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動情激素受體在乳癌生成進程之角色探討(第 2 年)
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（計畫名稱）

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一、中文摘要

乳癌為台灣女性第二常見的腫瘤，其發生率和致死率近十年內增加，且有年輕化之趨勢。基於癌細胞發生腋下淋巴或者是遠端組織器官的腫瘤轉移，往往對生命產生嚴重的威脅性。再者，癌細胞發生腋下淋巴轉移的預後評估較差，因此，對婦女乳癌而言，探討乳癌癌化進程乃至於發生淋巴轉移的機轉，便有其特殊意義的急迫性。本研究計畫中，我們嘗試在腫瘤細胞的發展過程中，以定量real time RT-PCR技術針對特定標的基因的表現量，探討與乳癌腋下淋巴轉移的關聯。我們從152例乳癌病患，透過雷射顯微擷取(laser capture microdissection)將其乳癌組織和乳部相鄰之正常組織檢體加以雷射分離後，進行real-time RT-PCR (qRT-PCR)，結果發現在乳癌預後的研究標記裏，(i) CCND1 (cyclinD1)和乳癌分期(stage)，(ii) CTNNB1 (β-catenin)和乳癌大小(tumor size) (iii) MTA-1 (Metastatic tumor antigen-1)和乳癌腋下淋巴轉移(lymphnode metastasis)呈正相關 (P<0.05)。另外，當乳癌細胞株以$siRNA_{ER}$進行基因靜默試驗，cyclin D1 和β-catenin 的表現會因為ER 的關閉而將低表現程度，這暗示著細胞週期調控(G1/S)的基因表現會與ER存在相關。再者，在ER關閉後的乳癌細胞株MCF-7中，其MTA-1的表現也受也受到明顯的抑制。針對多基因的複合性表現程度的探討，將有利於對台灣地區婦女乳癌發展進程提供完整重要的研究訊息，更可以做為國人婦女乳癌預後的評估指標。

關鍵字：乳癌，雷射顯微擷取，動情素受體蛋白，Cyclin D1，β-catenin，MTA-1

二、英文摘要

Metastasis is the major cause of mortality for breast cancer and knowledge of the molecular mechanism involved in the progression of breast carcinomas from primary stage to metastatic tumor is of particular clinical importance. Three candidate genes, *CCND1* (*CyclinD1*), *CTNNB1* (*β-catenin*) and *MTA-1* (*Metastatic tumor antigen-1*) have been considered as molecular markers in cancer prognosis evaluation. Real time reverse transcriptase-polymerase chain reaction (qRT-PCR) was used to detect both primary tumor and the corresponding tumor-adjacent normal tissues among 152 breast cancer patients. To support our hypothesis came from the observations that the associations between upregulated expression of the targeted genes of (i) *CCND1* (cyclin D1) and tumor staging, (ii) *CTNNB1* (β-catenin) and tumor size, and (iii) *MTA-1* (Metastatic tumor antigen-1) and auxiliary lymphnode metastasis (P<0.05)。On the basis of cell model, an increasing expression of β-catenin was seen in ER-negative Hs578 cells. By contrast, a decreased level MTA-1 was found in Hs578T and siRNA knockdown MCF-7 cells. Based on a method of standard and reproducible quantification, our results suggest that differential expression of *CCND1*, *CTNNB1* and *MTA-1* genes in tumor tissues can be regarded as suitable markers for prognostic evaluations with respect to tumor staging, size and lymphnode metastasis in breast cancer.

Key words：Breast cancer, Laser capture microdissection, ER, CCND1, β-catenin, MTA-1

報告內容

一、計畫緣由與目的

Tumorigenesis is a multistep process resulting from a series of genomic alterations which lead to the progressive disordering of the normal mechanisms controlling growth, death, and differentiation of the cell [Fearon and Vogelstein, 1990]. To account for the high frequency of genomic alterations required for tumor progression, it has been suggested that the genomes of cancer cells are unstable and that mutators cause these genomic instabilities (the "*mutator phenotype*" theory) [Leob, 1998]. Almost all known mutators, such as the genes repairing DNA damage, are involved in the formation of *irreversible* genetic mutations. However, clonal evolution during tumorigenesis, in particular, tumor metastasis, is characterized by unstable, phenotypic heterogeneity, which fluctuates too frequently to be mediated exclusively by these irreversible changes and their upstream mutators [Graff et al., 2000]. Furthermore, in metastatic lesions, re-expression of certain cancer-associated genes that have been lost during tumor initiation is sometimes essential for metastatic tumor cells to survive [Toyota et al., 1999]. For breast cancer development, the estrogen-stimulated growth in tumor cells as well as in normal cells requires the estrogen receptor (ER).

The human metastatic tumor antigen 1 (MTA1) is a constituent of the nucleosome-remodeling and -deacetylation complex. MTA1 was originally cloned by differential screening of the cDNA library from rat mammary adenocarcinoma metastatic cells [Toh et al., 1994]. It has been reported that MTA-1 expression is highly correlated with the invasion and metastasis of epithelial neoplasms [Hofer et al., 2004; Bagheri-Yarmand et al., 2004]. Recently, in the comparative analyses, it has been revealed that MTA1 mRNA differentially expressed in nonmetastatic and metastatic tumors, pointing out MTA1 is involved in enhancing the metastatic potential for malignant tumor cells [Nicolson et al, 2003]. Moreover, there were growing evidences suggested that MTA1 plays the crucial role as a corepressor of ligand-induced transactivation function of estrogen receptor-α (ER-α) in breast cancer cells [Kumar et al., 2002; Talukderet al., 2004].

