行政院國家科學委員會專題研究計畫　成果報告

FANCD2之活化與BRCT的交互作用及其FANCD2基因多形性與乳癌發生關聯之分子遺傳學研究

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1. 中文摘要

Fanconi 貧血症(Fanconi anemia)是一種關於染色體異常的體隱性遺傳疾病，臨床上常會伴隨著惡性細胞轉形的發生。*FANC* 群體複合蛋白會參與細胞因為染色體在分裂時可能發生交錯連結(interstrand crosslink)異常的修復作用。*BRCA1* 協同 DNA 雙股斷裂修復基因 *Rad51, Rad52, Nbs1* 和 *Mre11* 等，參與 DNA 同源染色體重組修復作用。最近，在細胞遺傳學的研究證據顯示，於乳癌的致癌機轉中，*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。然而，在乳癌細胞株 *HCC1937* (BRCT truncated protein)卻無法活化 *FANCD2* 進行染色體斷裂的修復。因此，本研究即是以酵母菌雙重雜交試驗來探討 *BRCA1* 之 *BRCT* 區域和 *FANCD2* 之間的交互作用。實驗中，我們首先對 *BRCT* 部份以定位突變建構此區域基因多型性的重組質體，並以核酸序列確認。另外，針對 *FANCD2* 藉由反轉錄作用，設計特定序列引子，將 *FANCD2* 基因區分為三個部分，並進行其基因片段的分子選殖。之後，將 *BRCT* 多型性基因片段各自連結於 *pGBT9* 質體(含 DNA 結合部位)和 *FANCD2* 的次選殖片段載入 *pACT2* 質體(含 DNA 活化部位)。最後，再將兩者以配對的方式分別殖入酵母菌（*LV40*）中，分析β-galactosidase (β-半乳糖酶)的酵素活性。現階段，我們已成功地完成了 15 個 *BRCT* 多形性變異基因的選殖和建構。再者，亦完整地選殖出 *FANCD2* 基因的三個次選殖部分，並完成其重組質體的建構。在後續的研究工作中，將針對 *BRCT* 多型性基因片段和 *FANCD2* 的次選殖片段分批共同殖入酵母菌中，評估分析酵母菌β-半乳糖酶的酵素活性，以確實釐清 *BRCA1* 與 *FANCD2* 真正作用的區段。台灣地區婦女乳癌的年輕化和高發生率已是一個不容忽視的公共衛生問題，其中因為染色體交錯異常亦常常是導致乳癌發生的關鍵原因之一。本實驗著眼於因為染色體異常的修復作用所可能造成細胞癌化的病理成因探討，其了解 *BRCT* 和 *FANCD2* 的作用機轉，除了能夠更進一步探討台灣近年來乳癌發生率高漲的可能致癌原因外，其後續對乳癌致癌診斷和治療的預後評估其貢獻將相當可觀。

關鍵字：乳癌、染色體雙股錯結、BRCA1、FANCD2、酵母菌雙重雜交

2. ABSTRACT

Fanconi anemia (FA) is an autosomal recessive disorder with diverse clinical symptoms and markedly predisposition to cell malignancy. FANCD2 can colocalize with BRCA1 in nuclear foci, associating with chromosomal damage repair. Underlying the rationale that FANCD2 can repair endogenous DNA interstrand crosslinks damage caused by estrogen exposure (the most important risk factor of breast cancer) and interact with *BRCA1*, showed that initiation of FANCD2 protein against hypersensitivity to such DNA break lesions. We thus, aimed at exploring the possible interaction between *BRCA1 BRCT* and *FANCD2* proteins using yeast two-hybrid system.

