行政院國家科學委員會專題研究計畫　成果報告

乳癌分子流行病學研究--BRCA1 與 FANCD2 基因多形性對 DNA 雙股錯結修復作用於乳癌發生所扮演之角色

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1．中文摘要

乳癌為台灣女性第二常見的腫瘤，其發生率近十年內增加三倍，且有年輕化之趨勢。研究指出，在乳癌的致癌機轉中，女性荷爾蒙的暴露是最重要的危險因子。而 DNA 氧化性傷害是荷爾蒙代謝過程中重要的內生性致變方式 (endogenous DNA mutagenesis)，所以對乳癌的發生而言，對抗因為氧化性傷害造成 DNA 雙股斷裂的同源染色體修復機轉將格外重要。其中，*FANCD2* 基因會受到 *BRCA1* 的活化並共同移位至細胞核中以修復雙股斷裂的染色體。文獻指出 BRCA1 會和 FANCD2 蛋白質發生免疫沉澱反應。因此，在本實驗中，我們收集 561 乳癌病例及 1126 健檢對照組進行病例對照組研究，實驗設計以 Mass array 檢定 *FANCD2* 的 $Leu^{714}Pro$ 和 Leu^{1366} silence mutation (T-to-G)基因分布頻率，來建立台灣地區華人婦女在 *FANCD2* 基因多形性的遺傳資料。統計分析結果發現：$Leu^{714}Pro$ 和 Leu^{1366} 基因型，在乳癌病患和對照組的分佈未達統計上的顯著差異。進一步採多變相分析，發現肥胖指數(BMI≧24)的乳癌患者中，$Leu^{714}Pro$ 和 Leu^{1366} 的基因型達統計上顯著的致癌危險因子 ($O.R._{Leu714pro}$=1.71；$O.R._{Leu1366(G)}$=1.96, P<0.05)。然而，將此二基因型考慮以加成作用和 FFTP、初經和停經等因子共同考慮後，並未達明顯的統計水準。總結而言，針對台灣地區的華人，*FANCD2* 之 $Leu^{714}Pro$ 和 Leu^{1366} 基因型並非為台灣婦女乳癌罹患之獨立易感受性危險因子。

關鍵字：乳癌、DNA 染色體雙股錯結、FANCD2、基因多型性

2. ABSTRACT

Fanconi anemia (FA) is an autosomal recessive disorder with diverse clinical symptoms and markedly predisposition to malignancies, especially acute myeloid leukemia, and lesser extent, solid tumors, such as head and neck squamous-cell carcinomas. FA complex protein forms a nuclear multiprotein, which is essential for the activation of FANCD2 by ubiquintination. FANCD2 can colocalize in nuclear foci, associating with chromosomal damage repair. To understand tumorigenic contribution associated with *FANCD2* participation in the DNA damage repair, especially for the association between genotypes and breast cancer risk may be modified by estrogen exposure, this present case-control study aims at knowing the oncogenesis of breast cancer using a Mass array technique to genotype the polymorphisms of FANCD2, i.e., $Leu^{714}Pro$ and Leu^{1366} silence mutation and evaluated the known risk factors by questionnaire in 561 sporadic breast cancer patients and 1126 age-matched controls in a Chinese population, Taiwan.

The genotype frequencies of Pro^{714} and Leu^{1366} (T allele) were 14.08% (79/561) and 84.1% (472/561) for cases and 11.99% and 82.9% for controls, respectively, which results did not reach statistically significant in univariate analysis (p=0.802 for Pro^{714}, and p=0.547 for Leu^{1366}, respectively). Further, in multivariate analysis, the modified elevated risk was observed in cancer patients having BMI over 24 who harbored either Leu^{714} allele (OR=1.71, 95% CIs, 1.21-2.43) or Leu^{1366} (G allele) (OR=1.96, 95% CIs, 1.10-3.50). However, there was no significant association between the elevated risk in cancer development and genotypic polymorphisms modified under estrogen exposure, i.e., early menarche or late postmenopausal statues.

In conclusion, the polymorphic alleles of *FANCD2*, Pro^{714} and Leu^{1366}, are not independent risk factors in the association with Chinese breast cancer development in Taiwan.

