CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Clinical specimens

During the period between September 2003 and December 2004, blood samples were collected from 31 Thai breast cancer patients. There were 5 breast cancer cases (P1, P17, P20, P35 and P39) with a family history of cancer from patients admitted to Songklanagarind Hospital. These patients aged between 20-80 years and did not receive any medical treatment. All of the patients had given informed consent before providing their blood samples. In addition, one healthy woman who is a daughter of the patient who was identified with the mutation of *BRCA1* in the study was included for examination of gene mutation.

3.1.2 Materials for genomic DNA

- Proteinase K (20 mg/ml)
- 6 M Sodium Chloride
- Isopropanol
- 70% Ethanol
- Sodium Dodecyl Sulphate (SDS)
- Lysis buffer (320 mM Sucrose, 5 M $MgCl_2.6H_2O$, 10 mM Tris-base and 1% Triton-X-100)
- SE buffer pH 8.0 (75 mM NaCl and 25 mM EDTA)
- TE buffer pH 8.0 (10 mM Tris-base and 0.1 mM EDTA)
- QIAquick Gel Extraction Kit (QIAGEN, Germany)

3.1.3 Materials for polymerase chain reaction (PCR)

- DNA template
- Forward and reverse primers
- $MgCl_2$
- dNTP (dTTP, dATP, dCTP and dGTP)
- *Taq* DNA polymerase
- PCR Buffer (20 mM Tris-HCl and 50 mM KCl)

3.1.4 Materials for agarose gel electrophoresis

- Agarose powder
- Electrophoresis buffer (2.42 g of Tris-base, 0.571 ml of glacial acetic acid and 1ml of 0.5 M EDTA, pH 8.0
- 6x Gel-loading buffer (0.25% Bromophenol blue and 40% sucrose)
- Ethidium Bromide

3.1.5 Materials for single-stranded conformation polymorphism

- Electrophoresis buffer (54 g of Tris-base, 27.5 g of boric acid and 20 ml of 0.5 M EDTA (pH 8.0))
- Acrylamide
- 2x SSCP gel-loading (formamide, 5% bromophenol blue, 5% xylene cyanol, 1M EDTA and 1M NaOH)
- *N, N'* methylene bis-acrylamide
- TEMED (*N,N,N',N'*- tetramethylethylenediamine)
- 40% Methanol
- 160 mM Nitric acid
- 10% Citric acid
- 10% Ammonium Persulfate
- 37% Formaldehyde
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- Glycerol
- Sodium Carbonate
- Silver staining solution (100 mg of Silver Nitrate ($AgNO_3$) and 50 ml of deionized water)

3.1.6 Materials for DNA sequencing

- PRISM™ Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems Ltd., USA)

3.1.7 PCR primers specific for *BRCA1* gene

The entire coding sequence of the *BRCA1* gene from each of 33 DNA samples were amplified using thirty-one *BRCA1* primer pairs as adapted from Munnes *et al*., 2000. Thirty-one PCR fragments amplified with the specified primer covered 22 exons of *BRCA1* gene. Coding sequence of the largest exon (exon 11) was amplified through 10 overlapping fragments. Lists of *BRCA1* primers pairs used in this work are shown in Table 3.1. All primers were ordered from QIAGEN® Operon®, Germany.

Table 3.1 Oligodeoxyribonucleotide primers used for PCR amplification and nucleotide sequences (adapted from Munnes *et al*., 2000).

Primer	Nucleotide position	Primer sequence (5'-3')	Annealing temperature (°C)	Size of product (bp)
BRCF 2 BRCR 2	4557 4764	GAAGTTGTCATTTTATAAACC TCTGTTCATTTGCATAGGAG	55	227
BRCF 3 BRCR 3	12842 13114	GTTGACTCAGTCATAACAGCTC GGAGTTGGATTTTTCGTTCTC	60	293
BRCF 5 BRCR 5	22116 22445	TCTTTTCATGGCTATTTGCC CCTGTATAAGGCAGATGTCCC	60	350
BRCF 6 BRCR 6	23730 23873	GGTTGATAATCACTTGCTGAG GCACTTGAGTTGCATTCTTGG	56	174
BRCF 7 BRCR 7	24381 24685	GAGCATACATAGGGTTTCTC CCTGGGCCACAGAGCAAGAC	56	324
BRCF 8 BRCR 8	28811 29051	CTGGCCAATAATTGCTTGAC CTTCCCAAAGCTGCCTACCAC	60	261
BRCF 9 BRCR 9	31316 31504	TACCTGCCACAGTAGATGCTC CCAGCTTCATAGACAAAGG	55	207

