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The *MDM2* and *CDKN2A* Copy-number-variation Influence the *TP53*-signature-score in Wild-type *TP53* Luminal Type Breast Cancer

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Abstract. *Background/Aim: The TP53-signature is a multi-gene signature that can predict TP53 structural mutations. It has presented remarkable ability to predict the prognosis of early-stage breast cancer. However, some samples presented discordance with the signature status and structure status. We aimed to investigate whether the mRNA expression levels or copy number variation (CNV) of MDM2 and CDKN2A influence the TP53-signature-score, subtype classification, and prognosis prediction in TP53 wild-type, luminal type early-stage breast cancer samples. Materials and Methods: We selected TP53 wild-type, luminal type early-stage breast cancer samples from The Cancer Genome Atlas (TCGA) and Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) cohorts. Then, we analyzed the correlation between the TP53-signature-score and mRNA expression levels or CNV of MDM2 and CDKN2A. Results: The samples with MDM2 copy number (CN) amplification or those with CDKN2A CN deep deletion presented higher TP53-signature-score. Moreover, samples with MDM2 CN amplification or those with CDKN2A CN deep deletion had more characteristics of the luminal B type. In addition, they showed lower estrogen response early score, which correlated with response to endocrine therapy in breast cancer. However, MDM2 and CDKN2A mRNA expression did not present the same tendency. Furthermore, samples with MDM2 CN amplification or those with CDKN2A CN deep deletion had a worse prognosis in METABRIC cohort. Conclusion: The MDM2 or CDKN2A CNV may be useful for classifying subtypes and predicting prognosis more accurately in TP53 wild-type, luminal type early-stage breast cancer patients.*

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Breast cancer is the most common occurring cancer among women worldwide. The subtype classification of breast cancer plays a pivotal role in its treatment and the prediction of prognosis.

Breast cancer can be divided into luminal, human epidermal growth factor receptor 2 (HER2)-like, and Basal-like subtypes based on the immunohistochemical (IHC) status of estrogen receptor (ER), progesterone receptor (PgR), HER2, and Ki-67 clinically (1). Luminal type can be further divided into luminal A and luminal B. Luminal A type is both ER and PgR positive, HER2-negative, with low expression levels of the protein Ki-67. On the other hand, luminal B type is ER-positive, either PgR- and HER2-positive or -negative, with high expression levels of Ki-67 (1).

Several multi-gene signatures have been developed to predict prognosis and classify the breast cancer subtypes more accurately, like OncotypeDX, MammaPrint, and PAM50 (2-4). Among them, PAM50 can divide breast cancer into Luminal A, Luminal B, Normal-like, HER2-enriched, and Basal-like, five intrinsic subtypes with fifty genes, more accurately than IHC classification (5).

Luminal type patients are treated mainly with endocrine therapy clinically, and have a better prognosis than patients with other subtypes (6). However, luminal B type has a worse prognosis and is less sensitive to endocrine therapy than luminal A type (7, 8).

TP53 is a tumor suppressor gene, which encodes the p53 protein. p53 can regulate the expression of various downstream genes to maintain genomic stability (9). Mutations in *TP53* can be found in the majority of solid tumors, and are correlated with tumorigenesis, tumor progression, and poor prognosis (10). The *TP53*-signature was constituted by differentially expressed genes (DEGs), which can predict the *TP53* structural mutation in the breast cancer cohort (11).

Yamaguchi *et al*. demonstrated that the *TP53*-signature-score had a remarkable ability to predict the prognosis of early-stage breast cancer compared to other pre-existing multi-gene signatures (12). They indicated that *TP53* structure mutated samples presented higher *TP53*-signature-score and defined them as *TP53*-signature mutant type. Interestingly, some patients presented with *TP53*-signature mutant type, although their *TP53* structure was wild type. This discordance was mainly observed in luminal B type samples, which were classified using PAM50, and in some patients who had *MDM2* mRNA over-expression.

MDM2 encodes an E3 ubiquitin ligase, MDM2 protein, which plays a critical role in regulating the normal function of p53 (13). It has been well studied that MDM2 and p53 form a negative feedback loop (14).

CDKN2A encodes p14ARF and p16INK4A proteins. Among them, p14ARF has been reported to bind to MDM2 and suppress its E3 ubiquitin ligase function, thereby stabilizing p53 (15). In addition, *CDKN2A* copy number (CN) loss and *MDM2* CN amplification are mutually exclusive (16).