Cell cyclin family spread out the divisions of the cell cycle and is responsible for the cell cycle stay during the replication of newly DNA synthesis and repairing of the damaged DNA [Vermeulen et al., 2003; Pawlik and Keyomarsi, 2004]. During cell cycle, D- and E-type cyclins underwent premature DNA synthesis in G1 phase. On the basis of molecular research, aberrant expression of cyclin D1 has been well documented in human breast cancer [Steeg and Zhou, 1998]. Similarly, over expression of cyclin D1 associated with breast cancer progression and served to be an earlier marker in cancer diagnosis has been demonstrated in comparative genome hybridization analysis [Lung et al., 2002]. In addition, cyclin D1 mRNA and protein are frequently over expressed in breast tumors but the gene is uncommonly amplified [Courjal et al.,1996; Bieche et al.,2002]

With regard to *β-catenin* gene, it partly belongs to be the membrane-bound cell growth-signaling complex that plays a role in cell adhesion, as well as in promotion of growth through activation of the *Wnt* signaling pathway [Moon et al., 2005]. Clinical evidences have

been appeared that β-catenin play a key role in the development of colorectal cancer and has been implicated in prostate and breast cancer as well [Chesire et al., 2002; Chesire et al., 2003; Giles et al., 2003; Hovanes et al., 2003; Li et al., 2002]. Induction for cell proliferation through oncogenic signaling has implicated that *β-catenin* releases and accumulates in the cytoplasm, translocates into the nucleus and promotes transcription, and consequently, activates many downstream genes are, such as *cyclin D1*, *c-myc* and *bcl* [Polsky D and Cordon-Cardo, 2003]. In recent, there had been reported that estrogen receptor (ER-α) and β-catenin precipitated within the same immunocomplexes by using human colon and breast cancer cells which implies these two signaling pathway reciprocally converge for the transactivation of cognate reporter genes [Kouzmenko et al., 2004].

In an effort to establish a methodological framework for analysis of molecules and mechanisms involved in this complex multistep process, in this study, we aimed at developed an experimental system with quantitatively real-time reverse transcription polymerase chain reaction (qRT-PCR) in detecting these three candidate genes in relation to the evaluation of tumor development. The effects of heterogeneity within a tumor were minimized by ensuring that genetic and phenotypic examinations are being carried out on the same tumor cells by using the method of laser capture microdissection (LCM) which allows a more precise evaluation of specific associations between genetic and pathological manifestation.

二、研究方法

Questionnaire

An experienced research nurse was assigned to administer a structured questionnaire to each breast cancer patients. The information collected included age at diagnosis, family history of breast cancer (first-degree relatives), history of breast biopsy, history of breast screening, age at menarche and/or menopause, parity, age at FFTP, number of pregnancies, history of breast feeding, use of oral contraceptives, HRT, history of alcohol consumption and cigarette smoking, ethnic background, residence area, family income, and education level. The BMI and menopausal status were also recorded.

Laser capture microdissection (LCM)

To ensure that tissue samples assayed consisted of >90% tumor cells, LCM was performed on routinely immunostained slides using a PixCell laser capture microscope (Arcturus Engineering, MountainView, CA) as described previously [Emmert-Buck, et al., 1996] with minor modification. Briefly, the stained, dehydrated tissue section was overlaid with a thermoplastic film mounted on an optically transparent cap. The visually selected areas (tumor cells) were bound to the membrane by short, low-energy laser pulses, resulting in focal melting of the polymer. The LCM captured cells were immersed in 50−100 μl of digestion buffer, containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 400 μg/ml proteinase K, and 1% Tween 20, and digested at 55°C overnight. After digestion, the enzyme was heat inactivated (95°C for 10 min), and the extractwas used directly for RNA isolation.

RNA preparation and detection of the targeted gene expressions by qRT-PCR

Total RNA was extracted from tumors and normal breast tissues of individual cases using an RNA extraction kit (RNeasy) (QIAGEN, Valencia, CA, USA). Stepwise, the RNA (1 μg in a volume of 5 μl) was reverse-transcribed for 70 min at 42°C using 5 units of Superscript II reverse transcriptase (Gibco-BRL, Gaithersburg, MD, USA) and 10 mM random primers of oligo(dT) 15 primer (Promega, Madison, WI) in a reaction volume of 20 μl. cDNA concentrations were determined by spectrophotometry. Substantially for qRT-PCR reaction, 25 ng of the total RNA-reverse transcribed cDNA product was subjected in totally 25 μl of Universal PCR Master Mix (Applied Biosystems), 900 nM forward primer, 900 nM reverse primer, 200 nM probe and nuclease-free water were added to a final volume of 50 μl. Amplification and detection steps were performed with the ABI Prism 7700 sequence detection system (Applied Biosystems) (Fig. 2). G6PD gene was used as the internal positive control in each qRT-PCR batch. The differential expression level of the relevant gene in cancer patient defines as the ratio when the tumor tissue and surrounding non-cancer tissue of breast were compared in each case.

Cell Culture and Reagents

The human breast cancer cell lines, MCF-7 and Hs578T were purchased from the American Type Culture Collection (Manassas,VA) and grown in Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies, Inc., Grand Island,NY) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 mg/ml streptomycin, and 100 units/ml penicillin. The cell lines were cultured at a constant temperature of 37°C in a 5% carbon dioxide-humidified atmosphere.

Transfection with siRNA of ER-a

The siRNA duplexes of ER-α siRNA was purchased from Santa Druz, Biotechnology Inc. (CA., USA). Cell line, MCF-7 (estrogen receptor positive) was transfected with siRNA using Oligofectamine protocol (Invitrogen-Life Technologies, Inc., La Jolla, CA). Briefly speaking, 24 h before transfection, the cells were seeded, without antibiotics, at 1 x 10^6 cells/60-mm dish, corresponding to 40–50% confluency at the time for siRNA transfection. The cells were then incubated for 4 h at 37°C with various concentrations of ER-α siRNA plus Oligofectamine in Opti-MEM I medium, then fresh culture medium containing 30% fetal bovine serum was added in the ratio of 1 volume/3 volumes of the transfection medium.

