CHAPTER 4
RESULTS

4.1 Clinical /pathological characteristics of the patients

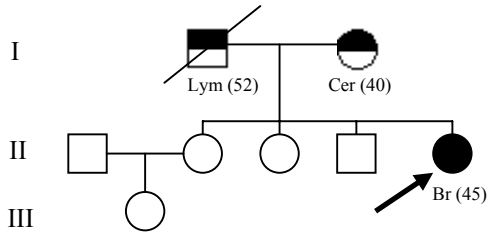
Between September 2003 and December 2004, blood samples were collected from 31 Thai patients diagnosed as affected with breast cancer (BrCa), age range 20-80 years, with no history of treatment by chemotherapy. There were 17 patients (ID 1, 2, 16, 17, 19, 20, 21, 22, 24, 25, 27, 28, 31, 32, 35, 39, 40) that presented with clinical features and had been admitted to Songklanagarind Hospital. Five of the 17 patients had a family history of cancer. The family history information and clinical features (pathologic classification, tumor grade and breast side) of each of the 17 patients are given in Appendix A. The other fourteen out of 31 patients did not have information of clinical features because they were volunteers in this project. The average age of 17 patients at diagnosis was 48 years (S.D. 11.3 years). The pathologic classification of the majority was invasive ductal carcinoma (inv.DC). The frequencies of clinical/pathological characteristics are summarized in Table 4.1.

Table 4.1 Percentage of the patients classified by clinical/pathological characteristics

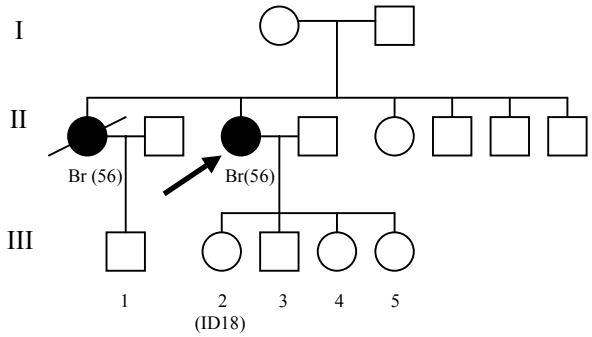
Type	Frequency	Percentage
Stage grouping		
I	6	35
IIA	5	29
IIIA	1	6
IIB	3	18
IIIB	2	12
Total	17	100
Breast side		
Right	11	65
Left	6	35
Total	17	100

Clinical pedigree data of these patient families (Fig. 4.1) are described below.

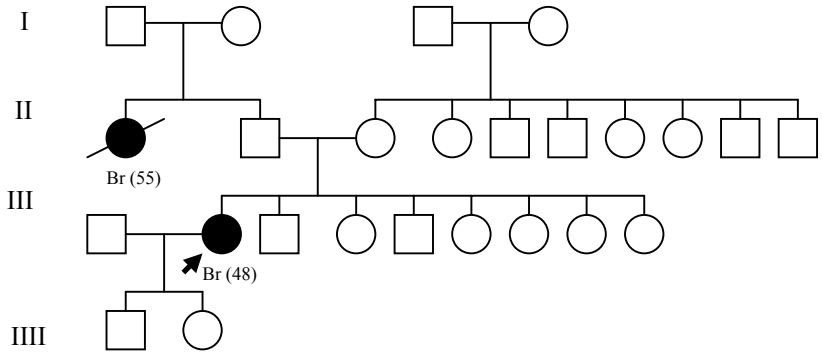
(a) Patient ID 1



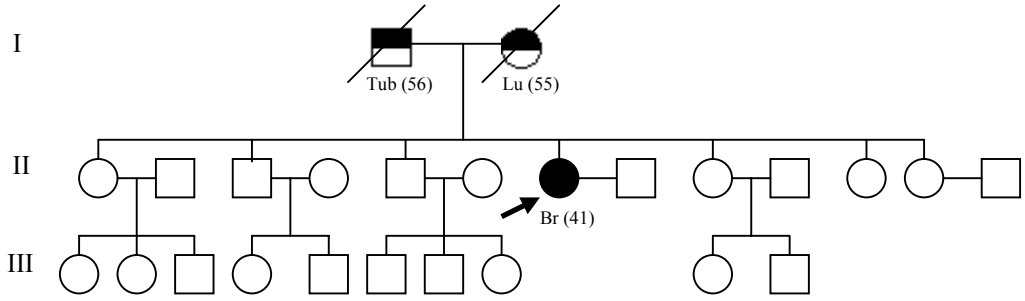
(b) Patient ID 17



(c) Patient ID 20



(d) Patient ID 35



(e) Patient ID 39

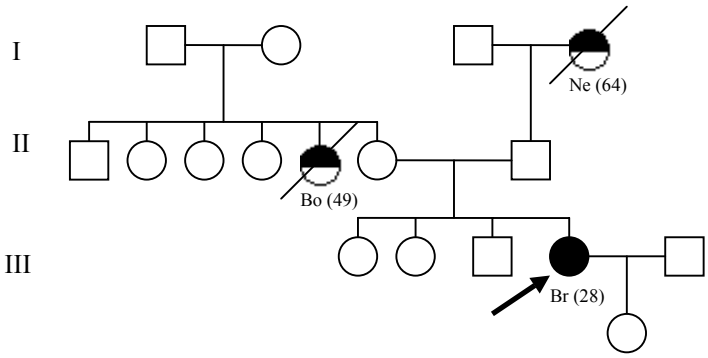


Fig. 4.1 The pedigrees of 5 patients with a family history of cancer. Black symbols are women affected with breast cancer. The upper-site-blackened symbols represent women affected with any other cancer or other disease. The ages at cancer diagnosis in patients are in brackets. The abbreviations are identified as follow, Bo, bone cancer; Br, breast cancer; Cer, cervical cancer; Lu, lung cancer; Lym lymphomatic cancer; Ne, neck cancer; Tub, tuberculosis; N, analyzed persons who do not have breast cancer. Probands are indicated by arrows.

In the family of patient ID 1, one lymphomatic, one cervical cancer and one BrCa occurred. In this family, the proband was diagnosed with BrCa at the age of 45, and whose father and mother affected with lymphomatic cancer and cervical cancer, respectively (Fig. 4.1a). In the family of patient ID 17, there were two BrCa cases. The proband and her deceased sister were diagnosed with BrCa at the age of 56. The proband's healthy daughter, who participated in this study at the age of 25, was designed as ID 18 (Fig. 4.1b). In the family of patient ID 20, two BrCa occurred. In this family, the proband was the niece of the deceased patient and diagnosed with BrCa at the age of 48 (Fig. 4.1c). Patient ID 35 had a father with tuberculosis and a mother with lung cancer. The proband was the daughter of the deceased patient and diagnosed with BrCa at the age of 41 (Fig. 4.1d). In the family of patient ID 39, one bone cancer, one neck cancer and one BrCa occurred. The proband was diagnosed with BrCa at the age of 28 (Fig. 4.1e).

4.2 Purity and concentrations of the genomic DNA

The genomic DNA was extracted from 31 blood samples collected at Songklanagarind Hospital. The purity and concentration of genomic DNA was estimated by measuring the absorbance of DNA solution at 260 nm (OD_{260}) and 280 nm (OD_{280}) with a spectrophotometer. The purity was determined from OD_{260}/OD_{280} ratios. Table 4.2 represented the purity and concentrations of genomic DNA.

Table 4.2 Summary of purity and concentrations of the genomic DNA

Patient ID	OD_{260}	OD_{280}	Purity (OD_{260} /OD_{280})	Concentration (ng/μl)
Normal	0.154	0.088	1.75	616
1	0.409	0.247	1.65	2,045
2	0.019	0.010	1.85	95
16	0.100	0.055	1.80	1,000
17	0.028	0.015	1.85	140
19	0.026	0.015	1.73	260
20	0.020	0.012	1.67	200
21	0.287	0.177	1.62	1,435
22	0.048	0.027	1.78	192
24	0.577	0.324	1.78	2,308
25	0.125	0.069	1.81	500
27	0.046	0.027	1.70	184
28	0.265	0.151	1.75	1,060
31	0.283	0.158	1.79	1,132
32	0.319	0.181	1.76	1,276
35	0.194	0.108	1.79	776
39	0.438	0.256	1.71	1,752
40	0.350	0.196	1.78	1,400
41	0.167	0.093	1.79	668
42	0.196	0.110	1.78	676
43	0.232	0.133	1.74	928
44	0.127	0.071	1.78	508
45	0.147	0.080	1.83	588
46	0.284	0.167	1.70	1,136
47	0.182	0.104	1.75	728
48	0.151	0.085	1.77	604
49	0.181	0.100	1.81	724
50	0.090	0.052	1.73	900
51	0.174	0.097	1.79	696
52	0.180	0.100	1.80	720
53	0.136	0.079	1.72	544
54	0.151	0.083	1.81	604

The genomic DNA was extracted from white blood cells by SDS-proteinase K treatment (Sambrook and Russell, 2001). Considerable yields of genomic DNA were obtained. The purity of genomic DNA was between 1.65-2.00, which was a satisfactory quality of genomic DNA for the Polymerase Chain Reaction Analysis (PCR).

4.3 PCR optimization for the amplification of *BRCA1* exons

Thirty-one pairs of primer were used in the screening for *BRCA1* mutations of 22 coding exons. The sizes of the product, annealing temperatures (Tm) and magnesium concentrations (Mg^{2+}) for successful PCR amplification were shown in Table 4.3.

Table 4.3 Summary of optimal conditions for PCR amplification of *BRCA1* exons.

Primer	Nucleotide position	Primer sequence (5'-3')	Tm (°C)	Mg^{2+} Concentration (mM)	Size of product (bp)
BRCF 2 BRCR 2	4557 4764	GAAGTTGTCATTTTATAAACC TCTGTTCATTTGCATAGGAG	55	1.5	227
BRCF 3 BRCR 3	12842 13114	GTTGACTCAGTCATAACAGCTC GGAGTTGGATTTTTCGTTCTC	60	1.5	293
BRCF 5 BRCR 5	22116 22445	TCTTTTCATGGCTATTTGCC CCTGTATAAGGCAGATGTCCC	60	1.5	350
BRCF 6 BRCR 6	23730 23873	GGTTGATAATCACTTGCTGAG GCACTTGAGTTGCATTCTTGG	56	1.5	174
BRCF 7 BRCR 7	24381 24685	GAGCATACATAGGGTTTCTC CCTGGGCCACAGAGCAAGAC	56	3.0	440
BRCF 8 BRCR 8	28811 29051	CTGGCCAATAATTGCTTGAC CTTCCCAAAGCTGCCTACCAC	60	1.5	261
BRCF 9 BRCR 9	31316 31504	TACCTGCCACAGTAGATGCTC CCAGCTTCATAGACAAAGG	55	1.5	207
BRCF 10 BRCR 10	32753 32914	CAGTTCTGCATACATGTAAC CCCACTCTCTTTTCAGTGCC	60	1.5	181
BRCF 11a BRCR 11a	33774 34158	GCCAGTTGGTTGATTTCCACC CCTTACTTCCAGCCCATCTG	60	1.5	404
BRCF 11b BRCR 11b	34046 34402	CATTACAGCATGAGAACAGCAG GCATTTGATTCAGACTCCCC	60	1.5	376
BRCF 11c BRCR 11c	34377 34762	GTTAGGTTCTGATGACTCACATG GTCTTTTGAACTGCCAAATCTGC	60	1.5	408
BRCF 11d BRCR 11d	34710 35080	GCGTAAAAGGAGACCTACATCAG GGTGGGCTTAGATTTCTACTGAC	60	1.5	393
BRCF 11e BRCR 11e	35032 35419	CTGAGGAGGAAGTCTTCTACCA GGGTCTTCAGCATTATTAGACAC	60	1.5	410
BRCF 11f BRCR 11f	35298 35680	CCCAATGGATACTTAAAGCCTTC GCTGAAGTTAACAAATGCACCT	60	1.5	405

Primer	Nucleotide position	Primer sequence (5'-3')	Tm (°C)	Mg^{2+} Concentration (mM)	Size of product (bp)
BRCF 11g	35637	GGGACTAATTCATGGTTGTTCC	60	1.5	418
BRCR 11g	36032	CCTAGAGCCTCCTTTGATAC			
BRCF 11h	36032	CCTAGAGCCTCCTTTGATAC	60	1.5	441
BRCR 11h	36450	GTTGCAAAACCCCTAATCTAAGC			
BRCF 11i	36430	GGGCCAAAATTGAATGCTATGC	60	1.5	400
BRCR 11i	36808	CTATTTCTTGGCCCCTCTTCG			
BRCF 11j	36862	GAAGAGCTTCCCTGCTTCCA	60	1.5	520
BRCR 11j	37360	GTAAAATGAGCTCCCCAAAAGC			
BRCF 12	37610	CCAGTCCTGCCAATGAGAAG	60	1.5	218
BRCR 12	37808	CCACACACACGCATGTGCAC			
BRCF 13	46032	CTTGTAGTTCCATACTAGGTG	62	1.5	376
BRCR 13	46388	GGTCCTTACTCTTCAGAAGG			
BRCF 14	52045	CAGTATTCTAACCTGAATTATCA	55	1.5	241
BRCR 14	52265	GATGTCAGATACCACAGCATC			
BRCF 15	54167	CACAATTGGTGGCGATGG	60	1.5	297
BRCR 15	54445	CTTTATGTAGGATTCAGAG			
BRCF 16	57362	CCAACACTGTATTCATGTACCC	60	1.5	475
BRCR 16	57815	GTCATTAGGGAGATACATATGG			
BRCF 17	60983	CTGAGCTGTGTGCTAGAG	62	1.5	247
BRCR 17	61210	TCGGCCTCCCAAAGTGCTGC			
BRCF 18	64713	GCTTCTTAGGACAGCACTTCC	56	1.5	250
BRCR 18	64945	CTCAGACTCAGCATCAGC			
BRCF 19	65278	GTGAATCGCTGACCTCTC	60	3.0	306
BRCR 19	65542	ATGAGCCACAGTGCAGGCCTGC			
BRCF 20	71522	GACGTGTCTGCTCCACTTC	56	2.0	210
BRCR 20	71713	TACAGAGTGGTGGGGTGAG			
BRCF 21	77578	CTCTCCATTCCCCTGTCCCTC	64	1.5	196
BRCR 21	77753	GCAATCTGAGGAACCCCCATC			
BRCF 22	79482	GAGGGCCTGGGTTAAGTATGC	64	1.5	172
BRCR 22	79633	TGTGTCCTCCCTCTCTGACTG			
BRCF 23	80968	ATGAAGTGCAGTTCCAGTAG	60	1.5	207
BRCR 23	81155	CTCAAGCACCAGGTAATGAG			
BRCF 24	82841	GAACTCATACAACCAGGACCC	60	1.5	275
BRCR 24	83096	ACTTTGTAAGCTCATTCTTG			

Determination for optimal condition of each parameter affecting PCR efficiency was discussed below.

4.3.1 Magnesium concentration

Magnesium concentration is a key factor affecting the performance of *Taq* DNA polymerase. Most exons were successfully amplified with the presence of 1.5 mM Mg^{2+}. Only few exons (exon 7, 19 and 20) were amplified with the presence of higher Mg^{2+} concentrations (2.0-3.0 mM).

