扮演 IDO 調控者的角色，此調控的機制是經由 HER2/Ras 路徑。因此，未來研究方向將可專注於找出 Ras 路徑訊號傳遞下游轉錄因子的確認，並深入瞭解 HER2 調控 IDO 基因表現及 HER2 過度表現腫瘤細胞誘發免疫逃脫之完整作用機轉，進而不只對於 IDO 基因倍增或 IDO 過度表現提供一條新的標靶治療策略，且在癌症的免疫治療提供一個新的治療遠景。

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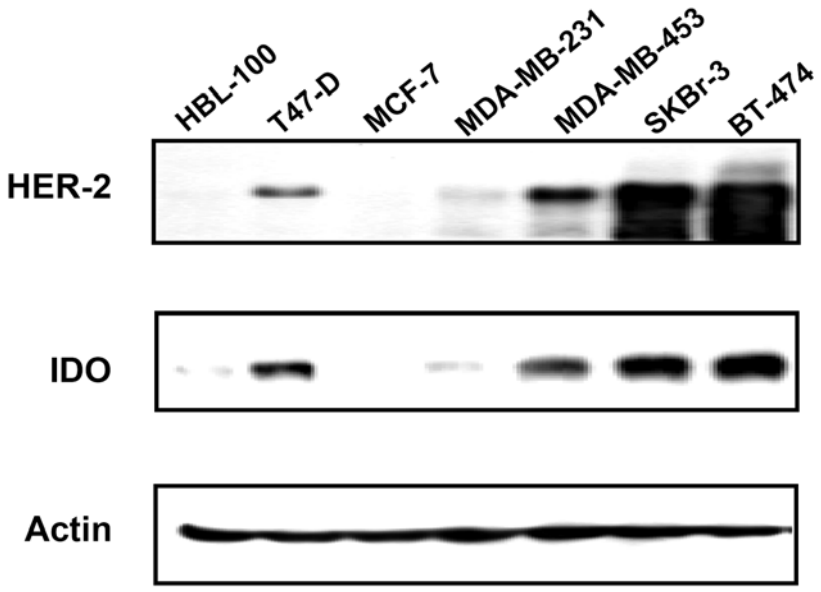
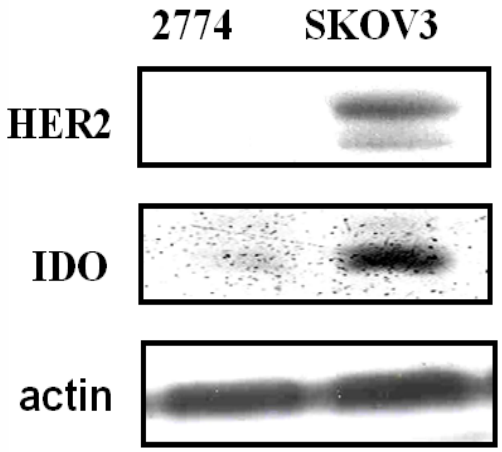
Figure 1

(A)



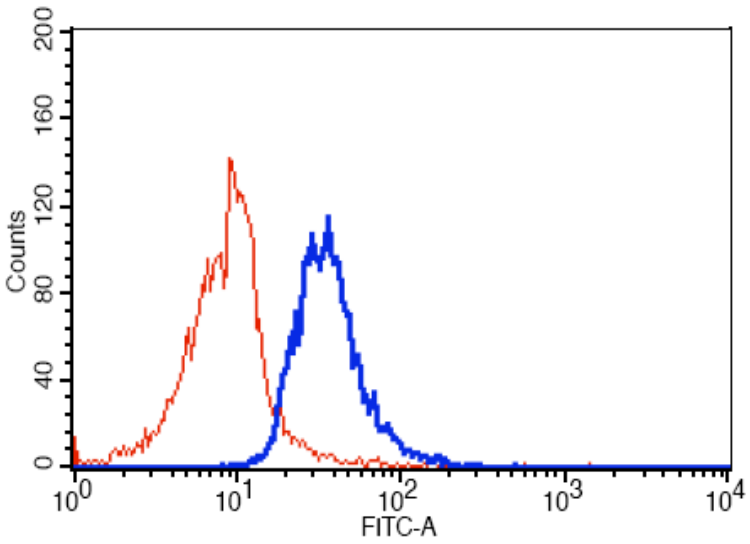
(A)HER2 enhances the IDO expression. HER-2 and IDO in various breast cancer cell lines were examined by western blotting.