Keywords：Breast Cancer、DNA crosslink、FANCD2、Genetic Polymorphism

3.計畫緣由與目的

Breast cancer is a major affliction of women in many countries. In Taiwan, breast cancer is the second common cancer in women and the incidence had a three-fold increase with continuing westernization and urbanization in the past decade. Fanconi anemia (FA) is a rare autosomal recessive disease characterized by skeletal defects, anemia, chromosomal instability and increased risk of leukemia (Fanconi, 1967; Huibregtse et al., 1985; D'Andrea & Grompe, 2003). The FA phenotype displays cellular and chromosomal hypersensitivity to clastogens, such as miotmycin C and diepoxybutane (DEB), which markedly enhance the forming of interstrand crosslinks (ICL) in DNA (Duckworth & Taylor, 1985; A.M.R.Carreau et al., 1999; Taniguchi and D'Andrea, 2002). Convincing evidences showed that the FA proteins may function in cellular processes, including cell cycle regulation, apoptosis, detoxification of reactive oxygen radicals and DNA repair (D'Andrea & Grompe, 1997; 2003). And thus, FA is regarded as an indicator for chromosome instability syndrome and the production of chromosomal damage by DEB. Briefly speaking, FANCE protein can interact with FANCC, FANCA and FANCG proteins, the FANCD1 and FANCD2 proteins function downstream after the FA protein complex conjugated (Kupfer et al., 1997; Garcia-Higuera et al., 1999; Waisfisz et al., 1999; Garcia-Higuera et al., 2000). Mono-ubiquitinated FANCD2 is activated through FA nuclear complex assembling, and which ubiquintinated protein is necessary for normal cellular repair against DNA breaks (Timmers et al., 2001; Garcia-Higuera et al., 2001). More importantly, activation of FANCD2 modified by mono-ubiquintination resulted in preferential localization within the nucleus where DNA is damaged. However, much less information is currently available regarding polymorphisms of *FANCD2* gene in Chinese, Taiwan. Lacks of notable sequence motifs and biological phenotypes of the single nucleotide polymorphisms (SNPs) of FANCD2 in the post-genomics era raised our attention to screen the frequency distributions of known FANCD2 SNPs, especially in Chinese population with female breast cancer. Therefore, n the present study, two nucleotide polymorphic sites of *FANCD2*, amino acid change from proline (Pro) to leucine (Leu) at 714^{th} residue (nucleotide mutation of T-to-C at 2141^{st}) and Leu^{1366} silence mutation (nucleotide mutation of T-to-G at 4098^{th}) will be subjected to genotyping analyses. Based on this molecular epidemiological study can provide us further understanding of the molecular mechanism of *FANCD2* associating with breast cancer patients. And, whether polymorphisms in the FANCD2 domain may be associated with an elevated risk of breast cancer, in addition, whether the association between genotypes and breast cancer risk may be modified by estrogen exposure will be addressed.

4.材料和方法

Study population. This case-control study will be part of an ongoing cooperative study aimed at understanding the causes of breast cancer in Taiwan (Lou *et al.*, 1997; Yang *et al*., 1997; Lo *et al.*, 1998; Huang *et al.*, 1999; Shen *et al.*, 2000). There were 561 breast cancer patients and 1126 healthy female controls enrolled in the present study. All breast cancer patients have pathologically confirmed primary breast carcinoma, and are diagnosed and treated at the National Taiwan University Hospital and Shin Kong Wu Ho-Su Memorial Hospital. To avoid any differential recall bias of previous disease history, we will purposely randomly select the controls from the health examination clinic of the same hospital during the same study period. The control subjects receive a 1.5 day comprehensive health examination, and show no evidence of breast cancer, any suspicious precancerous lesions of the breast, or other cancers.

Questionnaire. An experienced research nurse will be assigned to administer a structured questionnaire to both case and control subjects. The information collected will include 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 drinking alcohol and smoking cigarettes, ethnic background, residence area, family income and education level. The body mass index (BMI) and menopausal status are also recorded.

Laboratory analyses. A 10-mL sample of peripheral blood, collected in acetate-citrate dextrose, will be obtained from each breast cancer patient, prior to treatment, and from each control subject. The buffy coats of these specimens will be prepared immediately and stored at -80^{o}C until extraction of the genomic DNA. Genomic DNA is obtained by QIAamp DNA blood Mini kit (QIAGEN Inc., U.S.A.) then store at -20^{o}C until genotype analysis.

