行政院國家科學委員會專題研究計畫　成果報告

探討同時存在之不同組織型態乳癌的遺傳本質

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（一） 計畫中文摘要。(五百字以內)

乳癌並非單一疾病，可具有不同的組織形態。乳癌組織分類主要是基於腫瘤細胞的型態學，而非基於其癌細胞來源或基因變化。愈來愈多的研究顯示乳癌的致癌途徑並不一定是線性方式，而可能有不同的基因變化途徑。本研究希望能藉由雷射擷取術(Laser capture microdissection, LCM)的技術的建立，應用於瞭解在乳癌病人同時發生一個以上腫瘤時，其臨床病理表現的特徵及基因改變的途徑。

本研究已成功的建立在石蠟切片上，利用 LCM 技術，萃取 DNA 提供進一步的研究使用。然而，不同石蠟切片保存 DNA 的狀況，由於固定與保存的狀況會有很明顯的差異。我們收集 1998 年至 2005 年在台北醫學大學附設醫院與萬芳醫院病理科同時發生一個以上腫瘤的乳癌病人，共 20 例。經由覆閱其病理報告及病理切片，選擇 9 位病人共 21 個腫瘤進行石蠟切片的 DNA 萃取。其中 3 位病人乳癌組織的 DNA 保存良好，但其餘 6 位病人乳癌組織的 DNA 保存狀況不佳。在 3 位 DNA 保存狀況良好的乳癌石蠟切片，選取癌症與正常乳房組織進行 LCM，而且也順利完成 LCM 細胞的 DNA 萃取。

由於能順利抽取 DNA 的病例數少，我們決定改用 SNPs genotyping assay 進行研究。因為此技術耗材昂貴，限於有限的經費，故先選用一個病例進行研究。此病例為同時發生的兩側性乳癌，且具有不同的生物特性表現。而 SNPs genotyping assay 則委託中央研究院國家基因型鑑定中心李玲慧研究員實驗室執行，目前尚未有結果。未來將基於初步結果進行進一步的研究。

關鍵詞：乳癌, 雷射擷取術, 基因型分析

（二） 計畫英文摘要。(五百字以內)

Breast cancers are a group of heterogeneous diseases with different morphologic pictures and biologic behavior. The histological classification is based on the morphology of tumor cells, neither on histogenesis nor genetic nature. There is growing evidence that this very linear model of the pathogenesis of human breast cancer should be modified, in the sense that there may be several distinct genetic pathways. Our specific aims in this study are establishing the techniques of microdissection and subsequent analysis, evaluating the clinicopathological characteristics and the genetic nature in synchronous breast cancer.

We have successfully established the technique of DNA extraction of microdissected cells from paraffin-embedded section by Laser Capture Microdissection (LCM). However, the qualities of DNA extracted from paraffin-embedded tissue were quite variable in different paraffin blocks.

Totally 20 patients with synchronous multiple breast cancers from 1997 to 2005 in the Department of Pathology in Taipei Medical University Hospital and Municipal Wan-Fang Hospital were collected. After reviewing the pathological report and HE sections, 9 cases with 21 tumors were selected and submitted for DNA extraction from paraffin section. The qualities of extracted DNA were preserved in 3 out of 9 cases and degraded in the remaining 6 cases. LCM from normal and cancer areas was further performed in the 3 cases and the DNA was successfully extracted from the LCM cells for study.

Because of the limited number of cases with preserved DNA from archive paraffin section, we decided to perform SNPs genotyping assay instead of PCR amplification of LOH by microsatellite markers. The cost of SNP genotyping is expensive and we first chose one selective case because of limited budget. After reviewing these clinicopathological characteristics of the cases with preserved DNA, we chose a patient who had bilateral breast tumors with different biological markers for further SNPs genotyping assay. The extracted DNA was sent to the Dr. Lee's laboratory at National genotyping center at Academia Sinica for further SNP genotyping and the data is not available now. We will perform further study based on the result from SNP genotyping.

