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유전성 암 유전자에서 검출된 염기 변이의 해석을 위한 기능 연구 근거의 적용

Applying Functional Assay Evidence to Interpret Sequence Variants Identified in Hereditary Cancer Genes

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The demand for the interpretation of sequence variants identified by next-generation sequencing is gradually increasing in clinical laboratories. The American College of Medical Genetics and the Association for Molecular Pathology (ACMG/AMP) 2015 guidelines provide a basis for using functional assays as strong evidence for variant classification. However, it is challenging to use the evidence because the protein's function and the functional assays used to prove it are too diverse. Therefore, this study reviewed various functional assays that can aid in classifying sequence variants in clinical laboratories. This review focuses on the 1) general functional assays associated with basic protein functions and processing and 2) functional assays related to the specific pathogenic mechanisms of four genes (*TP53, BRCA1, CDH1,* and *PTEN*) associated with hereditary cancer.

Key Words: Functional assay, Variant interpretation, Hereditary cancer, Variant of uncertain significance

INTRODUCTION

The American College of Medical Genetics (ACMG) and the Association for Molecular Pathology (AMP) established standards and guidelines for the interpretation of sequence variants [1]. Criteria for classifying pathogenic or benign variants have been developed. However, some evidence, such as PS3/BS3, is somewhat ambiguous, and many laboratories have difficulty applying the criteria in variant interpretation. According to the ACMG/AMP guideline, PS3 and BS3 are defined by "well-established in vitro or in vivo functional studies supportive of a damaging effect on the gene or gene product" and "well-established in vitro or in vivo functional studies show no damaging effect on protein function or splicing," respectively [1].

Although functional studies can provide evidence to interpret a variant's effect on protein function, leading to the reclassification of variants of uncertain significance (VUS), detailed guidance on how functional evidence can be evaluated and applied has not been provided by the original ACMG/AMP guidelines. Brnich et al. [2] published a recommendation for applying the PS3/BS3 criterion to provide a more structured approach for evaluating functional evidence. Furthermore, Kanavy et al. [3] evaluated the comparative analysis of PS3/BS3 of six Variant Curation Expert Panels (*CDH1*, Hearing Loss, Inherited Cardiomyopathy-*MYH7, PAH,*

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PTEN, and RASopathy), and these studies can provide guidance for laboratories regarding the application of PS3/BS3 in variant interpretation.

Nevertheless, clinical laboratory practitioners are often unfamiliar with the various experimental procedures used for functional validation. We selected four genes (*TP53, BRCA1, CDH1,* and *PTEN*) associated with hereditary cancer for which PS3/BS3 criteria can often be considered when interpreting variants. We selected these four genes because most of their functional mechanisms did not overlap. In addition, a large number of VUS missense variants with insufficient evidence were observed in these genes despite ClinVar data suggesting the presence of numerous likely pathogenic and pathogenic missense variants. Therefore, in most cases, applying PS3/BS3 can serve as significant evidence to help VUS missense variants be classified as either pathogenic or benign. In this review, we discussed the literature on these genes and the functional assays mainly used to understand functional analysis. In Part I, we describe general assays associated with basic protein functions and processing. The functional assays introduced in Part I are summarized in Table 1. The functional assays related to the specific pathogenic mechanisms of individual genes are described in Part II. Recent ClinGen guidelines for PS3/BS3 interpretation in *TP53, CDH1,* and *PTEN* are summarized in Table 2 [4-6].

PART I

1. Gene expression and protein turnover assay

Several conditions must be met to assess the impact of a variant on the function of a gene. First, the protein encoded by the gene must be produced, as stated by the central dogma, and carried to the correct subcellular location. Finally, it must not be degraded before it can perform its function. Researchers carrying out functional studies must ensure that these conditions are met before making any hasty interpretations.

Several experimental methods can be used for that purpose proposed. As the central dogma states, a gene must undergo transcription to produce its corresponding mRNA, which in turn must be translated to generate its corresponding protein. The polymerase chain reaction (PCR) can measure the transcription step of the central dogma, while western blotting can prove translation. The correct subcellular localization can be visualized via immunofluorescence. Flow cytometry can also be used if the target organelle is the plasma membrane. Lastly, double fluorescence can be used to verify whether the protein is ubiquitylated and becomes prone to proteasomal degradation [7].

2. Transactivation assay

In the context of gene regulation, transactivation describes the increased expression of specific target genes through an intermediate transactivator protein binding to a response element (RE) located within the promoter or enhancer region. Therefore, transactivation assays can be used to evaluate transcription factor gene variants. They require REs of target genes upstream of either the target genes themselves or reporter genes, such as the green fluorescence protein (GFP), in addition to the transcription factor gene [8].