Western blotting analysis

Each protein extract was collected in the ice-cold TEGN lysis buffer containing 0.5% NP-40, 50mM Tris-Cl, 150 mM NaCl, 1 mM EDTA and 10% glycerol. The protease inhibitors including 1 μg/ml aprotinin, 0.5 μg/ml leupeptin and 100 μg/ml 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF) were added to the cell lysis suspension. The tissues were homogenized and the cell extracts were gently rotated at 4°C for 60 min. After centrifugation at 13,000 rpm for 15 min, the precipitate was discarded, and supernatant protein concentrations were determined by the Bio-rad protein assay kit (Bio-Rad laboratories, CA USA). Equal amounts of proteins (40–60 μg/well) were subjected to electrophoresis by 10-12% sodium

dodecyl sulfate-polyacrylamide gels. After electrophoresis, transfer of proteins from the gel onto polyvinylidene difluoride (PVDF) membranes, they were sequentially hybridized with specific primary antibodies (Santa Cruz, CA) and followed with a horseradish peroxidase-conjugated second antibody (Santa Cruz, CA). The protein bands were visualized using the chemiluminescence detection procedure according to the manufacturer's recommendation of Western blotting luminal reagent (Santa Cruz Biotechnology, CA., USA). To verify the equal protein loading and transfer, β-actin was used as the protein loading control. In addition, the intensity of the bands was scored and classified as positive and overexpressed for these protein expressions.

三、結果與討論

To evaluate the role of differential expressions of these tumor markers in association with breast cancer development, 152 patients histologically proven breast cancer underwent curative mastectomy at department of surgery, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan. The clinico-pathological characteristics of studied breast cancer patients were summarized in Table 1. Among them, 81.6% (124 out of 152) tissues were clinically staged as earlier tumor disease (Stage I and II) and 18.4% were tumor of poorer differentiation (Stage III and IV). Negative examination for estrogen receptor was found in 82 patients (53.9%), by contrast, 67 (44.1%) cases were ER-positive at the time of diagnosis, respectively. LNM was detected in 39.4% (60 out of 152) of patients. Additionally, all the patients have been approved by the ethics committee board of the department of surgery, Tri-Service General Hospital, National Defense Medical Center.

The paired primary tumor cells and corresponding adjacent non-tumor on the same patients of breast tissues were carefully collected by laser capture microdissection (Fig. 1A and 1B). Stepwise, those cDNA transcripts were all subjected into qRT-PCR analysis for examining the targeted genes, including *CCND1, CTNNB1,* and *MTA-1,* and β-actin gene was used as the internal control in each qRT-PCR batch. As shown in Table 2, up-regulated expression of *CCND1* was found in tumor tissues differed from their corresponding normal breast tissues in the same patient, showing a 6.84-fold risk in cancer patients with larger tumor size (T3 and T4) (P=0.048). Similarly, *MTA-1* gene is increased expression in cancer tissues of larger tumor sizes, however, this did not reach to statistically significant.

For the prediction of these three markers in tumor staging, we analyzed the up-regulated expression of *CCND1, CTNNB1,* and *MTA-1* in tumor laser-captured tissues compared to that of non-tumor parts. In univariate analysis, up-regulated expression of *CCND1* in tumor tissue was seen at a higher frequency in breast cancer patient having poorer stages (86.9%, 20/23) than that expressed in well-differentiated tumors (32.2%, 19/59) (P<0.0001). Similarly, increased expression of *CCND1* was significantly detected in tumor tissues of stage II (P=0.035) and stage IV (P=0.0034), respectively. On the other hand, neither *CTNNB1* nor *MTA-1* gene correlates with tumor staging of breast caner patients (Table 3).

As to tumor metastasis, we examined the association between these three genes and

breast cancer patients having positivity of lymph node. Higher percentage of up-regulated expression of MTA-1 gene was found in cancer patients with metastatic in their lymph nodes when compared to those who without lymphnode metastasis (54.7% vs 45.3%)(*P*=0.0007). However, either *CCND1* or *CTNNB1* gene dose not associate with the tumor status of positivity of lymphnode in breast cancer (*P*=0.485 for *CCND1* and *P*=0.394 for *CTNNB1*, respectively) (Table 4).

On the other hand, to discriminate the role of ER in contribution to tumor cell development, including tumor cell metastasis, we first measured the expression level of these three proteins. Immunoblot assays showed that a reduction in protein expression of MTA-1 was observed in Hs578T cells. Similarly, this reduced expression of MTA-1 was observed in siRNA ER-α knockdown MCF-7 cells in comparison with MCF-7 cells that were not treated with (Fig. 2). *β-catenin* gene, partly belongs to be the membrane-bound cell growth-signaling complex that plays a role in cell adhesion as well as in promotion of growth through activation of the *Wnt* signaling pathway [Moon et al., 2005]. Downregulation in the levels of β-cat protein results in decreased translocation of β-cat into nucleus, this associates with transcription factors of the T cell factor (TCF) and lymphocyte-enhancing factor (LEF) family to form multiprotein complexes that regulate genes important for proliferation. In Hs578T cells, b-cat protein expresses higher level than that expressed either in MCF-7 or in MCF-7 treated with ER-α siRNA (Fig. 2).

By using a cell model with transfection of siRNA ER-α knockdown, we further found that the reduced expression of β-catenin was associated with ER-α knockdown MCF-cells. As shown in Fig. 3, decreased expression of PI3-kinase p85 subunit is associated with knockdown of ER-α, which inactivates phosphorylation of Akt protein. Activation of Wnt signaling leads to inhibition of GSK-3 activity, resulting in accumulation of cytoplasmic β-catenin, which becomes available to bind the T cell factor (Tcf)/Lymphoid enhancer factor (Tcf/LEF) family of transcription factors. The inhibitory effect due to the lack expression of ER-α results in reduction of PI3-kinase/Akt and degradation of β-catenin. In addition, β-catenin-related downstream genes are activated in response to ER-α, such as *cyclin D1*. However, there was no significant decreased expression of cyclin D1 after MCF-7 cells with treatment of siRNA ER-a (Fig. 3). As to cyclin D1 expression in ER-a siRNA-treated MCF-7 cells may be due to some transcrption factors and kinase-related pathways, and further studies will necessitate to understand the real molecular functions of ER-a and b-catenin in modulation of cell cyclin D1 on growth regulation of breast cancer.