Key words: breast cancer, LCM, genotyping

成果報告

Introduction

Breast cancers are a group of heterogeneous diseases with different morphologic pictures and biologic behavior. The histological classification is based on the morphology of tumor cells, neither on histogenesis nor genetic nature (1). Is different morphology of breast cancers the result of cancer progression by a stepwise of genetic alterations or different distinct genetic pathway？ There is growing evidence that this very linear model of the pathogenesis of human breast cancer should be modified, in the sense that there may be several distinct genetic pathways (2,3). Based on the previous studies, some histological types of invasive breast cancer are genetically unique from invasive ductal carcinoma (IDC), and some are genetically similar to IDC and represent variant forms of IDC (4,5).

Breast cancer with different histological types in addition to IDC is not common. It may present with (1) a solitary tumor with different histology (mixed histological type such as mixed mucinous carcinoma), (2) multiple discrete tumors with different histological type in unilateral breast (multifocal or multicentric) or (3) bilateral breast cancers (synchronous or metachronous) with different histological types. The average incidence of bilateral breast cancer is 0.5-1% for synchronous and 3-7% for metachronous disease. There was a study using LOH analysis in 28 bilateral breast carcinomas on 14 chromosomal arms and showed the vast majority bilateral breast cancers are of independent clonal origin. Interestingly, strikingly similar allelotypes were detected in 3 of 6 synchronous bilateral breast cancers with the same or different histological type, but in none of 22 metachronous ones (6). We are interested in the clinicopathological features and genetic nature of synchronous breast cancer with different histological types. Our specific aims during this period are the followings: (1) establishing the techniques of microdissection and subsequent analysis in our department; (2) evaluating the clinicopathological characteristics of synchronous breast cancer with different histological type; (3) evaluating the genetic nature in synchronous breast cancer.

Material and method

Case selection

Cases meet one of the following criteria; 1) mixed histological type of breast cancer in a single tumor; 2) multifocal or multicentric breast cancers with different histological type in unilateral breast; or (3) bilateral breast cancer with different histologic type were selected. The selected cases had paraffin- embedded tissue material for histology and molecular analysis. The patients with single or multiple tumors with same histological type were collected for comparison.

Histopathological analysis

For histopathological study, the tissues were fixed in 10% buffered formalin and embedded in paraffin. Sections of 4-*um* thick were stained by HE (hematoxylin and eosin). The histological types of breast cancer are according with the WHO's classification.

Immunohistochemical analysis

For immunohistochemical analysis, paraffin sections of 4-*um* thick were stained using streptavidin-biotin-peroxidase complex method. Tumors are classified as ER, PR, or p53 positive when more than 10% of nuclei of tumor cells are stained positively. The HER2/neu interpretation is according to HERCEP test scoring system.

Microdissection and DNA extraction

To ensure to select pure population of tumor cells, different foci with different histology of tumor specimens were subjected to laser-based microdissection by a laser micromanipulator. Five-*um*-thick sections were collected on glass slide, briefly stained in HE, and unmounted. The selected area (including normal breast tissue and cancer tissue) were bound to the membrane by laser pulses. The cells were immersed in digestion buffer, containing 10mM Tris-HCL (pH 8.0), 1 mM EDTA, 400 *u*g/ml proteinase K, and 1% Tween 20, and digested at 55℃ overnight. After digestion, the enzyme was heat inactivated (95℃ for 10min), and the extract was used directly for PCR.

SNPs genotyping assay

This work will be performed by SNPs genotyping assay core laboratory. DNA extracted from the microdissected cells on paraffin section was analyzed.