3. Cell viability assay

Cell viability assays gauge how well or poorly cells proliferate by measuring an indicator of cell life or death. They can assess the physiological, structural, and functional aspects of cultured cells [9]. Cell life indicators include cell number, ATP content, DNA

Table 1. Summary of the general functional assays introduced in Part I

Mechanism	Endpoint	Example	Expected result in affected cell-lines
Gene expression and protein turnover	mRNA/protein level	PCR Western blot	Genetic material is not amplified. Protein band is absent.
Transactivation	Reporter gene expression level	Fluorescent reporter proteins, luciferase assays	Fluorescence is not detected.
Cell viability	Indicator of cell life or death	Colony formation assay Apoptosis assay	Colonies grow despite lack of cell anchorage. Cells are resistant to apoptosis.
Binding	Interaction between two molecules	Tetramerization assay	Protein band is detected at a different location on western blot.
Cell motility	Indicator of cell movement	Cell aggregation assay, cell invasion assay, wound closure assay	Cell adhesion loss and increased cell motility
Enzyme activity	Indicator of enzyme activity involved in a common pathway	Phosphatase assay	Varies depending on enzyme kinetics and inhibition in certain pathways.

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Table 2. PS3/BS3 interpretation suggested by ClinGen

Gene	Criteria	Specification
*TP53**	PS3_Strong	Transactivation assays in yeast (IARC classification based on data from Kato et al.) that demonstrate a low functioning allele (<20% activity) AND: - Evidence of a dominant-negative effect (DNE)+evidence of a LOF from Giacomelli et al. data OR - There is a second assay showing low function (colony formation assays, apoptosis assays, tetramer assays, knock-in mouse models, and growth suppression assays).
	PS3_Moderate	A) Transactivation assays in yeast (IARC classification based on data from Kato et al.) that demonstrate a partially functioning allele (>20% and ≤75% activity) AND: - Evidence of a DNE+evidence of a LOF from Giacomelli et al. data. OR - There is a second assay showing low function. *Do not use code with conflicting evidence.* B) No transactivation assays (IARC classification based on data Kato et al.) available BUT: - Evidence of a DNE+evidence of a LOF from Giacomelli et al. data. AND - There is a second assay showing low function. *Do not use code with conflicting evidence.*
	BS3_Strong	Transactivation assays in yeast (IARC classification based on data from Kato et al.) that show retained function (76–140% activity) or super-transactivation function AND: - No evidence of a DNE+no evidence of a LOF from Giacomelli et al. data. OR - There is a second assay, including colony formation assays, apoptosis assays, tetramer assays, growth suppression, and knock-in mouse models demonstrating retained function.
	BS3_Supporting	Transactivation assays in yeast (IARC classification based on data from Kato et al.) that demonstrate a partially functioning allele (>20% and ≤75% activity) AND: - No evidence of a DNE+no evidence of a LOF from Giacomelli et al. data. OR - There is a second assay demonstrating retained function. *Do not use code with conflicting evidence.*
CDH1†	PS3_Strong	RNA assay demonstrating abnormal out-of-frame transcripts. This rule can only be applied to demonstrate splicing defects.
	PS3_Supporting	RNA assay demonstrating abnormal in-frame transcripts. This rule can only be applied to demonstrate splicing defects.
	BS3_Strong	Functional RNA studies demonstrating no impact on transcript composition This rule can only be used to demonstrate a lack of splicing and can only be applied to synonymous, intronic, or non-coding variants. BS3 may be downgraded based on data quality.
PTEN†	PS3_Strong	Disease-Specific Well-established in vitro or in vivo functional studies supportive of a damaging effect on the gene or gene product. - Phosphatase activity <50% of wild-type - RNA, mini-gene, or other assays show impact on splicing
	PS3_Supporting	Disease-Specific; Strength Modified Abnormal in vitro cellular assay or transgenic model with a phenotype different from the wild-type that does not meet PS3.
	BS3_Strong	Disease-Specific Well-established in vitro or in vivo functional studies show no damaging effect on protein function. To be applied for missense variants with both lipid phosphatase activity AND results from a second assay appropriate to the protein domain demonstrating no statistically significant difference from the wild-type. For intronic or synonymous variants, RNA, mini-gene, or other splicing assays demonstrate no splicing impact.
	BS3_Supporting	Disease-Specific; Strength Modified In vitro or in vivo functional study or studies showing no damaging effect on protein function but BS3 not met.

**TP53* PS3/BS3 interpretation is suggested by Fortuno et al.; †*CDH1* PS3/BS3 interpretation is suggested by Lee et al.; ‡*PTEN* PS3/BS3 interpretation is suggested by Mester et al.

content, dehydrogenase activity, and membrane integrity. Cell death indicators include caspase activity, chromatin condensation, and phospholipid redistribution. Cell proliferation, colony formation, growth suppression, and apoptosis assays are examples of cell viability assays [10].