Although MDM2 and p14ARF can modulate the activity of p53, the relationship between the *TP53*-signature and *MDM2* or *CDKN2A* remains unknown, because not all genes in the *TP53*-signature are downstream genes of p53.

In this study, we aimed to analyze whether *MDM2* or *CDKN2A* mRNA expression levels or CNV influence the *TP53*-signature, and whether *MDM2* or *CDKN2A* mRNA expression levels or CNV influence the features of luminal types and prognosis in The Cancer Genome Atlas (TCGA) and Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) cohorts.

Materials and Methods

Clinical and transcriptomic data collection for breast cancer patients. TCGA (17) and METABRIC (18, 19) data were obtained from cBioportal (20, 21). We chose the female breast cancer patients who presented with *TP53* structure wild type and early-stage luminal A or luminal B type from TCGA (n=189) and METABRIC (n=596) cohorts (Table I). In the downloaded clinical data, luminal A type and luminal B type were classified using the PAM50 method.

Estimation of the TP53-signature-score and estrogen response early score. Expression data of the thirty-one genes comprising the *TP53*-signature were obtained from TCGA and METABRIC (Supplementary Figure S1). The *TP53*-signature-score was calculated using a previously described method (12). The estrogen response early score was calculated using the method described before that employed R package "GSVA" (22-24).

Definition of CNV. The CNV in TCGA and METABIC cohorts analyzed were displayed as -2, -1, 0, 1, and 2. Following the instructions in cBioportal, we defined "-2" as deep deletion, "-1" as loss, "0" as diploid, "1" as low-levels gain, and "2" as amplification (25).

Statistical analysis. The Spearman's rank correlation test was used to compare the relationship between the mRNA expression levels and signature score. The absolute values of r_s>0.2 were defined as significantly correlated. Kruskal–Wallis test and Mann–Whitney *U*-test were used to compare group means. The *p*-value of the Kaplan–Meier survival curves was calculated using the log-rank test. Fisher's Exact test was used for statistically analyzing patients' characteristics except for age. All statistical tests were performed using R software (version 4.0.1) and EZR software (26). All plots were constructed by using the EZR software. A *p*-value less than 0.05 was considered statistically significant. Statistically significant results are shown in bold in figures and tables.

Results

MDM2 and CDKN2A mRNA expression levels were not significantly correlated with the TP53-signature-score or the features of luminal B type. To determine whether *MDM2* or *CDKN2A* mRNA expression levels influence the *TP53*-signature-score, we analyzed the correlation between the *TP53*-signature-score and the mRNA expression levels of *MDM2* and *CDKN2A*. We found that neither *MDM2* nor *CDKN2A* mRNA expression levels showed a significant correlation with the *TP53*-signature-score (Figure 1A-D).

To study whether *MDM2* and *CDKN2A* mRNA expression levels influence the features of luminal B types, we analyzed the correlation between *MDM2* or *CDKN2A* mRNA expression levels and those of *ESR1*, *PGR,* and *MKI67* mRNA expression levels, which encode ERα, PgR, and Ki-67, respectively (27). We found that *MDM2* mRNA expression levels were not correlated with those of *ESR1* or *PGR* mRNA expression levels (Figure 2A, B, E and F). However, *MDM2* mRNA expression levels had a weak, positive correlation with those of *MKI67* mRNA expression levels (Figure 2C and G). We also analyzed the relationship between the *MDM2* mRNA expression levels and estrogen response early score, as it has been previously reported that a lower score may indicate resistance to endocrine therapy (20). We found that *MDM2* mRNA expression levels were not significantly correlated with estrogen response early score (Figure 2D and H).

In TCGA cohort, *CDKN2A* mRNA expression levels presented a weak, negative relationship with those of *ESR1* mRNA expression levels, but not in METABRIC cohort (Figure 3A and E). In addition, *CDKN2A* mRNA expression levels were not correlated with those of *PGR* or *MKI67* mRNA expression levels (Figure 3B, C, F and G). *CDKN2A* mRNA expression levels were not correlated with estrogen response early score in neither TCGA nor METABRIC cohorts either (Figure 3D and H).

MDM2 and CDKN2A CNV were correlated with the TP53-signature-score and the features of luminal B type. We analyzed whether *MDM2* and *CDKN2A* CNV influence the *TP53*-signature-score. In our results, samples with *MDM2*

Table I. *Clinical characteristics of the samples analyzed*.