4.3.2 Primer concentration

The concentration of primer in the 50 μl PCR reaction was varied from 0.1 to 0.5 μM. There was no different in the yield of the PCR products obtained with primer concentrations of 0.1, 0.3 or 0.5 μM (Fig. 4.2). However, high concentration of primer (0.5 μM) was applied to confer selectivity in the amplification when a complex template as genomic DNA was used.

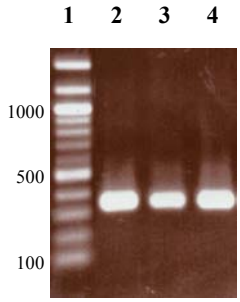


Fig. 4.2 The effect of primer concentration on PCR amplification of *BRCA1* gene using BRCF3 and BRCR3 primers. The PCR products (350 bp) were resolved by electrophoresis through 1% agarose gel and stained with ethidium bromide. Lane 1 is 100 bp DNA ladder. Lanes 2-4 were the PCR product profiles obtained with 0.1, 0.3 and 0.5 μM of primer concentration in 50 μl reaction, respectively.

4.3.3 Template concentration

Genomic DNA was a highly complex template for PCR amplification. Therefore high concentration of template (100 ng) was used with all pairs of primer.

4.3.4 PCR programs

In this work, amplification conditions were as described by Munnes *et al*., (2000). After the initial denaturation step at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 s, annealing for 45 s at temperatures (Tm) as listed in

Table 4.3 and extension step at 72 °C for 5 min were applied for each PCR amplification.

4.3.5 Annealing temperature

The optimal annealing temperature varied from the estimated melting temperature or Tm. Tm was calculated from the simple formula of 2 °C × (A+T) + 4 °C × (C+G), at which (A+T) is the number of A and T nucleotides in the primer sequence, and (C+G) is the number of C and G nucleotides. The annealing temperature was 1-2 °C below the calculated Tm for each primer. The annealing temperatures for each pair of primer were shown in Table 4.3. The pattern of PCR products amplified with each pair of primer at designated annealing temperature were shown in Fig. 4.3A-4.3E

A

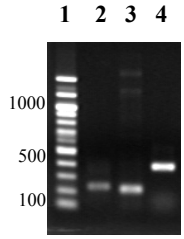


Fig. 4.3A The PCR products of *BRCA1* exons obtained at annealing temperature of 55 °C were run on a 1% agarose gel and stained with ethidium bromide. Lane1: 100 bp DNA ladder; lane2: exon 2 (227 bp); lane3: exon 9 (207 bp) and lane 4: exon 13 (376 bp)

B

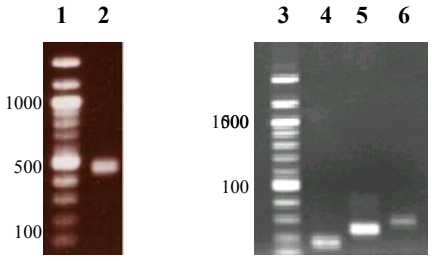


Fig. 4.3B The PCR products of *BRCA1* exons obtained at annealing temperature of 58 °C were run on a 1% agarose gel and stained with ethidium bromide. Lanes 1 and 3: 100 bp DNA ladder; lane2: exon 7 (440 bp); lane4: exon 6 (174 bp); lane5: exon 17 (247 bp) and lane6: exon 19 (306 bp)

C

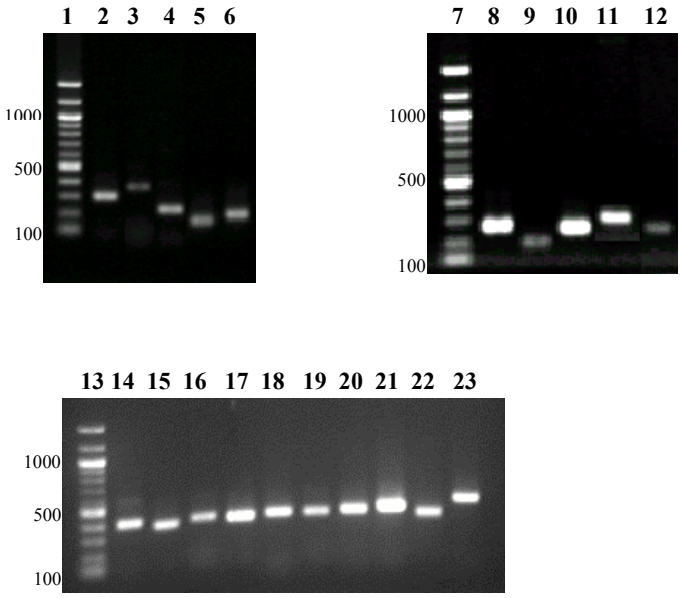


Fig. 4.3C The PCR products of *BRCA1* exons obtained at annealing temperature of 60 °C were run on a 1% agarose gel and stained with ethidium bromide. Lanes 1, 7 and 13: 100 bp DNA ladder; lane2: exon 3 (293 bp); lane3: exon 5 (350 bp); lane4: exon 18 (250 bp); lane5: exon 22 (172 bp); lane6: exon 23 (207 bp); lane8: exon 8 (261 bp); lane9: exon 10 (181 bp); lane10: exon 14 (241 bp); lane11: exon 15; lane12: exon 24 and lanes 14-23: exon 11a-11j, respectively.

D

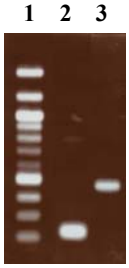


Fig. 4.3D The PCR products of *BRCA1* exons obtained at annealing temperature of 62 °C were run on a 1% agarose gel and stained with ethidium bromide. Lane1: 100 bp DNA ladder; lane2: exon 12 (218 bp) and lane3: exon 16 (475 bp)

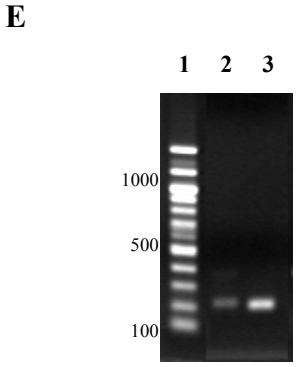


Fig. 4.3E The PCR products of *BRCA1* exons obtained at annealing temperature of 64 °C were run on a 1% agarose gel and stained with ethidium bromide. Lane1: 100 bp DNA ladder; lane2: exon 20 (210 bp) and lane3: exon 21 (196 bp)

4.4 Single-Stranded Conformation Polymorphism analysis of *BRCA1* exons

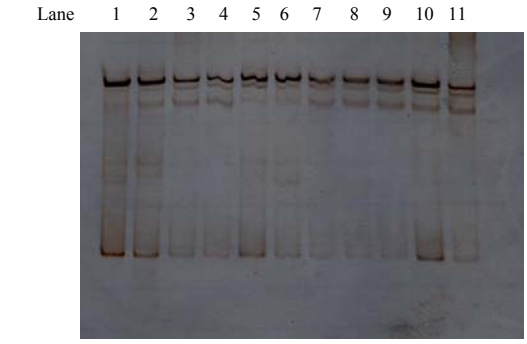
PCR-amplified *BRCA1* exons were subjected to SSCP analysis. The typical SSCP results for each exon of *BRCA1* of 31 Thai patients were shown in Fig. 4.4 - 4.19. Summary of selected patients whose exons exhibiting changes in electrophoretic mobility on 10% non-denaturing polyacrylamide gel was shown in Table 4.4.

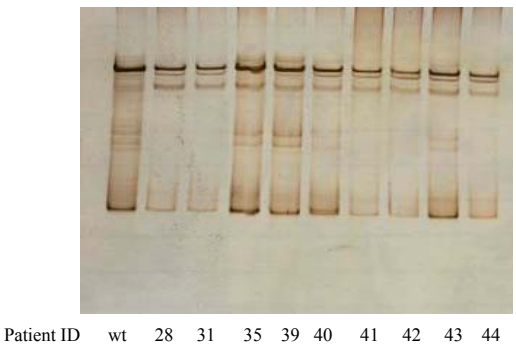
Table 4.4 Summary of selected patients whose exons exhibiting changes in electrophoretic mobility on 10% non-denaturing polyacrylamide gel

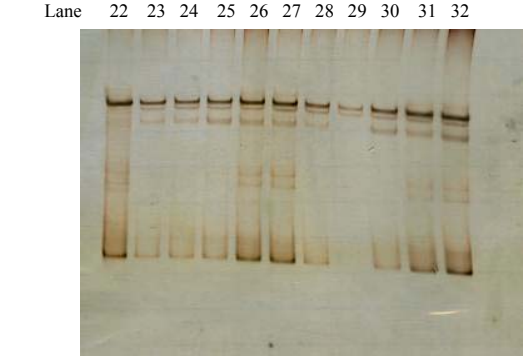
Exon	Patient ID

7	17
11a	54
11c	43 and 31
11e	40
11f	45 and 46
14	1 and 20

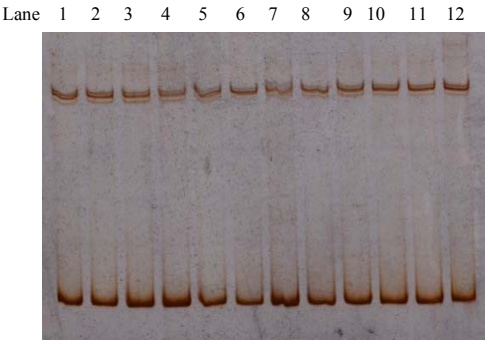
A

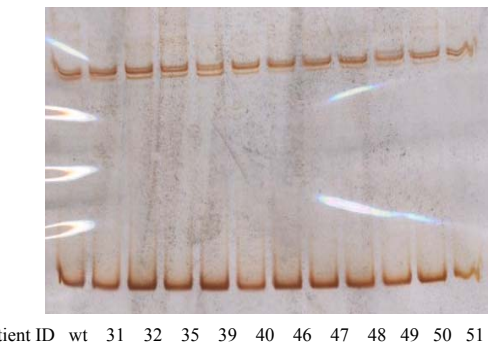






B





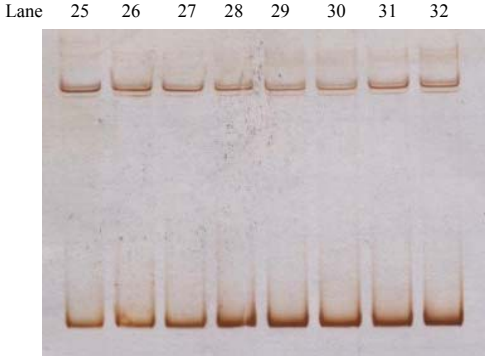


Fig. 4.4 SSCP analysis of the PCR fragment of *BRCA1* exon 2 (Panel A) and exon 3 (Panel B). Normal control person (wt) is presented in lanes 1, 12, 22 (A) and lanes 1, 13, 25 (B). The SSCP products were run on 10% non-denaturing polyacrylamide gel and visualized by silver staining.

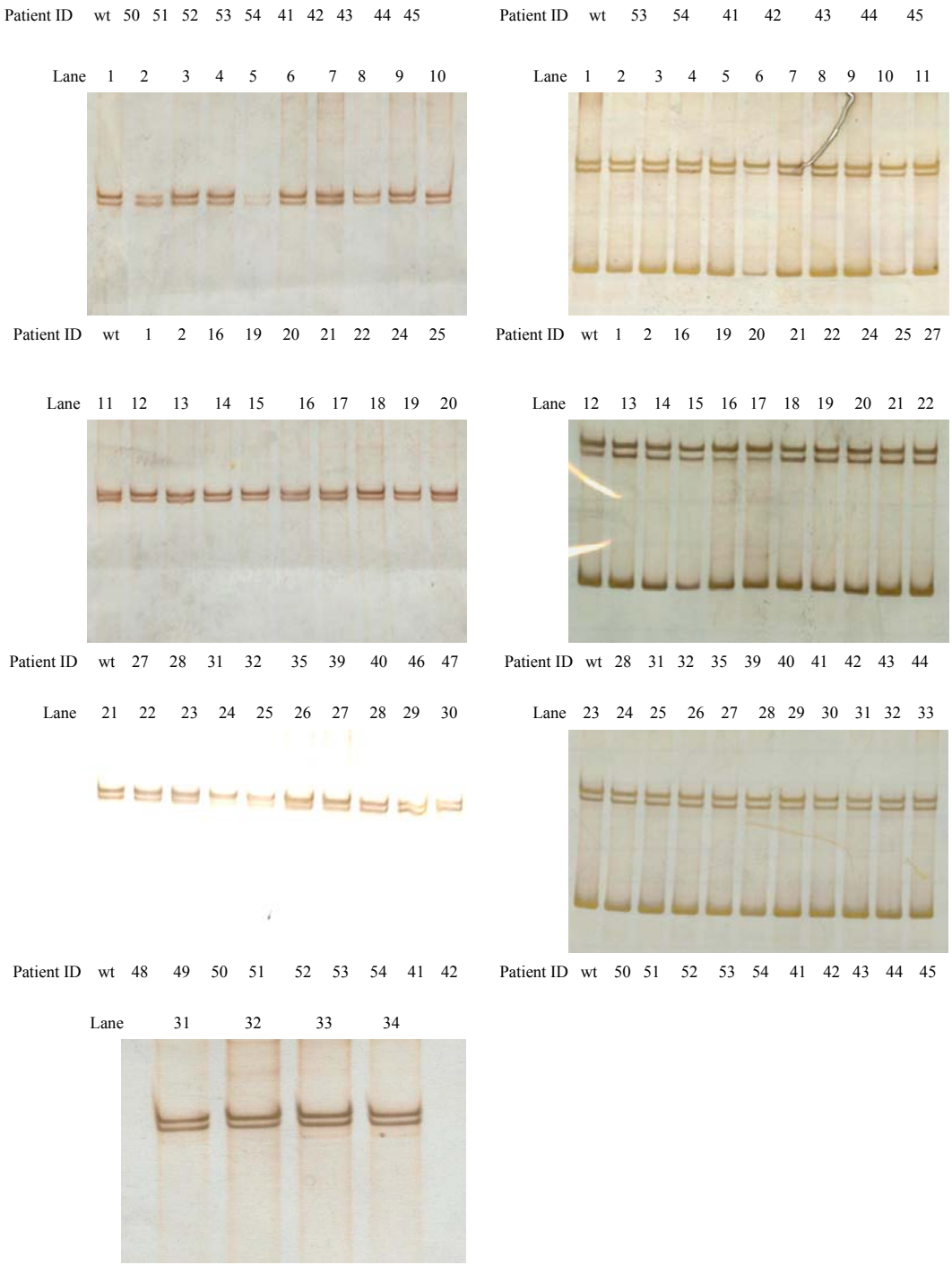


Fig. 4.5 SSCP analysis of the PCR fragment of *BRCA1* exon 5 (Panel A) and exon 6 (Panel B). Normal control person (wt) is presented in lanes 1, 11, 21, 31 (A) and lanes 1, 12, 23 (B). The SSCP products were run on 10% non-denaturing polyacrylamide gel and visualized by silver staining.