4. Binding assay

Binding assays are used to quantify interactions between two molecules, such as small molecule-proteins, protein–protein, and protein–DNA. Examples of binding assays include ATP-binding assays (small molecule-protein) and tetramerization assays (protein–protein) [11].

5. Cell motility assay

Motility is an essential cell feature. Thus, methods to study cell migratory behavior are valuable tools to observe cell characteristics, especially in cancer research, which includes migration and invasion through the extracellular matrix, intravasation into blood circulation, attachment to a distant site, and extravasation to form distant foci [12, 13]. Cell aggregation, cell invasion, and wound closure assays are well-known methods for observing cell motility. Cell aggregation assays have frequently been used to test cells' E-cadherin-dependent cell-cell adhesions, and assess the functionality of the complex in epithelioid cells [14]. A cell aggregation assay is a useful tool for distinguishing between invasive and non-invasive cell types. Cell invasion assays are different from cell migration assays in the field of experimental biology. Invasion is the movement of a cell through a 3D matrix that modifies the cell shape and interacts with the extracellular matrix [15]. Migration is the directed movement of cells on a 2D surface without an obstructive fiber network [15]. Invasion requires adhesion, proteolysis of extracellular matrix components, and migration [16]. Therefore, cell invasion assays help observe how invasive cells penetrate a barrier in response to chemoattractants or inhibiting compounds. The wound closure assay is the simplest method for determining the migration ability of collective cell migration [17]. In the wound closure assay, the migration of cells was measured as a closed distance over time and compared to a control. Observing single-cell lamellipodium formation, tail retraction, and directional movement may reveal any impaired migratory phenotypes [18].

6. Enzyme activity assay

Enzyme assays for the study of enzyme kinetics and enzyme inhibition help measure enzymatic activity. Assays to measure phosphatase activity are a type of enzyme assay. Phosphatase assays can be employed to study the catalytic activity of PTEN against phospholipid substrates [19].

PART II

We selected four genes causing hereditary cancer syndromes, each with different molecular roles: *TP53*, a transcription factor, *BRCA1*, which is involved in gene repair, *PTEN*, a phosphatase, and *CDH1*, an anchor protein. Part II reviews the functional assays used to evaluate the missense variants of these genes.

1. *TP53*

The tumor suppressor gene *TP53* encodes a 393 amino-acid-long transcription factor, the cellular tumor antigen p53 (p53) [20]. In response to DNA damage, oncogene activation, or hypoxia, various mechanisms stabilize p53 and ultimately inhibit p53 ubiquitination by Mdm2 [21]. Then, p53 becomes active by forming a homotetramer and transactivates downstream genes involved in apoptosis (*BAX, BBC3, NOXA, BID, FAS*, and *APAF1*), cell-cycle arrest (*PAK2* and *E2F1*), and senescence by binding to specific DNA sequences [21, 22]. p53 contains a DNA-binding domain (residues 94–312) and an oligomerization domain (residues 323–356) [23-25]. Functional assays used to evaluate *TP53* missense variants set their endpoints on either transactivation (transactivation assay), tetramerization (tetramerization assay), or p53 effector functions (colony formation, apoptosis, and growth suppression assays). The ClinGen *TP53* expert panel published guidelines to interpret *TP53* variants [4]. According to these guidelines, PS3 should be applied if: a) transactivation assays in yeast [26] demonstrated a low functioning allele (< 20% activity), and b) the growth suppression assays [27] showed evidence of a dominant-negative effect and loss of function (LOF) or another assay proved low function. In the following paragraphs, we review the assays mentioned in the guidelines.

1) Transactivation assay

Transactivation assays are used to investigate the effects of variants on the transactivation function of transcription factors. In functional studies that employed transactivation assays, transfected cell-lines were used to evaluate how efficiently *TP53* variants transactivate downstream genes. The expression of wild-type and *TP53* variants were controlled by the *ADH1* promoter, which is a constitutive promoter. Yeast cells and/or mammalian cell-lines (Saos-2 and H1299) have mainly been used [26, 28]. In addition to the human wild-type or mutated *TP53* gene, p53 REs of known downstream genes (e.g., *CDKN1A* and *MDM2* promoters, enhancer elements of *BAX, GADD45, TP53AIP1,* etc.) were inserted into a reporter plasmid. They were positioned upstream of reporter genes, such as GFP and Ds-Red, in yeast cells and luciferase in mammalian cells. The fluorescence intensities of each yeast strain expressing mutant *TP53* were compared to those of a yeast strain expressing wild-type *TP53* [26]. In studies using mammalian cell-lines, the relative luciferase activity of each strain was calculated [29],

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and the expression level of downstream gene products was measured directly by western blotting [11, 28]. Another study verified the presence of p53/p53 RE in the *MDM2* complex via an electrophoretic mobility shift assay instead of using fluorescent reporter proteins or luciferase assays [8]; it was found that some variants, such as p.Arg175His (NP_000537.3:p.R175H), abolished the transactivation function of all downstream genes regardless of which RE was used, and most variants, such as p.Pro177His (p.P177H) and p.Met243Val (p.M243V), affected the transactivation capacity differently depending on the REs [26].