Primer	Nucleotide position	Primer sequence (5'-3')	Annealing temperature (°C)	Size of product (bp)
BRCF 10	32753	CAGTTCTGCATACATGTAAC	60	181
BRCR 10	32914	CCCACTCTCTTTTCAGTGCC		
BRCF 11a	33774	GCCAGTTGGTTGATTTCCACC	60	404
BRCR 11a	34158	CCTTACTTCCAGCCCATCTG		
BRCF 11b	34046	CATTACAGCATGAGAACAGCAG	60	376
BRCR 11b	34402	GCATTTGATTCAGACTCCCC		
BRCF 11c	34377	GTTAGGTTCTGATGACTCACATG	60	408
BRCR 11c	34762	GTCTTTTGAACTGCCAAATCTGC		
BRCF 11d	34710	GCGTAAAAGGAGACCTACATCAG	60	393
BRCR 11d	35080	GGTGGGCTTAGATTTCTACTGAC		
BRCF 11e	35032	CTGAGGAGGAAGTCTTCTACCA	60	410
BRCR 11e	35419	GGGTCTTCAGCATTATTAGACAC		
BRCF 11f	35298	CCCAATGGATACTTAAAGCCTTC	60	405
BRCR 11f	35680	GCTGAAGTTAACAAATGCACCT		
BRCF 11g	35637	GGGACTAATTCATGGTTGTTCC	60	418
BRCR 11g	36032	CCTAGAGCCTCCTTTGATAC		
BRCF 11h	36032	CCTAGAGCCTCCTTTGATAC	60	441
BRCR 11h	36450	GTTGCAAAACCCCTAATCTAAGC		
BRCF 11i	36430	GGGCCAAAATTGAATGCTATGC	60	400
BRCR 11i	36808	CTATTTCTTGGCCCCTCTTCG		
BRCF 11j	36862	GAAGAGCTTCCCTGCTTCCA	60	520
BRCR 11j	37360	GTAAAATGAGCTCCCCAAAAGC		
BRCF 12	37610	CCAGTCCTGCCAATGAGAAG	62	218
BRCR 12	37808	CCACACACACGCATGTGCAC		
BRCF 13	46032	CTTGTAGTTCCATACTAGGTG	55	376
BRCR 13	46388	GGTCCTTACTCTTCAGAAGG		
BRCF 14	52045	CAGTATTCTAACCTGAATTATCA	60	241
BRCR 14	52265	GATGTCAGATACCACAGCATC		
BRCF 15	54167	CACAATTGGTGGCGATGG	60	297
BRCR 15	54445	CTTTATGTAGGATTCAGAG		
BRCF 16	57362	CCAACACTGTATTCATGTACCC	62	475
BRCR 16	57815	GTCATTAGGGAGATACATATGG		
BRCF 17	60983	CTGAGCTGTGTGCTAGAG	56	247
BRCR 17	61210	TCGGCCTCCCAAAGTGCTGC		
BRCF 18	64713	GCTTCTTAGGACAGCACTTCC	60	250
BRCR 18	64945	CTCAGACTCAGCATCAGC		
BRCF 19	65278	GTGAATCGCTGACCTCTC	56	306
BRCR 19	65542	ATGAGCCACAGTGCAGGCCTGC		
BRCF 20	71522	GACGTGTCTGCTCCACTTC	64	210
BRCR 20	71713	TACAGAGTGGTGGGGTGAG		
BRCF 21	77578	CTCTCCATTCCCCTGTCCCTC	64	196
BRCR 21	77753	GCAATCTGAGGAACCCCCATC		
BRCF 22	79482	GAGGGCCTGGGTTAAGTATGC	60	172
BRCR 22	79633	TGTGTCCTCCCTCTCTGACTG		
BRCF 23	80968	ATGAAGTGCAGTTCCAGTAG	60	207
BRCR 23	81155	CTCAAGCACCAGGTAATGAG		
BRCF 24	82841	GAACTCATACAACCAGGACCC	60	275
BRCR 24	83096	ACTTTGTAAGCTCATTCTTG		