Characteristic	TCGA		METABRIC	
	No. of samples	%	No. of samples	%
Samples	189	100%	596	100%
Diagnosed age (median)	29-89 (61)		28.04-90.43 (63.02)	
PAM50.subtype				
Luminal A	136	72.0%	397	66.6%
Luminal B	53	28.0%	199	33.4%
ER status				
Positive	184	97.4%	576	96.6%
Negative	3	1.6%	11	1.8%
Others	2	1.1%	9	1.5%
PgR status				
Positive	165	87.3%	454	76.2%
Negative	21	11.1%	142	23.8%
Others	3	1.6%	0	0.0%
HER2 status				
Positive	11	5.8%	24	4.0%
Negative	172	91.0%	572	96.0%
Others	6	3.2%	0	0.0%
Tumor stage				
T0	0	0.0%	1	0.2%
T1	72	38.1%	263	44.1%
T2	117	61.9%	332	55.7%
Lymph node				
Negative (N0 or 0)	122	64.6%	397	66.6%
Positive (N1 or 1~3)	67	35.4%	199	33.4%
MDM2 CNV				
Deletion (-1) and Diploid (0)	156	82.5%	530	88.9%
Low level gain (1)	24	12.7%	49	8.2%
High level amplification (2)	9	4.8%	17	2.9%
CDKN2A CNV				
Deep deletion (-2)	3	1.6%	7	1.2%
Loss (-1)	34	18.0%	84	14.1%
Diploid (0) and amplification (1,2)	152	80.4%	505	84.7%

TCGA: The Cancer Genome Atlas; METABRIC: Molecular Taxonomy of Breast Cancer International Consortium; ER: estrogen receptor; PgR: progesterone receptor; HER2: human epidermal growth factor receptor 2; CNV: copy number variation.

CN amplification or those with *CDKN2A* CN deep deletion presented higher *TP53-signature-score* than other CNV samples (Figure 4A-D).

There were no significant differences regarding *ESR1* mRNA expression levels (Figure 5A and E). However, the samples with *MDM2* CN amplification presented lower *PGR* mRNA expression levels (Figure 5B and F) and higher *MKI67* mRNA expression levels (Figure 5C and G). Estrogen response early score was lower in the samples with *MDM2* CN amplification in both TCGA and METABRIC cohorts, and was statistically significant in the METABRIC cohort (Figure 5D and H).

The samples with *CDKN2A* CN deep deletion presented higher *ESR1* mRNA expression levels in TCGA cohort, but not in METABRIC cohort (Figure 6A and E). They also presented lower *PGR* mRNA expression levels, but not higher *MKI67* mRNA expression levels (Figure 6B, C, F and G). The samples with *CDKN2A* CN deep deletion also presented lower estrogen response early score, but there were no statistically significant differences (Figure 6D and H). These results indicated that samples with *MDM2* CN amplification or those with *CDKN2A* CN deep deletion defines better the luminal B type than their mRNA expression levels. These samples may present resistance to endocrine therapy.

Samples with MDM2 CN amplification or those with CDKN2A CN deep deletion had worse prognosis. We compared the overall survival in TCGA and METABRIC cohorts with *MDM2* and *CDKN2A* CNV.

In the METABRIC cohort, the Kaplan–Meier survival curves revealed that samples with *MDM2* CN amplification