2) Colony formation assay

The colony formation assay is based on the fact that normal cells are prevented from anchorage-independent growth due to anoikis (a type of apoptosis triggered specifically by a lack of cell anchorage), while transformed cells are capable of proliferating without binding to a substrate. In this assay, cells are grown in a soft agar layer mixed with a cell culture medium resting on another layer containing a higher agar concentration. Studies employing this assay used the p53-null non-small-cell carcinoma cell line, H1299, to determine whether transfection with wild-type or mutant *TP53* inhibits colony growth. Non-transfected cell-lines and wild-type *TP53*-transfected cell-lines served as negative controls. Known pathogenic variants p.Ile254Thr (p.I254T) and p.Arg175His (p.R175H) were used as positive controls. The number of colonies was counted using a dissecting microscope and expressed as a percentage relative to the number of colonies of the p53-null strains [8, 10, 30]. p.Glu180Lys (p.E180K), p.Tyr234Cys (p.Y234C), p.Arg267Gln (p.R267Q), and p.Arg342Pro (p.R342P) produced a similar number of colonies as the positive controls [10].

3) Apoptosis assay

Apoptosis assays are based on the fact that apoptotic cells have reduced DNA content and undergo morphological changes making them distinguishable from viable cells via flow cytometry. In particular, the appearance of phosphatidylserine in the outer plasma membrane of early apoptotic cells due to a loss of plasma membrane asymmetry distinguishes early and late apoptotic cells [31]. In the reviewed study [10], H1299 cells co-transfected with a range of *TP53* variants and a GFP-expressing vector were stained with a combination of APC Annexin V and DAPI to assay for viable, early apoptotic, and late apoptotic or necrotic cells. Fluorescence intensities measured by flow cytometry in GFP-negative (non-transfected) versus GFP-positive (transfected) cells were compared [10]. The variant p.Arg342Pro (p.R342P) showed decreased number of apoptotic cells compared to wild-type *TP53*.

4) Tetramerization assay

A tetramerization assay evaluated missense variants within the oligomerization domain (residues 323–356). As p53 needs to form a homotetramer to function as a transcription factor, pathogenic variants that prevent tetramer formation or promote the formation of heterotetramers with p53 can exert a dominant-negative effect or act as gain-of-function mutations [32, 33]. H1299 and U2OS cell-lines transfected with either wild-type *TP53* or oligomerization domain mutants were grown and lysed for tetramerization assays. The lysates were divided into two groups: those treated with the protein crosslinking agent glutaraldehyde and those not treated with glutaraldehyde. Western blotting using the anti-p53 antibody of these lysates showed that p53 of all the strains not treated with glutaraldehyde existed as monomers, whereas glutaraldehyde-treated wild-type and p.Leu330Met (p.L330M) lysates formed a tetramer [11].

5) Growth suppression assay

Growth suppression assays aim to verify whether the mutated tumor suppressor genes confer resistance to small molecules and certain drugs, such as nutlin-3 and etoposide. In one study using such an assay, cell cultures at 50% confluence were transfected with either wild-type *TP53* or variant *TP53* forms and incubated with hygromycin B, an aminoglycoside antibiotic. The number of colonies were counted after 10 days of selection [34]. Giacomelli et al. [27] used nutlin-3 and etoposide. Nutlins are analogs of *cis*-imidazoline that disrupt the interaction between p53 and Mdm2 [35]. Thus, treatment with nutlin-3 did not affect p53-null strains but impaired the proliferation of p53-wild-type strains. Interestingly, although expression of the variant p.Pro278Ala (p.P278A) did not affect p53-null cells, it rendered p53-wild-type cells partially nutlin-3 resistant, indicating that this allele interferes with wild-type p53 in a dominant-negative fashion.

Etoposide is a DNA double-strand break-inducing agent that activates p53 and induces apoptosis in mouse thymocytes [36]. However, in other contexts, wild-type p53 allows DNA repair via cell-cycle arrest and prevents cell death from unresolved DNA dam-

age [37]; indeed, the authors found that wild-type p53 expression in p53-null cells prevented cell death upon etoposide treatment, whereas mutant p53 expression had no effect. Variant frequencies were measured after 12 days of incubation, and Z-scores were calculated for each variant. Evidence of a dominant negative effect (DNE) and LOF as defined by the ClinGen expert panel saw Z-scores of ≥0.61 and ≤-0.21 for p53-wild-type nutlin-3 and etoposide, respectively. Evidence of no DNE and no LOF was defined by Z-scores of <0.61 and >-0.21 for p53-wild-type nutlin-3 and etoposide, respectively [4].