3.2 Instruments

3.2.1 Agarose gel electrophoresis apparatus (EC 370, E-C Apparatus Company , USA)

3.2.2 Autoclave (HD-3D, Hirayama Company, Japan)

3.2.3 Freezer (Hotpack, Forma Scientific Company, USA)

3.2.4 Gel documentation equipment (1000, BIO-RAD Company, USA)

3.2.5 Hot air oven (Memmert GmbH Co., Germany)

3.2.6 Polyacrylamide gel electrophoresis apparatus (AE–6450 Dual mini slab kit, ATTO Company, Japan)

3.2.7 Polymerase chain reaction machine (GeneAmp 9600, Perkin-Elmer Company, USA)

3.2.8 Power supply (EC 135, E-C apparatus Company, USA and AE-8150 my power 500, ATTO, Company, JAPAN)

3.2.9 Refrigerated microcentrifuge (Kubota 1910, Kubota Company, JAPAN)

3.2.10 Shaking bath (SBO 50 BIO, Heto lab equipment Company, USA)

3.2.11 UV-spectrophotometer (Genesis 5, Spectronic Company, USA)

3.3 Methods

3.3.1 Genomic DNA extraction

3.3.1.1 Collection of blood samples

Five milliliters of blood samples from patients were collected into syringe and immediately transferred into falcon tube that contained five hundred microliters of ethylenediaminetetraamine solution (EDTA) as an anticoagulant. The tube was closed and the blood sample was mixed with EDTA solution by gently inversion several times. After this procedure, the blood cells were kept frozen at -20 ^{0}C until whole blood DNA extraction was performed.

3.3.1.2 Separation of white blood cells

The EDTA peripheral blood sample was allowed to stand at room temperature for 30 minutes. After that, blood samples were added with 15 ml of lysis buffer and mixed gently by inversion. The tube was placed on ice for 30 minutes, and inversely mixed while on ice, centrifuged at 9,000×g at 4 ^{0}C for 15 minutes. After that, supernatant was decanted. If the pellets were still red, additional 10 ml of cold lysis buffer was added and centrifuged again.

3.3.1.3 DNA extraction from white blood cells

The remaining white blood cell (WBC) pellet was resuspended in 2.5 ml of cold SE buffer, 6 µl of 20 mg/ml proteinase K and 125 µl of 20% SDS. The WBC suspension was incubated in a water bath overnight at 37 ^{0}C. On the second day, the WBC suspension was added with 1.4 ml of 6 M NaCl, vortexed at room temperature for 15 seconds and centrifuged at 9,000×g at room temperature for 15 minutes. The supernatant was transferred into a new falcon tube. The genomic DNA was precipitated with isopropanol. The DNA cluster was observed as a long and white like filament. A pastuer hook like pipette was used to fish out the DNA. The precipitated DNA was transferred to a 1.5 ml microcentrifuge tube. The DNA was washed with 70% ethanol and dried at room temperature for 30 minutes. The DNA was kept in 150 µl of TE buffer at 4 ^{0}C.

3.3.2 The purity and concentration of DNA

The purity and concentration of DNA was estimated by measuring the absorbance at 260 nm (OD_{260}) and 280 nm (OD_{280}) with a spectrophotometer. The ratio of OD_{260}/ OD_{280} was calculated to verify the purity of DNA. The ratio should be greater than 1.5 and the amount of DNA was calculated using following equation; 1 OD_{260} is equivalent to 50 µg/ ml DNA.