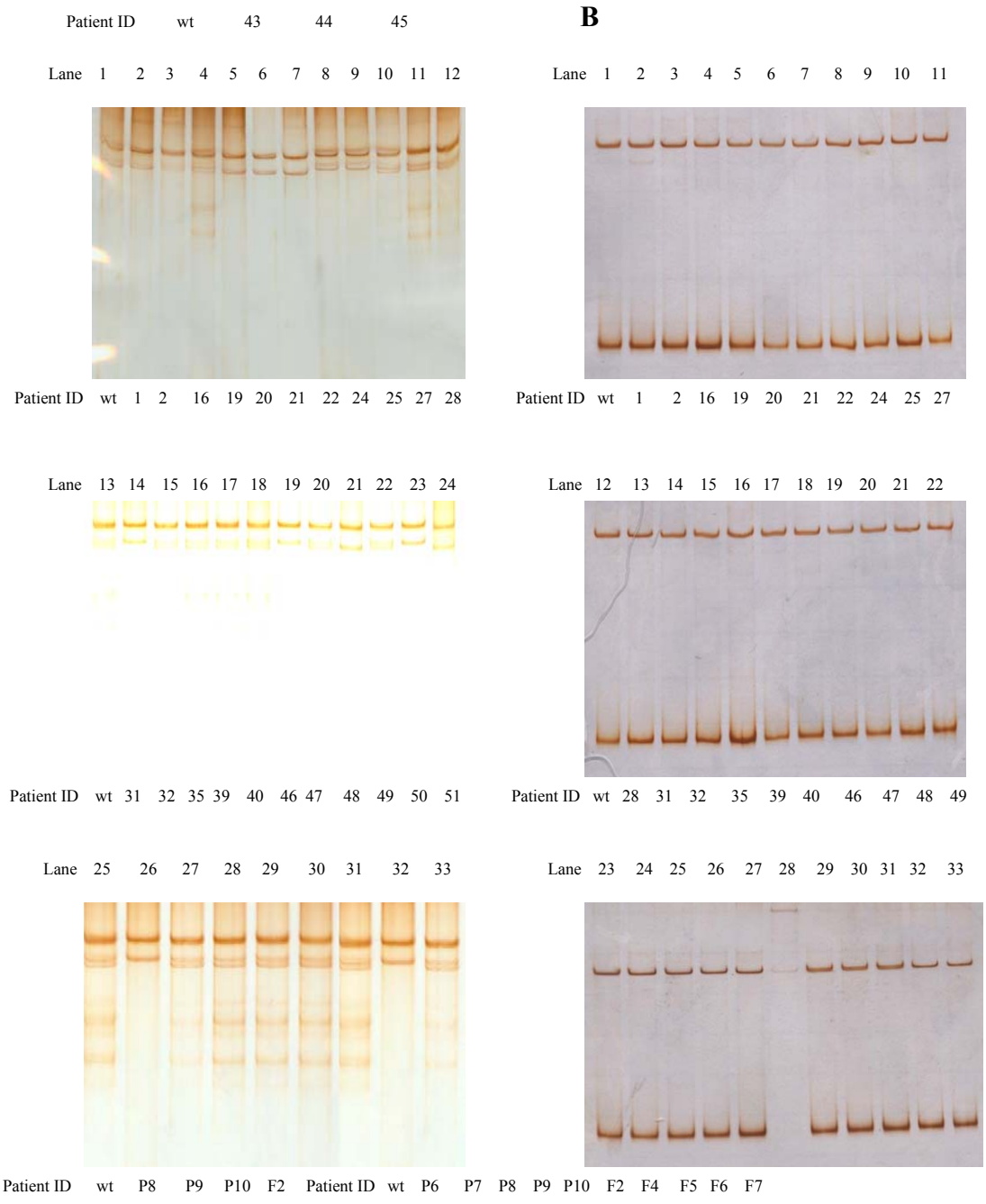


Fig. 4.6 SSCP analysis of the PCR fragment of *BRCA1* exon 7 (Panel A) and exon 8 (Panel B). Normal control person (wt) is presented in lanes 1, 13, 25 (A) and lanes 1, 12, 23 (B). The SSCP products were run on 10% non-denaturing polyacrylamide gel and visualized by silver staining.

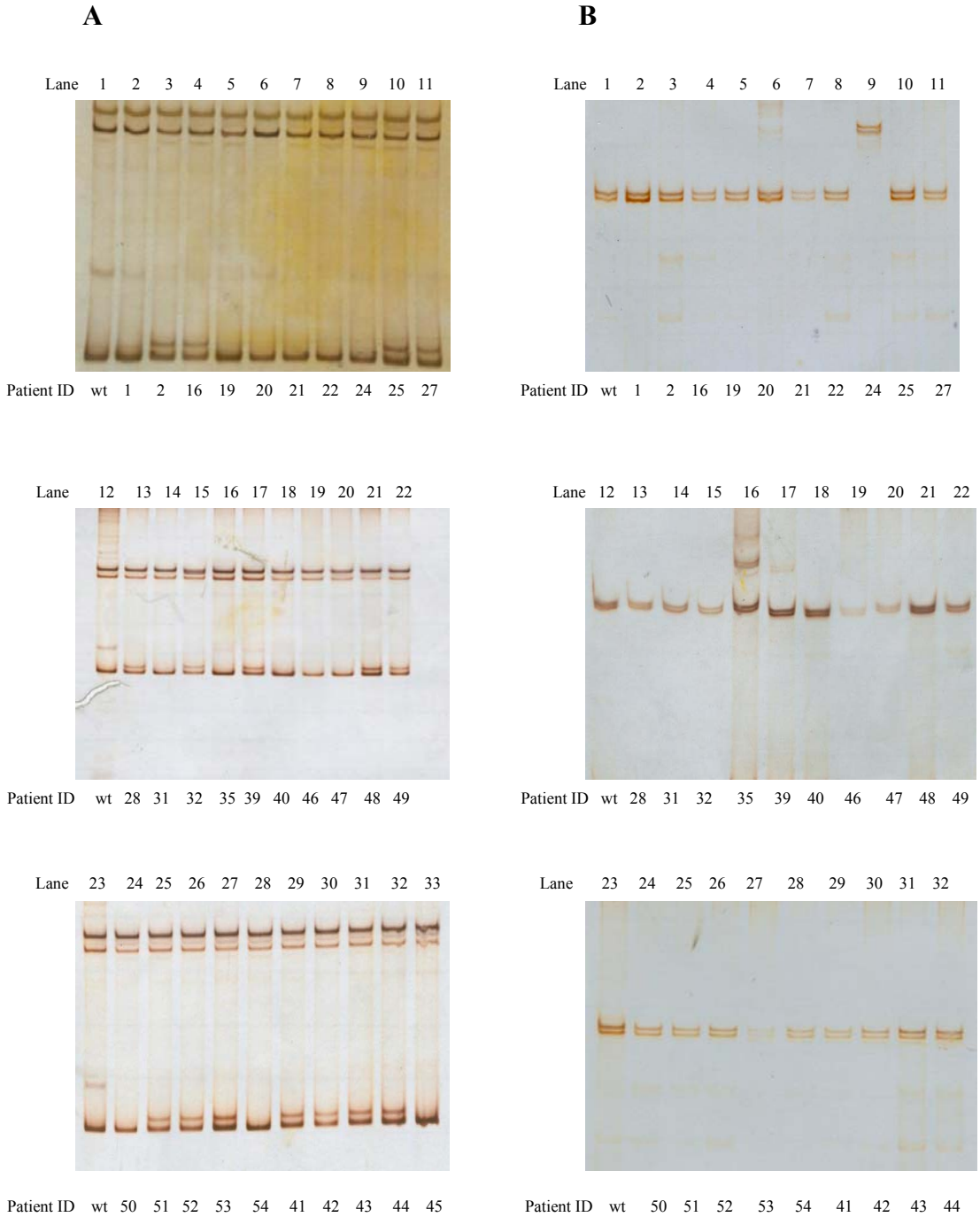


Fig. 4.7 SSCP analysis of the PCR fragment of *BRCA1* exon 9 (Panel A) and exon 10 (Panel B). Normal control person (wt) is presented in lanes 1, 12, 23 (A and B). The SSCP products were run on 10% non-denaturing polyacrylamide gel and visualized by silver staining.

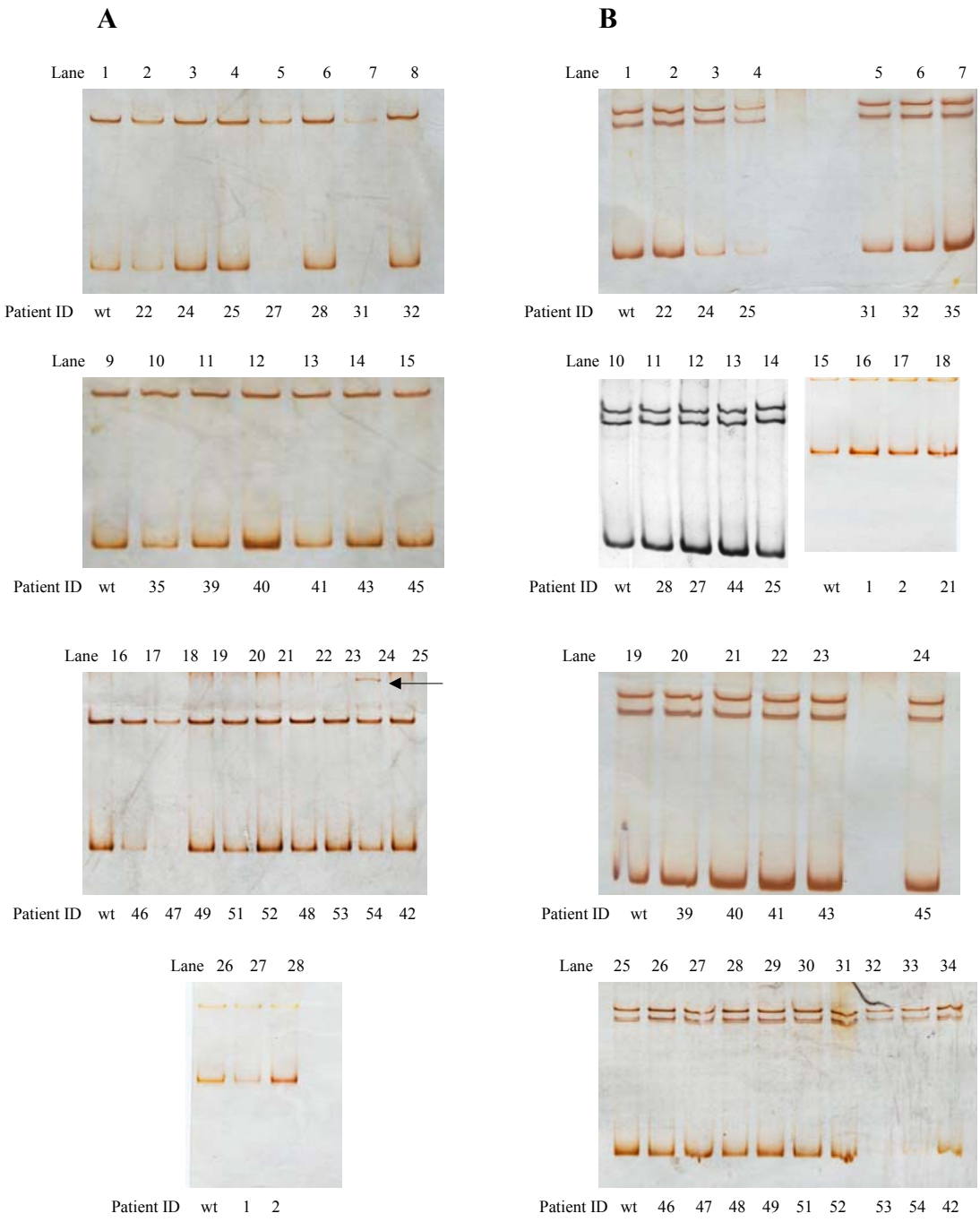


Fig. 4.8 SSCP analysis of the PCR fragment of *BRCA1* exon 11a (Panel A) and exon 11b (Panel B). Normal control person (wt) is presented in lanes 1, 9, 16, 26 (A) and lanes 1, 10, 19, 25 (B). Arrow indicated the different electrophoretic mobility of *BRCA1* exon 11a fragment of patient ID 54 with respect to the wild-type fragment (A). The SSCP products were run on 10% non-denaturing polyacrylamide gel and visualized by silver staining.

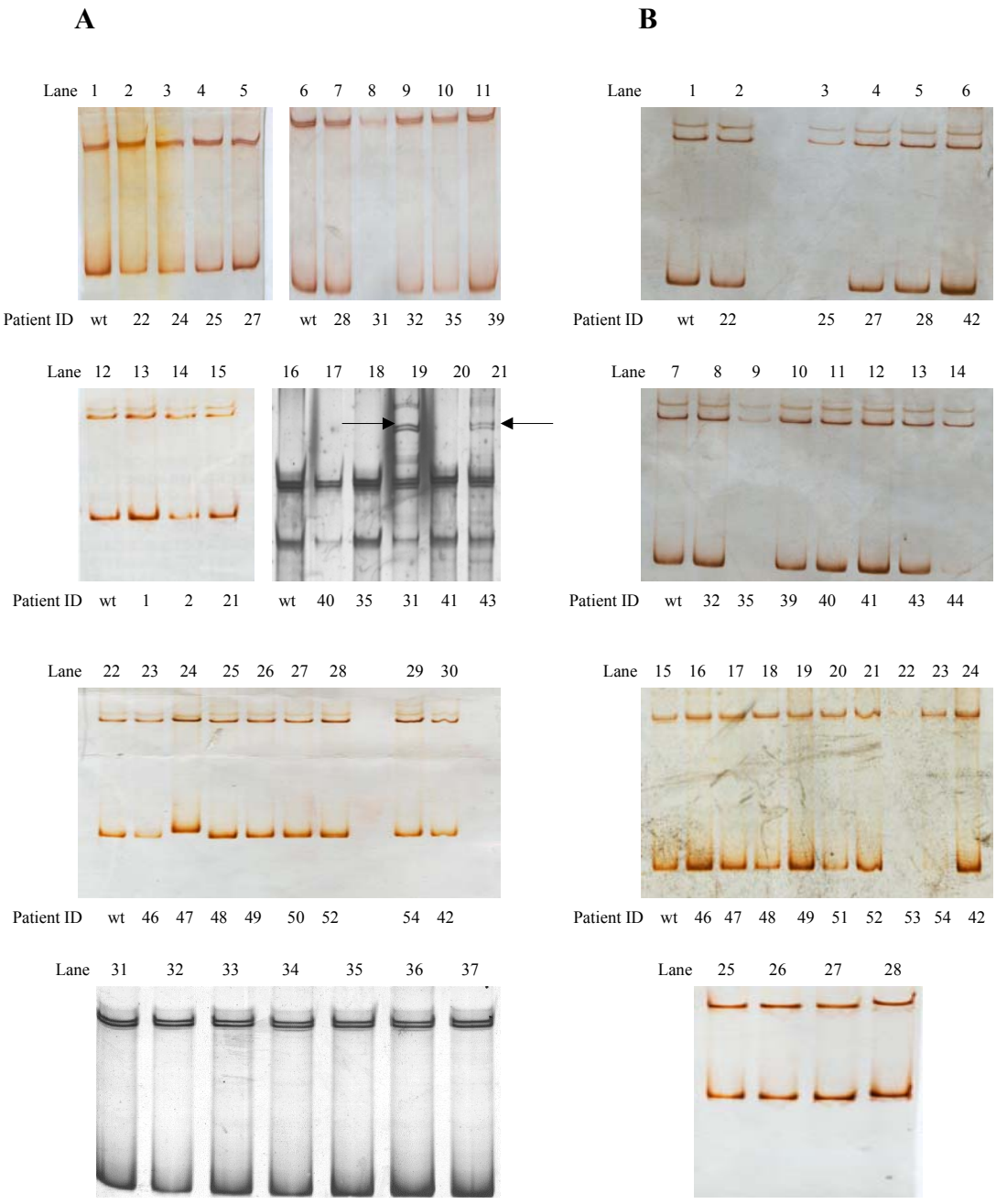


Fig. 4.9 SSCP analysis of the PCR fragment of *BRCA1* exon 11c (Panel A) and exon 11d (Panel B). Normal control person (wt) is presented in lanes 1, 12, 22, 31 (A) and lanes 1, 7, 15, 25 (B). Arrows indicated the different electrophoretic mobility of *BRCA1* exon 11c fragment of patient ID 31 and 43 with respect to the wild-type fragment (A). The SSCP products were run on 10% non-denaturing polyacrylamide gel and visualized by silver staining.