In the current study, we succeeded in constructing and sequencing 15 various polymorphic sites in *BRCT* region using site directed mutagenesis technique. On the other hand, the partial cDNA fragments of *FANCD2* were also separately subcloned into

3 portions. The *BRCT* clones, containing different polymorphic sites of C-terminus domains were further fused into vector *pGBT9*, having *Gal4* DNA binding domain (DBD). In addition, three *FANCD2* subclones have been individually reconstructed into vector *pACT2*, who has *Gal4* activating domain (AD). Followingly, the works at hand are going to separately cotransfected the polymorphic allele of *BRCT* and *FANCD2* subclones into yeast *LV40* cells. To fully understand tumorigenic contribution associated with interaction between individual *BRCT* and *FANCD2* in chromosomal damage repair, a yeast two-hybrid assay using β-galacosidease reporter gene will be used. We are now striving to obtain cotransfectants harboring *BRCT* and *FANCD2* subclones and examine their β-galacosidease activities in yeast. In summary, these data will provide a comprehensive understanding and characterizing the real interactions between the unique genetic polymorphism of *BRCT* acting on *FANCD2* fragment against DNA interstrand crosslink damage to breast cancer development. Also, this model will be further applied to explore the independent genetic risk factors in contribution to define the etiology of Chinese breast cancer development in Taiwan.

Keywords：Breast Cancer、interstrand crosslink、BRCA1、FANCD2、
Yeast two-hybrid assay

3.計畫緣由與目的

In the past fifteen years, breast cancer has remained the second most common cancer in Taiwanese women and the fourth leading cause of cancer mortality. More importantly, the incidence of Taiwanese breast cancer has increased more than two folds in the past two decades. Fanconi anemia (FA) is a rare autosomal recessive disease characterized by skeletal defects, anemia, chromosomal instability and increased risk of leukemia (Carreau et al., 1999; 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; Carreau et al., 1999; Taniguchi and D'Andrea, 2002). FA protein family consist at least eight complementation groups. FA complex proteins form the nuclear multiprotein, which is essential for the activation of FANCD2 by ubiqintination. 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).

Recently, evidence has been shown that BRCA1 is associated with the FA complex

via a molecular interaction with FANCD2. BRCA1 and FANCD2 can both colocalize in nuclear foci, which are associated with DNA damage and repair (Wang and D'Andrea, 2004). Study *in vitro* showed that the deficits of ICL DNA damage repair in tumor cell line *HCC1937* with MMC-treated unravels that BRCT domain of BRCA1 interacted with FA proteins, especially for the ubiquintinated form of FANCD2. However, to date, much less information is currently available regarding the potentiality of BRCT polymorphisms and *FANCD2* gene in breast cancer development as well as lacking of the knowledge of the roles between *BRCT* and *FANCD2* specifically against ICL DNA damage. We thus constructed the various polymorphic sites in *BRCT* region containing different polymorphic sites in the C-terminus domain of *BRCA1* gene using site directed mutagenesis technique. Besides, we separately cloned the partial cDNAs of *FANCD2*. Then, we cotransfected the mutated *BRCT* subclones and partial cDNA fragments of *FANCD2* into yeast *LV40* cells. A yeast two-hybrid assay using β-galacosidease reporter gene will be employed to measure relative contribution of individual *BRCT* clones associating with *FANCD2* in the restoration for DNA damage against DNA hypersensitive agents. Based on this molecular study would be not only of benefits in overviewing the network of DNA repair systems in contribution to the maintenance of chromosomal fidelity, but also give the more precise picture of repair in DNA interstrand crosslinks associated with *BRCA1* and *FANCD2* and a more comprehensive insight into breast tumorigenesis, resulting from the oxidative DNA damage.

4. 材料和方法

Construction of BRCT and FANCD2 subclones. BRCT domain is required for activation and leading to nucleus of FANCD2 ubiquintination. We focused on the BRCT domain, which has been defined to play an important role in transcription activation of *BRCA1*. Numerous BRCT domains harboring different single nucleotide polymorphisms or nonsense/frame-shift mutations identified in population or in cancer patients, respectively, were cloned using site directed mutagenesis technique. These *BRCT* fragments with different polymorphic site are used to examine various binding abilities to FANCD2 in the following experiments. As to the partial cDNA fragments of *FANCD2* had been amplified using mismatch PCR primers and the resultant amplification products containing the *EcoRI-BamHI* sites were obtained from normal healthy control cDNA libraries. All of the primer sequences are listed as follows.