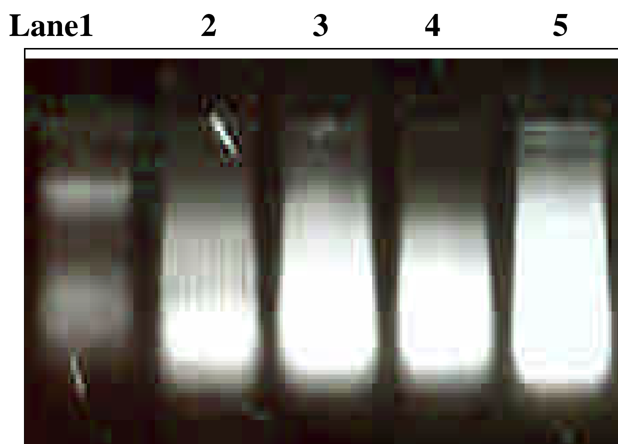
Result and Discussion

Establishing the techniques of microdissection and subsequent analysis in our department

We have successfully established the technique of DNA extraction of microdissected cells from paraffin-embedded section by Laser Capture Microdissection (LCM).

First, the qualities of DNA extracted from paraffin-embedded tissue were variable as shown in Fig. 1.

Figure 1. Variable qualities of DNA extracted from different paraffin-embedded tissues. The DNA is well-preserved in lane 1, partially degraded in lane 5 and less well-preserved in lane 2-4.

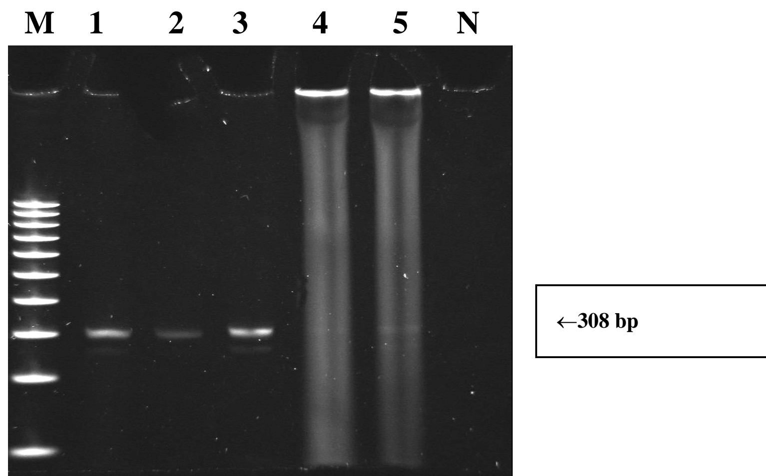


To check the qualities of DNA extracted form different paraffin-embedded tissues and LCM, we performed PCR amplification of ß-actin gene. The size of PCR product is 308bp. (Fig. 2)

Figure 2. PCR amplification of ß-actin gene (308bp) from DNA extracted from different paraffin-embedded tissues.

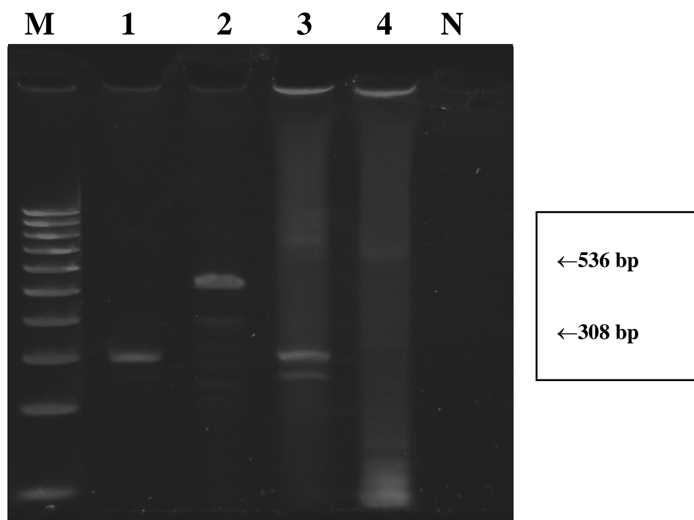
M: marker; Lane 1: the same DNA as shown in Fig. 1 lane 1. PCR product of ß-actin gene (308bp)