In the present study, we provide a more validated data, especially in the aspect of MTA-1 mRNA level, to prove the involvement of MTA-1 as a biomarker, being validated in risk assessment of breast cancer having lymphnode metastasis. Since it has been reported that MTA-1 over expresses in human tumor cell lines and tissues [Xue et al., 1998; Zhang et al., 1999; Mazumdar et al., 2001; Mahoney et al., 2002]. Enhanced expression of MTA-1 mRNA was also found in a variety of human cancerous tissues and carcinoma cell lines, including colorectal, gastric [Toh et al, 1997], or oesophageal [Toh et al, 1999] carcinomas and thymoma

[Sasaki et al, 2001]. Moreover, MTA-1 plays the crucial role as a corepressor of ligand-induced transactivation function of ER-α in breast cancer cells [Mazumdar et al., 2001; Kumar et al., 2003; Talukderet al., 2004; Acconcia et al., 2005]. Our results address the issue of estrogen receptor signaling in relation to breast cancer risk by using a pure colonal of cancer cells, and semiquantitatively examined the differential expression of MTA-1. Findings of this study can provide the earlier marker for prognostic evaluation in breast cancer with metastatic behavior. In conclusion, based on a method of standard and reproducible quantification, our results provide the evidences supporting that up-regulated expression of *CCND1* is associated with poorer differentiation of breast cancer, i.e. larger tumor size and higher tumor stage, and MTA-1 up-regulation is associated with lymph node metastasis of breast caner.

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五、計畫成果自評

本研究根據民國九十一年起至九十五年期間，自三軍總醫院婦女乳癌病患所建立起癌症組織資料庫，以雷射顯微擷取(laser capture microdissecion)分離癌組織和相鄰之正常組織後，再以反轉錄聚合酶連鎖反應將cDNA加以進行轉錄。利用定量Real-Time RT-PCR將乳癌患者之乳房病灶及其相鄰正常乳房上皮細胞為研究材料、結合臨床問卷調查，探討與淋巴移轉相關基因之表現程度差異的分子遺傳學研究，以釐清特定基因標記的表現量在婦女乳癌發展過程中所扮演的角色。我們發現*CCND1*基因的過度表現與乳癌之聲長大小和乳癌分期達顯著統計相關。再者，*MTA-1* 基因的表現也和乳癌之淋巴轉移有關，且達顯著統計差異 (OR=3.20, 95%CI=1.52-6.76; P=0.0007)。再者，經由探討乳癌細胞株因ER *siRNA*的基因關閉實驗，發現β-catenin蛋白質表現降低其表現是經由PI3-kinase/Akt的訊息調控。綜合而言，本研究結果著重於探討乳癌ER-α對於乳癌臨床預後評估的重要性。藉由對多基因的變項分析，更精準的以雷射捕獲組織檢體，外加以定量基因檢測分析，為國內建立起基因表現程度標記與乳癌發生、分期、腫瘤轉移和預後關聯性研究的資料庫。這些研究成果可提供解釋台灣近年來乳癌惡化病程的可能機轉和其相關聯的遺傳標記，有助於對乳癌致癌成因和乳癌治療的預後探討外；更可以進一步以此做為未來發展有效的乳癌治療策略。

六、圖表

Table 1. Clinicopathological characteristics of 152 breast cancer patients

Clinicopathology	Number	%
Tumor size		
T1	92	60.5
T2	53	34.9
T3+T4	7	4.6
Stage		
I	59	38.8
IIa+IIb	65	42.8
III	23	15.1
IV	5	3.3
Lymph node		
N0	91	59.9
N1	39	25.6
N2	21	13.8
N.A.	1	0.7
ER		
Negative	82	53.9
Positive	67	44.1
N.A.	3	2.0
PR		
Negative	79	52.0
Positive	70	46.0
N.A.	3	2.0

Table 2. The association between expression of individual *CCND1*, *CTNNB1* and *MTA-1* genes and tumor size in breast cancer

Gene profile	*CCND1*		aOR (95% CIs)	*P value*[#]
Tumor size	(T≧N)*	(T<N)		
T1	43 (46.7%)	49 (53.3%)	1.00 (Ref)	
T2	30 (56.6%)	23 (43.4%)	1.49 (0.71-3.11)	*P*=0.254
T3/T4	6 (85.7%)	1 (14.3%)	6.84 (0.76-156.77)	*P*=0.048
				Ptrend=0.097
	CTNNB1		aOR (95% CIs)	
Tumor size	(T≧N)*	(T<N)		
T1	50 (54.2%)	39 (45.8%)	1.00 (Ref)	
T2	30 (49.2%)	21 (50.8%)	1.11 (0.52-2.38)	*P*=0.762
T3/T4	3 (36.4%)	4 (63.6%)	0.59 (0.10-3.35)	*P*=0.497
				Ptrend=0.724
	MTA-1		aOR (95% CIs)	
Tumor size	(T≧N)*	(T<N)		
T1	35 (39.3%)	54 (60.7%)	1.00 (Ref)	
T2	26 (50.0%)	26 (50.0%)	1.54 (0.73-3.26)	*P*=0.218
T3/T4	5 (71.4%)	2 (28.6%)	3.86 (0.61-30.61)	*P*=0.098
				Ptrend=0.160

*, aOR was adjusted according to the combination with constant- and up-regulated expressions of the respective genes; #, $p<0.05$ represents statistically significant.