2. *BRCA1*

BRCA1 is a tumor suppressor gene, and mutations in *BRCA1* leads to the development of breast, ovarian, prostate, and pancreatic cancers. It encodes a protein of 1,863 amino acids that contains a RING domain at its N-terminus and tandem BRCT domains at its C-terminus [38]. BRCA1 interacts with BARD1 through the RING domain to form a complex that functions as an E3 ubiquitin ligase [39]. It also interacts with the phosphorylated abraxas, CtBP-interacting protein (CtIP), and BRCA1-associated carboxyl-terminal helicase (BACH1) through the BRCT domains to form complexes involved in the homologous recombination-mediated repair of double-strand breaks [40-42]. Although it is well known that BRCA1 functions in homologous recombination repair, it is also involved in cell-cycle checkpoint regulation, DNA replication, chromatin remodeling, transcription, centrosome regulation, and apoptosis [38]. Functional assays used to evaluate *BRCA1* missense variants include yeast small colony phenotype assays (SCP assays), protein binding assays, ubiquitin ligase assays, recombination assays, and centrosome amplification assays.

1) SCP assay

The SCP assay is based on the observation that *BRCA1* expression in yeast *Saccharomyces cerevisiae* inhibits its growth [43]. Although there is no yeast *BRCA1* homolog, the BRCT domain (1648–1863) is conserved in several yeast proteins, including Rad9 [44]. Rad9 is a checkpoint protein required for yeast cell-cycle arrest and transcriptional induction of DNA repair genes in response to DNA damage [45]. Authors who discovered this growth-suppressive phenotype of human *BRCA1* in yeast cells showed that while transfection with vectors containing *BRCA1* genes with deleted codons 1–302 and 1–1559 retained the ability to inhibit growth in yeast, *BRCA1* genes with deleted codons 1–1650 did not [43]. Therefore, it can be inferred that the SCP assay can evaluate missense variants in the BRCT domain. In another study [46], yeast cells were transfected with either an empty vector, wild-type *BRCA1*, or *BRCA1* variants encompassing various domains, including the BRCT domain. Results showed that transfection of yeast cell with an empty vector and *BRCA1* mutations located in the BRCT domain did not inhibit growth, while transfection with wild-type *BRCA1* and *BRCA1* variants not located within the BRCT domain did [46]. However, this finding was refuted when Millot et al. [47] demonstrated that mutations in the RING domain also restored the yeast proliferation rate. As additional truncation studies reported that expressing the BRCT domains alone was not sufficient to cause small colony formation, the authors argued that both the RING and BRCT domains were important but not essential for eliciting growth defects. Variants that affected SCP and thus, resulted in normal-sized colonies in this study were p.Met1Arg (NP_009225.1:M1R), Met18Thr (p.M18T), p.Glu33Ala (p.E33A), p.Cys39Tyr (p.C39Y), p.Cys44Tyr (p.C44Y), p.Cys47Phe (p.C47F), p.Ala1708Glu (p.A1708E), p.Pro1749Arg (p.P1749R), p.Met1775Arg (p.M1775R), and p.Ser1841Ala (p.S1841A).

2) Protein binding assay

Hetero-dimerization of BRCA1 with BARD1 via its RING domain is crucial for homologous recombination-mediated DNA repair. RING variants that disrupt dimerization result in the loss of tumor suppression [48, 49]. There are several ways to study protein–protein interactions, including co-immunoprecipitation and TAP-tag, protein arrays, mass spectrometry, yeast two-hybrid analysis, and split protein complementation assays [50]. Among these, one study used the latter and the split-GFP reassembly method [51]. Folding-reporter GFP (frGFP) was generated from the 5′ fragment (for residues 1–84) of EGFP and the 3′ fragment (residues 85–238) of GFPuv. These were fused with *BARD1* and *BRCA1*. Plasmids carrying these fusion genes were co-transfected into *E. coli* using the following combination: pET11- BARD1-NfrGFP/pM-RBAD-BRCA1-CfrGFP. Using this model, *BRCA1* mutations within the RING domain were evaluated by comparing the fluorescence of strains transfected with RING variants to those transfected with wild-type and negative controls. Among the variants tested, p.Val11Ala (p.V11A) and p.Met18Lys (p.M18K) were completely disrupted, while p.Leu52Phe (p.L52F) showed somewhat reduced re-

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assembly [51].

Another study used yeast two-hybrid analysis, where a yeast transcription factor was split into two fragments instead of a fluorescence protein [52]. In this study, the DNA-binding domain of Gal4 was fused to *BRCA1*, while the activation domain was fused to *BARD1*. The study was designed in a manner such that the binding of BRCA1 to BARD1 would transactivate the expression of a selectable reporter gene. Thus, yeast strains transfected with BRCA1 RING variants capable of binding to BARD1 would increase during selection, while those expressing nonfunctional variants would decrease. Their findings showed that the residues responsible for the coordination of the zinc ions were the most sensitive to missense variants, except p.His41 (p.H41) [52].

