



Original Article

Open Access

Detection of Germline Mutations in Breast Cancer Patients with Clinical Features of Hereditary Cancer Syndrome Using a Multi-Gene Panel Test

Hee-Chul Shin, MD, PhD¹
Han-Byoel Lee, MD²
Tae-Kyung Yoo, MD³
Eun-Shin Lee, MD²
Ryong Nam Kim, PhD⁴
Boyoung Park, MD, PhD⁵
Kyong-Ah Yoon, PhD⁶
Charny Park, PhD⁷
Eun Sook Lee, MD, PhD^{8,9}
Hyeong-Gon Moon, MD, PhD^{2,10}
Dong-Young Noh, MD, PhD^{2,10}
Sun-Young Kong, MD, PhD^{9,11}
Wonshik Han, MD, PhD^{2,10}

*A list of author's affiliations appears at the end of the paper.

Correspondence: Wonshik Han, MD, PhD
Department of Surgery, Seoul National
University Hospital, 101 Daehak-ro,
Jongno-gu, Seoul 03080, Korea
Tel: 82-2-2072-1958
Fax: 82-2-766-3975
E-mail: hanw@snu.ac.kr

Co-correspondence: Sun-Young Kong, MD, PhD
Genetic Counseling Clinic, Hospital,
Department of System Cancer Science,
Graduate School of Cancer Science and Policy,
Translational Epidemiology Branch, Research
Institute, National Cancer Center, 323 Ilsan-ro,
Ilsandong-gu, Goyang 10408, Korea
Tel: 82-31-920-1735
Fax: 82-31-920-1738
E-mail: ksy@ncc.re.kr

Received September 27, 2019

Accepted February 3, 2020

Published Online February 4, 2020

Purpose

Hereditary cancer syndrome means that inherited genetic mutations can increase a person's risk of developing cancer. We assessed the frequency of germline mutations using a next-generation sequencing (NGS)-based multiple-gene panel containing 64 cancer-predisposing genes in Korean breast cancer patients with clinical features of hereditary breast and ovarian cancer syndrome (HBOC).

Materials and Methods

A total of 64 genes associated with hereditary cancer syndrome were selected for development of an NGS-based multi-gene panel. Targeted sequencing using the multi-gene panel was performed to identify germline mutations in 496 breast cancer patients with clinical features of HBOC who underwent breast cancer surgery between January 2002 and December 2017.

Results

Of 496 patients, 95 patients (19.2%) were found to have 48 deleterious germline mutations in 16 cancer susceptibility genes. The deleterious mutations were found in 39 of 250 patients (15.6%) who had breast cancer and another primary cancer, 38 of 169 patients (22.5%) who had a family history of breast cancer (≥ 2 relatives), 16 of 57 patients (28.1%) who had bilateral breast cancer, and 29 of 84 patients (34.5%) who were diagnosed with breast cancer at younger than 40 years of age. Of the 95 patients with deleterious mutations, 60 patients (63.2%) had *BRCA1/2* mutations and 38 patients (40.0%) had non-*BRCA1/2* mutations. We detected two novel deleterious mutations in *BRCA2* and *MLH1*.

Conclusion

NGS-based multiple-gene panel testing improved the detection rates of deleterious mutations and provided a cost-effective cancer risk assessment.

Key words

Germline mutation, Next-generation sequencing, Breast neoplasms, Hereditary breast and ovarian cancer syndrome

Introduction

Hereditary cancer syndrome means that inherited genetic mutations can increase a person's risk of developing cancer. Specifically, certain genetic mutations can cause changes in the growth control of normal cells and cause them to become cancerous. Genetic mutations that promote cancer can be inherited if the mutations are present in germ cells. It is reported that inherited genetic mutations play a major role in 5% to 10% of all cancers. The most well-known genes associated with hereditary cancer syndrome are the *BRCA1/2* genes for hereditary breast and ovarian cancer syndrome (HBOC) and the *TP53* gene for Li-Fraumeni syndrome. Approximately 7% of breast and 13% of ovarian cancers are estimated to be due primarily to germline mutations in the *BRCA1/2* genes. The cumulative risks of breast and ovarian cancers in *BRCA1/2* mutation carriers are reported to be 72% (95% confidence interval [CI], 65 to 79) and 44% (95% CI, 36 to 53), respectively, in *BRCA1* carriers and 69% (95% CI, 61 to 77) and 17% (95% CI, 11 to 25), respectively, in *BRCA2* carriers [1]. The cumulative cancer risk associated with *TP53* mutation may be as high as 90% by the age of 60 years [2]. In addition to mutations in *BRCA1/2* and *TP53*, germline mutations in certain genes were associated with more than 50 hereditary cancer syndromes. Genetic tests for hereditary cancer syndromes can identify individuals and families at increased risk of developing cancer. Once individuals or families are identified for hereditary cancer syndrome, they can be referred for risk assessment and personalized management that may include intensive cancer surveillance, risk-reducing surgery and genetic counseling.

With the rapid progress that has been made in next-generation sequencing (NGS) technology, simultaneous sequencing of multiple genes has become available through multiple-gene panel testing, which is less expensive and more rapid than single-gene testing. Furthermore, multiple-gene panels using NGS technology have increased the detection rate of mutations compared to conventional gene-by-gene testing [3].

Currently, several commercial multiple-gene panels provide genetic information for hereditary cancer risk assessment. However, there are differences among ethnicities in cancer-susceptible germline mutations, and the assessment of germline mutations in all ethnic groups with clinical data is mandatory. In Korea and Asia, several studies evaluated the frequency of germline mutations, including *BRCA1/2* and/or other mutations associated with hereditary cancer syndrome. However, the results of most of the studies were not representative of the Korean and Asian population because of the relatively small number of patients included and the limited gene list evaluated.

In this study, we applied multiple-gene panel testing to 64 cancer susceptibility genes to examine the frequency of mutations and to assess the clinical value of NGS-based multiple-gene panel testing in breast cancer patients with clinical features of HBOC.

Materials and Methods

1. Patient selection

The study population included breast cancer patients with the following features of HBOC: (1) diagnosed with breast cancer and another primary cancer; (2) a family history that included at least two cases of breast cancer in first- or second-degree relatives; (3) bilateral breast cancer; or (4) breast cancer diagnosis before the age of 40 years and with collected blood samples in tissue bank in Breast Care Center, Seoul National University Hospital, Korea and Breast Cancer Center, National Cancer Center, Korea (CONSORT diagram) (S1 Fig.) Of the patients, 349 patients were admitted to Seoul National University Hospital, Korea, and 147 patients were admitted to the National Cancer Center, Korea, between 2002 and 2017. All patients consented to multi-gene panel testing for clinical research. Blood samples of the included patients were collected from each hospital and sent to a central laboratory for sequencing. The medical records were reviewed, and personal and family histories and pathologic data of cancer were recorded.

2. NGS assay

Genomic DNA was extracted from the participants' peripheral blood samples. Our panel included 64 hereditary cancer-predisposing genes (*ALK, APC, ATM, ATR, BAP1, BARD1, BLM, BMPR1A, BRCA1, BRCA2, BRIP1, CDH1, CDK4, CDKN2A, CHEK2, EPCAM, FAM175A, FANCA, FANCB, FANCC, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCL, FH, FLCN, GSTP1, HOXB13, KRAS, LIG4, MEN1, MET, MLH1, MRE11A, MSH2, MSH6, MUTYH, NAT1, NBN, NF1, PALB2, PALLD, PMS2, PRKAR1A, PRSS1, PTEN, RAD50, RAD51, RAD51C, RAD51D, RB1, RET, SDHB, SDHC, SDHD, SLX4, SMAD4, SPINK1, STK11, TP53, VHL, and XRCC2*) (Table 1). For mutation analysis, 64 gene-containing DNA fragments were enriched by solution-based hybridization capture and followed by sequencing with an Illumina NextSeq platform (Illumina, San Diego, CA) with the 150-bp paired-end read module. The target region included all coding exons. Capture probes were generated by Celeomics, Inc. (Seoul, Korea). The hybridization capture procedure was also performed