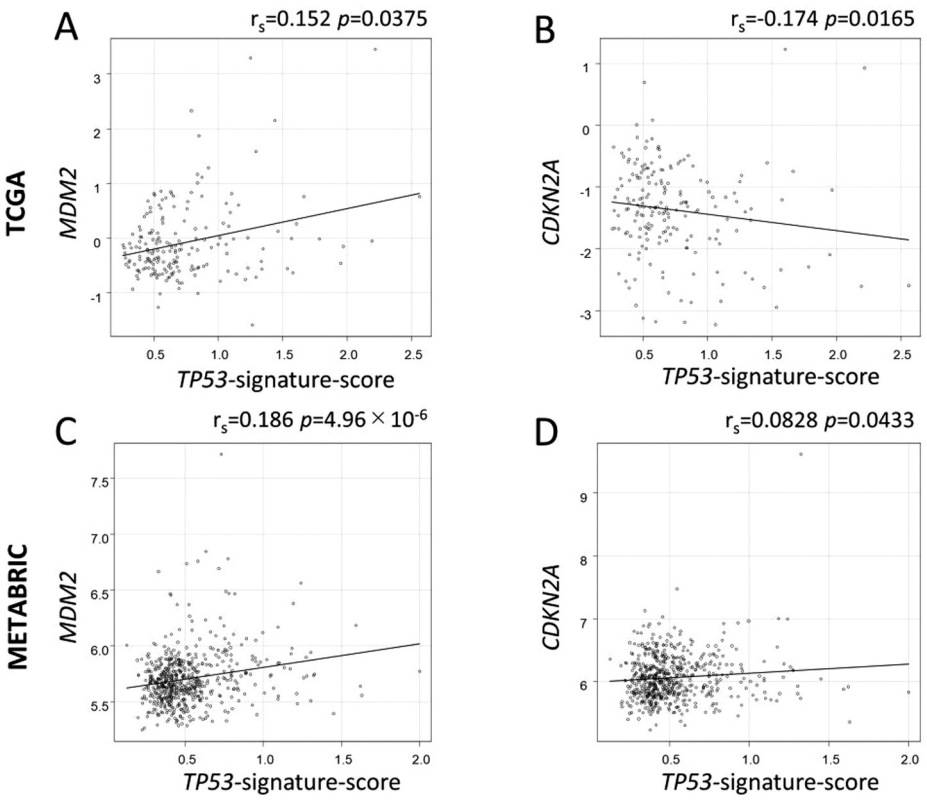

Figure 1. *Correlation between TP53-sigature-score and the mRNA expression levels of MDM2 and CDKN2A. Correlation plots of the TP53-signature-score with the mRNA expression levels of MDM2 and CDKND2A in TCGA (A, B) and METABRIC (C, D) cohorts. p-Values were calculated using the Spearman's rank correlation coefficient.*

or those with *CDKN2A* CN deep deletion presented worse prognosis (Figure 7A and B). The results of the samples with *MDM2* CN amplification were consistent with those in previous reports (28, 29).

However, there were no statistically significant differences in the median cut-off values of *MDM2* and *CDKN2A* mRNA expression levels (Supplementary Figure S2A-D). About 10% of the samples had *MDM2* CN gain and amplification in the METABRIC cohort, and 15% in the TCGA cohort (Supplementary Table S1 and Table S2). Therefore, we defined the samples with the top 10% and 15% of high *MDM2* mRNA expression as the high group and the others as the low group, and then compared their overall survival. However, no significant differences were found (Supplementary Figure S3A-D). Regarding the inconsistent results of *MDM2* mRNA expression levels and CNV, we found that some samples presented higher mRNA expression levels with CN not amplified, however, *MDM2* mRNA expression levels were comparable with the CNV in most of the samples (Supplementary Figure S4A, B).

About 15% of the samples had *CDKN2A* CN loss and deep deletion in the METABRIC cohort, and 20% in TCGA cohort (Supplementary Table S1 and Table S2). Therefore, we defined the samples with the bottom 15% and 20% of low *CDKN2A* mRNA expression as the low group and the others as the high group, and then compared overall survival between these two groups. There were no statistically significant differences in TCGA cohort (Supplementary Figure S5A, C). However, the low group presented poor prognosis in METABRIC cohort (Supplementary Figure S5B, D).

Discussion

In this study, we demonstrated that the CNV of *MDM2* or *CDKN2A*, but not their mRNA expression levels were correlated with the *TP53*-signature-score.

We also demonstrated that the samples with *MDM2* CN amplification or those with *CDKN2A* CN deep deletion presented more characteristic of luminal B type than their mRNA expression levels. Especially, the samples with *MDM2* CN amplification presented statistical significantly lower estrogen response early score in METABRIC cohort. A previous report demonstrated that MDM2 can degrade