was clearly demonstrated. Lane 2 and 3 were the DNA extracted from 2000 and 10000 pulse microdissected cells by LCM from the same paraffin-embedded tissue in lane 1. PCR products were also clearly demonstrated and the quantities of DNA were proportionate to the numbers of microdissected cells. Lane 4 was PCR of the less well-preserved DNA shown in Fig. 1 and no PCR product was seen. Lane 5 was PCR of 10000 pulse microdissected cells from tissue same as lane 4. No PCR product is seen. N: negative control



To further check the qualities of DNA extracted from paraffin-embedded tissue, PCR amplification of different size of products, β-actin gene (308bp) and β-globin gene (536bp), were performed (Fig. 3). Both PCR products of 308bp (lane 1) and 536 bp (lane 2) were clearly shown in DNA extracted from well-preserved tissue same as Fig. 1, lane 1; Only PCR product of 308 bp (lane 3), but not 536 bp (lane 4) was clearly shown in partially degraded DNA extracted from tissue same as Fig. 1, lane 5. M: marker; N: negative control.

Figure 3

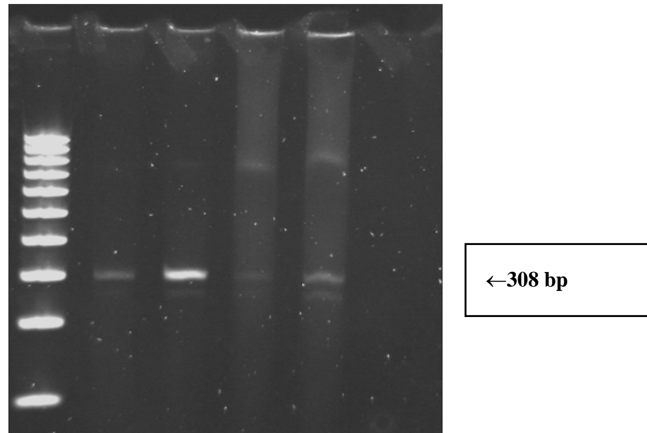


Because of the variable degree of DNA degradation in paraffin-embedded tissue, microsatellite marker assay can easily apply in those well-preserved DNA. In those partially degraded DNA, only limited microsatellite markers can use depending on the size of amplified fragments. If the DNA is too degraded for PCR, SNP (single nucleotide pleomorphism) genotyping assay is an alternative method for further study.

For determining the quantities of DNA, extracted from different paraffin-embedded tissues with different degree of degradation, for microsatellite marker assay or SNP, we can perform log-phase PCR in different cases (Figure 4). For example, lanes 1, 2 represent DNA extracted from same tissue (case 1) and lanes 3, 4 from the other same tissue (case 2). Lanes 1 and 3 represent the PCR products of 30 cycles and lanes 2 and 4 the products of 32 cycles. As shown in Figure 4, we need to use at least three times of DNA amount in case 2 than in case 1 for further microsatellite marker assay or SNP genotyping assay.

Figure 4 **Case 1** **Case 2**

M 1 2 3 4 N



Evaluating the clinicopathological characteristics of synchronous breast cancer

We collected the patients with synchronous multiple breast cancers from 1998 to 2005 in the Department of Pathology in Taipei Medical University Hospital and Municipal Wan-Fang Hospital. Totally 20 cases were collected. After reviewing the pathological report and HE sections, the following cases were selected and submitted for DNA extraction from paraffin section. The clinicopathological characteristics of these cases were summarized in Table 1.