(B)



(B) Increased expression of IDO in HER2-overexpressing ovarian cancer cell lines

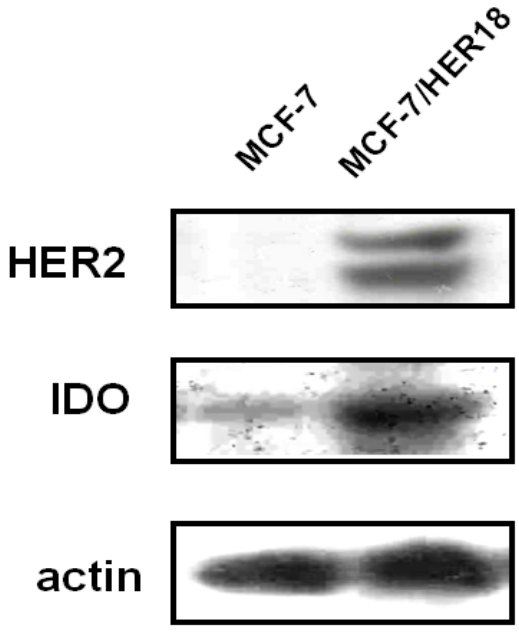
(C)



(C) MDA-MB-453 and MDA-MB-231 cells were stained for the IDO antibody and analyzed using FACS

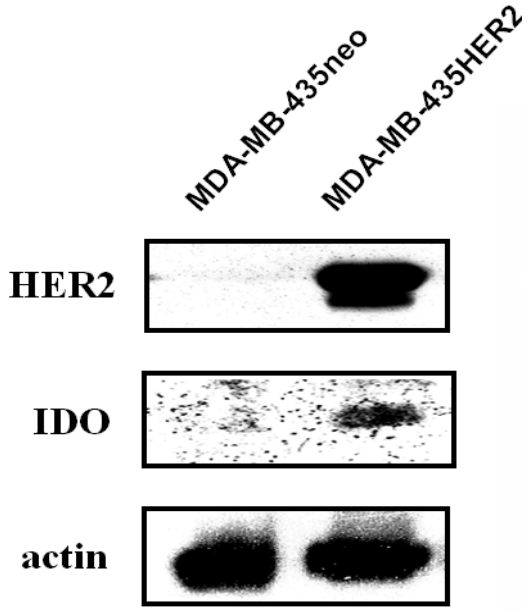
Figure 2

(A)



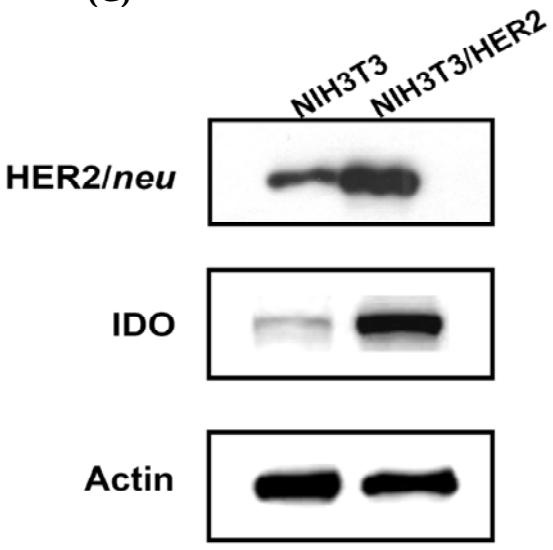
(A) Over-expression of IDO in HER2-transfected MCF-7/HER2 stable cell lines.

(B)



(B) Over-expression of IDO in HER2-transfected MDA-MB-435/HER2 stable cell lines.