Table 1. Hereditary cancer-predisposing genes in the multiple-gene panel test

| Gene | Breast | Ovarian | Colorectal | Endometrial | Gastric | Pancreatic | Melanoma | Prostate | Other |
|---------|--------|---------|------------|-------------|---------|------------|----------|----------|-------|
| ALK | - | - | - | - | - | - | - | - | O |
| APC | - | - | O | - | O | O | - | - | O |
| ATM | O | - | - | - | - | O | - | - | - |
| ATR | - | - | - | - | - | - | - | - | O |
| BAP1 | - | - | - | - | - | - | - | - | O |
| BARD1 | O | - | - | - | - | - | - | - | - |
| BLM | - | - | O | - | - | - | - | - | O |
| BMPR1A | - | - | O | - | O | O | - | - | O |
| BRCA1 | O | O | - | - | - | O | - | O | - |
| BRCA2 | O | O | - | - | - | O | O | O | - |
| BRIP1 | O | O | - | - | - | - | - | - | - |
| CDH1 | O | - | O | - | O | - | - | - | - |
| CDK4 | - | - | - | - | - | - | O | - | - |
| CDKN2A | - | - | - | - | - | O | O | - | - |
| CHEK2 | O | - | O | - | - | - | - | O | - |
| EPCAM | - | O | O | O | O | O | - | - | O |
| FAM175A | O | O | - | - | - | - | - | - | - |
| FANCA | O | - | - | - | - | - | - | - | O |
| FANCB | - | - | - | - | - | - | - | - | O |
| FANCC | O | - | - | - | - | - | - | - | O |
| FANCD2 | - | - | - | - | - | - | - | - | O |
| FANCE | - | - | - | - | - | - | - | - | O |
| FANCF | - | - | - | - | - | - | - | - | O |
| FANCG | - | - | - | - | - | - | - | - | O |
| FANCI | - | - | - | - | - | - | - | - | O |
| FANCL | - | - | - | - | - | - | - | - | O |
| FH | - | - | - | - | - | - | - | - | O |
| FLCN | - | - | - | - | - | - | - | - | O |
| GSTP1 | O | - | - | - | - | - | - | - | O |
| HOXB13 | - | - | - | - | - | - | - | O | - |
| KRAS | - | - | O | - | O | O | O | - | - |
| LIG4 | - | - | - | - | - | - | - | - | O |
| MEN1 | - | - | - | - | - | - | - | - | O |
| MET | - | - | - | - | - | - | - | - | O |
| MLH1 | - | O | O | O | O | O | - | - | O |
| MRE11A | O | - | - | - | - | - | - | - | - |
| MSH2 | - | O | O | O | O | O | - | - | O |
| MSH6 | - | O | O | O | O | O | - | - | O |
| MUTYH | - | - | O | - | - | - | - | - | O |
| NAT | - | - | O | - | - | - | - | - | O |
| NBN | O | - | - | - | - | - | - | O | - |
| NF1 | - | - | - | - | - | - | - | - | O |
| PALB2 | O | - | - | - | - | O | - | - | - |
| PALLD | - | - | - | - | - | - | - | - | - |
| PMS2 | - | O | O | O | O | O | - | - | O |
| PRKAR1A | - | - | - | - | - | - | - | - | O |
| PRSS1 | - | - | - | - | O | O | - | - | - |
| PTEN | O | - | O | O | - | - | - | - | O |
| RAD50 | O | O | - | - | - | - | - | - | - |
| RAD51 | O | O | - | - | - | - | - | - | - |

(Continued to the next page)

Table 1. Continued

| Gene | Breast | Ovarian | Colorectal | Endometrial | Gastric | Pancreatic | Melanoma | Prostate | Other |
|---------------|--------|---------|------------|-------------|---------|------------|----------|----------|-------|
| <i>RAD51C</i> | O | O | - | - | - | - | - | - | - |
| <i>RAD51D</i> | O | O | - | - | - | - | - | - | - |
| <i>RB1</i> | - | - | - | - | - | - | - | - | O |
| <i>RET</i> | - | - | - | - | - | - | - | - | O |
| <i>SDHB</i> | - | - | - | - | - | - | - | - | O |
| <i>SDHC</i> | - | - | - | - | - | - | - | - | O |
| <i>SDHD</i> | - | - | - | - | - | - | - | - | O |
| <i>SLX4</i> | - | - | - | - | - | - | - | - | O |
| <i>SMAD4</i> | - | - | O | - | O | O | - | - | O |
| <i>SPINK1</i> | - | - | - | - | - | - | - | - | - |
| <i>STK11</i> | O | O | O | O | O | O | - | - | O |
| <i>TP53</i> | O | O | O | O | O | O | O | O | O |
| <i>VHL</i> | - | - | - | - | - | - | - | - | O |
| <i>XRCC2</i> | O | - | - | - | - | - | - | - | - |

Table 2. Characteristics of patients with and without deleterious mutations

| Characteristic | Total | No. deleterious mutation | Deleterious mutation | p-value (χ^2) |
|---|------------|--------------------------|----------------------|----------------------|
| No. of patients | 496 (100) | 401 (80.8) | 95 (19.2) | |
| Age at diagnosis, median (range, yr) | 48 (19-80) | 49 (19-80) | 45 (22-72) | 0.027 ^a |
| Breast cancer stage | | | | |
| 0 | 32 (6.5) | 30 (7.5) | 2 (2.1) | 0.078 |
| I | 209 (42.1) | 170 (42.4) | 39 (41.1) | |
| II | 181 (36.5) | 138 (34.4) | 43 (45.3) | |
| III | 62 (12.5) | 52 (13.0) | 10 (10.5) | |
| IV | 10 (2.0) | 10 (2.5) | 0 | |
| Unknown | 2 (0.4) | 1 (0.2) | 1 (1.1) | |
| Risk factors for HBOC^a | | | | |
| Breast cancer with another primary cancer | 250 (50.4) | 211 (52.6) | 39 (41.1) | 0.052 |
| Family history of breast cancer (≥ 2 relatives) | 169 (34.1) | 131 (32.7) | 38 (40.0) | 0.187 |
| Bilateral breast cancer | 57 (11.5) | 41 (10.2) | 16 (16.8) | 0.075 |
| Breast cancer diagnosis at < 40 yr | 84 (16.9) | 60 (15.0) | 29 (30.5) | 0.022 |
| Two or more risk factors | 64 (12.9) | 42 (10.5) | 22 (23.2) | 0.002 |

Values are presented as number (%). HBOC, hereditary breast and ovarian cancer syndrome. ^aStatistical significance was evaluated by Student's t test.

according to the manufacturer's standard protocol. Genomic DNA was sheared via sonication. Biotynylated RNA oligonucleotide probes were hybridized with sheared DNA. Captured fragments were removed from solution via streptavidin-coated magnetic beads and subsequently eluted. The enriched fragment library was then subjected to polymerase chain reaction (PCR) amplification using primers specific to the linked Illumina adaptors. Resulting libraries were quantified via Agilent 2200 TapeStation before proceeding to

Illumina NextSeq platform. All samples were pooled into a single lane on a flow cell and sequenced together.

Raw FASTQ files were filtered using Trimmomatic ver. 0.33 and aligned with the genome of reference (GRCh37/hg19) using Burrows-Wheeler Aligner ver. 0.7.10. PCR duplicates, overrepresented sequences, and low-quality reads were removed. Realignment of insertions and deletions were performed using GATK. Reads with mapping quality of 0 were filtered out. If a read was able to be mapped at two

different places with an identical percentage, the mapping quality equaled zero. Otherwise, the read was mapped to the most identical region. When $\geq 90\%$ of mutation reads were biased to forward or reverse, this read was filtered out. The cutoff value of minimum supporting reads and minimum coverage was 2 and 8. The mean depth over target region was 660. The mean read size was 139 and mean uncovered ratio over target was 0.16%. Variant calling was performed with Samtools ver. 1.1 and Varscan ver. 2.4.0 (S2 Table).

3. Mutation analysis and variant classification

Variants were described according to the nomenclature recommendations of the Human Genome Variation Society (<http://www.hgvs.org/mutnomen>) and classified according to the following American College of Medical Genetics and Genomics recommendations: pathogenic (P), likely-pathogenic (LP), variants of unknown significance (VUS), likely-benign, and benign/polymorphism [4]. We used online databases, including the Human Gene Mutation Database, the Single Nucleotide Polymorphism Database, the 1000 Genome project, ClinVar, the Sorting Intolerant From Tolerant, Polymorphism Phenotyping-2, and the Korean Reference Genome Database, for *in silico* prediction of identified variants. Variants classified as P or LP were considered deleterious mutations.

4. Statistical analysis

Participant characteristics and sequencing results were summarized with descriptive statistics, which included medians, means, and standard deviations. The distributions of deleterious mutation according to the inclusion criteria were compared using Pearson's chi-square analysis and Student's t test. All p-values were 2-sided and a p-value less than 0.05 was considered significant. All statistical analyses were performed using IBM SPSS Statistics for Windows ver. 23.0 (IBM Corp., Armonk, NY).

5. Ethical statement

This study was approved by the Institutional Review Board of the Seoul National University Hospital (No. 1509-132-689) and National Cancer Center (No. NCCNCS13717). All participants in this study provided consent to this research.

Results

1. Study population

The clinical characteristics of the patients are shown in Table 2. The median age at diagnosis of cancer was 48 years (range, 19 to 80 years). In these patients, 390 patients (78.6%) had stage I or II disease. More than half of the patients (n=250, 50.4%) had another primary cancer, including ovarian cancer, stomach cancer, colon cancer, lung cancer, or other malignancy. In all, 169 patients (34.1%) reported that they had two or more first- or second-degree relatives with breast cancer. Fifty-seven patients (11.5%) had synchronous or metachronous bilateral breast cancer, and 84 patients (16.9%) were diagnosed with breast cancer at an age younger than 40 years. Sixty-four patients had two or more risk factors for HBOC (e.g., bilateral breast cancer and breast cancer diagnosis < 40 years old).