3) E3 ubiquitin ligase assay

The BRCA1/BARD1 complex functions as an E3 ubiquitin ligase [39]. Therefore, E3 ubiquitin ligase activity may also be a BRCA1 functional assay endpoint. In one study using such a functional assay, a fusion protein of BARD1 (residues 26–126) and BRCA1 (residues 2–304) capable of auto-ubiquitination in vitro was used in a phage display assay [52]. BARD1-BRCA1 fusion proteins with different variants of BRCA1 were expressed at the C-terminus of the bacteriophage T7 coat protein. The multiple phage strains displaying BRCA1 variants were incubated in ubiquitination reactions (containing E1, E2, FLAG-tagged ubiquitin, and ATP). Under such conditions, phages carrying active *BRCA1* variants became ubiquitinated and were collected using anti-FLAG beads. After washing, bound phages were eluted by competition with a FLAG polypeptide, re-amplified in *E. coli*, and used in the subsequent selection round. Phage DNA was extracted and sequenced after five selection rounds. The variant frequency before and after each round was used to calculate the selected versus input phage DNA ratio of each variant, which was then used to obtain the slope of log2 ratios over the five selection rounds [52].

4) Recombination assay

The aforementioned assays can only evaluate missense variants in either the BRCT or RING domains. Thus, assays capable of investigating the pathogenicity of variants located throughout *BRCA1* were needed. As mentioned above, BRCA1 plays a crucial role in homologous recombination. Assays developed to observe the impact of BRCA1 variants on homologous recombination in yeast cells have been used in several studies [46, 49, 53, 54]. The diploid RS112 strain used in these studies contains the *HIS3* gene separated by the *LEU2* marker on the same chromosome and *ade2-40* and *ade2-101* on two separate homologous chromosomes. Thus, intrachromosomal recombination leads to *HIS3* reversion and *LEU2* loss, while interchromosomal recombination results in a functional *ADE2* gene. The *HIS3* and *ADE2* genes make it convenient to select recombinant yeast cells; the *HIS3* gene enables colonies to grow in a medium lacking histidine, and the *ADE2* gene makes colonies white in a medium lacking adenine [55]. Studies using this assay transfected RS112 yeast cells with *BRCA1* variants under the galactose-inducible promoter *GAL1p*. Methyl methanesulfonate (MMS) was added to the galactose medium at different doses to promote homologous recombination [53]. In another study using this assay, the authors discovered that p.Met18Thr (p.M18T), p.Cys24Arg (p.C24R), p.Cys27Ala (p.C27A), p.Thr37Arg (p.T37R), p.Cys39Tyr (p.C39Y), p.His41Arg (p.H41R), p.Cys44Phe (p.C44F), p.Cys47Gly (p.C47G), p.Cys61Gly (p.C61G), and p.Cys64Gly (p.C64G) had deleterious effects [49].

5) Transactivation assay

Although homologous recombination is the main function of BRCA1, it also functions as a transcription factor. Therefore, transactivation assays, which have been used to study *TP53* function, have also evaluated *BRCA1* variants in numerous studies [56-60]. These studies revealed that p.Leu1407Pro (p.L1407P), p.Thr1685Ile (p.T1685I), p.Ala1708Glu (p.A1708E), p.Ala1752Pro (p.A1752P), p.Met1775Arg (p.M1775R), p.Gly1788Val (p.G1788V), p.Val1809Phe (p.V1809F), and p.Trp1837Arg (p.W1837R) had defective transcriptional transactivation functions.

6) Centrosome amplification assay

In addition to its numerous nuclear functions, BRCA1 also has cytoplasmic roles. Having exactly two centrosomes is crucial for the proper segregation of chromosomes in dividing cells. BRCA1 regulates centrosome amplification through its E3 ubiquitin ligase activity by ubiquitylating gamma-tubulin [7] and a gamma-tubulin adapter protein [61], thus preventing centrosome reduplication during the same cell cycle [62]. Functional studies using centrosome amplification assays used GFP-tagged centrins or anti-pericentrin antibodies to visualize centrosomes. Subsequently, the proportion of cells with abnormal numbers of centrosomes in

strains transfected with a range of *BRCA1* alleles were counted [63, 64]; it was reported that the p.Met18Thr (p.M18T), p.Cys24Arg (p.C24R), p.Cys27Ala (p.C27A), p.Cys39Tyr (p.C39Y), p.His41Arg (p.H41R), p.Ile42Val (p.I42V), p.Cys44Phe (p.C44F), and p.Cys47Gly (p.C47G) variants had deleterious effects.