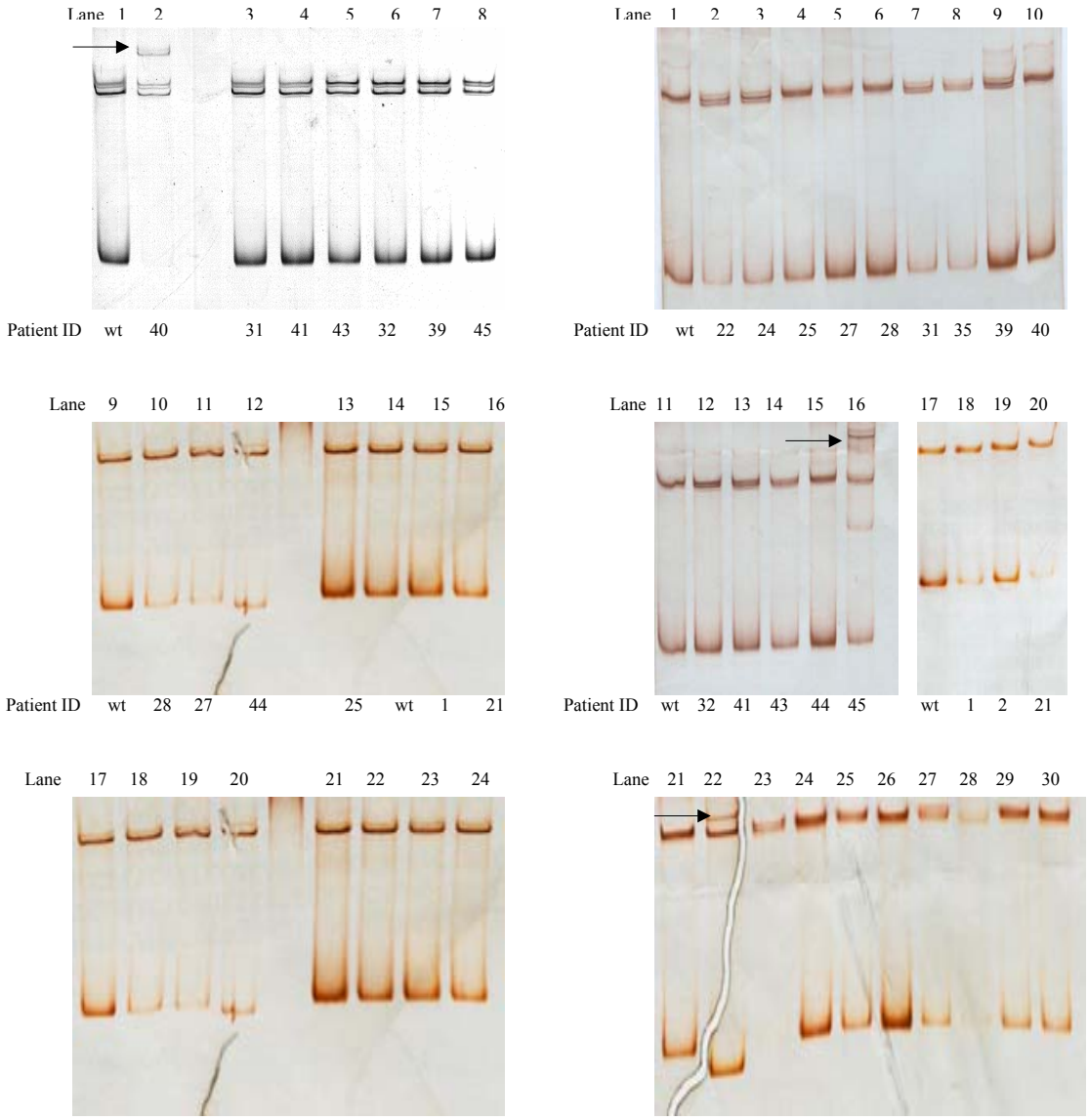
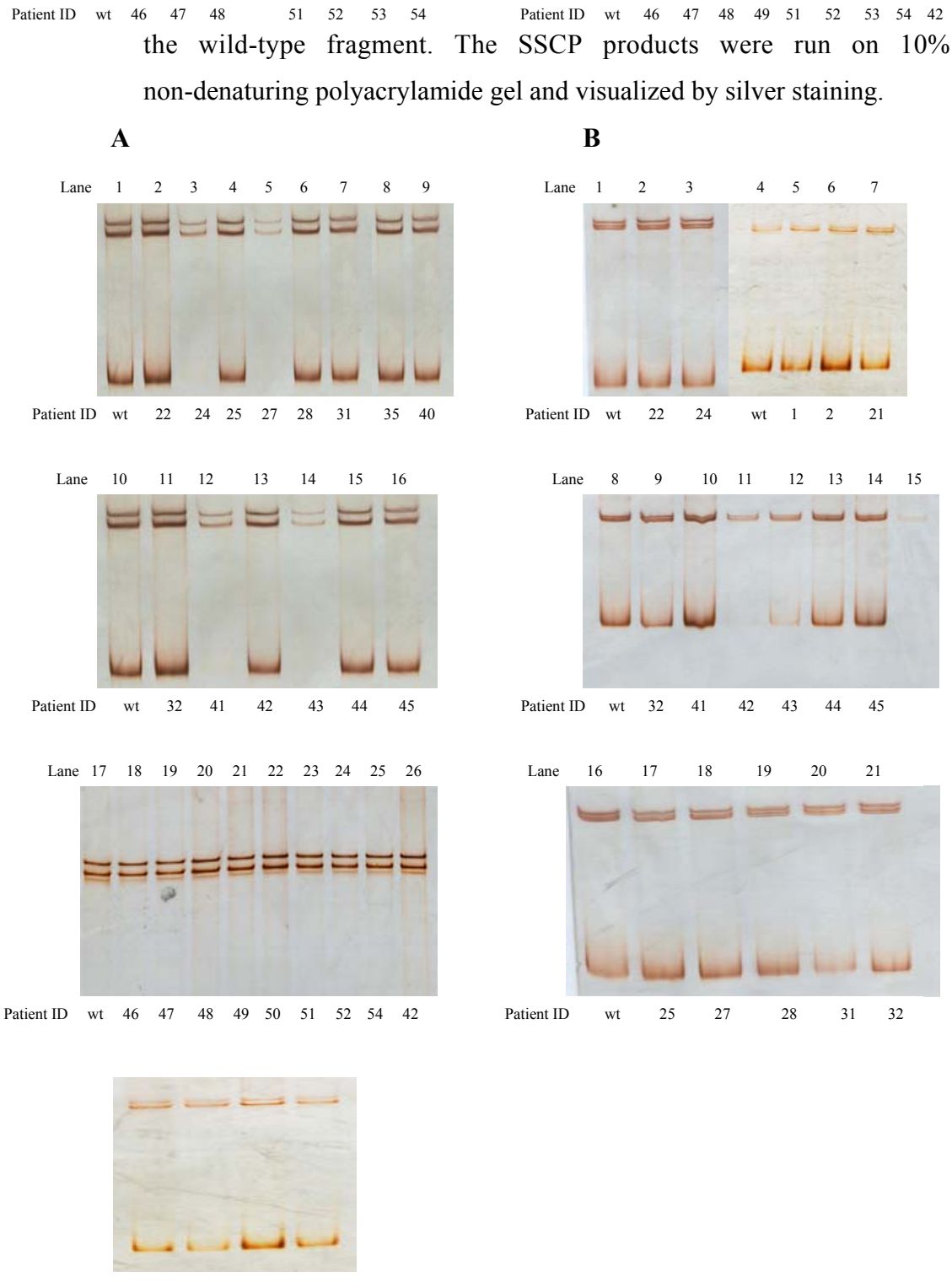


Fig. 4.10 SSCP analysis of the PCR fragment of *BRCA1* exon 11e (Panel A) and exon 11f (Panel B). Normal control person (wt) is presented in lanes 1, 9, 17 (A) and lanes 1, 11, 21 (B). Arrows indicated the different electrophoretic mobility of *BRCA1* exon 11e fragment of patient ID 40 (A) and *BRCA1* exon 11f fragment of patient ID 45, 46 (B) with respect to the wild-type fragment. The SSCP products were run on 10% non-denaturing polyacrylamide gel and visualized by silver staining.



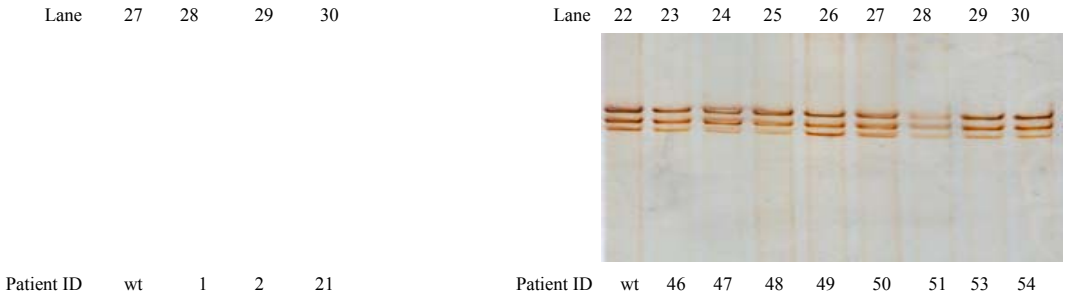
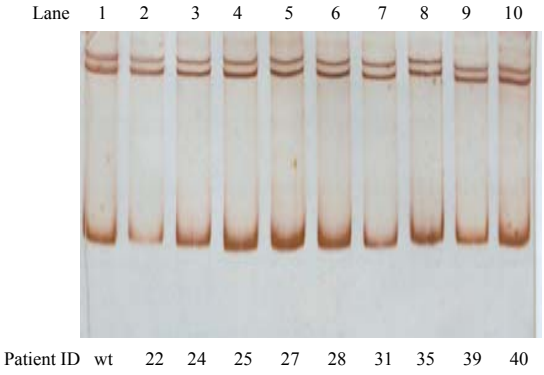
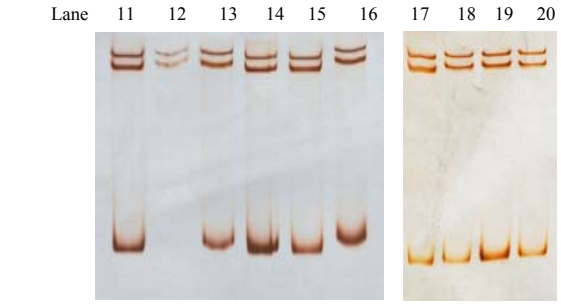


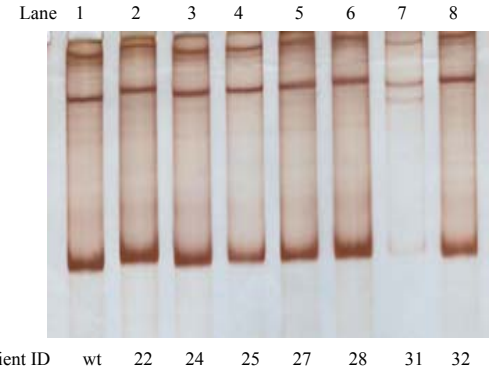
Fig. 4.11 SSCP analysis of the PCR fragment of *BRCA1* exon 11g (Panel A) and exon 11h (Panel B). Normal control person (wt) is presented in lanes 1, 7, 17, 27 (A) and lanes 1, 8, 16, 22 (B). The SSCP products were run on 10% non-denaturing polyacrylamide gel and visualized by silver staining.

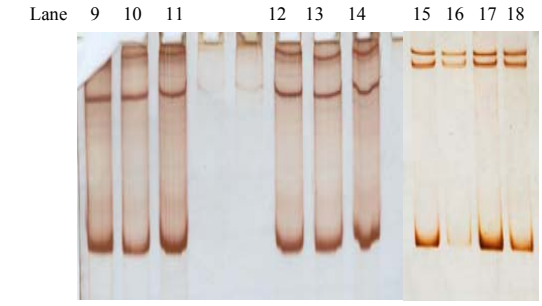
A

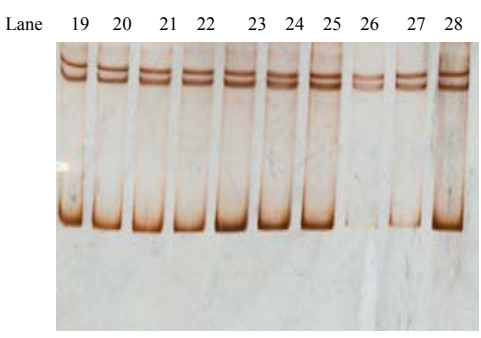


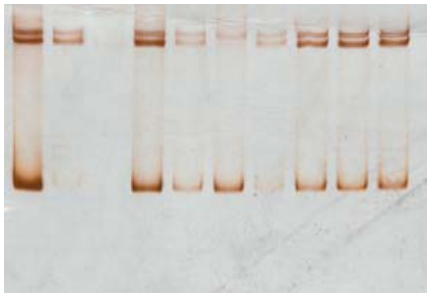


B









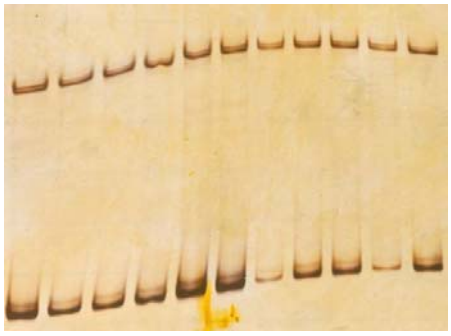
Patient ID wt 46 48 49 51 52 53 54 42

Patient ID wt 46 47 48 49 51 52 53 54 42

Fig. 4.12 SSCP analysis of the PCR fragment of *BRCA1* exon 11i (Panel A) and exon 11j (Panel B). Normal control person (wt) is presented in lanes 1, 7, 11, 26 (A) and lanes 1, 9, 15, 25 (B). The SSCP products were run on 10% non-denaturing polyacrylamide gel and visualized by silver staining.