2. Frequency of deleterious mutations

Table 2 summarizes the characteristics of patients with and without deleterious mutations. Of all 496 patients, 95 patients (19.2%) were found to have deleterious germline mutations of cancer susceptibility genes and 401 patients (80.8%) were not detected to carry deleterious mutations. The breast cancer stage was not different between the two groups (p=0.078). The proportions of risk factors, including breast cancer with another primary cancer, family history of breast cancer, and bilateral breast cancer were also not different between the

Table 3. The percentage of breast cancer subtypes according to *BRCA1/2* mutations

| Subtype | Patients without <i>BRCA1/2</i> mutations | Patients with <i>BRCA1</i> mutation | p-value (χ^2) | Patients with <i>BRCA2</i> mutation | p-value (χ^2) |
|-----------------|---|-------------------------------------|----------------------|-------------------------------------|----------------------|
| Luminal A | 235 (64.6) | 5 (20.8) | < 0.001 | 15 (65.2) | 0.825 |
| Luminal B | 32 (8.5) | 0 | | 1 (4.3) | |
| HER2-enriched | 23 (6.3) | 1 (4.2) | | 1 (4.3) | |
| Triple-negative | 75 (20.6) | 18 (75.0) | | 6 (26.1) | |

Values are presented as number (%). HER2, human epidermal growth factor receptor 2.

Table 4. List of deleterious mutations identified in patients

| Gene | Mutation | Affected transcript | Affected protein | Case No. |
|----------------------|----------------------------|----------------------------|-------------------|----------|
| BRCA1 | Frameshift insertion | NM_007294.3:c.3627dup | p.Glu1210Argfs*9 | HOPE_112 |
| | | | | HOPE_131 |
| | | | | HOPE_191 |
| | | | | HOPE_309 |
| | | | | HOPE_421 |
| | | | | HOPE_454 |
| | | | | HOPE_502 |
| | Nonsense mutation | NM_007294.3:c.4981G>T | p.Glu1661* | HOPE_287 |
| | | | | HOPE_11 |
| | Nonsense mutation | NM_007294.3:c.5080G>T | p.Glu1694* | HOPE_129 |
| | | | | HOPE_429 |
| | | | | HOPE_478 |
| | Frameshift deletion | NM_007297.3:c.1575del | p.Glu525Aspfs*16 | HOPE_399 |
| | Frameshift deletion | NM_007294.3:c.1961del | p.Lys654Serfs*47 | HOPE_118 |
| | Missense mutation | NM_007294.3:c.5339T>C | p.Leu1780Pro | HOPE_226 |
| | | | | HOPE_337 |
| | | | | HOPE_356 |
| | Nonsense mutation | NM_007294.3:c.3991C>T | p.Gln1331* | HOPE_57 |
| | Nonsense mutation | NM_007294.3:c.928C>T | p.Gln310* | HOPE_10 |
| | Frameshift insertion | NM_007294.3:c.1511dup | p.Lys505* | HOPE_65 |
| HOPE_309 | | | | |
| Frameshift deletion | NM_007294.3:c.923_924del | p.Ser308Lysfs*11 | HOPE_502 | |
| | | | HOPE_36 | |
| Frameshift deletion | NM_007294.3:c.3700_3704del | p.Val1234Glnfs*8 | HOPE_270 | |
| | | | HOPE_61 | |
| Nonsense mutation | NM_007294.3:c.5445G>A | p.Trp1815* | HOPE_351 | |
| | | | HOPE_280 | |
| Nonsense mutation | NM_007294.3:c.390C>A | p.Tyr130* | HOPE_72 | |
| | | | HOPE_168 | |
| Frameshift insertion | NM_007294.3:c.1511dup | p.Lys505* | HOPE_182 | |
| | | | HOPE_190 | |
| Frameshift deletion | NM_007294.3:c.923_924del | p.Ser308Lysfs*11 | HOPE_269 | |
| | | | HOPE_501 | |
| BRCA2 | Splice donor variant | NG_005905.2:c.5467+1G>A | p.= | HOPE_501 |
| | Frameshift deletion | NM_000059.3:c.700del | p.Ser234Profs*7 | HOPE_229 |
| | | | | HOPE_468 |
| | Frameshift deletion | NM_000059.3:c.3096_3111del | p.Lys1032Asnfs*6 | novel |
| | Frameshift insertion | NM_000059.3:c.9253dup | p.Thr3085Asnfs*26 | HOPE_64 |
| | Missense mutation | NM_000059.3:c.8023A>G | p.Ile2675Val | HOPE_407 |
| | Nonsense mutation | NM_000059.3:c.1399A>T | p.Lys467* | HOPE_57 |
| | | | | HOPE_91 |
| | | | | HOPE_177 |
| | Frameshift deletion | NM_000059.3:c.4092_4093del | p.Ile1364Metfs*3 | HOPE_355 |
| | Nonsense mutation | NM_000059.3:c.8140C>T | p.Gln2714* | HOPE_14 |
| Nonsense mutation | NM_000059.3:c.9076C>T | p.Gln3026* | HOPE_456 | |
| Frameshift deletion | NM_000059.3:c.5576_5579del | p.Ile1859Lysfs*3 | HOPE_465 | |
| | | | | HOPE_133 |

(Continued to the next page)

Table 4. Continued

| Gene | Mutation | Affected transcript | Affected protein | Case No. |
|---------------|----------------------|----------------------------|-------------------|--|
| | Nonsense mutation | NM_000059.3:c.7480C>T | p.Arg2494* | HOPE_5 HOPE_31 HOPE_80 HOPE_114 HOPE_307 HOPE_345 HOPE_389 HOPE_479 |
| | Frameshift deletion | NM_000059.3:c.2798_2799del | p.Thr933Argfs*2 | HOPE_350 |
| | Nonsense mutation | NM_000059.3:c.8951C>G | p.Ser2984* | HOPE_359 |
| | Frameshift deletion | NM_000059.3:c.3195_3198del | p.Asn1066Leufs*10 | HOPE_33 HOPE_488 |
| | Frameshift deletion | NM_000059.3:c.3744_3747del | p.Ser1248Argfs*10 | HOPE_158 HOPE_233 HOPE_274 HOPE_281 HOPE_352 |
| | Frameshift deletion | NM_000059.3:c.755_758del | p.Asp252Valfs*24 | HOPE_372 |
| <i>BRIP1</i> | Nonsense mutation | NM_032043.2:c.2392C>T | p.Arg798* | HOPE_485 |
| <i>CDH1</i> | Missense mutation | NM_004360.4:c.2494G>A | p.Val832Met | HOPE_23 HOPE_28 HOPE_33 HOPE_78 HOPE_192 HOPE_222 HOPE_288 HOPE_319 |
| <i>CHEK2</i> | Nonsense mutation | NM_007194.3:c.409C>T | p.Arg137* | HOPE_162 |
| | Nonsense mutation | NM_001005735.1:c.1684C>T | p.Arg562* | HOPE_310 |
| <i>FANCA</i> | Frameshift deletion | NM_000135.3:c.3720_3724del | p.Glu1240Aspfs*36 | HOPE_125 |
| | Frameshift deletion | NM_000135.2:c.2546del | p.Ser849Phefs*40 | HOPE_66 |
| <i>MLH1</i> | Frameshift insertion | NM_000249.3:c.1758dup | p.Met587Hisfs*6 | HOPE_315 |
| | Nonsense mutation | NM_000249.3:c.849T>A | p.Tyr283* | HOPE_378 novel |
| <i>MRE11A</i> | Missense mutation | NM_005591.3:c.140C>T | p.Ala47Val | HOPE_285 |
| <i>MSH2</i> | Frameshift deletion | NM_000251.2:c.229_230del | p.Ser77Cysfs*4 | HOPE_394 |
| <i>MUTYH</i> | Nonsense mutation | NM_001128425.1:c.55C>T | p.Arg19* | HOPE_225 |
| <i>NBN</i> | Missense mutation | NM_002485.4:c.511A>G | p.Ile171Val | HOPE_264 HOPE_421 HOPE_470 |
| <i>RAD51</i> | Missense mutation | NM_002875.4:c.449G>A | p.Arg150Gln | HOPE_24 HOPE_35 HOPE_231 HOPE_266 HOPE_324 HOPE_335 HOPE_418 |

(Continued to the next page)

Table 4. Continued

| Gene | Mutation | Affected transcript | Affected protein | Case No. |
|---------------|-------------------|----------------------|------------------|-------------------|
| <i>SPINK1</i> | Missense mutation | NM_003122.4:c.101A>G | p.Asn34Ser | HOPE_14 |
| | | | | HOPE_105 |
| | | | | HOPE_144 |
| | | | | HOPE_179 |
| | | | | HOPE_413 |
| <i>TP53</i> | Missense mutation | NM_000546.5:c.566C>T | p.Ala189Val | HOPE_33 |
| | | | | HOPE_395 |
| | | | | HOPE_396 |
| | Missense mutation | NM_000546.5:c.638G>A | p.Arg213Gln | HOPE_290 |
| | | | | Missense mutation |