Table 1. The clinicopathological characteristics of breast cancers

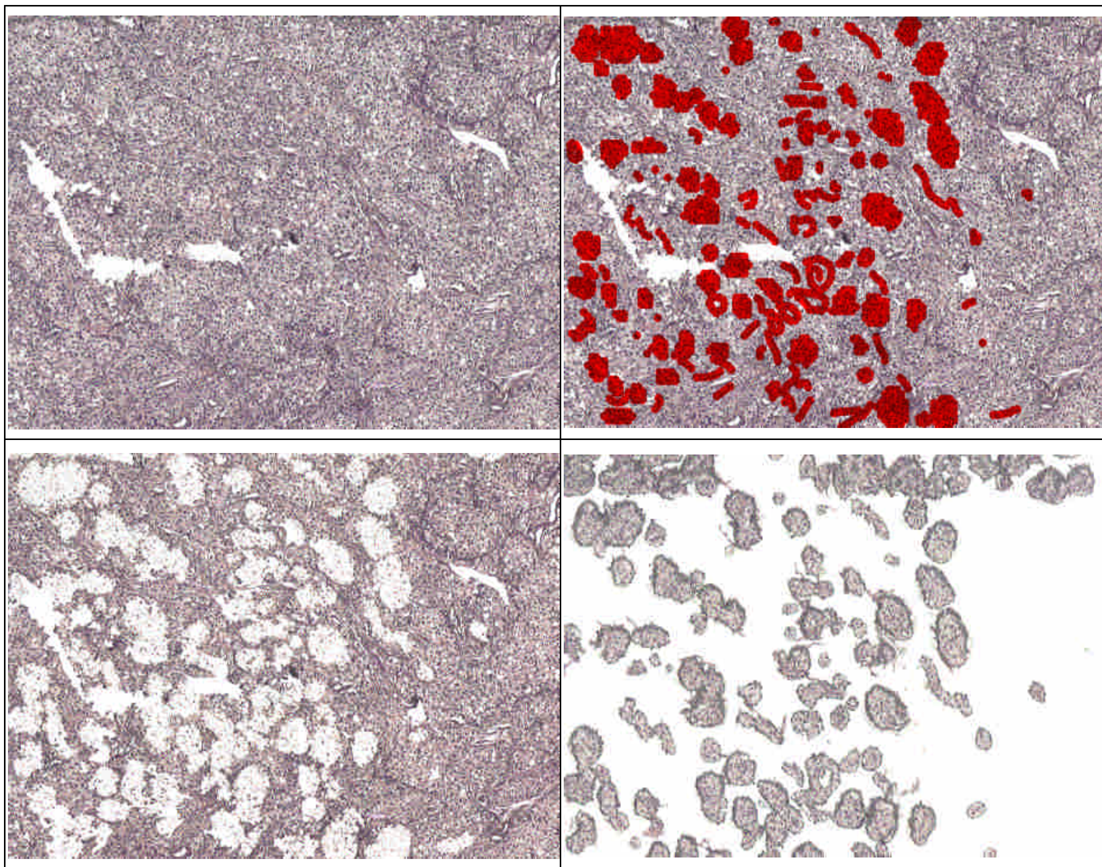
No.	Tumor No.	Laterality	Type	Size (cm)	Grade	LN	ER	PR	Neu	DNA	LCM
1	3	Left	IDC	2.4	2	+	+	+	-	Fail	
		Right	Papillary DCIS	3	1	-	+	+	-	Fail	
		Right	Tubular	1.5	1	-	+	+	-	Fail	
2	2	Right	MFH	10.5		-				Fail	
		Left	Invasive papillary	11.5		+	-	-		Fail	
3	2	Right	Mucinous ca	2.5	1	+	+	-		Fail	
		Right	IDC	2	2	+	+	-		Fail	
4	2	Right	DCIS	2		-				Fail	
		Left	Invasive papillary	2.5		-				Fail	
5	2	Right	IDC	3.5	3	+	-	-	+	Fail	
		Right	IDC	1.8	3	-	-	-	+	Fail	
6	2	Left	IDC	1.4	2	-	+	-	+	OK	OK