Primers designing for N-terminus of FANCD2 with 512 amino acids:

5'- ATA GGA TCC ATG GTT TCC AAA AGA AGA CTG TCA

5'- ATA CTC GAG GAC AGC ATT CAT CAT CAT AGC AGA

Primers designing for middle region of FANCD2 with 512 amino acids:

5’- ATA GAA TTC TGC CTT AGT GAC CCA TAT CTG CAG

5’- ATA CTC GAG TGA ATG TTC TCC AGG TGG TTA CAC

Primers designing for C-terminus of FANCD2 with 492 amino acids:

5’- ATA GGA TCC CTG ACA CCT CCT ATT GCC AGG AGA

5’- ATA CTC GAG TGG AGG GAA TGG AAA TGG GCA TAG

PCR amplification products containing *EcoRI-BamHI* sites will be adapted into vector, pCR2.1. Of the following cloning strategies, those recombinant plasmids harbored either *FANCD2* subclones or *BRCT* mutated clones will be further constructed and trasnfected, respectively. The mutated *BRCT* cDNA fragments are prepared to subclone into *pGBT9* plasmid (DNA binding domain, DBD), while the partial cDNAs of *FANCD2* are subjected to subclone into *pACT2* vector (activating domain, AD).

Transfection conditions. Cotransfection of the shuttle vectors were performed according to the protocol described Durfee et al. (1993) with some modification. Plasmids harbored different *BRCT* and *FANCD2* subclones will be cotransfected into yeast *LV40* yeast strain using lithium acetate reagent with 100 ug salmon sperm DNA carrier. Transfectants were incubated with THULL broth complexes with 25 ug/ml ampicillin. After transfection, yeast cells were grown onto THULL agar plate within 40ug/ml X-gal and incubated three days for screening the blue colony. After that, the co-transfected yeasts were harvested after 1-2 days incubation, and individual colony was selected and analyzed for the interaction assay.

β-galcotosidase reporter gene assay. To answer the question whether the *FANCD2* interacts with specific component in *BRCT* domain, the yeast two-hybrid system is used to examine the differential interactions between *BRCA1* and *FANCD2* fragments harboring different *BRCT* polymorphisms with nonsense/frame-shift mutations and *FANCD2*. The interactions between *pACT2/FANCD2* (prey) and *pGBT9/BRCT* subclones (bait) were measured by the reporter gene of β-galacosidase activity. The blue color is to be calculated using microplate reader at wavelength of 540nm, representing the intensity of binding efficiency between the BRCT variations and FANCD2.

5. 結果與討論

To the best of our knowledge, deficits of the repair mechanism against ICLs was manifested with the spontaneous chromosome breakage and IR sensitivity in HCC1937, BRCA1 double knockout cells (*BRCA1*-/-) (Wang and D’Andrea, 2004). Tumorigenic contribution of *BRCA1 BRCT* domain in breast cancer is specifically associated with its participation in DNA interstrand crosslinks against oxidative damage by activation of FANCD2, imposing a selective microenvironment to breast epithelium cells that have reduced ICL-repaired capacity due to the specific genotype. As to our previous research