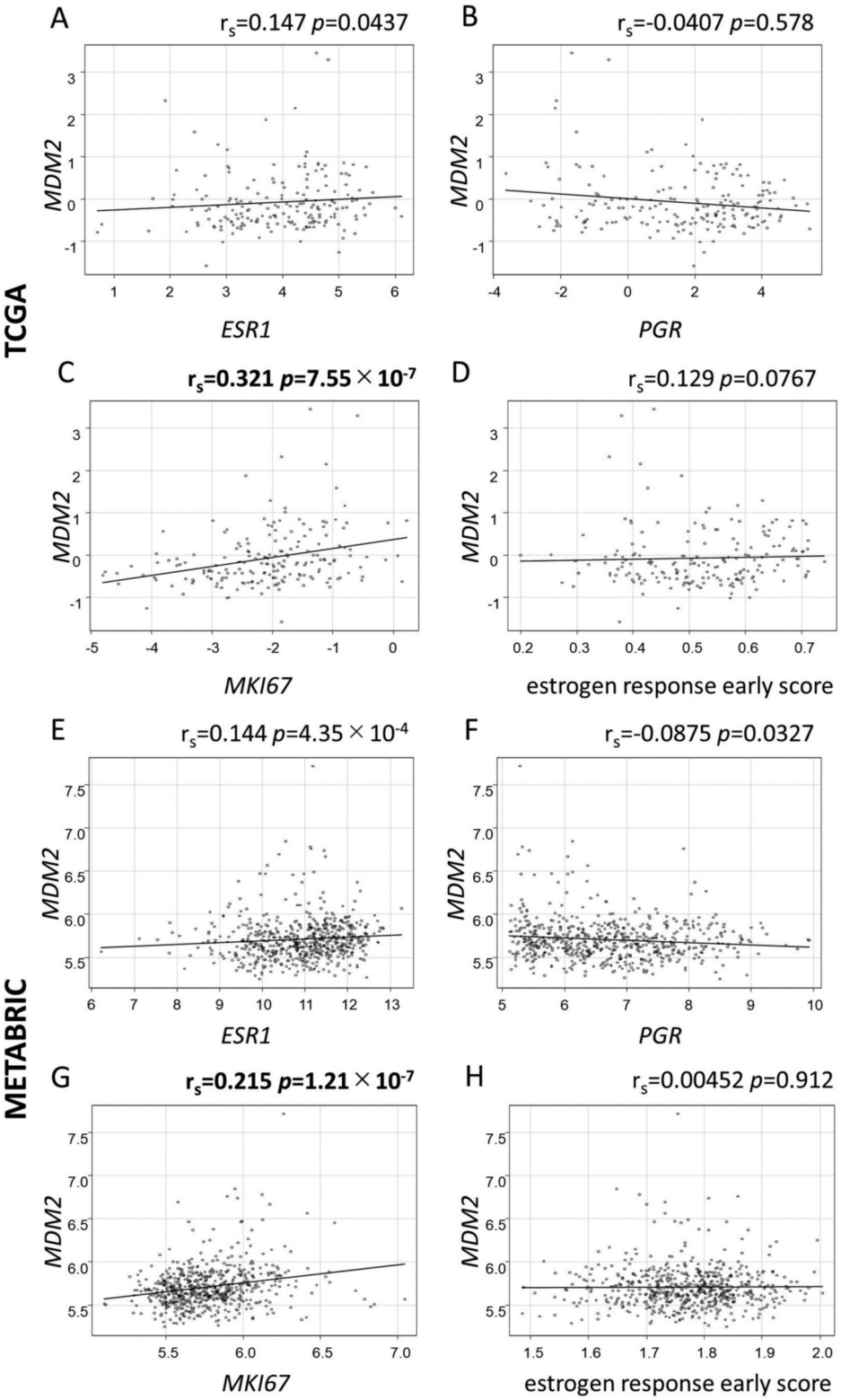

Figure 2. *Correlation between MDM2 mRNA expression levels and the mRNA expression levels of ESR1, PGR, MKI67, and estrogen response early score. (A-C, E-G) Plots showing correlations between MDM2 mRNA expression levels and those of typical genes for luminal type (ESR1, PGR, MKI67) in both TCGA and METABRIC cohorts. (D, H) Plots showing correlations between MDM2 mRNA expression levels and the estrogen response early score. p-Values were calculated using Spearman's rank correlation coefficient.*