3. *CDH1*

The cadherin 1 (*CDH1*) gene is a tumor suppressor gene located on chromosome 16q22.1 that transcribes a 120-kDa protein called epithelial cadherin (E-cadherin) [65]. E-cadherin belongs to a family of transmembrane glycoproteins called cadherins, which mediate calcium-dependent cell adhesion to form organized tissues by complexing with another set of cytosolic proteins called catenins [66-68]. E-cadherin is necessary for cell proliferation, cell adhesion, cell polarity, and epithelial-mesenchymal transition [68].

Germline mutations in *CDH1* are associated with hereditary diffuse gastric cancer and lobular breast cancer [69, 70]. The risks associated with *CDH1* mutations are reportedly >70% for gastric cancer and up to 40% for lobular breast cancer in women [71]. More than 150 *CDH1* mutations have been identified, approximately 80% of which are truncating, and the remaining 20% are missense mutations [72, 73].

LOF through *CDH1* mutation inactivation or promoter methylation disrupts the cadherin-catenin complex and results in cell adhesion loss, causing increased cell motility, uncontrollable cell growth and division, and metastatic ability of the tumor [65, 74-79]. The pathogenic role of non-truncating mutations in the *CDH1* gene has not yet been established. Thus, functional studies using cell aggregation, cell invasion, and wound closure assays have been performed.

1) Aggregation/Invasion assay

Cell aggregation depends on cell-cell adhesion, and the cadherin-catenin complex is necessary between epithelial cells [14, 80]. Downregulation of the complex is often observed in tumor cells during tumor progression. It is associated with high tumor infiltrative and metastatic abilities due to cell adhesion loss and increased cell motility [81-83]. Aggregation and collagen invasion assays can be used to evaluate *CDH1* mutation effects on the function of E-cadherin, which promotes homotypic cell-cell adhesion and suppresses cell invasion.

Chinese hamster ovary (CHO) cells are often utilized because they do not express *CDH1* [84]. According to Suriano et al. [84] CHO cells transfected with the wild-type *CDH1* construct displayed cell-to-cell aggregation in an aggregation assay. CHO cells expressing *CDH1* mutations, such as NP_004351.1:p.Ala634Val (p.A634V) or p.Thr340Ala (p.T340A) failed to aggregate. Corso et al. [85] evaluated the p.Arg224Cys (p.R224C) missense mutation as non-pathogenic using an aggregation assay, resulting in the ability to form a compact aggregate of CHO cells. Brooks-Wilson et al. [86] tested three missense mutations, p.Trp409Arg (p.W409R), p.Arg732Gln (p.R732Q), and p.Ala298Thr (p.A298T), using both aggregation and collagen invasion assays. The smaller particle diameter measurements after incubation and higher invasion index percentages compared to a wild-type support that all three mutants were pathogenic.

2) Wound closure assay

A wound closure assay, also called wound healing assay, is a simple method to test cell motility. Removing the cells from an area through mechanical, thermal, or chemical damage creates a cell-free area in a confluent monolayer [87]. This assay is usually performed under conditions of suppressed cell proliferation. Introducing a cell-free area next to the cell monolayer induces cell migration into the gap. Suriano et al. [88] used an in vitro wound closure assay to test p.Ala617Thr (p.A617T), p.Thr340Ala (p.T340A), p.Ala634Val (p.A634V), and p.Val832Met (p.V832M) mutations compared to wild-type and mock cells. According to the results, p.Ala617Thr (p.A617T) and wild-type cells showed similar cell motility. p.Thr340Ala (p.T340A) and p.Ala634Val (p.A634V) showed high cell motility. p.Val832Met (p.V832M) and mock cells showed very low cell motility, failing to migrate unidirectionally due to low polarization. However, these results caused the destabilization of the E-cadherin adhesion complex, implying that motile capability is neither necessary nor sufficient for cells to invade.

4. *PTEN*

The phosphatase and TENsin homolog (*PTEN*) deleted on chromosome 10 is a classical tumor suppressor gene located on chromosome 10q23.31. This gene encodes a 403-amino acid multifunctional protein that retains lipid and protein phosphatase activity [89]. The lipid phosphatase activity of *PTEN* downregulates AKT phosphorylation, which increases p27 expression. Protein phosphatase activity downregulates MAPK phosphorylation, de-

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creasing cyclin D1 expression levels [90, 91]. PTEN is primarily localized to the cytoplasm and/or membrane-bound nucleus [92].

The *PTEN* gene is a well-known negative regulator of the phosphatidylinositol 3 kinase (PI3K)/AKT pathway in the cytoplasm. *PTEN* dephosphorylates phosphatidylinositol (3,4,5)-trisphosphate (PIP3) to phosphatidylinositol 4,5-biphosphate (PIP2) to prevent unchecked cell survival and proliferation by hampering all AKT/mTOR axis-controlled downstream functions [93-95]. In addition, PTEN protein phosphatase activity resists the action of focal adhesion kinase and Shc to modulate complex pathways affecting cell migration [94, 96]. In the nucleus, *PTEN* downregulates MAPK phosphorylation and cyclin D1 to arrest cell-cycle progression [91].