experiences, the apparent uncommon frequency distribution of BRCA1 polymorphisms found in Caucasian population were detected in our population showing that there would be other genotypic profiles associated with the development of breast cancer in our population. To completely understand the etiological role of *BRCA1*-associated *FA* protein in response to DNA damage from DNA hypersensitive agents in breast tumorigenesis, there would be required more evidences that evaluate interactions between *BRCT* and FA complementation groups, especially for the activation of *FANCD2* mono-ubiquintination. Our previous molecular epidemiological study comprehensively address the most possible relevant issues regarding genotypic associations with cell oncogenesis, including the examining the genotype frequencies of *FANCD2 Pro*^{714} and *Leu*^{1366} in 561 sporadic breast cancer patients and 1126 age-matched controls in a Chinese population. However, both did not show an independent risk factor in the association with Chinese breast cancer development in Taiwan. We further issued a cancer genetic study and strived to identify the interplaying roles of *BRCA1* and *FANCD2* genes in interstrand crosslink damage. Molecular cloning strategy for the *BRCT* polymorphic alleles and partial *FANCD2* DNA fragments were designed and cloned. We constructed the *BRCT* subclones using site directed mutagenesis technique, yielding *BRCA1* clones with full BRCT (exons 16-24), N-terminus of BRCT (exons 16-21), C-terminus of BRCT (exons 21-24) and individual clones harboring mutation/polymorphism at amino acid residues 1708, 1756, 1775 and 1853 (Fig. 1). Amino acid residue in the conserved region at 1775th residue is the most frequent found in the *BRCA1* C-terminus (Jensen et al., 1996; Langston and Malone, 1996). Moreover, tyrosine residue with the substitution of stop codon, leading to a truncated protein only 11 amino acid from the C-terminal end that is associated with very early onset breast cancer (Friedman et al., 1994; Hayes et al., 2000). As a result, a total of 15 *BRCT* expression clones were utilized for further re-subcloning into vector *pGBT9* containing with DNA binding domain) (Fig.1). Detailed sequencing analyses of the *BRCT* subclones were examined and shown in Fig. 2. On the other hand, the cDNA subclones of *FANCD2* gene were separately characterized into three portions, including 512 amino acids from N-terminus; 512 amino acids located in the middle part which containing the lysine residue at 561 position and proline residue at 713 position, and the C-terminus with 492 amino acids of *FANCD2*. The reasonably *Gal4* AD fusion of the *FANCD2* DNA fragments into plasmid *pACT2* were completely subcloned. To explore the phenomena that *BRCT-FANCD2* is essential for the interaction and activation of mono-ubiquntinated form of FANCD2 in transfected breast HCC1937 cells, we thus, co-transfected and expressed the *BRCT* and *FANCD2* gene clones into yeast *LV40* cells, and the transfectants were subjected to yeast-two hybrid assay by estimating β-galacosidase reporter gene. However, till yet we have not completely performed all the *BRCT* subclones

co-transfected with *FANCD2* subgenes into yeast and thus fail to show the whole data of differential expression of all transfectants tested in this study. To confirm the mechanisms in protein interactions between *BRCT* and *FANCD2* genes, further evidences are needed to be investigated.

Based on our hypothesis that tumorigenic contribution of the roles between BRCA1 and FANCD2 in breast cancer is specifically associated with its potential role in participation in DNA interstrand crosslinks against oxidative damage, and oxidative lesions caused by estrogen exposure in breast epithelium would impose a selective microenvironment to the breast epithelium cells. Exploring the directing role between *FANCD2* and *BRCT* polymorphic alleles in endogenous oxidative DNA damage and breast cancer development was manifested with chromosomal abnormalities associating with estrogen exposure. To our knowledge, those three partial FANCD2 cDNA subclones presented here are the first investigated from the literatures yet. In addition, the *BRCT* mutated suclones are collaborated internationally, i.e. collaborated with Dr. Monteria's Lab works. Once such BRCT-FANCD2 protein-interacting model established, it can be further applied in predicting potentially 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; Taniguchi et al., 2002; Timmer et al., 2001; Tomposon and Schild, 2001; Venkitaraman, 2002a; 2002b). In summary, this study can yield the valuable clues reemphasizing into the association in responding to the roles of *FANCD2* gene and *BRCT* polymorphisms in breast tumorigenesis, and even in the capacity for DNA repair and interstrand crosslink damage linked to environmental hormonal exposure.