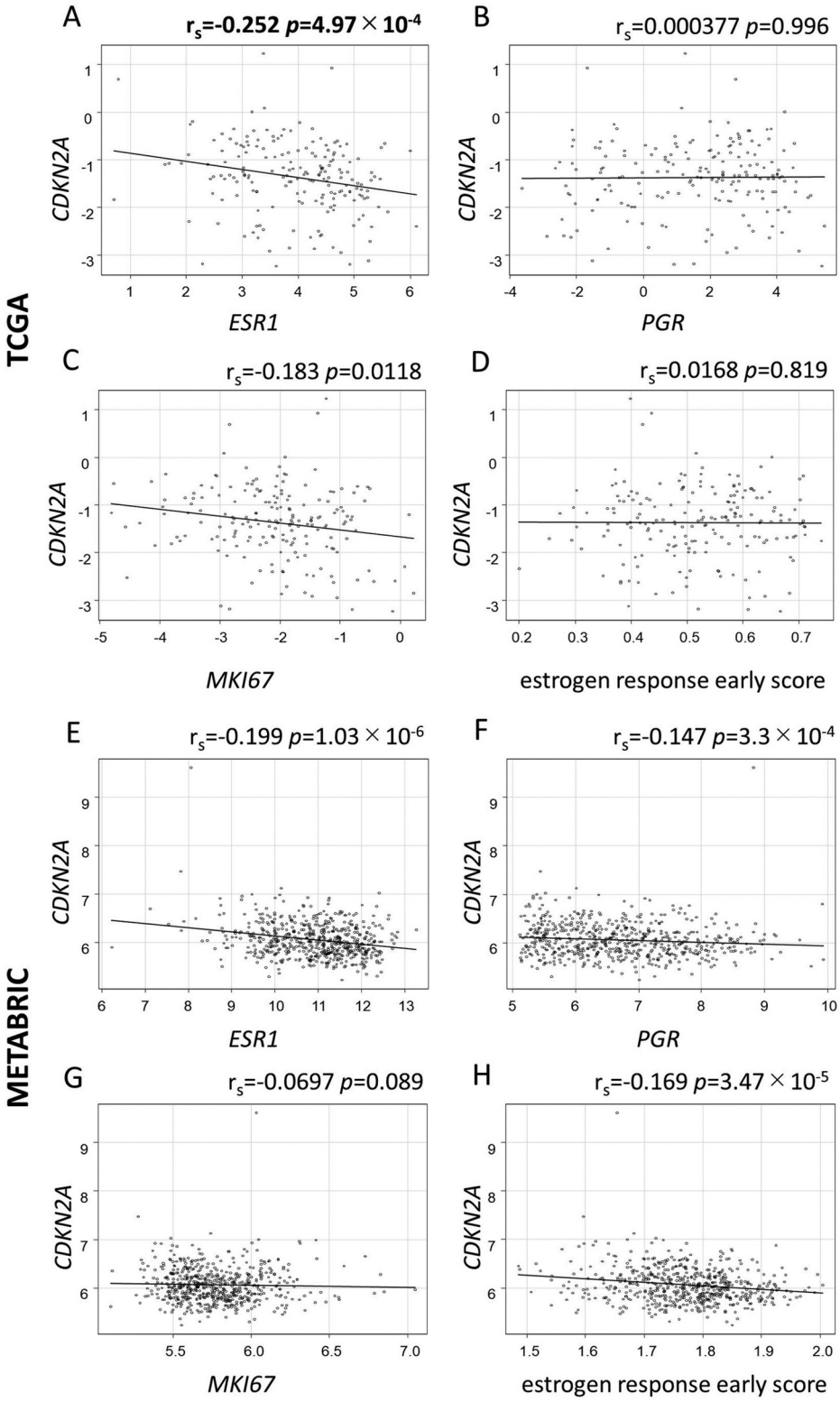

Figure 3. *Correlation between CDKN2A mRNA expression levels and the mRNA expression levels of ESR1, PGR, MKI67, and estrogen response early score. (A-C, E-G) Plots showing correlations between CDKN2A mRNA expression levels and those of typical genes for luminal type (ESR1, PGR, MKI67) in both TCGA and METABRIC cohorts. (D, H) Plots showing the correlations between CDKN2A mRNA expression levels and the estrogen response early score in both TCGA and METABRIC cohorts. p-Values were calculated using Spearman's rank correlation coefficient.*