Two nucleotide polymorphic sites of *FANCD2* cDNA, Leu^{1366} silence mutation (nucleotide mutation of T-to-G at 4098th base) and amino acid change from proline (Pro) to leucine (Leu) at 714th residue (nucleotide mutation of T-to-C at 2141st base) will be analyzed. The single nucleotide polymorphisms (SNP) of *FANCD2* (*Leu^{714}Pro*) and (*Leu^{1366}, silence mutation*) were genotyped by a Mass array system (SEQUENOM, Inc., San Diego, CA), based on the primer extension protocol. The PCR primers and extension primers for these 2 SNPs were designed using Spectro-Designer software (SEQUENOM, Inc.).

Statistical analysis. Univariate and multivariate analyses will be used to determine the risk factors for breast cancer in this series of study subjects, and the ORs and corresponding 95%CIs will be estimated. In the present study, deficits of DNA interstrand crosslink repair gene of *FANCD2*, indicated by genotypic polymorphisms associated with reduced interstrand crosslink repair capacity. And, the risk modification of FANCD2 associated with the biologically effective dose of estrogen exposure, i.e. within different levels of estrogen exposure, measured by total years of estrogen exposure, the number of years between menarche and first full-term

pregnancy, is hypothesized to contribute to enhance breast cancer risk. Therefore, women harboring additional high-risk *FANCD2* genotypic polymorphisms are considered to be at higher risk of breast cancer.

5.結果與討論

The major risk factors of breast cancer were reviewed by a detail questionnaire. Multivariate analysis of risk factors in the questionnaire showed that the body mass index (BMI≧24), younger age at menarche were independent significant risk factors. As to the genotypic polymorphisms of *FANCD2* gene, the frequency distributions in respect to Pro^{714} was 14.08% (79/561) for cases and 11.99% (135/1126) for controls, and Leu^{1366} silence mutation (G homozygous allele) was uncommon rare of one individual for cases and 12 individuals for controls, which were all not statistically significant in univariate analysis (Table 1). Possible limitation of this molecular epidemiological approach is that the biological significance of some polymorphisms has remained unclear. Therefore, biological plausibility and availability of multigenic model in association with breast cancer development will be the major consideration of this proposed study to decide the polymorphic sites to be assayed. We grouped together patients showing homozygous and heterozygous of Pro^{714} allele or homozygous and heterozygous of Leu^{1366} (*G* allele) as a combined polymorphic population in the following data analysis. In multivariate analysis, we found that individuals having BMI over 24 got increased relative risk of 1.78-fold and 1.96-fold in respect to subjects harboring Leu^{714} and Leu^{1366} polymorphisms, whereas, a reverse association was found in individuals carrying Leu^{714} homozygous variant in the population of early menarche of 0.60-fold relative risk. In addition, there was no significant association between the age of FFTP, menopause statuses and FANCD2 polymorphisms examined in this study (Table 2 and 3). Supposing that slow repairing activities of intrestrand crosslink associated with polymorphic allele of Leu^{714} and Leu^{1366} (G allele) phenotypes, one additional risk genotypes will have the elevated risk in the development of breast cancer. Though we did find an increasing trend of risk susceptibility in breast cancer, however this did not reach statistically significant (Table 4). Furthermore, we were also aware of the modification of the environmental risk, estrogen exposure, on breast cancer development underlying the chromosomal aberration resulting from genotypic polymorphisms of FANCD2 (Yager and Liehr, 1996). Results of consistency showed that there was an increasing trend of breast cancer risk, associated with the number of the genotypic polymorphisms of FANCD2 (Table 5), however, those analysis did not reach statistically significant. It can be postulated that possible genetic factors of chromosomal aberrations by other genes mentioned in the previous studies, such as *BRCA1*, *BRCA2*, *Nbs1*, *Rad51* and *Mre11* (Futaki and Liu, 2001; Venkitaraman, 2002a; 2002b; Taniguchi et al., 2002). Therefore, these Fanconi anemia-associated genes will be adjusted by multivariate analyses including the information of their published polymorphisms in logistic regression model. And, *FANCD2* genotypes interacted with the genes responsible for the chromosomal segregation and within different levels of estrogen exposure, the

number of years between menarche, first full-term pregnancy and postmenopausal status will be needed to be addressed in future. In summary, this study yields the valuable clues reemphasizing into the association between genotypic polymorphisms of *FANCD2* and breast tumorigenesis, and even in the capacity for DNA repair and interstrand crosslink damage linked to estrogen exposure.