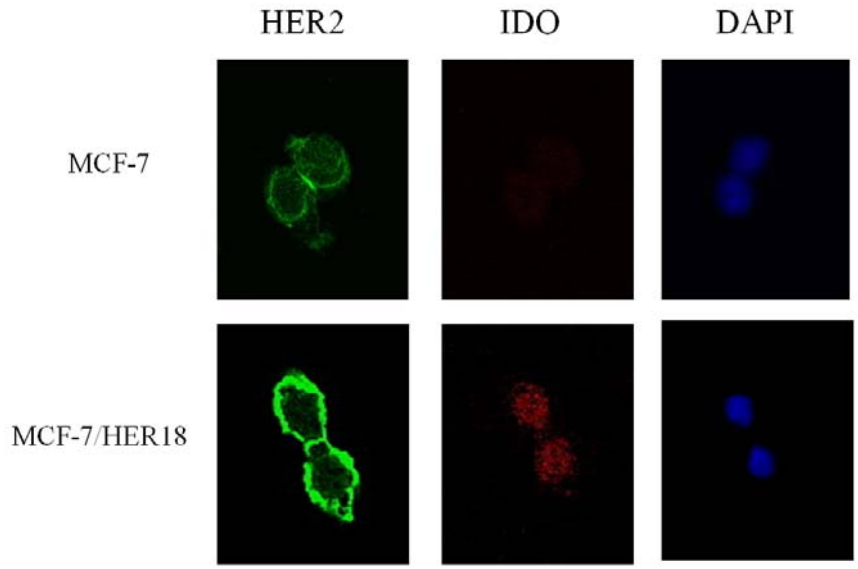
(C)



(C). NIH3T3 cells and their HER2 stable transfectants were stained for the HER2, IDO and Actin antibody and analyzed using Western blot.

Figure 3

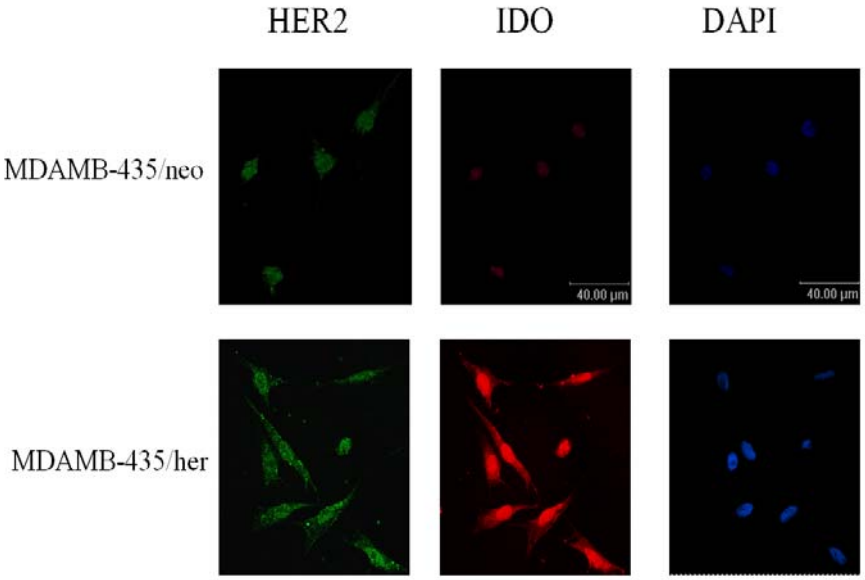
(A)