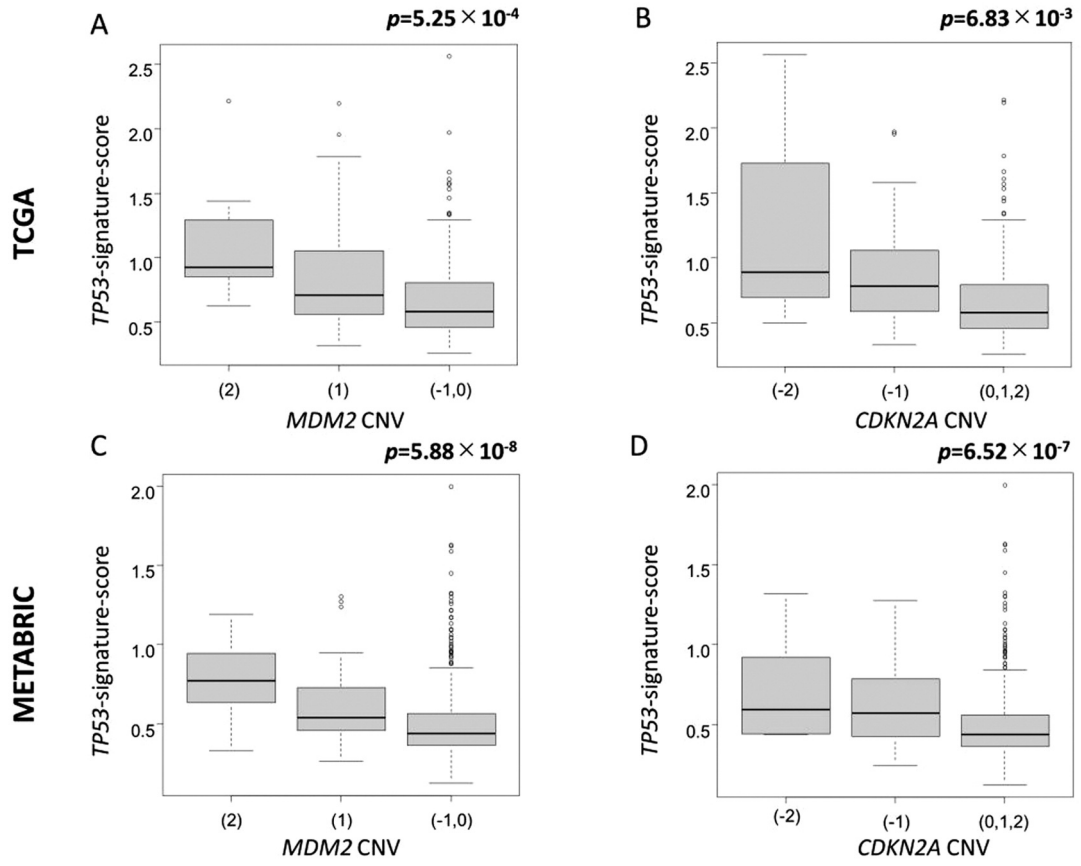

Figure 4. *The TP53-signature-score is associated with the CNV of MDM2 and CDKN2A. (A, C) Boxplots of the comparison of the TP53-signature-score with the MDM2 CNV [(2) amplified, (1) gain, (-1, 0) not amplified] in both TCGA and METABRIC cohorts. (B, D) Boxplots of the comparison of the TP53-signature-score with the CDKN2A CNV [(-2) deep deletion, (-1) loss, (0, 1, 2) not deleted] in both TCGA and METABRIC cohorts. p-values were calculated using Kruskal–Wallis test and Mann–Whitney U-test. CNV: Copy number variation.*

ERα (30). According to this relationship between MDM2 and ERα, we assumed that the amplified functional MDM2 destroys the ERα and results in a decrease in the dependency of the ER signal.

In regards to the inconsistent results of *MDM2* mRNA expression levels and CNV, the splice variants of *MDM2* were considered. It has been reported that *MDM2* has several types of splice variants, and some of them have distinct functions from full-length MDM2 (31-33). However, many *MDM2* splice variants functions are still not yet completely understood. Therefore, further research is required.

In contrast to *MDM2*, the *CDKN2A* mRNA expression levels presented the same tendency with their CNV to some extent. However, these results indicate that it is exceedingly difficult to set a common cut-off value for mRNA expression levels in clinical settings with different cohorts.

As this was a retrospective study, there are also some limitations. The major limitation was that we analyzed only two big cohorts. Moreover, the proportion of the samples that presented *MDM2* CN amplification or *CDKN2A* CN deep deletion was less than 5%, therefore, the available data were limited.

Despite the small number of samples with *MDM2* CN amplification or *CDKN2A* CN deep deletion, they are still worth measuring. Especially samples with *MDM2* CN amplification have been reported to be correlated with a worse outcome after immune checkpoint inhibitor treatment in a pan-cancer analysis (34). We believe that they should be one of the indicators for considering a treatment plan more accurately.

In conclusion, our results demonstrated that the *MDM2* or *CDKN2A* CNV may be more useful than their mRNA expression levels for classifying the subtypes and predicting the prognosis more accurately in *TP53* wild-type, luminal type early-stage breast cancer patients.