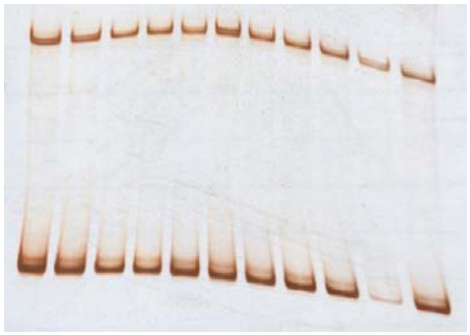
A

Lane 1 2 3 4 5 6 7 8 9 10 11



Patient ID wt 1 2 16 19 20 21 22 24 25 27

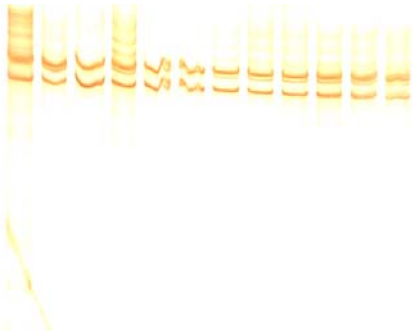
Lane 12 13 14 15 16 17 18 19 20 21 22



Patient ID wt 28 31 32 35 39 40 46 47 48 49

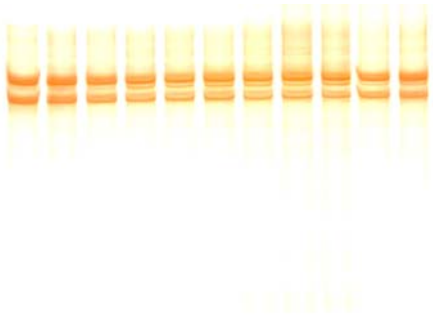
B

Lane 1 2 3 4 5 6 7 8 9 10 11 12



Patient ID wt 1 2 16 19 20 21 22 24 5 27 28

Lane 13 14 15 16 17 18 19 20 21 22 23



Patient ID wt 31 32 35 39 40 46 47 48 49 50

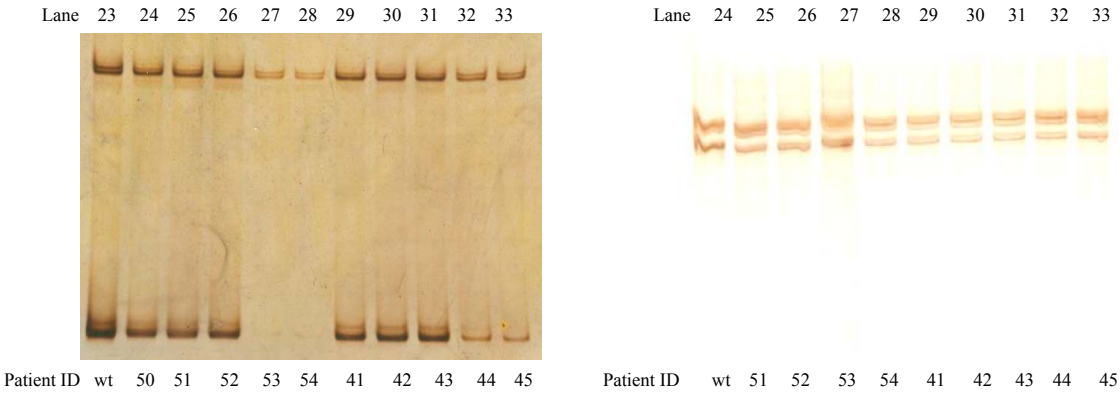
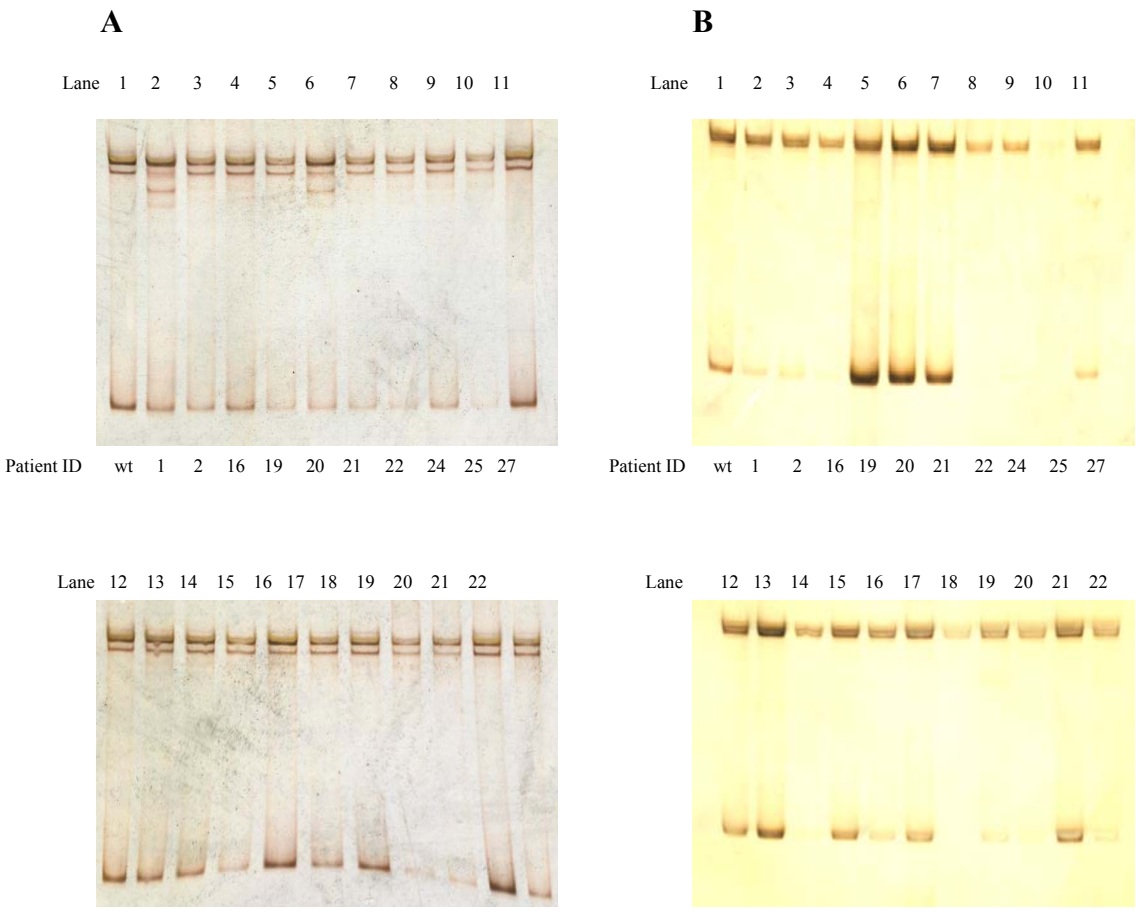


Fig. 4.13 SSCP analysis of the PCR fragment of *BRCA1* exon 12 (Panel A) and exon 13 (Panel B). Normal control person (wt) is presented in lanes 1, 12, 23 (A) and lanes 1, 13, 24 (B). The SSCP products were run on 10% non-denaturing polyacrylamide gel and visualized by silver staining.



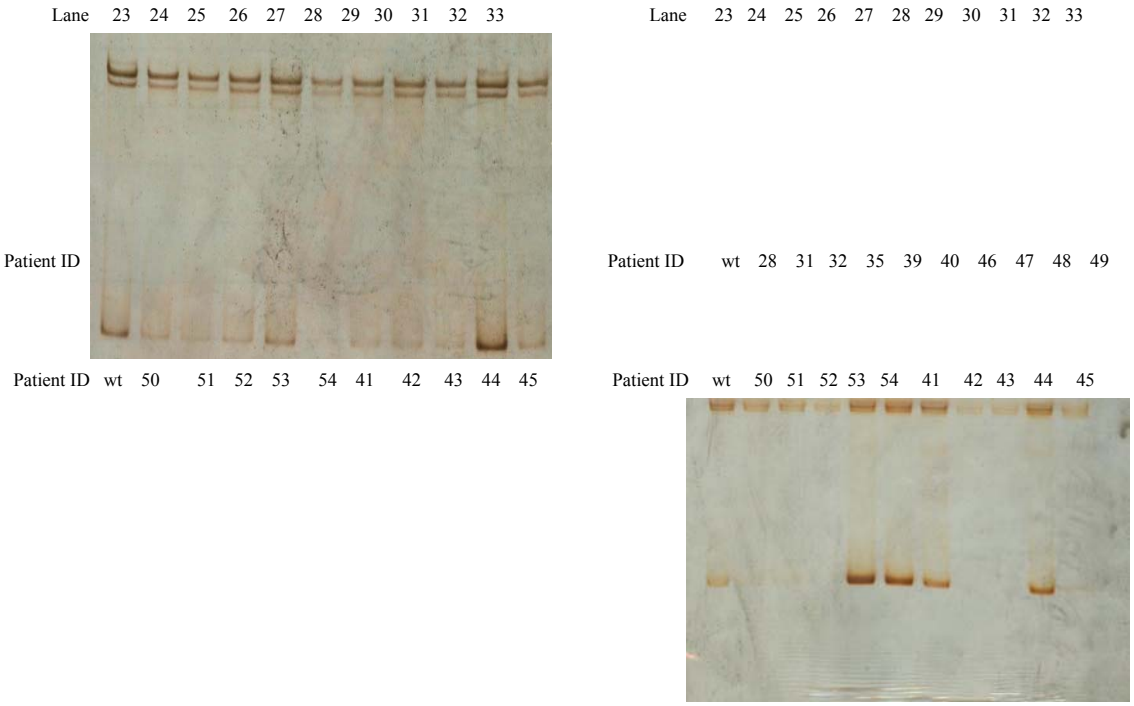
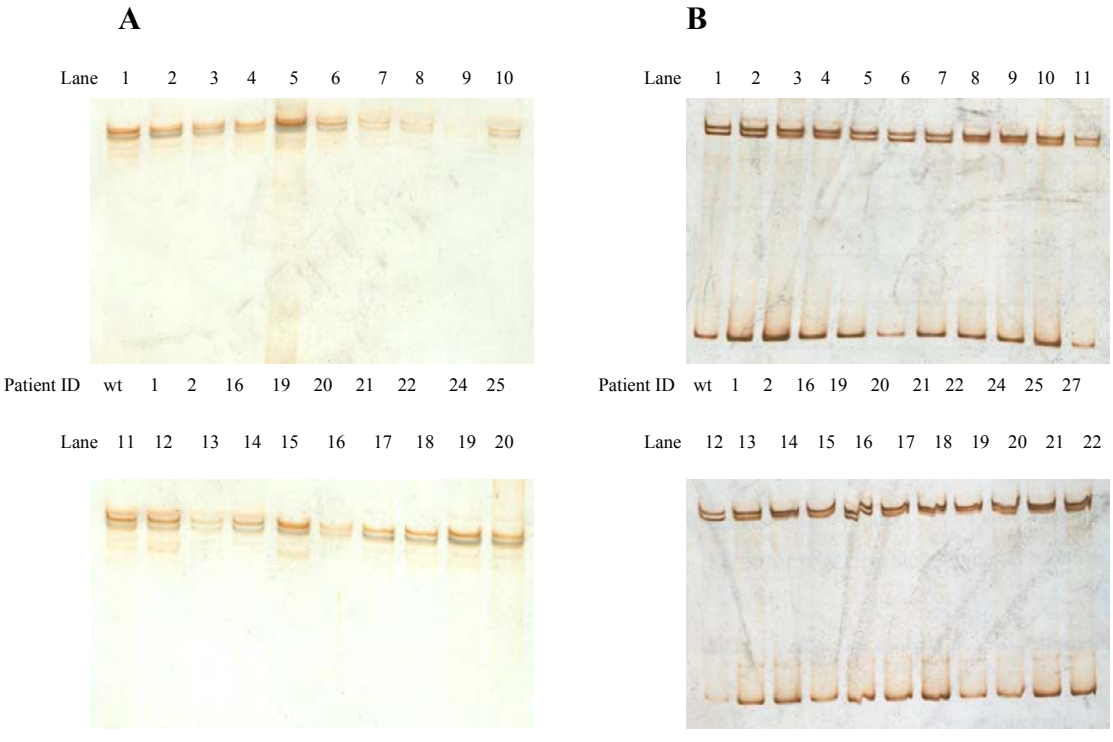


Fig. 4.14 SSCP analysis of the PCR fragment of *BRCA1* exon 14 (Panel A) and exon 15 (Panel B). Normal control person (wt) is presented in lanes 1, 12, 23 (A and B). The SSCP products were run on 10% non-denaturing polyacrylamide gel and visualized by silver staining.



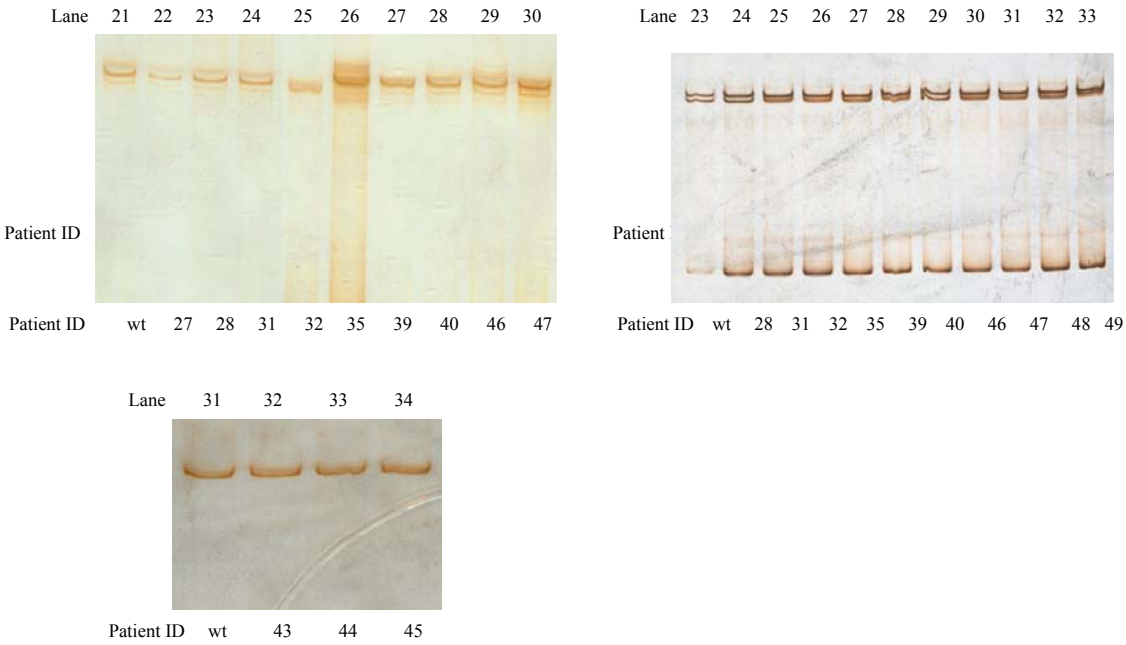
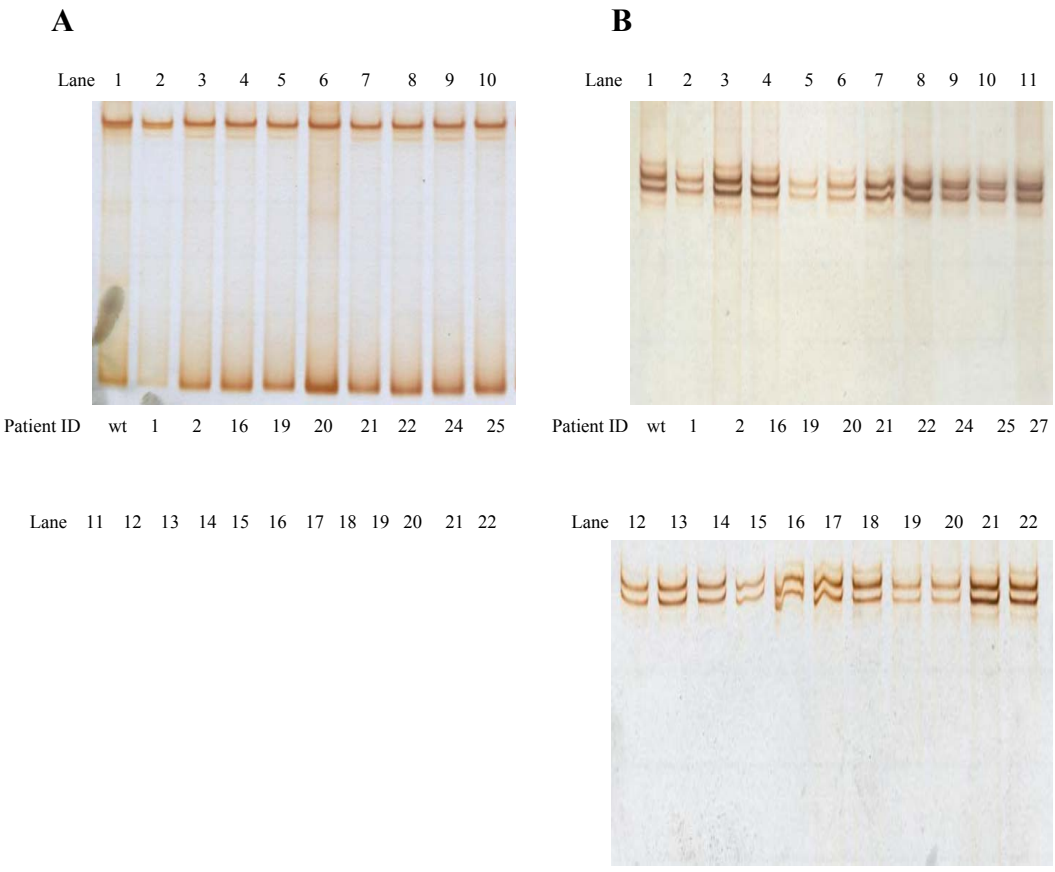