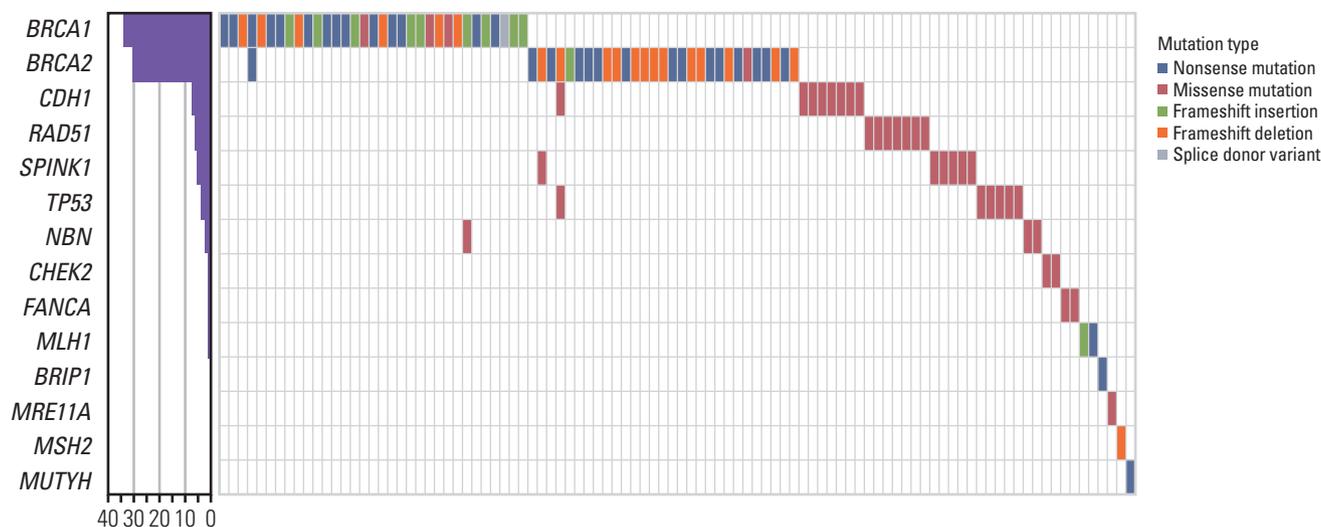


Fig. 1. Summary of 48 deleterious mutations in 95 patients. Deleterious *BRCA1* and *BRCA2* mutations were detected in 30 patients and 31 patients, respectively. Non-*BRCA1/2* germline mutations were found in 38 patients including *CDH1*, *RAD51*, *SPINK1*, *TP53* and so on.

groups. However, the proportion of patients with deleterious mutations were higher in patients who were diagnosed with breast cancer at younger than 40 years old than patients with another risk factors. Breast cancer diagnosis at young age was associated with a higher rate of deleterious mutations ($p=0.022$). Furthermore, having two or more risk factors for HBOC was also associated with a higher rate of deleterious mutations ($p=0.001$).

Breast cancers can be divided into four major subtypes depending on hormone receptor (HR) and human epidermal growth factor receptor 2 (HER2) status that have different clinical outcomes and responses to therapy a: luminal A (HR+ and HER2-), luminal B (HR+ and HER2+), HER2-enriched (HR- and HER2+), and triple-negative (HR- and

HER2). Table 3 showed the percentage of subtypes in 410 patients whose immunohistochemistry data were available. The percentage of patients with *BRCA1* mutations was different with patients without *BRCA1/2* mutations ($p < 0.001$). Seventy-five percent of patients with *BRCA1* mutations were triple-negative breast cancer, whereas 20.8% of patients were HR (+) breast cancer including luminal A and luminal B subtypes. In contrast, the percentage of subtypes in *BRCA2* mutations was not statistically different with patients without *BRCA1/2* mutations ($p=0.825$).

Table 4 and Fig. 1 summarize 48 deleterious mutations found in 95 patients. Of these patients with deleterious mutations, 60 patients (12.1%) had *BRCA1/2* mutations: 31 in *BRCA1* and 30 in *BRCA2*. Patients HOPE_309 and HOPE_502

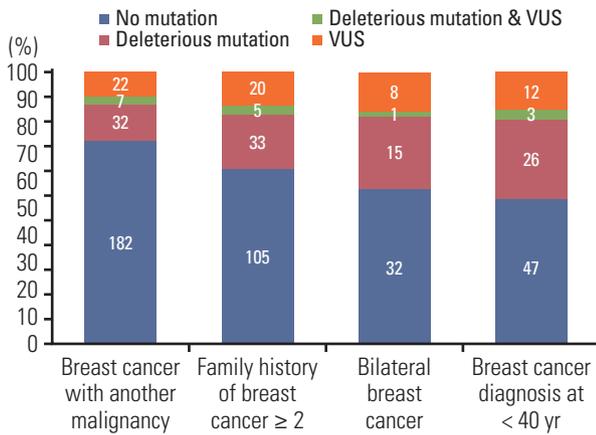


Fig. 2. The proportion of deleterious mutations according to risk factors of hereditary cancer syndrome. The highest proportion of deleterious mutations were found in breast cancer patients who were diagnosed at < 40 years old and the lowest were found in breast cancer patient with another primary cancer. VUS, variants of unknown significance.

had 2 *BRCA1* mutations and patient HOPE_57 carried both *BRCA1* and *BRCA2* mutations. In addition, 38 patients (7.7%) had cancer susceptibility gene mutations other than *BRCA1/2*: 35 patients had non-*BRCA1/2* mutations and three patients had both a *BRCA1/2* mutation and a non-*BRCA1/2* mutation (HOPE_14 had *BRCA2* and *SPINK1* mutations; HOPE_33 had *BRCA2*, *CDH1*, and *TP53* mutations; and HOPE_421 had *BRCA1* and *NBN* mutations). Most of the deleterious mutations were found in *CDH1* (n=8, 8.4%), *RAD51* (n=7, 7.4%), *SPINK1* (n=6, 6.3%), *TP53* (n=5, 5.3%) and *NBN* (n=3, 3.2%). The remaining patients had deleterious mutations in *CHEK2*, *FANCA*, *MLH1* (n=2 of each, 2.1%), *BRIP1*, *MRE11A*, *MSH2*, and *MUTYH* (n=1 of each, 1.1%).

The proportion of deleterious mutations varied according to risk factors. The deleterious mutations were found in 39 of 250 patients (15.6%) who had breast cancer and another primary cancer, 38 of 169 patients (22.5%) who had a family history (≥ 2 relatives) of breast cancer, 16 of 57 patients (28.1%) who had bilateral breast cancer, and 29 of 84 patients (34.5%) who were diagnosed with breast cancer at younger than 40 years old (Fig. 2). Furthermore, the distributions of the cancer susceptibility genes were different according to risk factors (Fig. 3). In breast cancer patients with another primary cancer, *BRCA1/2* and non-*BRCA1/2* mutations accounted for 52.3% and 47.7% of mutations, respectively. The non-*BRCA1/2* mutations comprised *CDH1* (11.4%), *SPINK1*

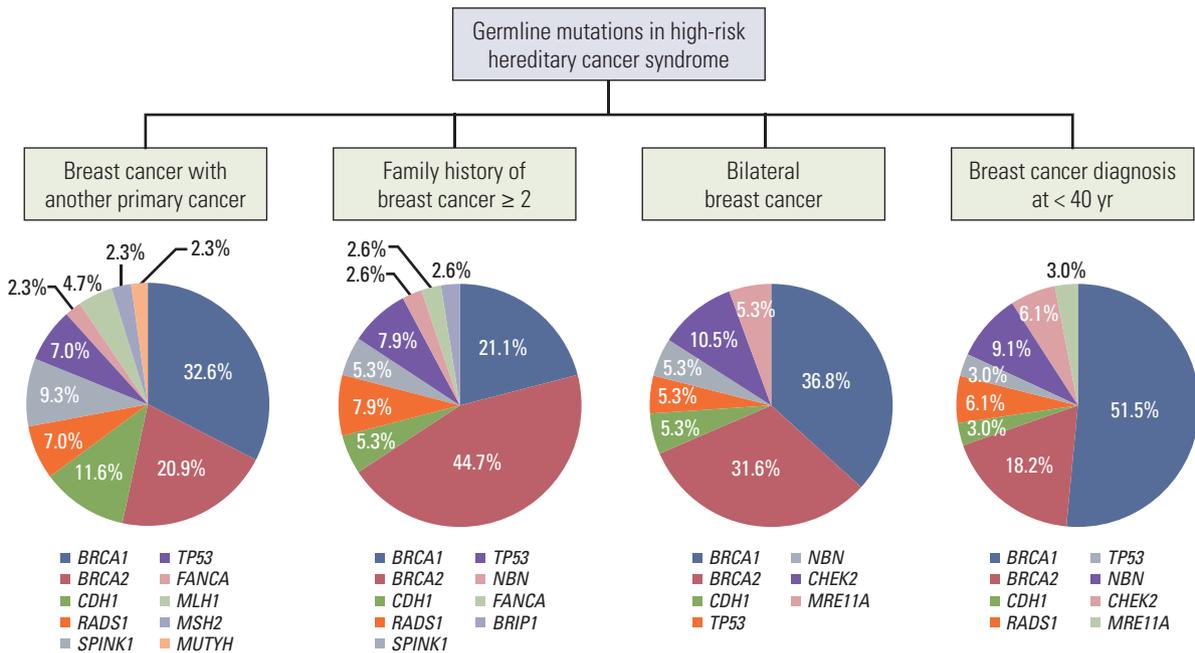


Fig. 3. The distributions of the cancer susceptibility genes according to risk factors hereditary cancer syndrome. The proportion of *BRCA1/2* mutations were relatively small in breast cancer patients with another primary cancer compared with patients with other risk factors.