6. 計畫成果自評

在乳癌致癌危險因子中，女性雌激素的暴露在代謝過程中扮演著極為關鍵的危險因子，內生性雌激素會直接或間接的導致 DNA 的變異，因此對乳癌的發生而言，對抗環境荷爾蒙的暴露傷害造成 DNA 雙股斷裂和染色體的錯結的修復機轉將格外重要。*BRCA1* 會協同 *Rad51, Rad52, Nbs1* 和 *Mre11* 等基因來參與對抗 DNA 雙股斷裂傷害的修復過程；再者，2003 年 D'Andrea 等人就細胞遺傳學的研究指出，*BRCA1* 之 *BRCT* 可能會與 *Fanconi anemia* (*FANCD*) 群體複合蛋白作用、活化並共同移位至細胞核中修復染色體交錯斷裂的遺傳物質錯誤。本研究則是著眼於 *BRCA1* 和 *FANCD2* 兩者間的交互作用，探討有關乳癌病例中 BRCA1 之 BRCT 區域基因多形性變異與 FANCD2 蛋白之間的關係。在本研究中，我們已成功的完成了 15 個 *BRCT* 多形性變異基因的選殖和建構。再者，亦完整地將 *FANCD2* 基因區分為三處次選殖部分並完成其重組質體的建構，回顧文獻至今，這將是最新的實驗設計。為了能夠更加詳細釐清此二者蛋白的作用關係，我們在後續的研究中，將針對 *BRCT* 多型性基因片段和 *FANCD2* 的次選殖片段殖入酵母菌（*LV40*）中，進行酵母菌雙重雜交試驗(yeast two-hybrid assay)，以確實釐清 *BRCA1* 與 *FANCD2* 真正作用的區段及 BRCT

對 FANCD2 蛋白活化的功能性分析。除了能夠更進一步探討台灣近年來乳癌發生率高漲的可能致癌機轉，本實驗更首度著眼於因為染色體鏈結結構錯誤的修復作用異常所可能造成細胞癌化的病理成因探討，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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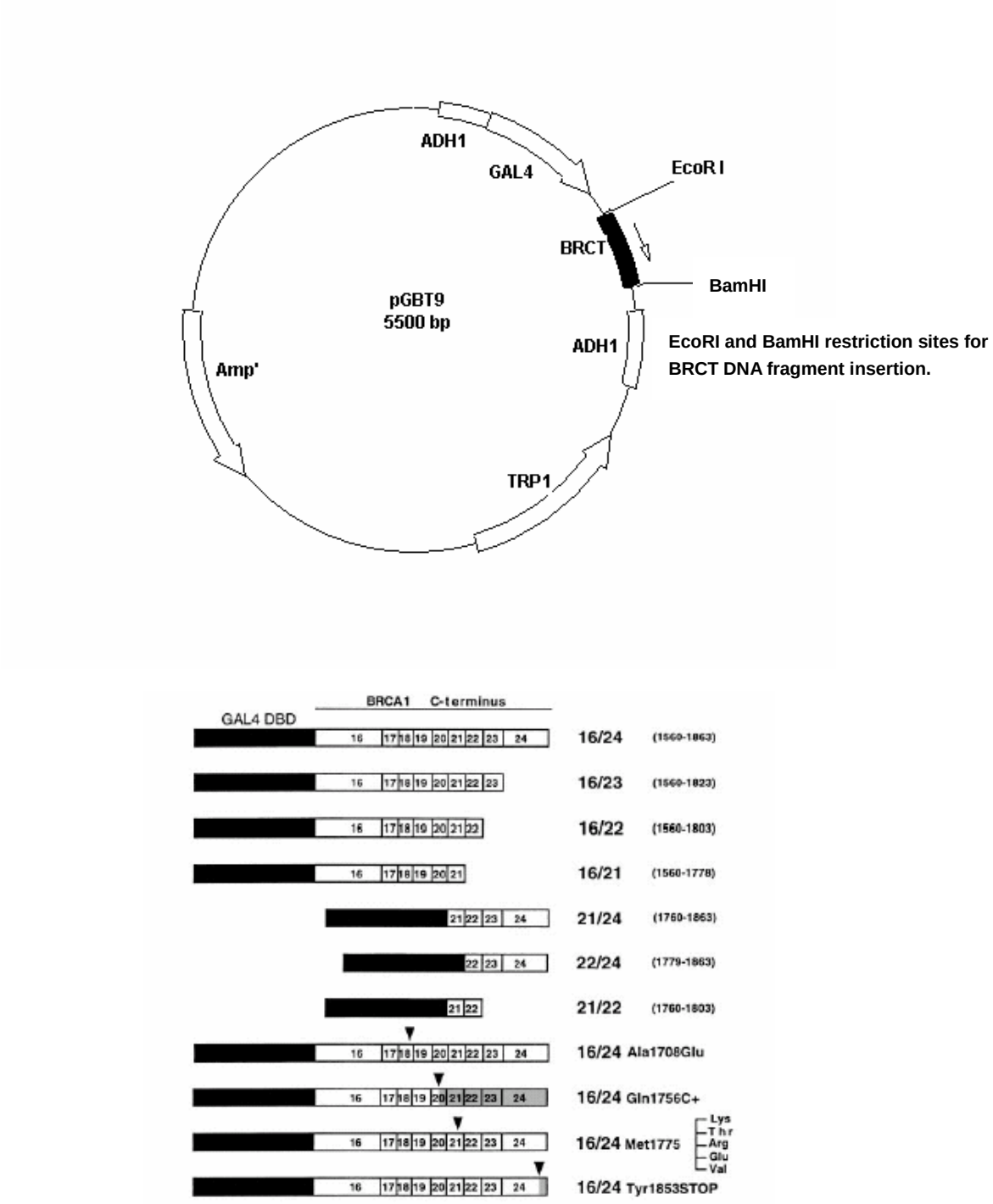