Fig. 4.15 SSCP analysis of the PCR fragment of *BRCA1* exon 16 (Panel A) and exon 17 (Panel B). Normal control person (wt) is presented in lanes 1, 11, 21, 31 (A) and 1, 12, 23 (B). The SSCP products were run on 10% non-denaturing polyacrylamide gel and visualized by silver staining.



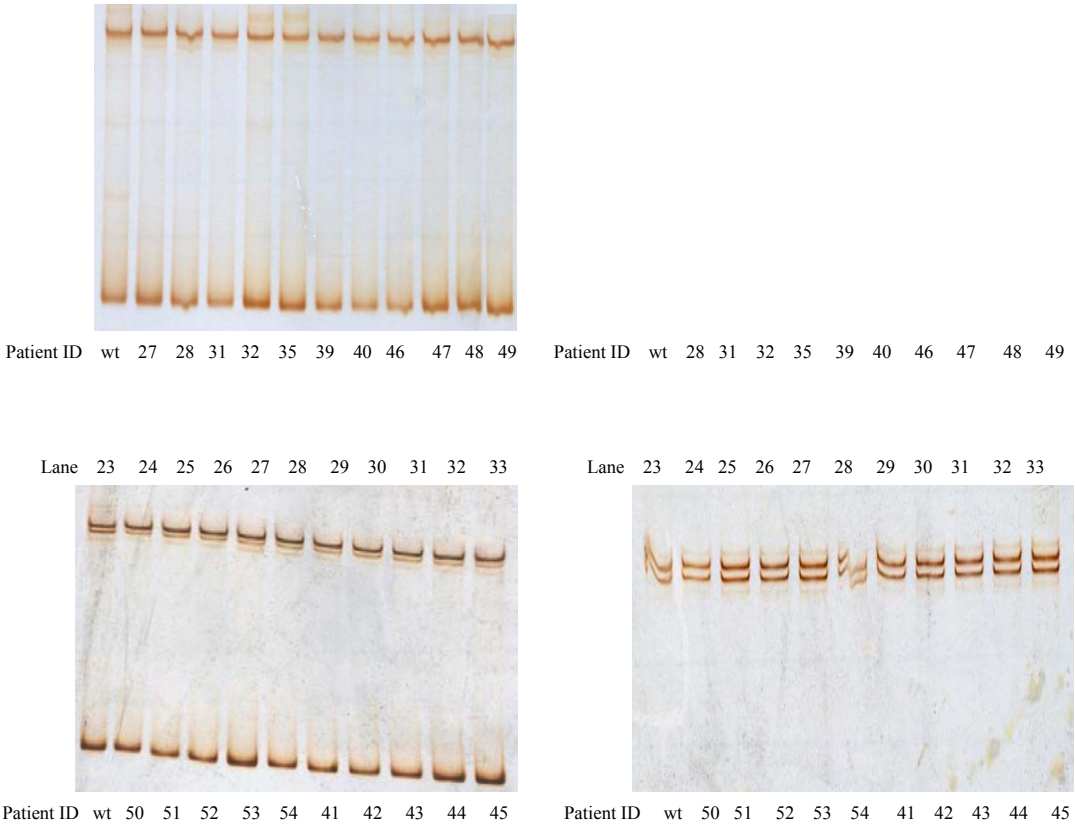
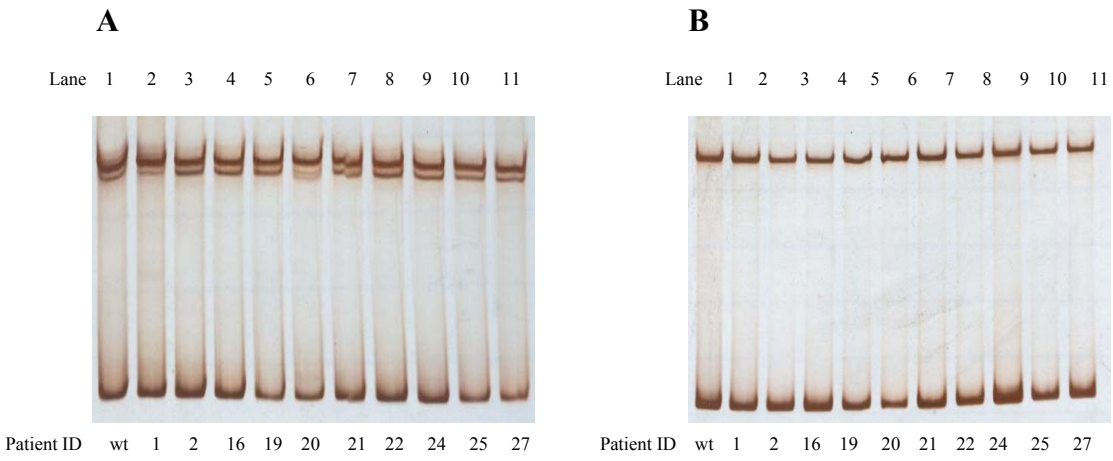


Fig. 4.16 SSCP analysis of the PCR fragment of *BRCA1* exon 18 (Panel A) and exon 19 (Panel B). Normal control person (wt) is presented in lanes 1, 11, 23 (A) and 1, 12, 23 (B). The SSCP products were run on 10% non-denaturing polyacrylamide gel and visualized by silver staining.



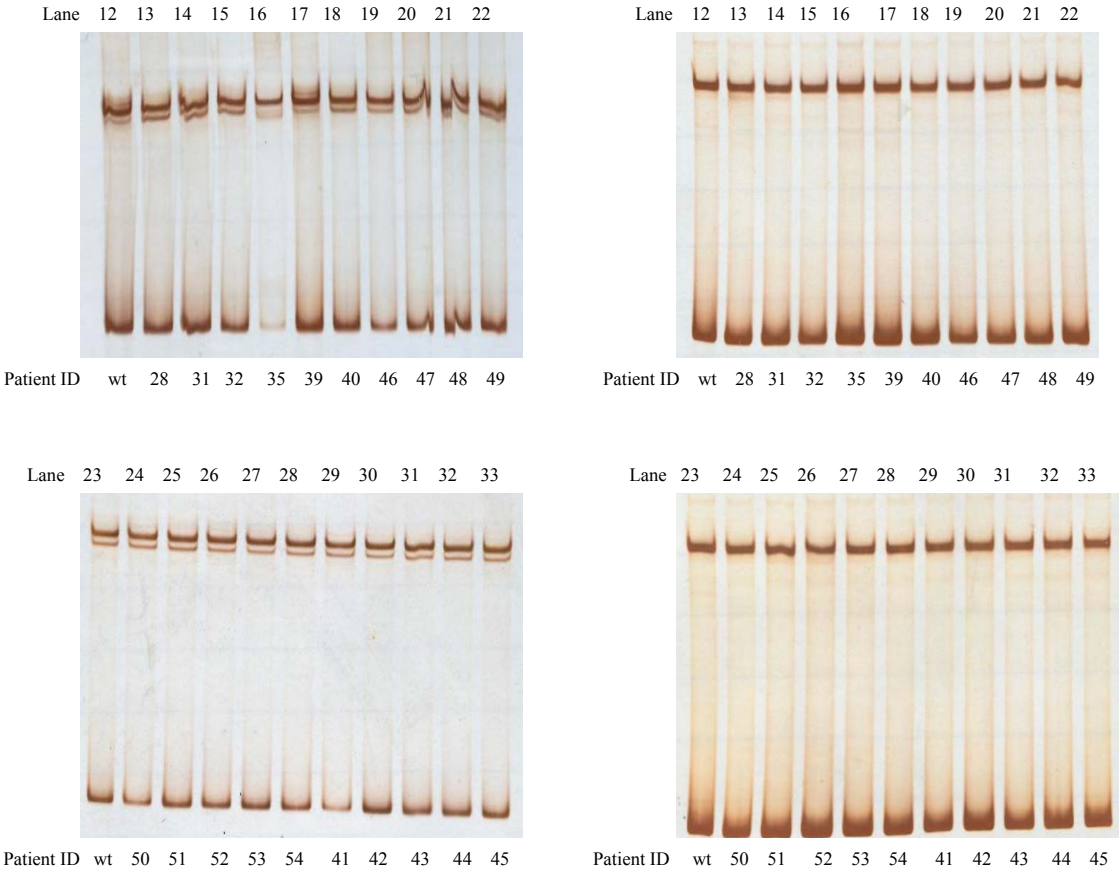
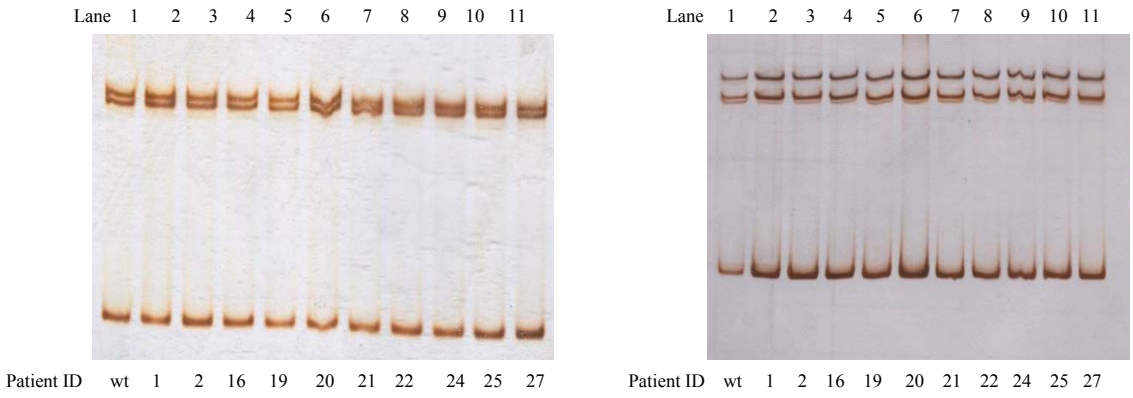


Fig. 4.17 SSCP analysis of the PCR fragment of *BRCA1* exon 20 (Panel A) and exon 21 (Panel B). Normal control person (wt) is presented in lanes 1, 12, 23 (A and B). The SSCP products were run on 10% non-denaturing polyacrylamide gel and visualized by silver staining.

A **B**



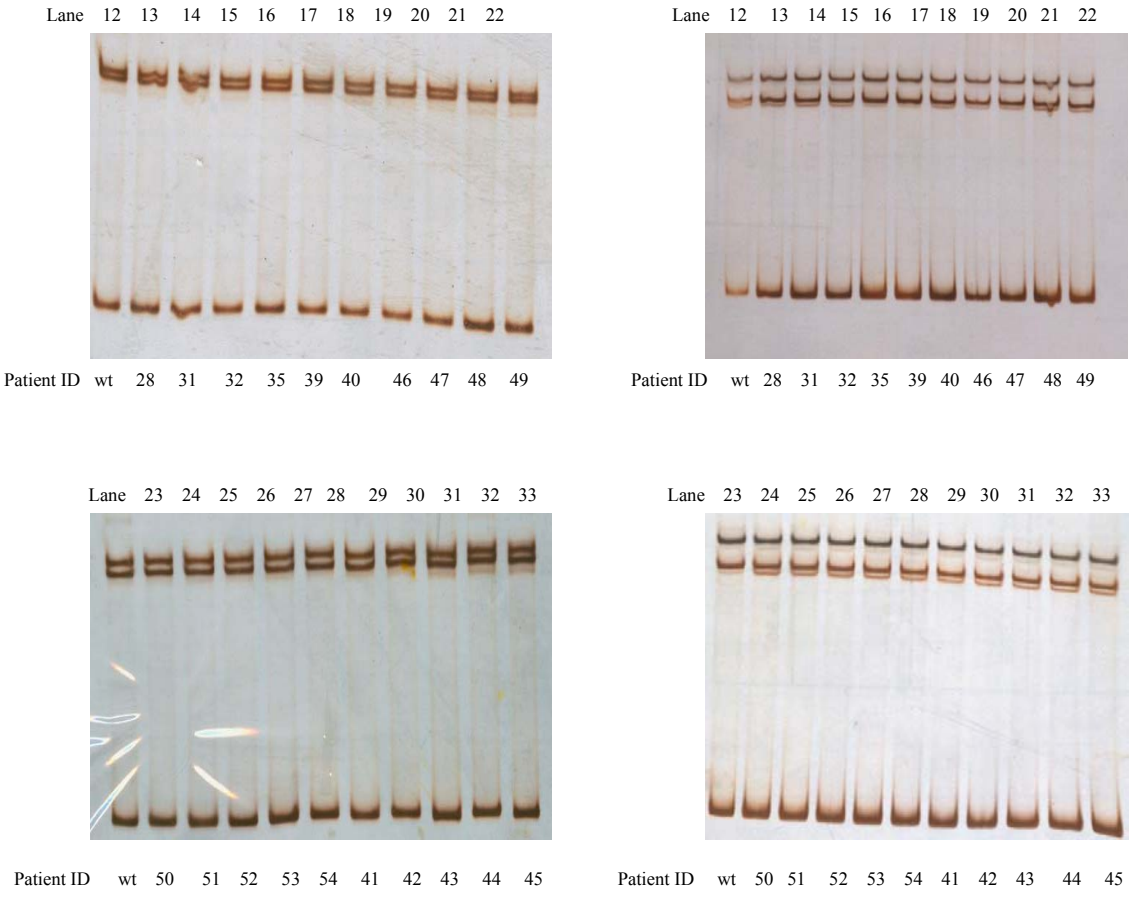
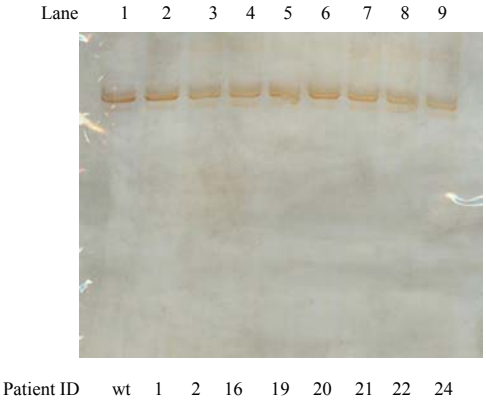


Fig. 4.18 SSCP analysis of the PCR fragment of *BRCA1* exon 22 (Panel A) and exon 23 (Panel B). Normal control person (wt) is presented in lanes 1, 12, 23 (A and B). The SSCP products were run on 10% non-denaturing polyacrylamide gel and visualized by silver staining.