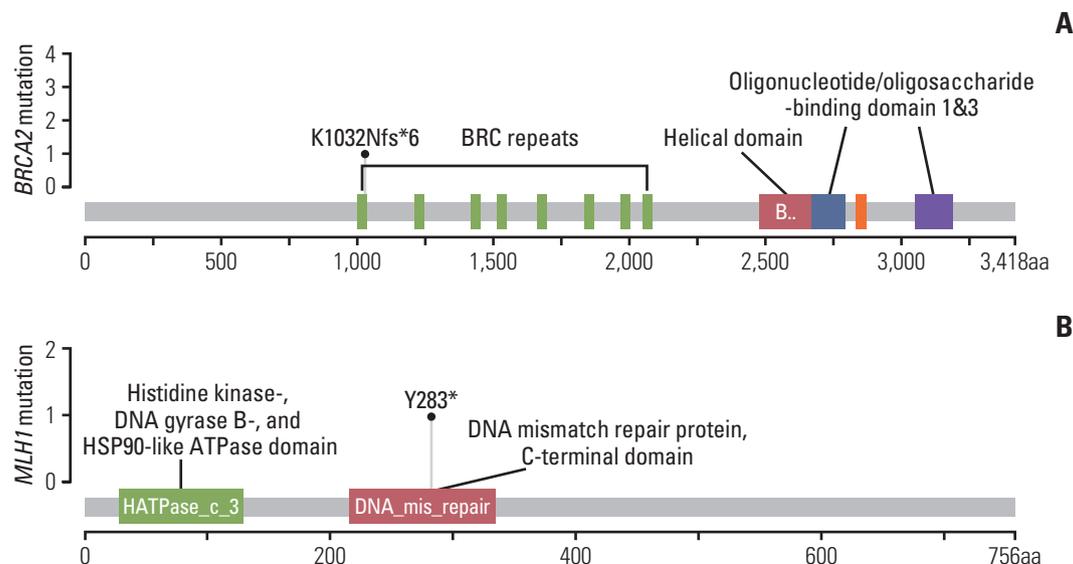


Fig. 4. Novel deleterious mutations mapped on corresponding protein structures. The impact of mutatinos were predicted in *in silico* analysis. (A) NM_000059.3:c.3096_3111del (p.Lys1032Asnfs*6) in *BRCA2*. (B) NM_000249.3:c.849T>A (p.Tyr283*) in *MLH1*.

(9.1%), *RAD51* (6.8%), and *TP53* (6.8%) mutations. In breast cancer patients with a family history of breast cancer, 65.8% carried a *BRCA1/2* mutation. In 34.2% of non-*BRCA1/2* mutations, 7.9% had *RAD51* and *TP53* mutations, and 5.3% had *CDH1* and *SPINK1* mutations. In bilateral breast cancer patients, 68.4% carried a *BRCA1/2* mutation. Among the 31.6% who had non-*BRCA1/2* mutations, *CHEK2* (10.5%) were found frequently and 5.3% of patients had *CDH1*, *TP53*, *NBN*, and *MRE11A* mutations. In patients diagnosed with breast cancer at younger than 40 years old, 62.1% carried *BRCA1/2* mutations and 37.9% carried non-*BRCA1/2* mutations including *RAD51*, *NBN*, *CHEK2*, *CDH1*, *TP53*, *PTEN*, *FANCA*, and *MRE11A* mutations.

In 64 hereditary cancer-predisposing genes, we found deleterious mutations in 16 genes, including *BRCA1/2*. However, we did not find deleterious mutations in the remaining 48 genes.

3. Novel deleterious mutations

We detected two novel deleterious mutations that were not previously reported: NM_000059.3:c.3096_3111del (p.Lys1032Asnfs*6) in *BRCA2* and NM_000249.3:c.849T>A (p.Tyr283*) in *MLH1*. The NM_000059.3:c.3096_3111del in *BRCA2* is identified in patient HOPE_468. This mutation encodes a truncated non-functional protein in the domain of the BRC repeats, interfering with cellular response to DNA damage (Fig. 4A). The NM_000249.3:c.849T>A in *MLH1* is identified in patient HOPE_378 and is also predicted to encode a non-

functional protein, leading to the disruption of an important functional domain, such as the MutL C-terminal domain (Fig. 4B). The impact of both mutations were predicted deleterious mutations in *in silico* prediction.

4. Frequency of VUS

A total of 333 missense mutations were identified in 64 genes. After *in silico* prediction by database and bioinformatics analysis to evaluate pathogenicity, most of the missense mutations were classified as benign or likely-benign. Mutations with conflicting interpretations of pathogenicity but suspicion of being deleterious were classified as VUS. A total of 20 VUS were identified in 67 patients (13.5%) (Table 5). In 15 patients, deleterious mutation and VUS were found concurrently. The proportion of VUS differed among the risk factors for HBOC (Fig. 2). VUS was identified in 11.6% of breast cancer patients with another primary cancer, 14.8% of patients with a family history of breast cancer, 15.8% of bilateral breast cancer patients, and 17.0% of patients who were diagnosed with breast cancer younger than 40 years old. Additionally, 13 patients with VUS also had a concurrent deleterious mutation (HOPE_33, 66, 105, 115, 133, 182, 222, 233, 264, 280, 454, 468, and 501).

Table 5. Variants of uncertain significance strongly suspected of being deleterious mutations

| Gene | Mutation | Affected transcript | Affected protein | Case No. | | | |
|----------|-------------------|--------------------------|------------------|-------------------|-----------------------|--------------|----------|
| ALK | Missense mutation | NM_004304.4:c.3260C>T | p.Thr1087Ile | HOPE_163 | | | |
| | | | | HOPE_264 | | | |
| ATR | Missense mutation | NM_001184.3:c.3637A>G | p.Ser1213Gly | HOPE_33 | | | |
| | | | | HOPE_204 | | | |
| BLM | Missense mutation | NM_000057.3:c.2371C>T | p.Arg791Cys | HOPE_468 | | | |
| | | | | HOPE_387 | | | |
| | | | | HOPE_393 | | | |
| BRCA1 | Missense mutation | NM_007294.3:c.154C>T | p.Leu52Phe | HOPE_79 | | | |
| | | | | HOPE_105 | | | |
| | | | | HOPE_187 | | | |
| | | | | HOPE_232 | | | |
| | | | | HOPE_233 | | | |
| BRCA2 | Missense mutation | NM_007294.3:c.3448C>T | p.Pro1150Ser | HOPE_200 | | | |
| | | | | Missense mutation | NM_000059.3:c.7522G>A | p.Gly2508Ser | HOPE_115 |
| | | | | | | | HOPE_487 |
| HOPE_306 | | | | | | | |
| CDH1 | Missense mutation | NM_004360.4:c.1018A>G | p.Thr340Ala | HOPE_124 | | | |
| | | | | HOPE_133 | | | |
| | | | | HOPE_218 | | | |
| | | | | HOPE_436 | | | |
| CHEK2 | Missense mutation | NM_001005735.1:c.1240C>T | p.His414Tyr | HOPE_476 | | | |
| | | | | HOPE_164 | | | |
| | | | | HOPE_242 | | | |
| FANCD2 | Missense mutation | NM_001018115.2:c.2480A>C | p.Glu827Ala | HOPE_466 | | | |
| | | | | HOPE_34 | | | |
| | | | | HOPE_66 | | | |
| | | | | HOPE_142 | | | |
| | | | | HOPE_214 | | | |
| FANCD2 | Nonsense mutation | NM_001018115.1:c.1318C>T | p.Gln440* | HOPE_347 | | | |
| | | | | HOPE_415 | | | |
| FANCE | Missense mutation | NM_021922.2:c.991C>G | p.Leu331Val | HOPE_172 | | | |
| FANCI | Missense mutation | NM_001113378.1:c.1111A>G | p.Ser371Gly | HOPE_26 | | | |
| | | | | HOPE_25 | | | |
| | | | | HOPE_86 | | | |
| | | | | HOPE_113 | | | |
| | | | | HOPE_164 | | | |
| | | | | HOPE_202 | | | |
| | | | | HOPE_217 | | | |
| | | | | HOPE_246 | | | |
| | | | | HOPE_280 | | | |
| | | | | HOPE_342 | | | |
| HOPE_468 | | | | | | | |
| FH | Missense mutation | NM_000143.3:c.302G>A | p.Arg101Gln | HOPE_501 | | | |
| | | | | HOPE_145 | | | |
| | | | | HOPE_182 | | | |
| | | | | HOPE_198 | | | |
| LIG4 | Missense mutation | NM_001098268.1:c.2586T>A | p.His862Gln | HOPE_439 | | | |
| | | | | HOPE_182 | | | |
| | | | | HOPE_291 | | | |

(Continued to the next page)