Furthermore, *PTEN* germline mutations are often observed in PTEN hamartoma tumor syndromes, such as Cowden syndrome, Lhermitte-Duclos disease, Bannayan–Riley–Ruvalcaba syndrome, and Proteus syndrome in autosomal dominant inheritance patterns. Although they are different disease entities, they commonly have hamartomatous tumors [97, 98]. In contrast to classical tumor suppressor models, which require complete inactivation to induce cancer, the haploinsufficiency of *PTEN* is enough for tumor growth [99, 100]. More than half of *PTEN* mutations are truncating, and approximately 35% are missense mutations [101].

1) Phosphatase activity

Most missense mutations are clustered around the phosphatase domain. Therefore, an assay to measure phosphatase activity is useful for the functional analysis of *PTEN*. Han et al. [101] tested 42 missense mutations using a phosphatase assay. Of the 42 mutations, 38 showed eliminated or reduced phosphatase activity. Mighell et al. [102] evaluated the effects of *PTEN* mutations on lipid phosphatase activity. Among the 7,244 single amino acid *PTEN* variants tested, 2,273, including 1,789 missense mutations, showed reduced lipid phosphatase activity. Although genotype-phenotype matching should be further discussed, no alteration or loss of phosphatase activity observed in the phosphatase assay can serve as evidence for BS3 or PS3.

2) PTEN/pAKT expression level

PTEN dephosphorylates PIP3, preventing the downstream pathway of AKT phosphorylation. Therefore, cells with *PTEN* mutations demonstrate elevated levels of PIP3 and phosphorylated AKT (pAKT) [103]. pAKT levels can be affected by PTEN phosphatase activity. Thus, decreased PTEN expression levels have also been correlated with pathogenic *PTEN* variants [104]. Spinelli et al. [105] measured PTEN and pAKT expression levels from seven *PTEN* mutations identified in autism and five mutations in PTEN hamartoma tumor syndrome. PTEN and pAKT expression levels were investigated by immunoblotting with total cell lysates. The results showed that all five mutations in PTEN hamartoma tumor syndrome appeared to inhibit AKT signaling directly, but seven *PTEN* mutants in autism retained the ability to suppress cellular AKT signaling.

CONCLUSION

This study is intended to help clinical laboratories apply functional evidence criteria when interpreting the sequence variants

Table 3. Literature on functional assays used in each gene

Gene	Transactivation assay	Cell viability assay	Binding assay	Cell motility assay	Enzyme activity assay	HDR	Centrosome
TP53	7878469, 30224644, 31081129, 17690113, 24816189	24816189, 12509279, 24677579, 19693097, 8479523	17690113				
PTEN		26504226		26504226	10866302, 29706350, 21828076, 26504226, 19915616, 25875300, 28263967, 25527629		
CDH1				12588804, 21106365, 15235021, 14500541, 22470475, 18772194, 12944922, 16924464			
BRCA1	32656256, 15689452, 10811118, 19493677, 8942979, 18087219	11301010, 18680205	23628597, 18493658		18493658	8939848, 22172724, 25823446, 27484786	18087219, 18927495, 15923272

Literatures are represented using PMID number.
Abbreviation: HDR, homology directed repair.

found in clinical genetic testing. For this purpose, four cancer susceptibility genes with different mechanisms of action (*TP53, BRCA1, CDH1*, and *PTEN*) were chosen to include as many functional assays as possible. Then, various experimental designs that used the same or similar functional assays were introduced. In Table 3, we added the literature referenced in Part II and the literature that used the functional assays presented in this review but not referenced in Part II. Future studies are required for the genes and diseases not covered in this review.

요 약

임상검사실에서 차세대염기서열분석 검사로 검출된 염기 변이의 해석에 대한 요구가 점차 증가하고 있다. 2015년 ACMG/AMP 지침에서 기능 연구 결과를 변이 분류의 강력한 근거로 제시하고 있다. 하지만 단백질의 기능과 이를 증명하기 위해 사용되는 기능 연구가 매우 다양하여 해당 근거를 적용하는 데 어려움이 있다. 따라서 본 연구에서는 다양한 기능 연구 결과를 검토하여 임상검사실에서 염기 변이를 분류하는 데 도움을 주고자 하였다. 본 논문에서는 1) 단백질의 기본적인 기능 및 처리와 연관된 일반적 기능 연구 및 2) 유전성 암과 연관된 4개 유전자(*TP53, BRCA1, CDH1, PTEN*)의 특정 병인 기전과 관련된 기능 연구에 초점을 맞추었다.

Conflicts of Interest

None declared.

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