Fig. 1. Structure of transfection clones. *BRCA1* clones with full BRCT (exons16-24), N-terminus of BRCT (exons 16-21), C-terminus of BRCT (exons21-24) and individual clones harboring mutation/polymorphism at amino acid residues 1708, 1756, 1775 and 1863. As a result, a total of 15 *BRCT* expression clones utilized for transfection and subjected to yeast-two hybrid assay.

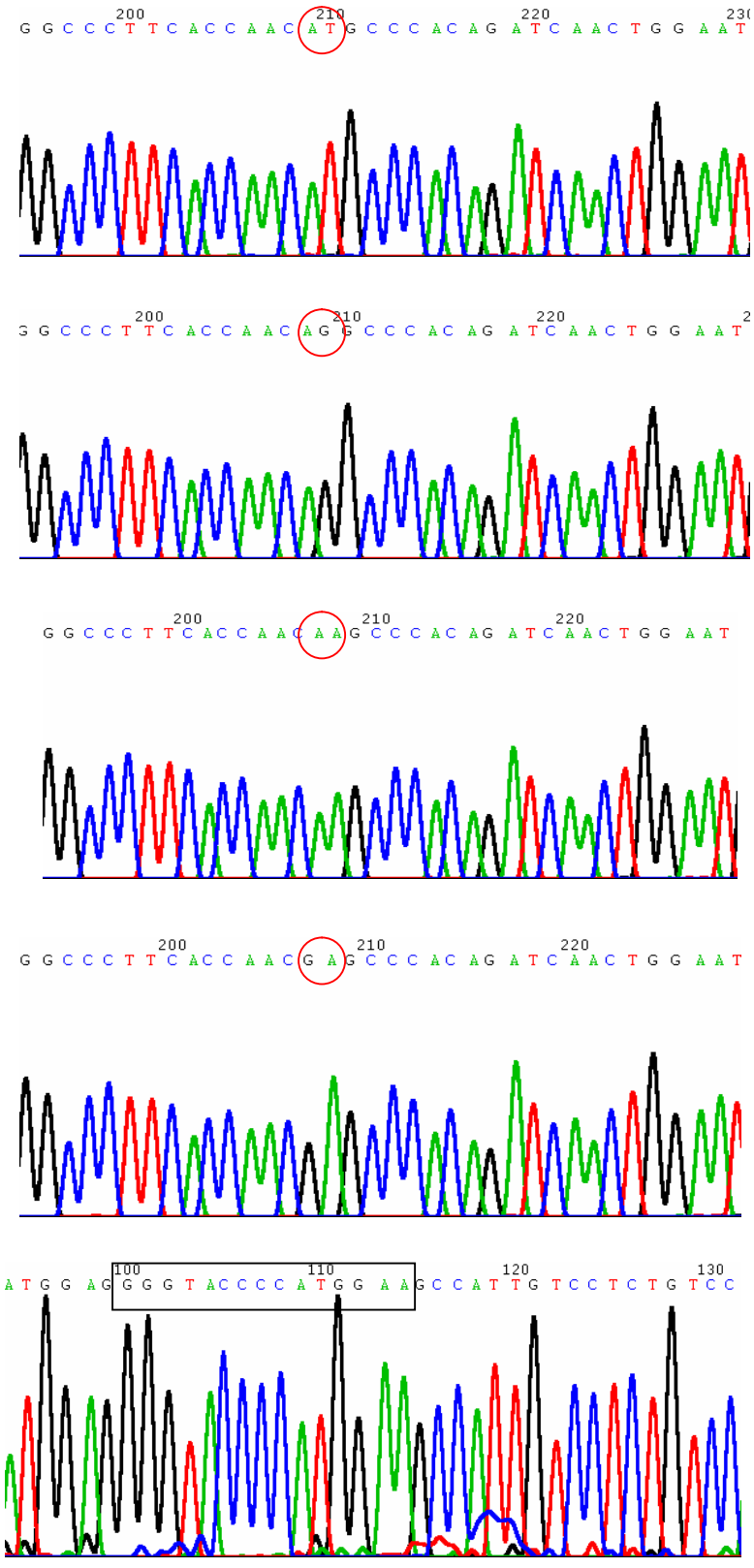
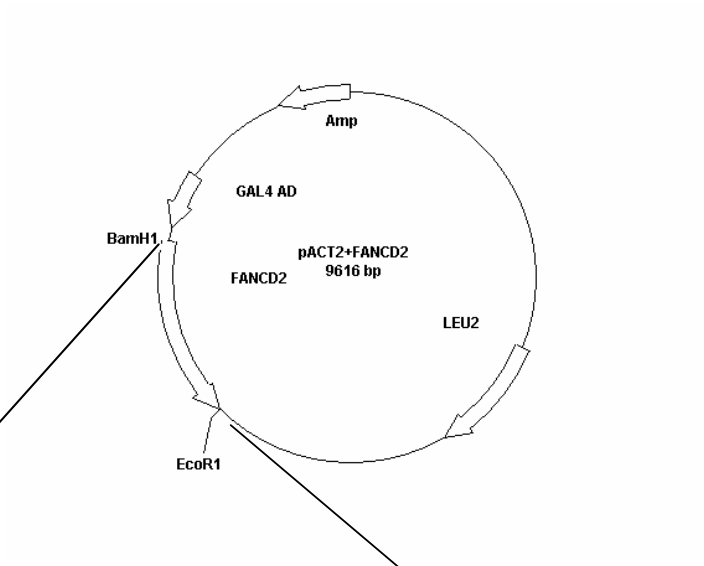


Fig. 2. The partial sequencing data with the targeted nucleotide substitutions in *BRCT* cDNA. Open red circles represented the different polymorphic sites at 1775th amino acid and open black square represented insertion of five amino acids in 1822nd in *BRCT* domain, respectively. These recombinant plasmids were subcloned into p*GBT9* vector (bait) for the evaluation of the interactions between *BRCT* and *FANCD*2 using yeast two-hybird assay.



Full length of *FANCD2* gene with 4,476 nucleotides encoded a protein of 1,492 amino acids

N-terminus of FANCD2 with 512 amino acids (1536 bps)
222nd amino acid (kinase phosphorylation of serine residue for S-phase checkpoint response in *FANCD2*)

M-terminus of FANCD2 with 512 amino acids (1536 bps)
561st amino acid (mono-ubiquintination of lysine residue in *FANCD2*)

C-terminus of FANCD2 with 492 amino acids (1476 bps)

Fig. 3. Full length and partial DNA fragments of *FANCD2* gene re-subcloned into plasmid *pACT2* (prey). Arrows showed here indicated the functional amino acid residues in *FANCD2*.