6.計畫成果自評

本研究根據民國九十年起至九十二年期間在臺大醫院和新光醫院外科乳癌病患所建立起大規模的基因資料庫，嘗試針對乳癌發生的病理成因加以探討。我們以協調染色體正常功能有關的 *FANCD2* 基因多型性為研究題材、結合相關系統性的臨床問卷調查，採單變相和多變相分析與乳癌發生病理關聯的分子遺傳學研究。希望藉此研究，能夠釐清因為 *FANCD2* 基因多型性變異導致染色體分裂異常對婦女乳癌發展所扮演的角色。

計劃執行中，在實驗的設計上，我們發現 FANCD2 基因型 Leu^{714} 和 Leu^{1366} 基因型(G allele)對乳癌致癌危險上雖具有保護的作用，但未達統計顯著水準。當體重肥胖指數大於 24 時，此二基因型則具有明顯的保護作用，且達到統計顯著相關。此外，針對動情激素的暴露而言，我們亦以初經年齡和停經年齡做為其內生性環境危險因子的暴露指標。但初步分析未能觀察出動情激素暴露時間與 *FANCD2* 基因多型性變異的關聯。探討可能的原因是與部分婦女尚未到達停經的時期，其有關停經年紀的問卷資料，仍需持續的追蹤調查。綜合言之，此基因資料庫是針對台灣地區華人的乳癌發生所建立的分子流行病學研究。除能夠部份解答台灣近年來乳癌發生率暴漲的致癌機轉，本實驗更首度著眼於因為染色體異常所可能造成細胞癌化的成因探討。藉由本篇乳癌研究領域的規模和深度，亦可隨時選擇對於華人有特別意義的基因型繼續探討，其後續對乳癌致癌成因和乳癌治療的預後評估的貢獻將相當可觀。

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Table 1. Frequency distributions of the genotypic polymorphisms of *FANCD2* in Chinese female breast cancer patients, Taiwan.

	Cases (%) (n=561)	Controls (%) (n=1126)	OR (95% CIs)	aOR (95% CIs) *	P value
Leu^{714th} Pro					
Leu/Leu	246 (43.85)	501 (44.49)	1.00		
Pro/Leu	236 (42.07)	490 (43.52)	0.98 (0.78-1.23)		
Pro/Pro	79 (14.08)	135 (11.99)	1.19 (0.86-1.66)	1.03 (0.83-1.27)	P=0.802
Leu allele (T allele)	64.9	66.3	1.00		
Pro allele (C allele)	35.1	33.7	1.41 (0.82-2.42)		

	Cases (%) (n=561)	Control (%) (n=1122)	OR (95% CIs)	aOR (95% CIs) *	P value
Leu^{1366th} (silence mutation)					
Homozygous T	472 (84.1)	931 (82.9)	1.00		
Heterozygous T/G	88 (15.8)	179 (15.9)	0.97 (0.73-1.29)		
Homozygous G	1 (0.1)	12 (0.02)	0.16 (0.01-1.12)	0.92 (0.69-1.22)	P=0.547
T allele	92.0	91.0	1.00		
G allele	8.0	9.0	0.88 (0.29-2.62)		

*, combined genotypes with C-to-T mutation at nucleotide 2141st and T-to-G mutation 4098th position, respectively.

Adjusted odds ratios are considered as the group of combined risk susceptibility in association with breast cancer.

Table 2. Breast cancer risk (aOR) associated with endogenous environmental factors stratified by individual *FANCD2* genotypic polymorphisms.