Table 3. The association between expression of individual *CCND1*, *CTNNB1* and *MTA-1* genes and tumor size in breast cancer

Gene profile	*CCND1*		aOR (95% CIs)	*P value*[#]
Stage	(T≧N)*	(T<N)		
I	19 (32.2%)	40 (67.8%)	1.00 (Ref)	
IIa+IIb	38 (58.5%)	27 (41.5%)	2.96 (1.33-6.33)	*P*=0.0035
IIIa+IIIb	20 (86.9%)	3 (13.1%)	14.04 (3.34-68.21)	*P* <0.0001
IV	4 (80.0%)	1 (20.0%)	8.42 (0.72-212.34)	*P*=0.0034
				Ptrend<0.001
	CTNNB1		aOR (95% CIs)	
Stage	(T≧N)*	(T<N)		
I	32 (54.2%)	27 (45.8%)	1.00 (Ref)	
IIa+IIb	30 (49.2%)	31 (50.8%)	0.82 (0.37-1.78)	*P*=0.815
IIIa+IIIb	8 (36.4%)	14 (63.6%)	0.48 (0.16-1.47)	*P*=0.155
IV	2 (40.0%)	3 (60.0%)	0.56 (0.06-0.40)	*P*=0.528
				Ptrend=0.543
	MTA-1		aOR (95% CIs)	
Stage	(T≧N)*	(T<N)		
I	19 (32.2%)	40 (67.8%)	1.00 (Ref)	
IIa+IIb	30 (47.6%)	33 (52.4%)	1.94 (0.86-4.28)	*P*=0.083
IIIa+IIIb	14 (60.9%)	9 (39.1%)	3.27 (1.18-10.08)	*P*=0.018
IV	3 (60.0%)	2 (40.0%)	3.16 (0.38-30.04)	*P*=0.212
				Ptrend=0.077

*, aOR was adjusted according to the combination with constant- and up-regulated expressions of the respective genes; #, $p<0.05$ represents statistically significant.

Table 4. Frequency distribution of differential expression status of *CCND1*, *CTNNB1* and *MTA-1* genes and estimated odds ratio (OR) in relation to breast cancer risk with LNM

mRNA expression[a]	LNM-positive	LNM-negative	OR (95% CIs)	*P* value[b]
CCND1				
Constant/Down-regulated (T≦N)	39 (41.5%)	55 (58.5%)	1.00 (Ref)[c]	
Up-regulated (T>N)	20 (35.7%)	36 (64.3%)	0.78 (0.37-1.64)	*P*=0.485
CTNNB1				
Constant/Down-regulated (T≦N)	30 (41.1%)	43 (58.9%)	1.00 (Ref)[c]	
Up-regulated (T>N)	25 (34.2%)	48 (65.8%)	0.75 (0.36-1.54)	*P*=0.394
MTA-1				
Constant/Down-regulated (T≦N)	23 (27.4%)	61 (72.6%)	1.00 (Ref)[c]	
Up-regulated (T>N)	35 (54.7%)	29 (45.3%)	3.20 (1.52-6.76)	*P*=0.0007

a, three statuses for the evaluation of mRNA expression were categorized as described in Materials and methods. b, $p<0.05$, represents statistically significant. C, Ref, reference group.

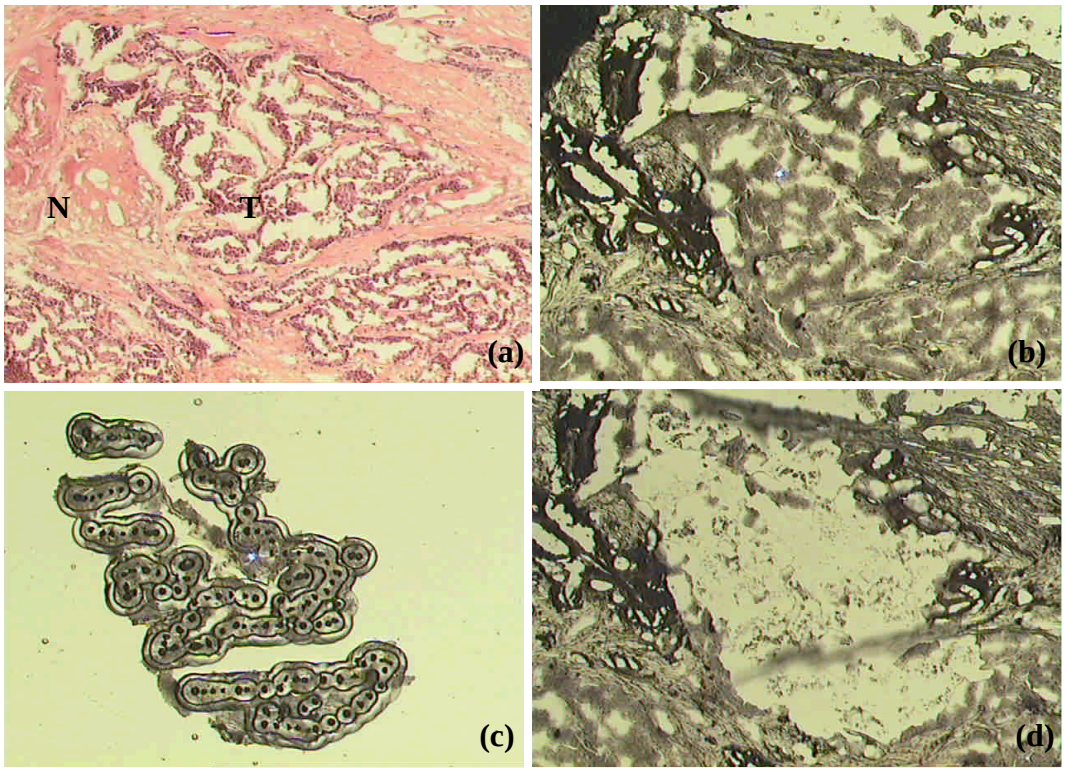

Fig. 1A. Isolation of breast tumor cells using laser capture microdissection technique. (a) hematoxylin and eosin stain of the breast cancer resection. T, tumor and N, tumor-adjacent normal part; (b) combined images of a serial section of (a) with the cancer cells marked for LCM; (c) Post-LCM image of laser capture and (d) LCM captured cancer cells. Cancer tissues collected from breast cancer patient who was clinically characterized T2, stage IV and positivity of lymphnode metastasis (N2).