Table 5. Continued

| Gene | Mutation | Affected transcript | Affected protein | Case No. | | | |
|--------------|-------------------|-------------------------|------------------|-------------------|-----------------------|-------------|----------|
| <i>MSH2</i> | Missense mutation | NM_000251.2:c.14C>A | p.Pro5Gln | HOPE_186 | | | |
| | | | | HOPE_209 | | | |
| | | | | HOPE_222 | | | |
| | Missense mutation | NM_000251.2:c.1255C>A | p.Gln419Lys | HOPE_35 | | | |
| | | | | HOPE_88 | | | |
| | | | | HOPE_98 | | | |
| | | | | HOPE_232 | | | |
| | | | | HOPE_237 | | | |
| | | | | HOPE_414 | | | |
| | | | | HOPE_435 | | | |
| | | | | HOPE_462 | | | |
| <i>MSH6</i> | Missense mutation | NM_000179.2:c.3772C>G | p.Gln1258Glu | HOPE_144 | | | |
| | | | | HOPE_442 | | | |
| | | | | HOPE_490 | | | |
| <i>PALB2</i> | Missense mutation | NM_000179.2:c.2503C>G | p.Gln835Glu | HOPE_244 | | | |
| | | | | Missense mutation | NM_024675.3:c.2509G>A | p.Glu837Lys | HOPE_291 |
| | | | | | | | HOPE_293 |
| | | | | HOPE_358 | | | |
| <i>PTCH1</i> | Start lost | NM_001083603.2:c.1A>G | p.Met1? | HOPE_89 | | | |
| | | | | HOPE_463 | | | |
| | | | | HOPE_481 | | | |
| <i>TP53</i> | Missense mutation | NM_001126114.2:c.847C>T | p.Arg283Cys | HOPE_187 | | | |

Discussion

Patients who carry deleterious mutations are considered to be at high risk for developing cancer, and depending on the target organ, tailored surveillance programs or prophylactic risk-reducing surgery are recommended for decreasing cancer-related mortality. Currently, the National Comprehensive Cancer Network (NCCN) guidelines provide principles of genetic risk assessment and surveillance recommendations for various types of cancer. For example, women with *BRCA1/2* mutations are at high risk for breast and ovarian cancers and they are recommended to undergo magnetic resonance imaging (MRI) of the breast for screening and to discuss options for risk-reducing mastectomy or salphingo-oophorectomy.

We found that 19.2% of breast cancer patients who had clinical features of HBOC had deleterious mutations of cancer susceptibility genes. The breast cancer stage was not different between patients with and without deleterious mutations, nor were the proportions of risk factors for HBOC (breast cancer with another primary cancer, family history of breast cancer in two or more first- or second-degree relatives, and bilateral breast cancer). However, there was a signifi-

cantly higher rate of patients who were diagnosed with breast cancer at an age younger than 40 years among deleterious mutation-positive patients ($p=0.022$) (Table 2, Fig. 2). Furthermore, having two or more risk factors for HBOC was also associated with deleterious mutations ($p=0.001$).

Among 496 patients who were tested by the multiple-gene panel for cancer susceptibility genes, 60 patients (12.1%) were *BRCA1/2* positive, which was similar proportion to that reported in Western countries [5]. A previous study that included *BRCA1/2*-negative Korean breast cancer patients with features of hereditary breast cancer found that only 2.5% of non-*BRCA1/2* deleterious mutations were detected: *CHEK2* (0.4%), *PALB2* (0.9%), *MRE11* (0.4%), and *RAD50* (0.9%) [6]. Another study including Western patients reported that deleterious mutations were found only in 1.7% of 1994 familial breast cancer patients: *PALB2* (1.3%), *TP53* (0.3%), *CDH1* (0.05%), and *ATM* (0.05%) [7]. These studies reported that the frequency of deleterious mutations in each gene was less than 1% and concluded that a small portion of hereditary breast cancer was associated with non-*BRCA1/2* germline mutations. However, Li et al. [8] detected 11.5% non-*BRCA1/2* mutations, including *ATM*, *CDH1*, *CHEK2*, *PALB2*, *PTEN*, *STK11*, and *TP53* in 660 cases of familial breast cancer in a Western population. Ricker et al. [3] reported that

multiple-gene panel testing increased the detection rate of deleterious mutations from 8.6% to 15.6% compared with a conventional gene-by-gene approach. Furthermore, they reported that there were no significant differences in the mutation rates according to race or ethnic groups [3]. We identified 35 patients (8.0%) who had non-*BRCA1/2* deleterious mutations. These mutations included *CDH1* in seven patients (1.6%), *RAD51* in seven patients (1.6%), *SPINK1* in five patients (1.1%), and *TP53* in four patients (0.9%). The remaining patients had deleterious mutations in *CHEK2* (0.5%), *FANCA* (0.5%), *MLH1* (0.5%), *NBN* (0.5%), *BRIP1* (0.2%), *MRE11A* (0.2%), *MSH2* (0.2%), and *MUTYH* (0.2%). These results show that multiple-gene panel testing helps to increase the mutation detection rate compared to the conventional *BRCA* test alone. The results of previous studies and of our study are compatible with the suggestion of NCCN guidelines that multiple-gene testing may be more efficient and cost-effective for cancer risk assessment for patients with a high probability of hereditary cancer syndrome.

Germline *CDH1* mutations among the most frequently detected deleterious non-*BRCA1/2* mutations in our study. *CDH1* mutation is known to be associated with invasive lobular carcinoma and diffuse gastric cancer [9,10]. The NCCN guidelines recommend that women with the *CDH1* mutation receive regular breast examinations with annual mammogram and breast MRI, as well as prophylactic total gastrectomy or regular esophagogastroduodenoscopy with multiple random biopsy. In this study, we found eight patients with *CDH1* mutation (NM_004360.4:c.2494G>A), 7 patients with *CDH1* mutation only, and one patient (HOPE_33) with *CDH1* and another mutations (*BRCA2* NM_000059.3:c.3195_3198del and *TP53* NM_000546.5:c.566C>T). Patient HOPE_33 carried *BRCA2*, *CDH1*, and *TP53* mutations; she developed bilateral breast cancer at 33 years of age. Two patients with only the *CDH1* mutation had a family history of breast cancer in two or more first- or second-degree relatives. Five patients with only the *CDH1* mutation had breast cancer with another primary cancer including leukemia (HOPE_23), stomach cancer (HOPE_28), colon cancer (HOPE_222), cervical cancer (HOPE_192), and thyroid cancer (HOPE_319). Patients with the *CDH1* mutation should have been recommended to receive close surveillance for contralateral breast cancer and stomach cancer. Further, family members of patient HOPE_28, who already had stomach cancer, should undergo genetic testing and receive close surveillance for breast and stomach cancers.

The *RAD51* gene has a key role in the repair of DNA double-strand breaks through homologous recombination [11]. Germline mutation of *RAD51* is known to cause congenital mirror movement which is characterized by involuntary movements of one side of the body that mirror intentional movements on the opposite side [12]. In addition to this con-

genital neurologic disorder, *RAD51* mutation is associated with the development of malignancy, in including breast cancer and pancreatic cancer [13,14]. A previous study found that the proteins *BRCA2* and *PALB2* control the function of *RAD51*, yielding structural change for cancer susceptibility [15]. In this study, we found eight patients with *RAD51* NM_002875.4:c.449G>A. All patients with *RAD51* mutation were *BRCA1/2* negative. Three patients (HOPE_24, 35, and 335) had breast cancer and another primary cancer, including lung cancer, brain tumor, and thyroid cancer. Another three patients (HOPE_231, 324, and 418) had a family history of breast cancer in two or more relatives. One patient (HOPE_266) had breast cancer at an age younger than 40 years.

Germline mutation of *SPINK1* has been associated with hereditary pancreatitis by inhibiting the function of *SPINK1* protein and causing cellular damage by activated trypsin [16]. Several studies found that the *SPINK1* mutation was associated with pancreatic cancer [17]. In our study, 6 patients carried the deleterious *SPINK1* mutations (NM_003122.4:c.101A>G). One patient (HOPE_14) had both breast cancer and pancreatic cancer and found to carry both *SPINK1* and *BRCA2* mutations (NM_000059.3:c.4092_4093del). Another three patients had breast cancer and an additional primary cancer, including stomach cancer, cervical cancer, and common bile duct cancer. The remaining two patients had a family history of breast cancer. However, *SPINK1* mutation is not rare despite of deleterious mutation. According to 1000 Genome Project Phase 3, allele frequency of this mutation is 0.003 in American, 0.008 in East Asian and 0.014 in South Asian. Because the allele frequency is relatively high in Asian population, this mutation is thought to have low penetrance in Asian population. Patient HOPE_14 who carried *SPINK1* and *BRCA2* mutations developed breast cancer in 2002 and pancreatic cancer in 2005. Considering that the *BRCA2* mutation is also known for increasing risk of pancreatic cancer and high minor allele frequency of *SPINK1* mutation (NM_003122.4:c.101A>G), the main cause of breast and pancreatic cancer in patients HOPE_14 was *BRCA2* mutation, not *SPINK1* mutation [18].