Factors	Genotypes	Cases	Controls	OR [b]	OR [c]
		No（%）	No（%）	（95% CI）	（95% CI）
BMI	**Leu^{714}/Pro**				
≦24	TT	130(23.7)	361(32.1)	1.00 (--)	1.00 (--)
≦24	TC/CC	172(31.3)	413(36.8)	1.18(0.90-1.55)	1.21(0.90-1.61)
＞24	TT	111(20.2)	138(12.3)	1.78(1.28-2.48) *	1.71(1.21-2.43)*
＞24	TC/CC	136(24.8)	211(18.8)	1.40(1.03-1.90) *	1.31(0.95-1.80)
	Leu^{1366} silence				
≦24	TG/GG	44(8.0)	139(12.4)	1.00 (--)	1.00 (--)
≦24	TT	258(47.0)	634(56.7)	1.31(0.90-1.90)	1.41(0.94-2.12)
＞24	TG/GG	42(7.7)	52(4.6)	2.01(1.16-3.46)*	1.96(1.10-3.50)*
＞24	TT	205(37.3)	294(26.3)	1.75(1.18-2.58)*	1.75(1.15-2.68)*
FFTP	**Leu^{714}/Pro**				
≦26	TT	161(32.0)	281(28.2)	1.00 (--)	1.00 (--)
≦26	TC/CC	196(39.0)	335(33.7)	1.01(0.77-1.33)	0.98(0.74-1.28)
＞26	TT	60(11.9)	162(16.3)	0.77(0.53-1.11)	0.74(0.51-1.07)
＞26	TC/CC	86(17.1)	217(21.8)	0.80(0.58-1.11)	0.79(0.57-1.09)
	Leu^{1366} silence				
≦26	TG/GG	58(11.5)	95(9.6)	1.00 (--)	1.00 (--)
≦26	TT	299(59.4)	518(52.3)	0.97(0.68-1.41)	1.00(0.69-1.45)
＞26	TG/GG	20(4.0)	75(7.6)	0.51(0.28-0.94)*	0.52(0.28-0.96)*
＞26	TT	126(25.1)	303(30.6)	0.83(0.56-1.24)	0.84(0.56-1.26)

[a] The aORs and 95%CIs for breast cancer associated with BMI and first full-term pregnancy were calculated in a multivariate logistic regression model containing age, family history of breast cancer, and years of FFTP (>26 years *vs.* ≦ 26 years) before first full-term pregnancy. [b] OR was adjusted with age；[c] OR was adjusted with age、BMI and FFTP. *, p＜0.05, statistically significant.

Table 3. Breast cancer risk (aOR) associated with the duration of estrogen exposure stratified by individual *FANCD2* genotypic polymorphisms.

Factors	Genotypes [b]	Cases	Controls	OR [c]	OR [d]
		No（%）	No（%）	（95% CI）	（95% CI）
Early menarche	**Leu^{714}/Pro**				
＞13	TT	168(30.6)	281(25.0)	1.00 (--)	1.00 (--)
＞13	TC/CC	199(36.3)	381(33.9)	0.88(0.67-1.14)	0.83(0.63-1.09)
≦13	TT	72(13.1)	218(19.4)	0.67(0.48-0.94)*	0.60(0.41-0.86)*
≦13	TC/CC	110(20.0)	244(21.7)	0.89(0.66-1.21)	0.89(0.64-1.23)
	Leu^{1366} silence				
＞13	TG/GG	56(10.2)	109(9.7)	1.00 (--)	1.00 (--)
＞13	TT	311(56.7	550(49.1)	1.16(0.81-1.66)	1.15(0.79-1.68)
≦13	TG/GG	30(5.5)	82(7.3)	0.90(0.52-1.55)	0.80(0.44-1.45)
≦13	TT	152(27.7)	379(33.8)	0.97(0.66-1.42)	0.97(0.64-1.45)
postmenopause	**Leu^{714}/Pro**				
≦50	TT	95(30.5)	118(30.5)	1.00 (--)	1.00 (--)
≦50	TC/CC	110(35.3)	142(36.7)	0.94(0.65-1.36)	1.01(0.68-1.50)
＞50	TT	47(15.1)	64(16.5)	0.75(0.47-1.21)	0.87(0.53-1.42)
＞50	TC/CC	60(19.2)	63(16.3)	0.95(0.60-1.51)	0.96(0.59-1.54)
	Leu^{1366} silence				
≦50	TG/GG	32(10.3)	49(12.7)	1.00 (--)	1.00 (--)
≦50	TT	173(55.5)	209(54.3)	1.25(0.76-2.05)	1.41(0.84-2.39)
＞50	TG/GG	18(5.8)	22(5.7)	0.99(0.46-2.17)	1.02(0.45-2.31)
＞50	TT	89(28.5)	105(27.3)	1.07(0.62-1.83)	1.24(0.71-2.20)