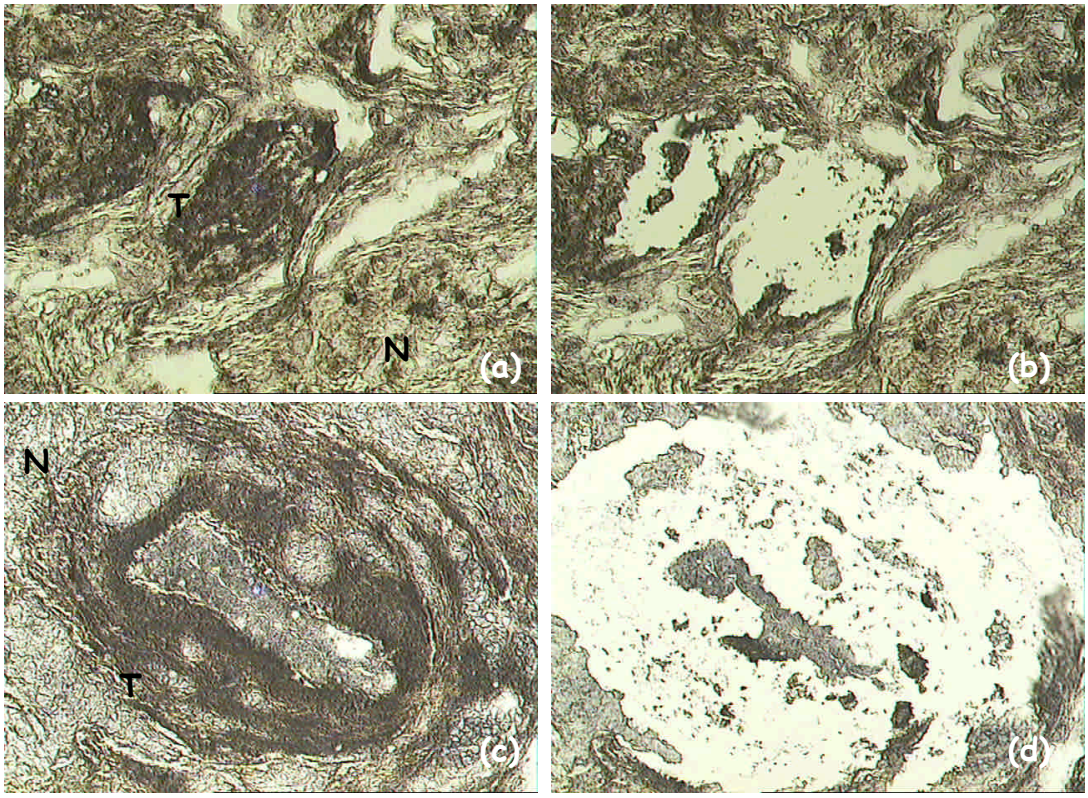

Fig. 1B. Isolation of breast tumor cells using laser capture microdissection technique. (a) and (c) are breast cancer tissue resections before LCM treatment. T, tumor and N, tumor-adjacent normal part; (b) and (d) are Post-LCM image of laser capture. Cancer tissues collected from breast cancer patients who were characterized as T1, stage I and negativity of lymphnode metastasis (N0).

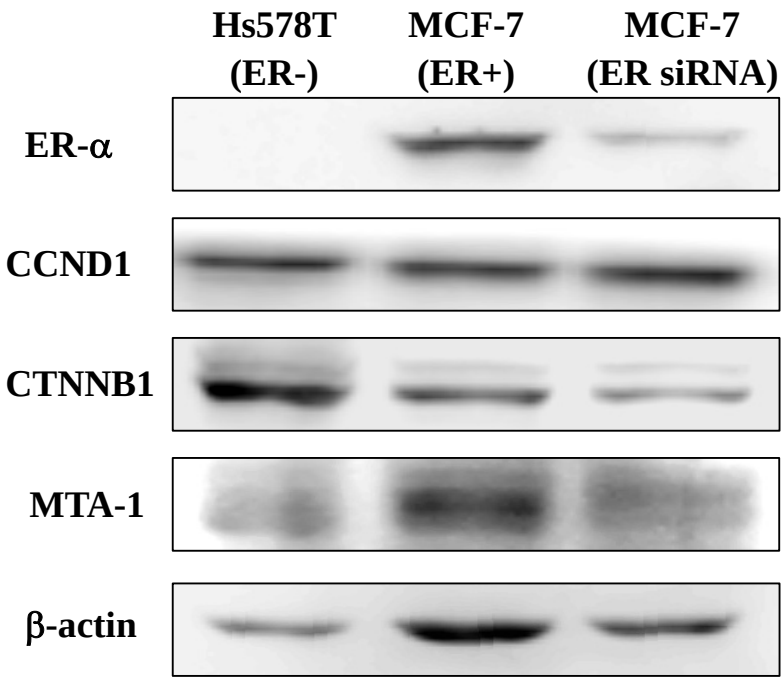

Fig. 2. Expression levels of the targeted proteins in ER-negative (Hs578T cells), ER-positive (MCF-7 cells) and *ER* siRNA knockdown MCF-7 cells. Cell lysates were immunoblotted with respect to the indicated antibodies.