Germline mutation of *TP53* is known as Li-Fraumeni syndrome. This mutation is associated with multiple cancers including breast cancer, soft tissue sarcoma, acute leukemia, brain tumor, adrenal carcinoma, and colon cancer. For this reason, Li-Fraumeni syndrome patients have a poor prognosis. Once this syndrome was revealed to be associated with a germline mutation of *TP53*, it became possible to detect carriers of inherited *TP53* mutations. Currently, individuals with a *TP53* mutation are recommended to undergo targeted surveillance, depending on individual medical history and family history. Villani et al. [19] reported that individuals with *TP53* mutation who received intensive surveillance with colonoscopy, whole body MRI, breast MRI, brain MRI,

skin examination, and physical examination showed improved overall survival compared with individuals who did not receive surveillance ($p=0.013$). This result supports the effectiveness of a tailored surveillance program for increasing survival rates and is beneficial to individuals with deleterious mutations. In our study, five patients were identified to carry *TP53* mutations (NM_000546.5:c.566C>T, NM_000546.5:c.638G >A, and NM_000546.5:c.743G>A). As mentioned, HOPE_33 carried *BRCA2*, *CDH1*, and *TP53* mutations and had bilateral breast cancer at a young age. HOPE_395 and 396 carried *TP53* mutation (NM_000546.5:c.566C>T) and suffered from breast cancer and thyroid cancer and had at least two relatives with a history of breast cancer. HOPE_290 had *TP53* mutation (NM_000546.5:c.638G>A) and a family history of breast cancer in at least two relatives. HOPE_115 had *TP53* mutation (NM_000546.5:c.743G>A) and had breast cancer and lung cancer. Family members of patients with *TP53* mutation need to undergo genetic testing to find out whether they are carriers of the *TP53* mutation or not. Depending on the results of genetic testing, *TP53* mutation carriers, as well as patients with *TP53* mutation, should consider clinical intensive surveillance for early detection of cancer and improved long-term survival.

Surveillance and risk-reducing strategies for patients with germline mutations of Lynch syndrome (*MLH1*, *MSH2*, *MSH6*, *PMS2*, and *EPCAM2*), such as colonoscopy, prophylactic hysterectomy, and bilateral salpingo-oophorectomy should be considered. In our study, three patients were found to have deleterious mutations in *MLH1* and *MSH2* (NM_000249.3: c.1758dup, NC_000003.11:c.849T>A, and NM_000251.2:c.229_230del). Patient HOPE_315 who carried *MLH1* mutation (NM_000249.3:c.1758dup) had primary breast, colon cancer, and lung cancer. Patient HOPE_394 who had *MSH2* mutations (NM_000251.2:c.229_230del) had primary breast and colon cancers. Patient HOPE_378 who carried novel deleterious mutation in *MLH1* (NM_000249.3:c.849T>A) had breast cancer and hepatocellular carcinoma. Because Lynch syndrome is an inherited disorders that increases the risk of various type of cancer, particularly in colorectum, endometrium, ovary, stomach, small bowel, liver, bile duct, upper urinary tract, and brain, these three patients' family members should have intensive surveillance for colon cancer and genetic testing for germline mutations of Lynch syndrome.

It is well known that *BRCA1* and *BRCA2* mutation carriers showed differences in tumor histopathology. A large proportion of breast cancer in women who carry a *BRCA1* mutation exhibited a triple-negative breast cancer. Previous study including Korean familial breast cancer patients reported that triple-negative breast cancer was diagnosed in 57.1% of *BRCA1* mutation carriers [20]. In contrast with *BRCA1* mutations, luminal A and luminal B subtype of breast cancer was found in 83.0% in breast cancer patients who carried *BRCA2*

mutations [21]. Our results showed that 75.0% of *BRCA1* mutation carriers were triple-negative breast cancer and 69.5% of *BRCA2* mutation carriers were HR-positive breast cancer including luminal A and B subtype, which are concordant with previous studies (Table 3).

The proportions and distributions of deleterious mutations in *BRCA1/2* negative patients were quite different in this Asian population than in a previously reported Western population. Maxwell et al. [22] reported that Caucasian and African American breast cancer patients who were *BRCA1/2* negative and had early-onset breast cancer (< 40 years old at diagnosis) carried 11% of non-*BRCA1/2* deleterious mutations. The deleterious mutations were *ATM* (25.8%), *CHEK2* (32.3%), *TP53* (12.9%), and *MRE11A* (6.5%). The remaining mutations were *MSH6*, *CDKN2A*, *MUTYH*, *BARD1*, *BRIP1*, *NBN*, and *RAD50* (3.2%). The majority of deleterious mutations in our study in *BRCA1/2*-negative and early-onset breast cancer patients were *NBN* (30.0%), *RAD51* (20.0%), and *CHEK2* (20.0%) (Fig. 3). Recently, Li et al. [23] reported the results of germline mutations among Chinese patients with features of hereditary breast cancer. They found that 16.9% of included patients carried *BRCA1/2* mutations and 6.8% of patients had non-*BRCA1/2* mutations including *TP53*, *PALB2*, *CHEK2*, *ATM*, *BARD1*, *BRIP1*, *CDH1* and *RAD50*. Recent studies reported that mutations in *PALB2* and *RAD51C* were found to be an important cause of HBOC [24,25]. Additionally, *CDH1* mutations were not found in the Western study but detected in the Chinese study. Although we did not find the *ATM*, *PALB2*, and *RAD51C* mutations in any of our study patients, we should have caution to interpret sequencing results of these important genes for HBOC.

We detected 2 novel deleterious mutations that have not been previously reported: NM_000059.3:c.3096_3111del (p.Lys-1032Asnfs*6) in *BRCA2* and NM_000249.3:c.849T>A (p.Tyr-283*) in *MLH1*. The p.Lys1032Asnfs*6 mutation in *BRCA2* was identified in patient HOPE_468. This mutation encodes a truncated non-functional protein in the domain of the BRC repeats (Fig. 4A). The human tumor suppressor protein *BRCA2* plays a key role in recombinant DNA repair. *BRCA2* recruits *RAD51* to sites of DNA damage through interaction with eight conserved motifs of approximately 35 amino acids, the BRC repeats, although the specific function of each repeat remains unclear [26]. The mutation of *BRCA2* p.Lys-1032Asnfs*6 is thought to interfere with cellular response to DNA damage, resulting in malignant transformations. The p.Tyr283* mutation in *MLH1* is found in patient HOPE_378 and is also predicted to encode a non-functional protein, leading to the disruption of important functional domain like MutL C-terminal domain (Fig. 4B). The subunits of *MLH1* and *PMS2* make the MutL α complex, which plays an essential role in mismatch repair [27]. A defect in *MLH1* is associated with mismatch repair and results in microsatellite

instability and spontaneous mutation rate. The family history of patients with novel deleterious mutations and genetic tests of family members are required to determine the clinical impact of these newly identified mutations.

In this study, 67 patients (13.5%) were shown to have 20 VUS in 18 genes (Table 5). Compared with other studies, the rate of VUS in this study was relatively low. This could be because we excluded most of the missense mutations with conflicting interpretations and considered benign or likely-benign. We only considered mutations as VUS when mutations had conflicting interpretation of pathogenicity but a suspicion of being deleterious. Most of the VUS will be re-categorized as benign or deleterious. Until the significance is fully understood, VUS should not be used for making clinical decisions. It is also important to reduce the number of VUS in clinical practice. Potential deleterious mutations can be selected by mutation frequency analysis and *in silico* analysis. Recently, Findlay et al. [28] used saturation genome editing to assay single-nucleotide variants in exons that encode functional domains of *BRCA1*. They found that functional effects of saturation genome editing were almost perfectly concordant with established assessments of pathogenicity. The saturation genome editing will be useful for accurate classification of VUS in clinically actionable genes.

The application of multi-gene panel testing has been rapidly increasing in clinical practice, especially in the evaluation of germline mutations which are associated with cancer susceptibility. The identification of deleterious mutations in cancer susceptibility genes in individuals with a high risk for hereditary cancer can improve the effectiveness of personalized surveillance, leading to early detection or prophylactic treatment of hereditary cancer in both individuals and their family members. Intensive surveillance for early detection and prophylactic treatment is directly linked with better survival in patients with deleterious mutations.

However, there are limitations to multi-gene panel testing. The prevalence of pathogenic mutations and VUS vary across races and ethnicities. Furthermore, the penetrance and phenotype of mutations are different among individuals. Detection of a deleterious mutation does not always mean an individual will develop cancer, and conversely, a negative result from a multi-gene panel test does not mean an individual has no risk of getting cancer. Although two novel mutations were found to be pathogenic because of their functioning effect on protein level in *in silico* analysis, these mutations were not clinically verified. Additional investigation including family history and targeted genetic tests of family members are required to determine the clinical impact of these newly identified mutations.

Another limitation is that we could not compare sequencing results between NGS and Sanger sequencing with same blood sample because of shortage of sample amount. The

NGS-based multi-gene panel testing have weakness point in detecting mutations compared with Sanger sequencing including uncovered area, large insertion/deletion, and copy-number variation. Although these weaknesses can be overcome by technical improvement, clinical implication including long-term outcomes should be discussed carefully.

Last limitation is that the attitude and knowledge gaps of physicians who provide care for individuals who undergo genetic testing for a disease. One survey reported that, although most physicians received formal genetic education and agreed that genetic tests are clinically useful for assessing disease risk, they were not confident about interpreting test results and were not prepared for managing individuals at high risk for genetic disease. For these reasons, genetic education and genetic counseling, as well as the appropriate and accurate interpretation of results, are important for the effective clinical application of risk management strategies. Stadler et al. [29] proposed that the results of germline genetic testing using multi-gene panels, including cancer-related findings and other incidental findings, should be integrated with traditional risk assessments, such as personal and family histories, to establish cancer and non-cancer risk management and follow-up plans. The paradigm shift toward personalized and precision medicine requires the incorporation of NGS technologies into clinical practice.