DNA concentration (µg/ ml or ng/µl) = OD_{260} x 50 x dilution factor

3.3.3 Screening for *BRCA1* mutations

3.3.3.1 DNA amplification

The purified genomic DNA was amplified by polymerase chain reaction (PCR). The standard procedure for a PCR reaction was performed in a 50 µl volume. It contained 1x PCR buffer (1.5 mM of $MgCl_2$ and 10 mM Tris-HCl pH 8.4), 1.5 to 3 mM of $MgCl_2$, 200 µM dNTPs, 0.5 µM each of the PCR primers, 2.5 U *Taq* DNA polymerase (QIAGEN, USA), 100 ng of genomic DNA and sterile distilled water to make up of 50 µl. The mixture was contained in PCR tube and placed in a thermal cycler (Perkin-Elmer GeneAmp 9600). The conditions of the thermal cycle are shown in Table 3.2

Table 3.2 The conditions of thermal cycle

	Step	Temperature (^{0}C)	Time	Number of cycle
1	Denaturing	94	5 minutes	1
2	Denaturing Annealing Extension	94 depend on each of BRCA1 exons 72	30 seconds 45 seconds 45 seconds	30
3	Extension Holding	72 4	10 minutes ∞	1

3.3.3.2 Analysis of the exon-intron 7 boundary sequence of patient ID 17 and the patient's daughter

The exon-intron 7 boundary was screened using the human genomic DNA of patient ID 17 and the patient's daughter. The exon-intron 7 boundary sequence was shown in Fig. 3.1. Oligonucleotide primers, designed from the human *BRCA1* genomic DNA, were used for primer extension PCRs to sequence patient ID 17 and the patient's daughter to determine the intron 7 sequence surrounding the exon 7. Five oligonucleotide primer pairs were used to amplify cover interested

exon-intron 7 boundary (Table 3.3). Amplification was carried out as follows: initiation denaturation at 94 ^{0}C for 5 min, then 94 ^{0}C for 30 s, annealing depend on each of *BRCA1* exon-intron 7 boundary primer for 45 s and extension 72 ^{0}C for 45 s for 30 cycles, followed by 1 cycle of 94 ^{0}C for 30 s, annealing depend on each of *BRCA1* exon-intron 7 boundary primer for 45 s and extension 72 ^{0}C for 10 min. The PCR-amplified fragments were directly sequenced using the same primers and dry terminators (PRISM^{TM} Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit) and an ABI 377 DNA sequencer (Applied Biosystems Ltd., USA).

Exon-intron 7 boundary sequence

24361 cattgtctta acacaacaaa gagcatacat agggtttctc ttggtttctt tgattataat tcatacattt Intron 6

24431 ttctctaact gcaaacataa tgttttccct tgtattttac ag **atgcaaac agctataatt ttgcaaaaaa**

Exon 7

24501 **ggaaaataac tctcctgaac atctaaaaga tgaagtttct atcatccaaa gtatgggcta**

24561 **cagaaaccgt gccaaaagac ttctacagag tgaacccgaa aatccttcct tg**|gtaaaacc atttgttttc

24631 ttcttcttct tcttcttctt ttctttttttt tttctttttt ttttttgaga tggagtcttg ctctgtggcc caggctagaa

Intron 7

24711 gcagtcctcc tgccttagcc cccttagtag ctgggattac aggcacgcgc caccatgcca ggctaatttt

24781 tgtattttta gtagagacgg ggtttcatca tgttggccag gctggtctcg aactcctaac ctcagggtgat

24851 ccacccacct cggctcccca aattgctggg attacaggtg tgagccactg tgcccggccg gtaaaaccat

24921 tttcatttat tctggcaaca tctctttatt gagcattgtg|

Fig. 3.1 The exon-intron 7 boundary sequences in the *BRCA1* gene. The exon 7 is indicated by boldface, while intron 7 sequences is indicated by vertical bars.

Table 3.3 Oligodeoxyribonucleotide primers used for PCR amplification of exon-intron 7 boundary and nucleotide sequence

Primers	Nucleotide position	Primer sequence (5'-3')	Annealing temperature (°C)	Size of product (bp)
Fw Rv 1	24381 24801	GAGCATACATAGGGTTTCTC GGTTTCATCATGTTGGCCAG	56	440
Fw Rv 2	24381 24869	GAGCATACATAGGGTTTCTC CAAATTGCTGGGATTACAGGTG	60	510
Fw 1 Rv 1	24555 24801	GGGCTACAGAAACCGTGCC GGTTTCATCATGTTGGCCAG	58	260
Fw 1 Rv 2	24555 24869	GGGCTACAGAAACCGTGCC CAAATTGCTGGGATTACAGGTG	60	337
Fw 2 Rv 1	24591 24801	TGAACCCGAAAATCCTTCCTTG GGTTTCATCATGTTGGCCAG	58	230