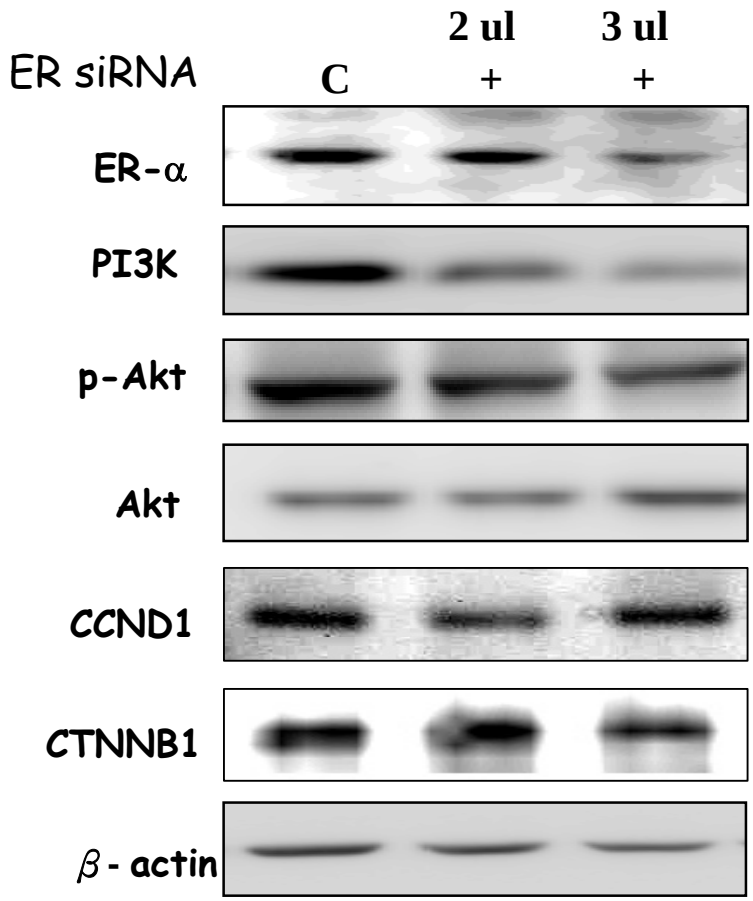

Fig. 3. Differential expression of the targeted proteins with respect to *ER* siRNA (2 ul and 3 ul) treatment in MCF-7 cells and treatment with vehicle (C). Cell lysates were immunoblotted with the specific antibodies as shown. β-actin was used as the protein loading control.

出席國際學術會議心得報告

計畫編號	95-2314-B-040-030-MY2
計畫名稱	動情激素受體在乳癌生成進程之角色探討
出國人員姓名 服務機關及職稱	鄭鈞文 中山醫學大學生化暨生物科技研究所副教授
會議時間地點	April 12-16, 2008 ; San Diego, CA, USA
會議名稱	Annual Meeting 2008, American Association for Cancer Research
發表論文題目	Detection of CCND1 and β-catenin amplification using laser capture microdissection coupled with real-time polymerase chain reaction in female breast cancer

一、參加會議經過

2008 年美國癌症醫學研究會研討會 (2008 Annual Meeting , American Association for Cancer Research) 於二千零八年四月十二日至十六日，為期五天，假美國加州聖地牙哥市召開。本次大會有來自全球各癌症醫學相關學術機構、業界之專業研究人員共聚一堂，與會人士共超過一萬四千餘人。本次所參與發表的論文摘要共計有五千七百餘篇，於五天的會議中分別以口頭發表、討論及論文海報張貼等方式同時進行。年會進行中，特地將本屆 AACR 終身成就獎頒發給德國海德堡 Deutsches Krebsforschungszentrum, Harald zur Hausen 博士，表彰他致力於病毒與癌症發生之病理學研究。Harald zur Hausen 博士就人類乳突狀病毒感染和子宮頸癌發生，Epstein-Barr DNA 和 Burkitt's lymphoma 對於鼻咽癌罹患的病理學，是為研究病毒與癌症發生關連的先驅者，其研究貢獻卓越。另外，1993 年與 Dr. Richard 共同榮獲諾貝爾醫學獎 Phillip A. Sharp 博士，現任麻省理工學院癌症醫學研究中心主任，他發現核糖核酸的接合(Splicing)現象。專題演講中，他以 short RNAs 為主提，闡釋 RNA 分子在癌症發生所扮演的角色。大會 Kirk A. Landon-AACR Lecture 的獎項則是頒發給 Arnold J. Levine 博士。因為他致力於研究腫瘤抑制基因 p53，揭開 p53 的基因功能和癌症的關係。也藉由 p53 基因多形性，說明 p53 腫瘤抑制蛋白功能活性變異對癌細胞增生的調控機制和癌症進程的影響。另外，Steven R. Tannenbaum 博士以化學生合成硝基胺的衍生物，模擬人體內生性生合成含氮化物對細胞 DNA 的傷害，與導致發炎作用所引發癌症的致癌機。因此，Steven R. Tannenbaum 博士獲得大會給予第二屆 AACR Award for Outstanding Achievement in Chemistry in Cancer Research Lecture 的殊榮。而癌症流行病學的研究中，Robert N. Hoover, M.D., Sc.D., 以遺傳流行病學的觀點，探究環境暴露的致癌危險，也同為大會頒發給予 AACR 本年度 Award Lecture for Research Excellence 的殊榮。再者，在這次大會所邀請的演講中，