(A)(B) HER2 enhances IDO expression demonstrated by immunofluorescent staining

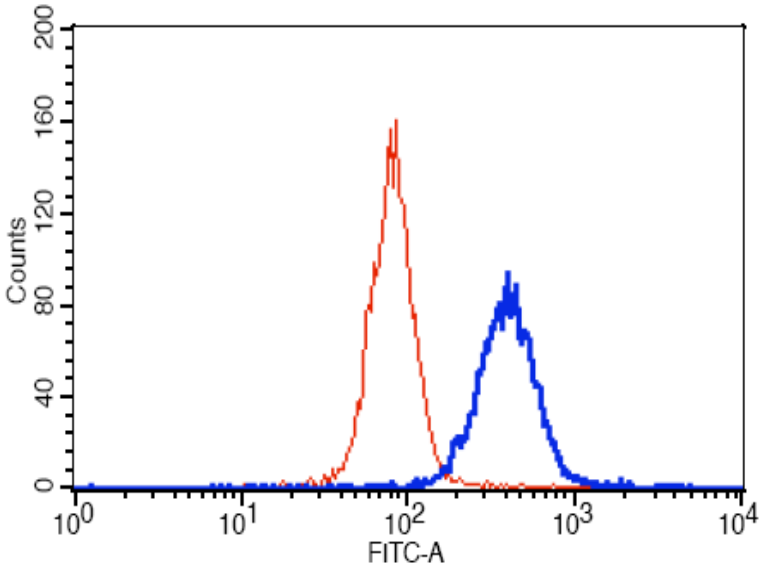
(A) MCF-7 and MCF-7/HER2 cells were stained with anti-HER2 (green) and anti-IDO (red) antibodies and examined under a confocal microscope

(B)



(B) 435/neo and 435/HER2 cells were stained with anti-HER2 (green) and anti-IDO (red) antibodies and examined under a confocal microscope

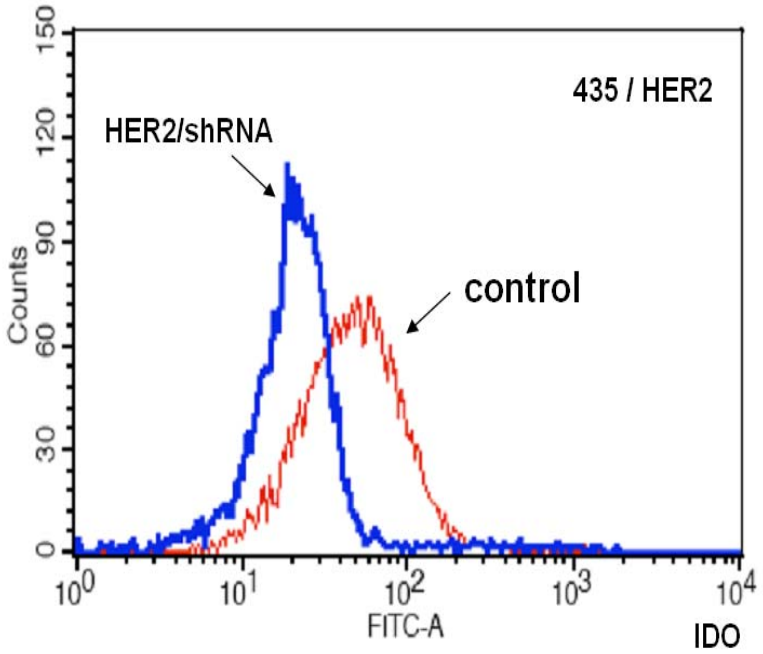
(C)



(C) MDA-MB-435 and their HER2 stable transfectants cells were stained for the IDO antibody and analyzed using FACS.

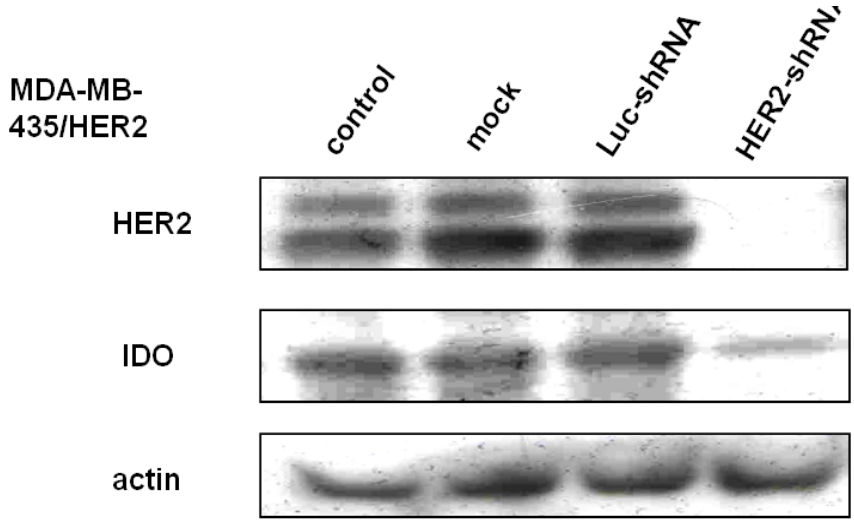
Figure 4

(A)