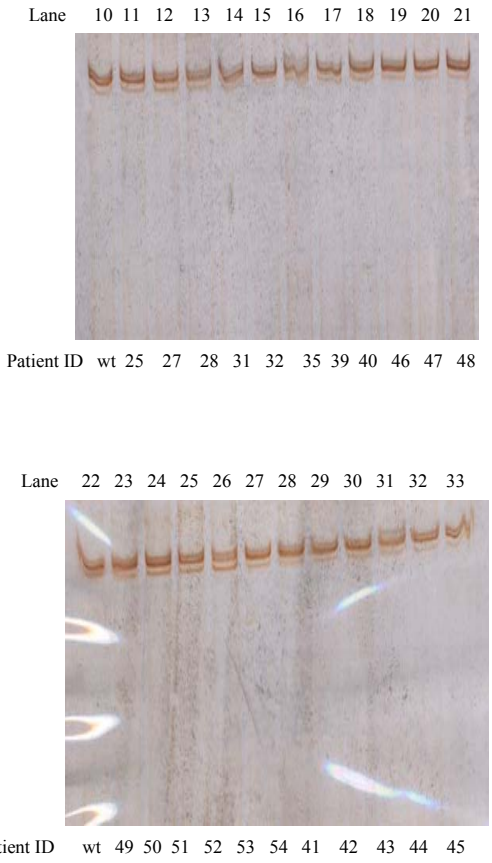
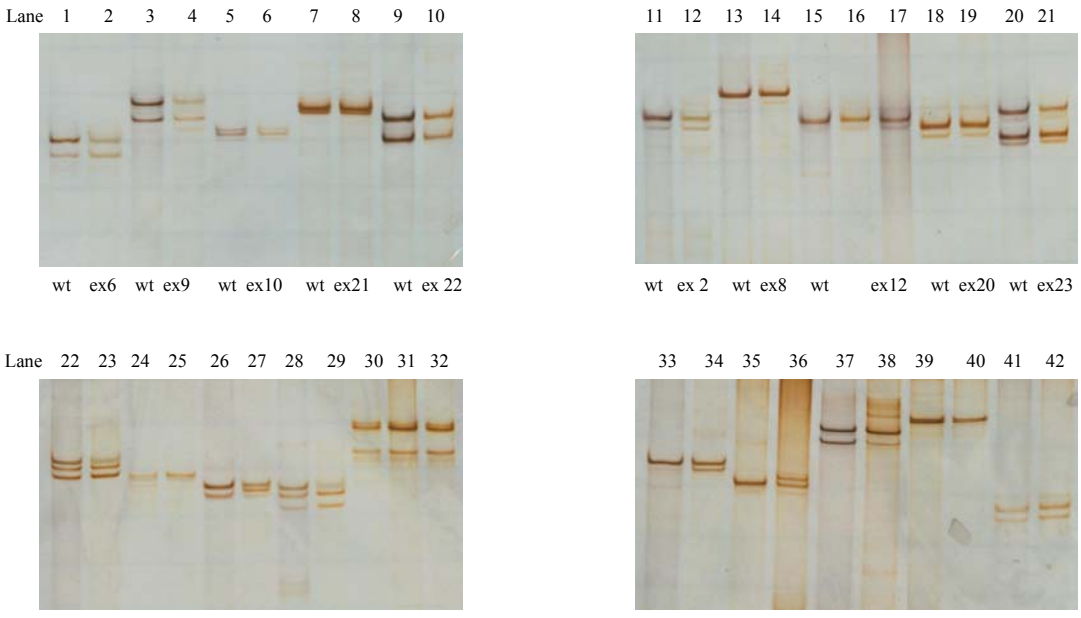


Fig. 4.19 SSCP analysis of the PCR fragment of *BRCA1* exon 24. Normal control person (wt) is presented in lanes 1, 10 and 22. The SSCP products were run on 10% non-denaturing polyacrylamide gel and visualized by silver staining.



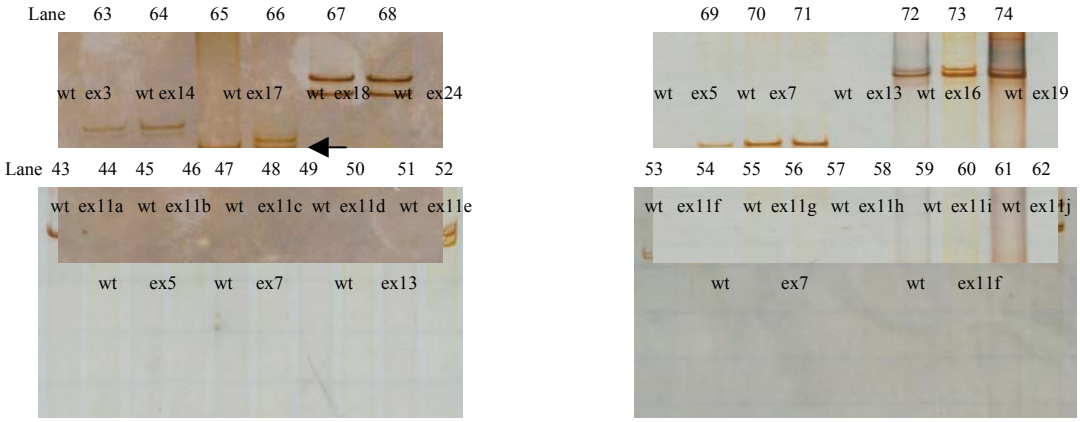
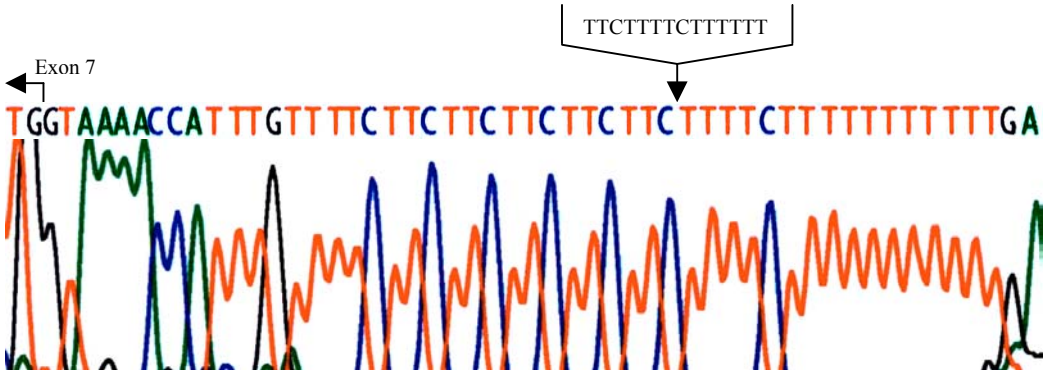


Fig. 4.20 SSCP analysis of the PCR fragment of *BRCA1* exons of patient ID 17. Arrow indicated the different electrophoretic mobility of *BRCA1* exon 7 fragment with respect to the wild-type fragment. The SSCP products were run on 10% non-denaturing polyacrylamide gel and visualized by silver staining. Wt stands for normal control person, ex stands for exon.

4.5 Direct sequencing of *BRCA1* exons

PCR products of exon fragments with observed change in electrophoretic mobility were further analyzed by direct sequencing using PRISM^{TM} Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit and the Applied Biosystems ABI 377 sequencer.

A



B

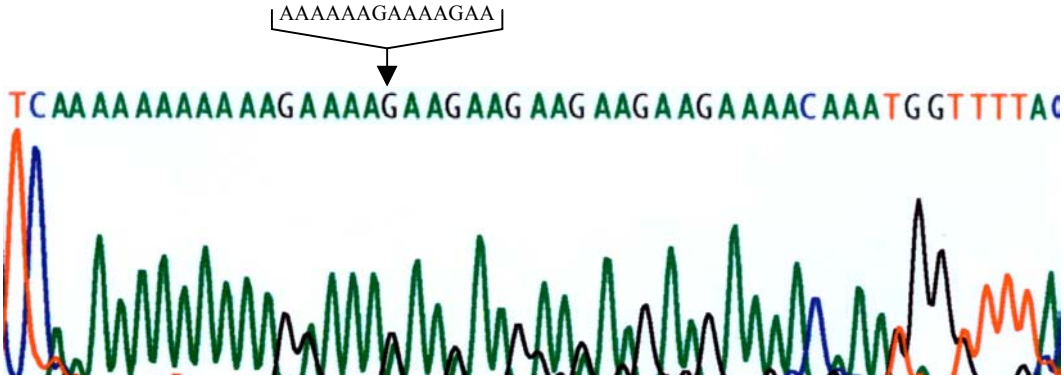


Fig. 4.21 Genomic sequences of the *BRCA1* IVS7+34_47 del TTCTTTTCTTTTTT deletion. *BRCA1* exon 7 - intron 7 boundary in the sense and antisense strand sequences of patient ID 17. (A) Sense sequence of *BRCA1* IVS7+34_47delTTCTTTTCTTTTTT deletion. (B) Antisense sequence of *BRCA1* IVS7+34_47delTTCTTTTCTTTTTT deletion.

The above results showed sequence alteration in electropherogram of exon 7 from patient ID 17. Sequencing of the *BRCA1* gene in the proband's genomic DNA revealed the absence of the non-coding intervening sequence at position +34 to +47 downstream the exon 7 of the *BRCA1* gene, resulting in the deletion of the TTCTTTTCTTTTTT sequences (g.24646_24659delTTCTTTTCTTTTTT, corresponding to the traditional nomenclature IVS7+34_47delTTCTTTTCTTTTTT).

Pedigree of patient ID 17 was shown in Fig. 4.22. The family history revealed a deceased sister (II-1) who had been diagnosed with breast cancer in her 56s. The proband's parents (I-1 and I-2) and younger sister (II-5) are unaffected. Patient's ID17 has a daughter (III-2). Based on the family history of patient ID17, we would expect a risk imposed on the daughter due to maternal transmission. Hence, it was of great interest to further analyse *BRCA1* mutations in the proband's daughter, ID 18 (III-2).

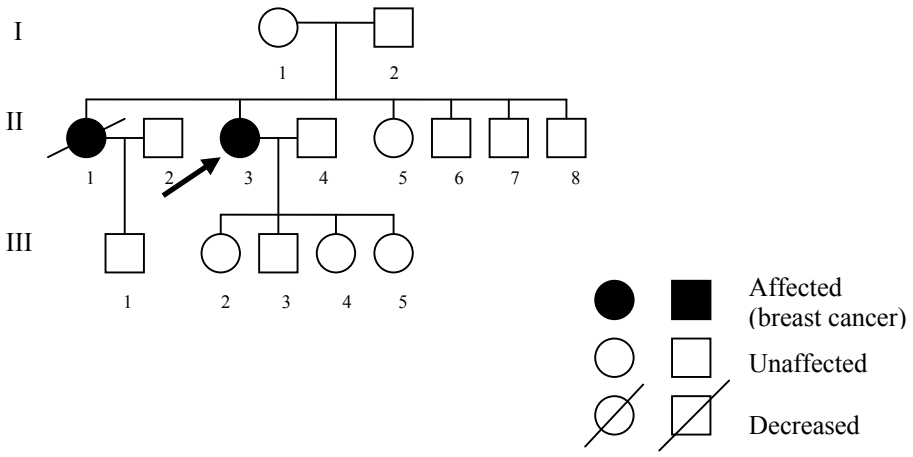


Fig. 4.22 Circle and square denote female and male individuals, respectively, while the slash marks indicate the deceased. Blackened shapes are affected individuals, and the proband (II-3) is designated with a left arrow with breast cancer at the age of 56. Individual II-1 developed breast cancer in her 56s and deceased at the age of 58. The proband's daughter (III-2) was a healthy female.

4.6 Single-Stranded Conformation Polymorphism (SSCP) analysis of patient's healthy daughter (ID 18)

Electrophoretic mobility shift of bands were observed in exon 7 and exon 9 of ID 18 (Fig. 4.23) and whose identities subsequently confirmed by direct sequencing. Further analysis of the patient's healthy daughter genomic DNA showed the heterozygous sequences, compared with the sense strand sequence of the proband with a substitution of C for T at nucleotide position of 24650 in intron 7, corresponding to the traditional nomenclature IVS7+38T>C. In addition, deletion of the *BRCA1* gene in the non-coding intervening sequence of the patient's healthy daughter genomic DNA was found. In the sense strand, the deletion in the non-coding intervening sequence at +50 to +63 downstream the exon 7 of the *BRCA1* gene was identitied, resulting in the deletion of the TTCTTTTTTTTTT (g.24662_24675 delTTCTTTTTTTTTT, corresponding to the traditional nomenclature IVS7+50_63

delTTCTTTTTTTTTTT) and in the antisense strand, the deletion in the noncoding intervening sequence at the +34 to +47 was detected, resulting in the deletion of the AAGAAAAGAAAAAAA sequences, of which identical to deleted sequence observed in the patient ID 17 (Fig. 4.24).