專題研究報告涵蓋層次廣泛且深入。其中，癌症莖細胞(cancer stem cells, CSC)的研究領域如雨後春筍般，有許多場特別演講和討論會分別舉行。因為癌症莖細胞被認為與癌症的發展有著密切的關聯，而以癌症莖細胞為研究標的，可以為將來癌症治療模式提供新的思考。同時，在這次會議期間邀請到多位研究學者，就近幾年癌症分子醫學的進展提出關鍵的研究報告。其中 Johns Hopkins University School of Medicine, Dr. Carol W. Greider 針對端粒酶作用和端粒結構異常與癌症發生的關連；Dr. Kornelia Polyak 以 Molecular mechanism underlying breast tumor heterogeneity 為題，精細地剖析癌細胞株的演繹模式(clonal evolution models)，以不同癌細胞源自於不同 cell clone，詳加以考慮相異基因的特殊剖面做為臨床治療的觀點，而提出癌莖細胞所具有其特殊基因異質性為 breast tumor heterogeneity 源由的假說。以新的癌症組織研究觀點闡述癌症分期、發展，以致於癌症的臨床治療預後評估等等的專題報告發表，都為我們研究室即將針對乳癌細胞生成病理機轉和相關蛋白訊息啟動調控提供了非常寶貴的研究資訊。

會議舉行期間，邀請現今世界各知名實驗室的主持人，就其專業研究領域給予專題演講、教育訓練課程和會議研討等。其研究內容涵蓋更為廣泛，包含有細胞生長訊息傳遞、細胞死亡和老化、轉錄活化因子和基因表現調節、基因受損和修復作用、致癌基因體學、血管生成、腫瘤生成、腫瘤侵襲和轉移機轉、生物資訊學、癌至遺傳標記、代謝與致癌、基因靜默轉殖、腫瘤內分泌、microRNA、和抑癌基因的表現差異、分子流行病學、癌症臨床研究治療、藥物治療抗性，癌症免疫學、基因治療與預防等各項專題研究報告。更值得一提的是在本屆年會中，更向下紮根往高中的學生宣導癌症的教育課程，引導並開放給與高中生來認識癌症，其課程區分為(1)了解癌症 (Understanding Cancer)、(2)癌症防治的關鍵(Keys to Cancer Prevention)、(3)來自癌症存活者的訊息(A Message from a Cancer Survivor)和(4)癌症研究需要你的努力(Cancer Research Needs You)等四項專題來加以介紹。針對癌症進行深入淺出的講演、期使能夠讓更多的學生體會到癌症預防、定期健康檢查和臨床治療的體認，這是非常值得讓國內學界來加以重視的發展。

二、與會心得

此次前往美國癌症醫學研究學會年會之國內學者亦有許多人，其中來自中央研究院、台大、陽明，北醫、國防、長庚、中國醫大等各大專院校醫學院及醫學研究中心之癌症研究學者。其研究領域涵蓋有腫瘤分子生物學、癌症莖細胞醫學、分子流行病學和臨床藥理等癌症醫學之相關研究課題。藉由會議舉行期間能夠與國內、外研究先進進行學術交流，針對腫瘤生成暨細胞癌化發生的成因、遺傳基因變異、細胞程序化死亡訊息傳遞、癌症臨床標記之研究、腫瘤之基因治療和各種癌症致癌感受性危險相關因子之統計分析等各項專題，進行學術經驗的交流、分享彼此研究技術和心得。這對於腫瘤癌症方面之初學者即將踏入此領域，或是此領域多年資深

研究工作者，皆有莫大助益，同時也可以拓展個人的研究視野和深度，實為難能可貴的進修機會。本屆美國癌症醫學研究學會年會中，我們以「CCND1 為標的基因，探討基因表現和乳癌進程發展的分期評估」為主題，透過雷射捕獲分析技術(laser capture microdissection) 從病人癌組織和癌相鄰之正常組織分別擷取，再以定量 qRT-PCR 的表現來做為乳癌的分期關聯性分析。藉由 cyclin D1 和β-catenin 等基因共同表現的重要性，這些標記蛋白之表現程度在癌組織的高低差異，將被視為乳癌基因體學的前置試驗依據。再者，我們發現 cyclin D1 與乳癌患者發生腋下淋巴轉移亦有關聯，這些攸關癌化過程的訊息結合臨床組織之分期，進行多變相分析評估結果，將可針對台灣婦女乳癌之年輕化特色，完成初步的研究報告並進行發表。摘要發表期間，同各國學者就乳癌做廣泛的討論並且交換研究心得，實獲益匪淺；更可以成為在將來對台灣地區婦女乳癌的相關研究，給予更明確的發展方向。

另外，值得一提的是，在今年大會的摘要發表中，也著重於以 micro RNA (miRNA)的基因體合併分析、結合生物和電腦資訊，以快速的基因檢定針對癌症臨床標記、蛋白質功能分析和應用於臨床癌症藥物篩檢有了更精闢的發展。從組織或是血液中研究 miRNA 的差異性表現，不但可以了解到基因從另一層次被逆向調控於腫瘤抑制基因抑或是致癌基因對癌細胞的進程發展，將這些觀念加以應用研發，可以更有效率地判斷出與癌症生成或癌症治療藥物的機制，這也極可能是未來癌症研究的重要議題。現今，在我們的實驗室也陸續投諸許多的人力、心力和資源著手進行此方向的研究，冀望能夠透過對 miRNA 的研究，找出對台灣婦女乳癌發生更有意義的研究標記。

三、會後建議：

在這次學術研究年會中，本人也發現國內、外的研究先進在癌症領域鑽研相當長的時間，許多先進學者的研究群除了指導自己的碩、博士班研究生、博士後研究員，兼具該研究單位對內、對外的合作伙伴，也都有跨院校及國際合作的對象，所以加快實驗室各項專業技術的進步。除此之外，更結合了電腦化與生物資訊的交流，因此解決問題的效率相對提高，這是為什麼他們能夠走在學術尖端的理由。另一方面，在參與此次國際性的研討會議中，也深刻感受到亞洲各國在癌症學術領域的研究也較以往有更具深度，尤其是以韓國、印度和中國大陸的進步特為顯著。許多的研究人員展現出強烈的企圖心，都將成為未來國內癌症學術研究上的競爭者。衷心希望學術的同仁能密切地合作，為國內的學術研究向下紮根，方能與其他國家的研究並駕齊驅，同為癌症預防治療之研究盡力。