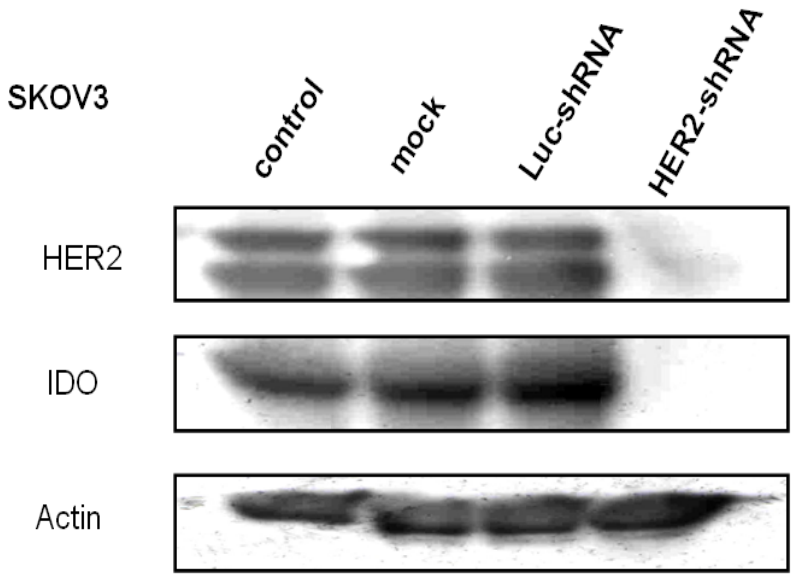
(A) MDA-MB-435/HER2 cells were treated with HER2 siRNA and subjected for FACS analysis.

(B)



(B) HER2-shRNA reduces IDO level in HER2-overexpressing cells MDA-MB-435/HER2 cells were transfected with HER2-shRNA.

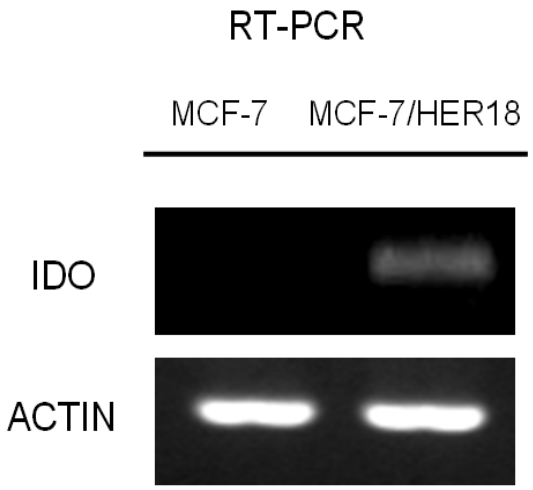
(C)



(C) HER2-shRNA reduces IDO level in HER2-overexpressing SKOV3 cells were transfected with HER2-shRNA