To the best of our knowledge, this is the largest study to include Korean breast cancer patients with clinical features of HBOC and examine the frequency and characteristics of germline mutations in *BRCA1/2* and non-*BRCA1/2* cancer susceptibility genes.

We analyzed germline mutations from 496 breast cancer patients of Asian ethnicity with clinical features of HBOC using NGS-based multi-gene panel testing. Overall, 95 patients (19.2%) were found to carry 48 deleterious germline mutations in 16 cancer susceptibility genes. Of these 95 patients, 60 patients (63.2%) had *BRCA1/2* mutations, 38 patients (40.0%) had non-*BRCA1/2* mutations and three patients (3.2%) had both *BRCA1/2* and non-*BRCA1/2* mutations. The NGS-based multi-gene panel test improved the detection rates of deleterious mutations and provided a cost-effective cancer risk assessment compared with a gene-by-gene approach.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (<https://www.e-crt.org>).

Conflicts of Interest

Hee-Chul Shin, Han-Byoel Lee and Wonshik Han had stocks of DCGEN Co. Ltd.

Acknowledgments

This research was supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute, funded by the Ministry of Health & Welfare, Republic of Korea (grant number: HCl14C1277, HI18C2282).

Author Details

¹Department of Surgery, Seoul National University Bundang Hospital, Seongnam, ²Department of Surgery, Seoul National University Hospital, Seoul, ³Department of Surgery, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul,

⁴Center for Medical Innovation, Biomedical Research Institute, Seoul National University Hospital, Seoul, ⁵National Cancer Center Graduate School of Cancer Science and Policy, Goyang, ⁶College of Veterinary Medicine, Konkuk University, Seoul, ⁷Clinical Genomics Analysis Branch, Research Institute, National Cancer Center, Goyang, ⁸Center for Breast Cancer, Hospital, National Cancer Center, Goyang, ⁹Translational Cancer Research Branch, Division of Translational Science, National Cancer Center, Goyang, ¹⁰Cancer Research Institute, Seoul National University, Seoul, ¹¹Genetic Counseling Clinic, Hospital, Department of System Cancer Science, National Cancer Center Graduate School of Cancer Science and Policy, Goyang, Korea

References

- Kuchenbaecker KB, Hopper JL, Barnes DR, Phillips KA, Mooij TM, Roos-Blom MJ, et al. Risks of breast, ovarian, and contralateral breast cancer for BRCA1 and BRCA2 mutation carriers. *JAMA*. 2017;317:2402-16.
- Mai PL, Khincha PP, Loud JT, DeCastro RM, Bremer RC, Peters JA, et al. Prevalence of cancer at baseline screening in the national cancer institute Li-Fraumeni syndrome cohort. *JAMA Oncol*. 2017;3:1640-5.
- Ricker C, Culver JO, Lowstuter K, Sturgeon D, Sturgeon JD, Chanock CR, et al. Increased yield of actionable mutations using multi-gene panels to assess hereditary cancer susceptibility in an ethnically diverse clinical cohort. *Cancer Genet*. 2016;209:130-7.
- Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med*. 2015;17:405-24.
- Hall MJ, Reid JE, Burbidge LA, Pruss D, Deffenbaugh AM, Frye C, et al. BRCA1 and BRCA2 mutations in women of different ethnicities undergoing testing for hereditary breast-ovarian cancer. *Cancer*. 2009;115:2222-33.
- Kim H, Cho DY, Choi DH, Oh M, Shin I, Park W, et al. Frequency of pathogenic germline mutation in CHEK2, PALB2, MRE11, and RAD50 in patients at high risk for hereditary breast cancer. *Breast Cancer Res Treat*. 2017;161:95-102.
- Thompson ER, Rowley SM, Li N, McInerney S, Devereux L, Wong-Brown MW, et al. Panel testing for familial breast cancer: calibrating the tension between research and clinical care. *J Clin Oncol*. 2016;34:1455-9.
- Li J, Meeks H, Feng BJ, Healey S, Thorne H, Makunin I, et al. Targeted massively parallel sequencing of a panel of putative breast cancer susceptibility genes in a large cohort of multiple-case breast and ovarian cancer families. *J Med Genet*. 2016;53:34-42.
- Fitzgerald RC, Hardwick R, Huntsman D, Carneiro F, Guilford P, Blair V, et al. Hereditary diffuse gastric cancer: updated consensus guidelines for clinical management and directions for future research. *J Med Genet*. 2010;47:436-44.
- Masciari S, Larsson N, Senz J, Boyd N, Kaurah P, Kandel MJ, et al. Germline E-cadherin mutations in familial lobular breast cancer. *J Med Genet*. 2007;44:726-31.
- Cousineau I, Abaji C, Belmaaza A. BRCA1 regulates RAD51 function in response to DNA damage and suppresses spontaneous sister chromatid replication slippage: implications for sister chromatid cohesion, genome stability, and carcinogenesis. *Cancer Res*. 2005;65:11384-91.
- Depienne C, Bouteiller D, Meneret A, Billot S, Groppa S, Klebe S, et al. RAD51 haploinsufficiency causes congenital mirror movements in humans. *Am J Hum Genet*. 2012;90:301-7.
- Martin RW, Orelli BJ, Yamazoe M, Minn AJ, Takeda S, Bishop DK. RAD51 up-regulation bypasses BRCA1 function and is a common feature of BRCA1-deficient breast tumors. *Cancer Res*. 2007;67:9658-65.
- Maacke H, Jost K, Opitz S, Miska S, Yuan Y, Hasselbach L, et al. DNA repair and recombination factor Rad51 is overexpressed in human pancreatic adenocarcinoma. *Oncogene*. 2000;19:2791-5.
- Buisson R, Dion-Cote AM, Coulombe Y, Launay H, Cai H, Stasiak AZ, et al. Cooperation of breast cancer proteins PALB2 and piccolo BRCA2 in stimulating homologous recombination. *Nat Struct Mol Biol*. 2010;17:1247-54.
- Witt H, Luck W, Hennies HC, Classen M, Kage A, Lass U, et al. Mutations in the gene encoding the serine protease inhibitor, Kazal type 1 are associated with chronic pancreatitis. *Nat Genet*. 2000;25:213-6.
- Midha S, Sreenivas V, Kabra M, Chattopadhyay TK, Joshi YK, Garg PK. Genetically determined chronic pancreatitis but not alcoholic pancreatitis is a strong risk factor for pancreatic cancer. *Pancreas*. 2016;45:1478-84.

18. Zhen DB, Rabe KG, Gallinger S, Syngal S, Schwartz AG, Goggins MG, et al. BRCA1, BRCA2, PALB2, and CDKN2A mutations in familial pancreatic cancer: a PACGENE study. *Genet Med.* 2015;17:569-77.
19. Villani A, Shore A, Wasserman JD, Stephens D, Kim RH, Druker H, et al. Biochemical and imaging surveillance in germline TP53 mutation carriers with Li-Fraumeni syndrome: 11 year follow-up of a prospective observational study. *Lancet Oncol.* 2016;17:1295-305.
20. Seong MW, Kim KH, Chung IY, Kang E, Lee JW, Park SK, et al. A multi-institutional study on the association between BRCA1/BRCA2 mutational status and triple-negative breast cancer in familial breast cancer patients. *Breast Cancer Res Treat.* 2014;146:63-9.
21. Krammer J, Pinker-Domenig K, Robson ME, Gonen M, Bernard-Davila B, Morris EA, et al. Breast cancer detection and tumor characteristics in BRCA1 and BRCA2 mutation carriers. *Breast Cancer Res Treat.* 2017;163:565-71.
22. Maxwell KN, Wubbenhorst B, D'Andrea K, Garman B, Long JM, Powers J, et al. Prevalence of mutations in a panel of breast cancer susceptibility genes in BRCA1/2-negative patients with early-onset breast cancer. *Genet Med.* 2015;17:630-8.
23. Li JY, Jing R, Wei H, Wang M, Xiaowei Q, Liu H, et al. Germline mutations in 40 cancer susceptibility genes among Chinese patients with high hereditary risk breast cancer. *Int J Cancer.* 2019;144:281-9.
24. Antoniou AC, Casadei S, Heikkinen T, Barrowdale D, Pylkas K, Roberts J, et al. Breast-cancer risk in families with mutations in PALB2. *N Engl J Med.* 2014;371:497-506.
25. Li N, McInerney S, Zethoven M, Cheasley D, Lim BW, Rowley SM, et al. Combined tumor sequencing and case-control analyses of RAD51C in breast cancer. *J Natl Cancer Inst.* 2019;111:1332-8.
26. Carreira A, Kowalczykowski SC. Two classes of BRC repeats in BRCA2 promote RAD51 nucleoprotein filament function by distinct mechanisms. *Proc Natl Acad Sci U S A.* 2011;108:10448-53.
27. Kadyrov FA, Dzantiev L, Constantin N, Modrich P. Endonucleolytic function of MutLalpha in human mismatch repair. *Cell.* 2006;126:297-308.
28. Findlay GM, Daza RM, Martin B, Zhang MD, Leith AP, Gasperini M, et al. Accurate classification of BRCA1 variants with saturation genome editing. *Nature.* 2018;562:217-22.
29. Stadler ZK, Schrader KA, Vijai J, Robson ME, Offit K. Cancer genomics and inherited risk. *J Clin Oncol.* 2014;32:687-98.