[a] The aORs and 95%CIs for breast cancer development associated with menopause statues were calculated in a multivariate logistic regression model containing age, family history of breast cancer, and years of menarche (>13 years *vs.* ≦ 13 years) and menopausal statues (>50 years *vs.* ≦50 years); [b] represented as combined genotypes of heterozygous and homozygous variant type; [c] OR was adjusted with age；[d] OR was adjusted with age、BMI and FFTP, * p＜0.05, statistically significant.

Table 4. Breast cancer risk (aOR and 95% CI) associated with the number of putative high-risk genotypes of FANCD2 genotypic polymorphisms of *Leu*^{1366} silence mutation and *Pro*^{417}*Leu* missense mutation.

No. genotypic polymorphisms of FANCD2[a]	Cases (n=561)	Controls (n=1122)	P value
0	193 (34.40%)	373 (33.24%)	
1	332 (59.18%)	684 (60.96%)	
2	36 (6.42%)	65 (5.80%)	X^2=0.588; P=0.745

a, The women harboring the same numbers of high-risk genotypes of FANCD2 polymorphisms. The risks were estimated using the women harboring the wild type of *FANCD2* genotypes as the reference.

Table 5. Modification of breast cancer risk by environmental risk factors associated with different numbers of *FANCD2* genotypic polymorphisms

Environmental factors	Sum of the mutant genotypes[a]	Cases No. (%)	Controls No. (%)	OR[b] (95% CI)
BMI	Leu^{714}/Pro, Leu^{1366} silence			
≦24	0	24(4.4)	89(8.0)	1.0
	1	126(23.0)	321(28.7)	1.32(0.78-2.25)
	2	152(27.7)	363(32.4)	1.58(0.94-2.67)
＞24	0	28(5.1)	37(3.3)	1.97(0.96-4.02)
	1	97(17.7)	115(10.3)	2.28(1.29-4.01)*
	2	122(22.2)	194(17.3)	1.63(0.95-2.80)
FFTP	Leu^{714}/Pro, Leu^{1366} silence			
≦26	0	33(6.6)	65(6.6)	1.0
	1	153(30.4)	244(24.6)	1.27(0.79-2.06)
	2	171(34.0)	304(30.7)	1.11(0.69-1.79)
＞26	0	16(3.2)	48(4.8)	0.74(0.36-1.55)
	1	48(9.5)	141(14.2)	0.81(0.47-1.40)
	2	82(16.3)	189(19.1)	1.01(0.61-1.69)
Menarche	Leu^{714}/Pro,Leu^{1366} silence			
＞13	0	39(7.1)	67(6.0)	1.00
	1	146(26.6)	255(22.8)	1.00(0.62-1.60)
	2	182(33.2)	337(30.1)	0.91(0.57-1.45)
≦13	0	13(2.4)	59(5.3)	0.41(0.18-0.91)*
	1	76(13.8)	181(16.2)	0.80(0.48-1.35)
	2	93(16.9)	221(19.7)	0.89(0.54-1.46)
Postmenopause	Leu^{714}/Pro,Leu^{1366} silence			
≦50	0	18(5.8)	34(8.8)	1.00
	1	91(29.2)	99(25.7)	1.85(0.93-3.68)
	2	96(30.8)	125(32.5)	1.64(0.83-3.23)
＞50	0	11(3.5)	15(3.9)	1.25(0.46-3.44)
	1	43(13.8)	56(14.6)	1.43(0.67-3.03)
	2	53(17.0)	56(14.6)	1.58(0.76-3.31)

[a] with one additional mutant genotype of *FANCD2* gene (*0 vs. 1 vs.2*), stratified by years of estrogen exposure, [b] adjusted OR was stratified with age, * $p<0.05$, statistically significant.