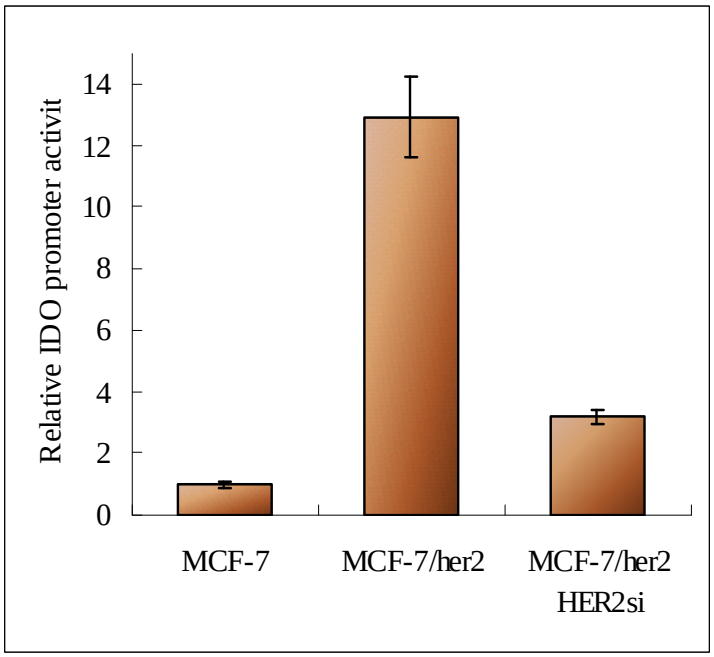
Figure 5

(A)



(A) Amplification of IDO mRNA in HER2-overexpression cell line.

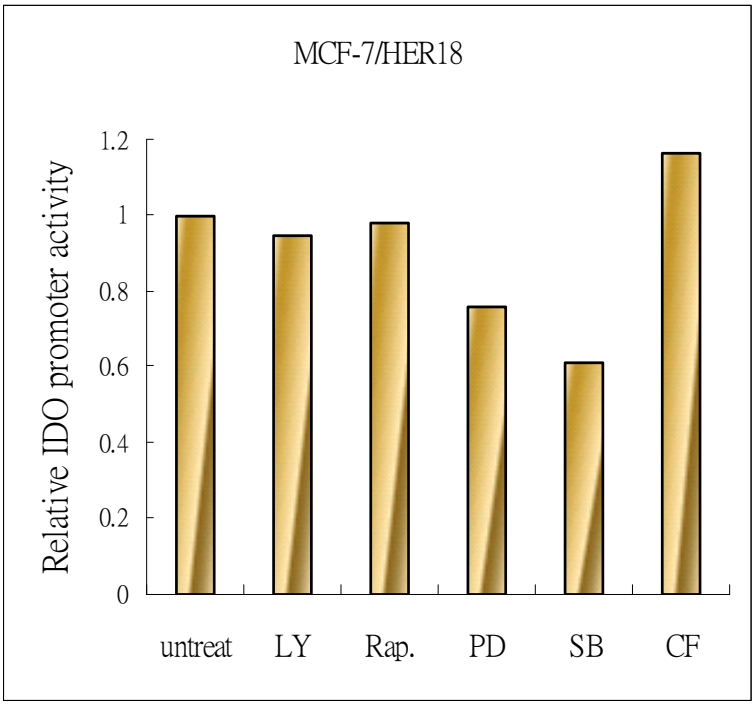
(B)



(B) Luciferase activities from MCF-7/HER2 cells showed increase of IDO promoter activity compared with the MCF-7 cells. However, HER2-shRNA may reduce IDO promoter activity.

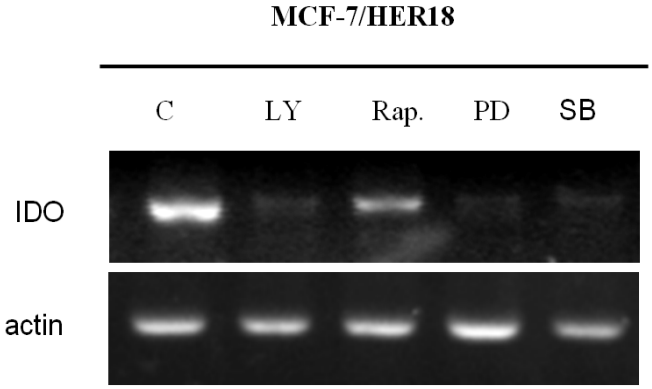
Figure 6

(A)



(A) Effect of HER2 signaling pathway on IDO promoter activity

(B)



C ：untreat

SB ：JNK inhibitor

LY ：LY294002, PI3K inhibitor

PD ：PD98059, MEK1 inhibitor

Rap. ：Rapamycin, mTOR inhibitor

(B) Effect of HER2 signaling pathway on IDO mRNA expression