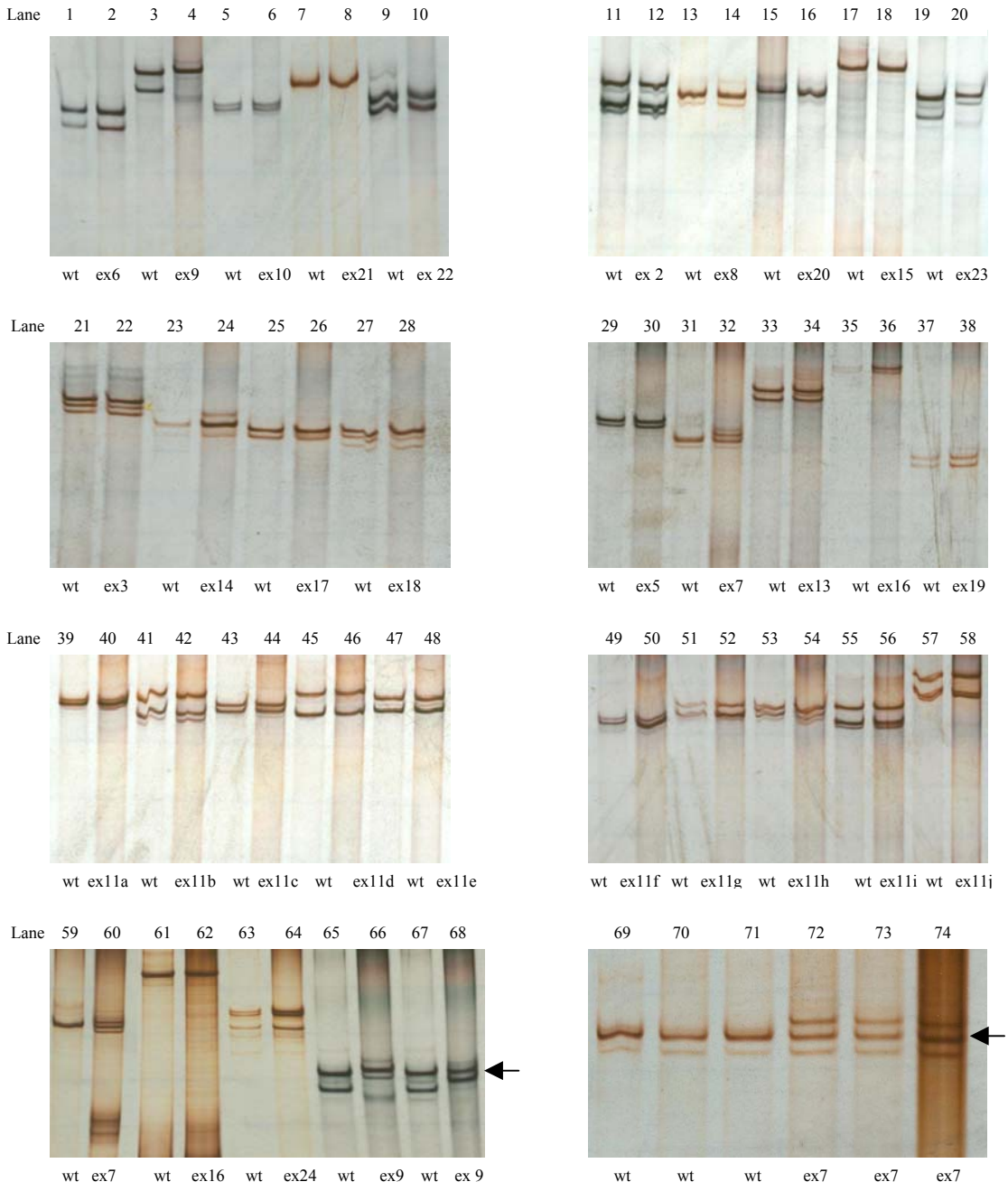
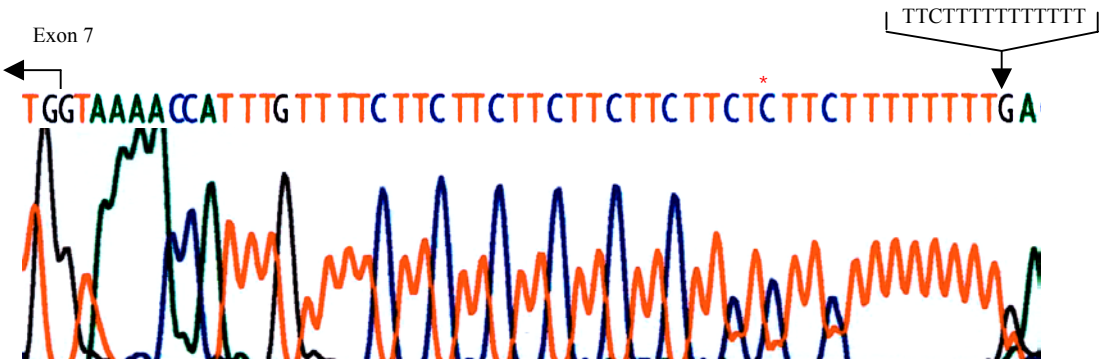


Fig. 4.23 SSCP analysis of the PCR fragment of *BRCA1* exons of ID 18. Arrows indicated the different electrophoretic mobility of *BRCA1* exon 7 or exon 9 fragment with respect to the wild-type fragment. The SSCP products were run on 10% non-denaturing polyacrylamide gel and visualized by silver staining. Wt stands for normal control person and ex stands for exon.

A



B

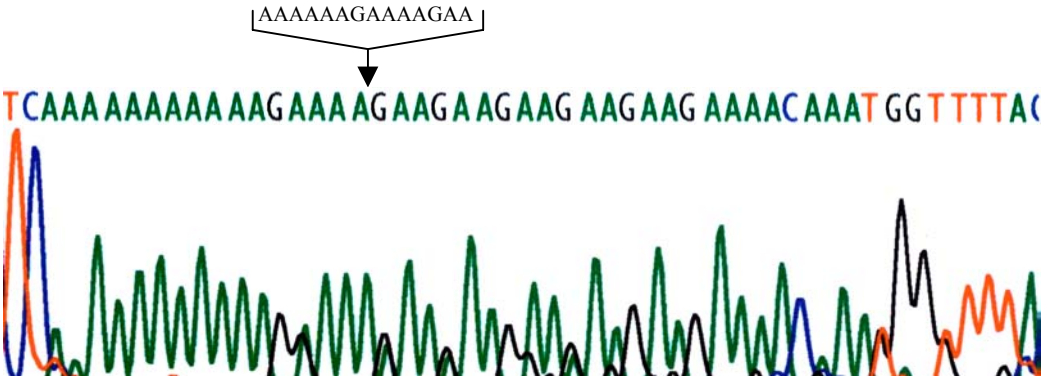


Fig. 4.24 Genomic sequences of the *BRCA1* exon-intron 7 boundary in the sense and antisense strands of the patient ID17's healthy daughter. (A) Sense sequence of *BRCA1* IVS7+50_63delTTCTTTTTTTTTTT deletion. (B) Antisense sequence of *BRCA1* IVS7+34_47delAAGAAAAGAAAAAA deletion. Base substitution at IVS7+38T>C in the sense strand of the patient's healthy daughter is identified with an asterisk.

4.7 Sequence analysis of the exon-intron 7 boundary of patient ID 17 and the patient's daughter (ID 18)

The exon-intron 7 boundary sequence was analyzed using primers (Fw1 and Rv1) (page 41). On SSCP analysis, electrophoretic mobility of PCR products of ID 17 (Fig. 4.25A) and ID 18 (Fig. 4.25B) was different from that of normal healthy Thai control group. The exon-intron 7 boundary sequence was consequently verified by direct sequencing (Fig. 4.26 and Fig. 4.27).

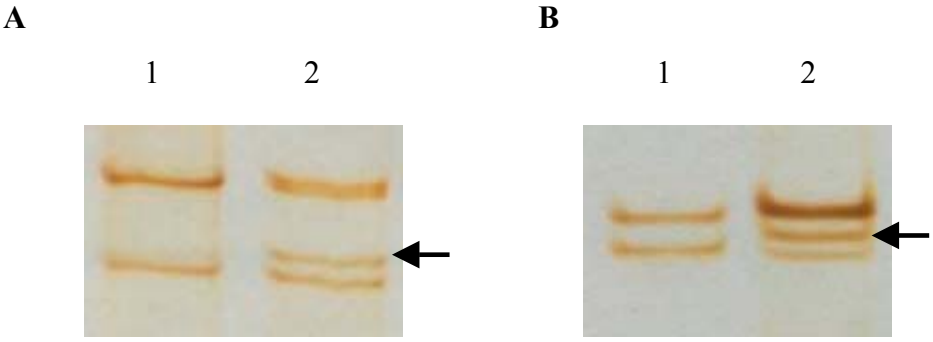


Fig. 4.25 Single-stranded conformation polymorphism of polymerase chain reaction products with silver staining. (A) A shift band (arrow) was found on the gel from patient ID 17 noncoding intervening 7 (lane 2). (B) A shift band (arrow) was found on the gel from patient's daughter (ID 18) noncoding intervening 7 (lane2). Lane1 was normal healthy Thai control group.

4.8 *Pfu* DNA polymerase on patients whose exons exhibiting changes in SSCP analysis

Prior studying of amplification of *BRCA1* exons were performed by PCR-SSCP analysis. The study showed that 1/31 of the DNA fragment mobility shifts were detected with SSCP, but only 9/31 of these contained mutations when confirmed by direct sequencing. We expected that this result may be occurred by the PCR using the DNA polymerase from *Thermus aquaticus* (*Taq* DNA polymerase). *Taq* DNA polymerase used in this technique will make errors during DNA synthesis and

reaction conditions. The sensitivity of PCR procedure depends on the fidelity level of the polymerase used to catalyze the PCR. Therefore, we searched for an enzyme with high fidelity, *Pfu*. It is the thermostable DNA polymerase derived from *Pyrococcus furiosus* (Fiala and Stetteer, 1986). The 3'-5' exonuclease activity of polymerase reduces the accumulation of frameshift and misinsertion mutations (Bebenek *et al*., 1990). Prior testing of *Pfu* reported an error rate of 1.6×10^{-6} per base pair (Lundberg *et al*., 1991). The fidelity of *Pfu* is significantly higher than the other commonly used thermostable polymerases: *Taq* has error rate of 10^{-4} and *Vent* has an error rate of 10^{-5} (Andre *et al*., 1997). Including, the genomic sequences in intron 7 of patient ID 17 and patient's healthy daughter (ID 18) have trinucleotide repeat sequences (TTC) with 7 units. This structure might be potential for mutations in amplified products arising with the higher than normal sequence. Thus, genomic DNA of patients whose exons exhibiting changes in SSCP analysis were amplified using *Pfu* DNA polymerase. In order to determine whether 3'-5' exonuclease activity can diminish error rate and increase the fidelity of synthesis of trinucleotide repeat sequences during the PCR.

The sequence of PCR amplified products were identical as in the previous observation. No mutations were found in the patient ID 54 in exon 11a, ID 43 and 31 in exon 11c, ID 40 in exon 11e, ID 45 and 46 in exon 11f and ID 14 in exon 1 and 20, respectively. However, alterations in *BRCA1* sequence were found in patient ID 17 (IVS7+50_63delTTCTTTTCTTTTTT) and patient's healthy daughter (ID 18) (IVS7+38T > C and IVS7+50_63delTTCTTTTTTTTTT on the sense strand and IVS7+34_47delAAGAAAAGAAAAAA on the antisense strand).

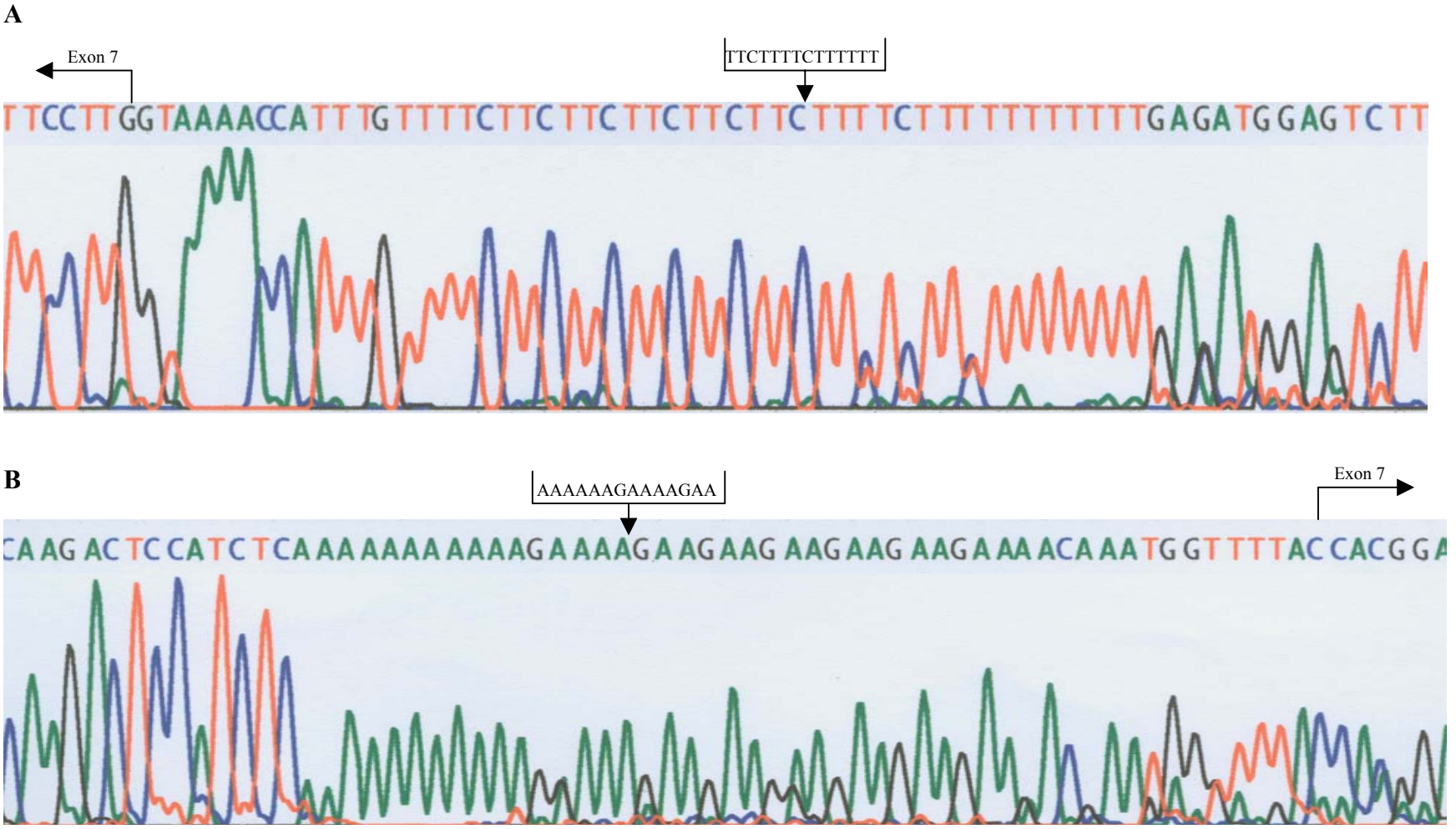


Fig. 4.26 Genomic sequence of the *BRCA1* IVS7+34_47 delTTCTTTTCTTTTTT deletion. *BRCA1* exon-intron 7 boundary using *Pfu* DNA polymerase in the sense and antisense strand sequences of the patient ID 17. (A) Sequence analysis of the sense *BRCA1* IVS7+34_47 delTTCTTTTCTTTTTT deletion. (B) Sequence analysis of the antisense *BRCA1* IVS7+34_47 delTTCTTTTCTTTTTT deletion.

Fig. 4.27 Genomic sequence of the *BRCA1* exon-intron 7 boundary using *Pfu* DNA polymerase in the sense and antisense strand sequences of the healthy patient's daughter. (A) Sequence analysis of the sense *BRCA1* IVS 7+50_63 delTTCTTTTTTTTTTT deletion. (B) Sequence analysis of the antisense *BRCA1* IVS 7+34_47 delAAGAAAAGAAAAAA deletion. Base substitution at IVS 7+38 T > C (red star) in the sense strand of the healthy patient's daughter.