		Right	IDC	1.2	3	-	+	+	-	OK	OK
7	4	Left	IDC	3.0	1	-	+	+	-	OK	OK
		Right	IDC	2.0	1	-	+	+	-	OK	OK
		Right	DCIS+FA	2.0		-	+	+	-	OK	OK
		Right	IDC	0.7	1	-	+	+	-	OK	OK
8	2	Right	IDC	1.8			+	-	-	OK	OK
		Right	IDC	0.6						OK	OK
9	2	Left	IDC	1.7	3	+	+	+	-	Fail	
		Left	IDC	1.6	3					Fail	

IDC: invasive ductal carcinoma; DCIS: ductal carcinoma in situ; FA: fibroadenoma; LN: lymph node; DNA: the quality of DNA extracted from paraffin section; LCM: the quality of DNA from laser captured microdissected cells from paraffin sections

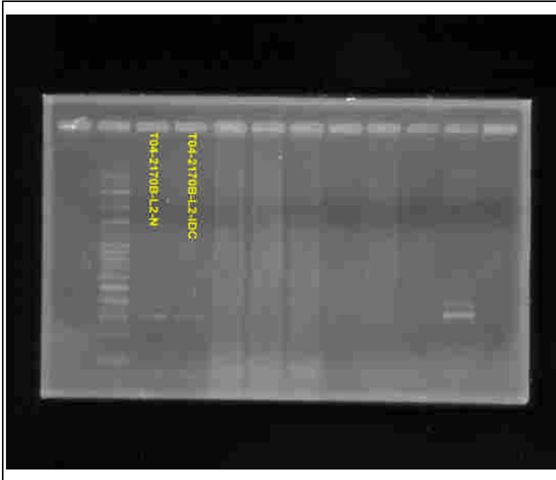
The qualities of extracted DNA from paraffin sections were preserved in 3 out of 9 cases and degraded in the remaining 6 cases. LCM was further performed in the 3 cases with preserved DNA and the DNA was successfully extracted from the LCM cells from the paraffin section. The processes of LCM from case No. 6 were illustrated in Figure 5.

Figure 5. The left upper figure represented the original HE section in IDC area of right breast. The IDC cells were selected and marked in right upper figure. The selected cells were microdissected by LCM in the left lower figure. After LCM, the microdissected cells on the cap were shown in the right lower figure.



We performed DNA extraction from the microdissected cells on the cap. The quality and

quantity of DNA was confirmed by PCR amplification of ß-actin gene (308bp) and shown in Figure 6. Lane 2 and 3 represented the PCR product of DNA extracted from microdissected cell of normal and IDC cancer cells.



Evaluating the genetic nature in synchronous breast cancer

Because of the limited number of cases with preserved DNA from archive paraffin section, we decided to perform SNPs genotyping assay instead of PCR amplification of LOH by microsatellite markers. SNPs may cause changes of gene expressions or may be close to more complicated and unknown genetic variation sites and serve as useful markers to genetic studies. A new high-density genotyping assay, the GeneChip Human mapping 10K Array (Affymetrix) is available recently. SNPs genotyping assay permits the simultaneous genotyping of 11,560 SNPs, spaced throughout the human genome at a median intermarker distance of 210 kb. After reviewing these cases with preserved DNA, we chose the patient No. 6 for further SNPs genotyping assay because the bilateral tumors had different immunophenotypes. The extracted DNA was sent to the Dr. Lee’s laboratory at National genotyping center at Academia Sinica for further SNP genotyping and the data is not available now.

In summary, we have successfully established the technique of DNA extraction from archive paraffin sections by LCM. The extracted DNA can further applied for PCR amplification for alleolotyping or SNP genotyping. Because of the degree of DNA degradation from archive paraffin section was beyond our expectation, only limited cases with preserved DNA were collected. The cost of SNP genotyping is expensive and we first chose one selective case because of limited budget. We will perform further study based on the result from SNP genotyping.

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