3.3.3.3 Agarose gel electrophoresis

The size of the fragments of *BRCA1* exons were checked for quality in 1.0% (w/v) agarose gel electrophoresis. A 1.0% (w/v) agarose solution was prepared using 0.5x TAE buffer melted and poured on a plastic tray. A plastic comb was then placed in the gel. After the agarose gel was completely set (30-45 minutes at room temperature), the comb was carefully removed and the gel was installed on the platform in the electrophoresis chamber containing 0.5x TAE buffer. The solution of *BRCA1* exons was mixed with gel loading dye and slowly loaded into the slots of the submerged gel using an automatic micropipette. Electrophoresis was carried out at a constant 80 volts for 40-45 minutes, or until the running dyes had migrated the necessary distance. Next, the gel was stained with 0.5 μg/ml of ethidium bromide (EtBr) solution for 5 minutes and destained with distilled water for 15 minutes. After that the DNA pattern was observed using a UV light box (Gel Doc model 1000, BIO-RAD, USA) and the photograph was taken and printed out.

3.3.3.4 Single-Stranded Conformation Polymorphism

3.3.3.4.1 Preparation of non-denaturing polyacrylamide gel

Assembled glass plates of the vertical mini-slab gel set according to the manufacture's detailed instructions. Both glass plates were carefully cleaned with distilled water and absolute ethanol and dried before assembly. Ten percent non-polyacrylamide gel was prepared as the mixture shown in the Table 3.4.

Table 3.4 Preparation of 10% non - denaturing polyacrylamide gel

Reagent	One gel (ml)	Two gel (ml)
40% acrylamide : bisacrylamide (49:1)	2.5	5.0
5 x TBE buffer	2.0	4.0
Glycerol	0.5	1.0
Distilled water	4.39	9.78
10% Ammonium persulphate	0.07	0.14
TEMED	0.004	0.008

The gel mixture was poured into the space between the glass-plates sandwich by using a syringe and a comb inserted immediately at the topside in order to make sample wells without allowing air bubbles to become trapped under the teeth. The gel was allowed to polymerize for at least 30 minutes. After polymerization, the glass-plates were assembled upon the electrophoresis set and the comb removed.

3.3.3.4.2 Sample preparation

The sample mixture contained 2 µl of each PCR product, 6 µl of distilled water and 2 µl of 2x SSCP gel-loading solution. The PCR product mixture was denatured at 95 ^{0}C for 10 minutes and allowed to stand in ice for PCR reannealing for 5 minutes.

3.3.3.4.3 Polyacrylamide gel electrophoresis

After applying 10 μl of PCR reannealing into a well of the non-denaturing polyacrylamide gel, electrophoresis was performed in 1x TBE buffer at room temperature at 70 volts, 15 mA for about 4 hours.

3.3.3.4.4 Silver staining of gel

The gel was transferred into a plastic box or flat bottomed chamber. The electrophoresed gel was fixed in 50 ml of 40% methanol for 10 minutes. Every step in silver staining was shaken. Then, 40% methanol was discarded, the gel was soaked in 50 ml of 160 mM HNO_3 for 6 minutes. The nitric acid was discarded and the gel was rinsed and washed twice for 5 minutes, each with distilled water. The gel was stained in 50 ml of 0.2% silver nitrate for 20 minutes and the staining solution was discarded into a waste box. The gel was washed twice with distilled water for 50 minutes each and soaked with 50 ml of the developer solution for 4-10 minutes, in order to develop the silver staining. The gel was shaken gently until the DNA bands became visible. Once the DNA bands were clearly seen, 50 ml of stop solution was immediately added, followed by shaking for 5 minutes. The gel was washed with distilled water for 5 minutes and finally dried with gel dryer for one day.

3.4 Data Analysis

Descriptive statistic was performed to calculate basic information of patients as age at diagnosis, clinical and pathological characteristics.