Supplementary Material

Supplementary material can be obtained at: https://www.dropbox.com/sh/ndv7g89vuxnw0hs/AABkq7LPZUU-bOxJ2GQ7DvDoa?dl=0

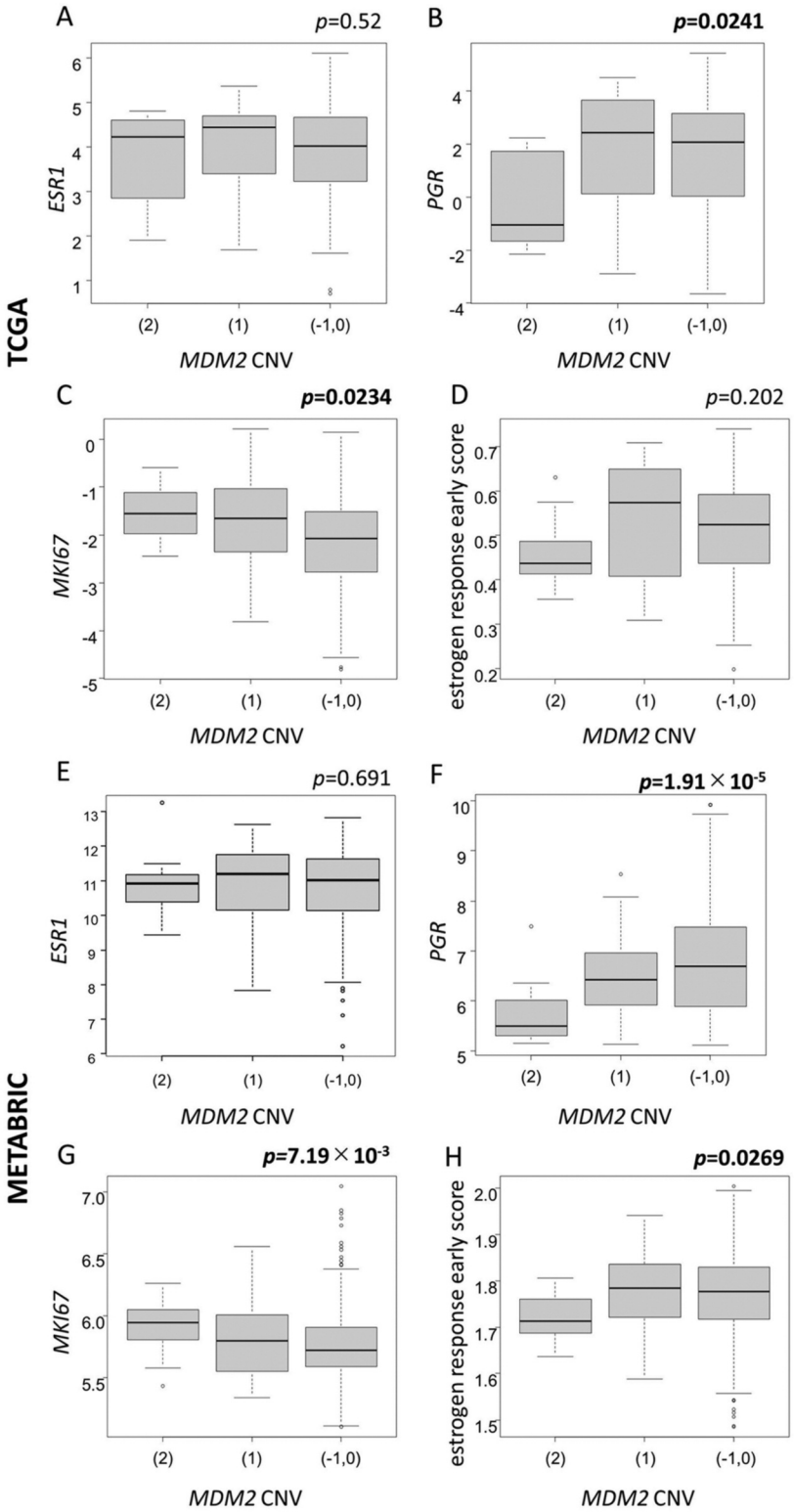

Figure 5. *The association between the MDM2 CNV and the mRNA expression levels of ESR1, PGR, MKI67, and estrogen response early score. (A-C, E-G) Boxplots of the comparison of typical genes for luminal type (ESR1, PGR, MKI67) with the MDM2 CNV [(2) amplified, (1) gain, (-1, 0) not amplified] in both TCGA and METABRIC cohorts. (D, H) Boxplots of the comparison of the estrogen response early score with the MDM2 CNV [(2) amplified, (1) gain, (-1, 0) not amplified] in both TCGA and METABRIC cohorts. p-Values were calculated using Kruskal–Wallis test and Mann–Whitney U-test. CNV: Copy number variation.*

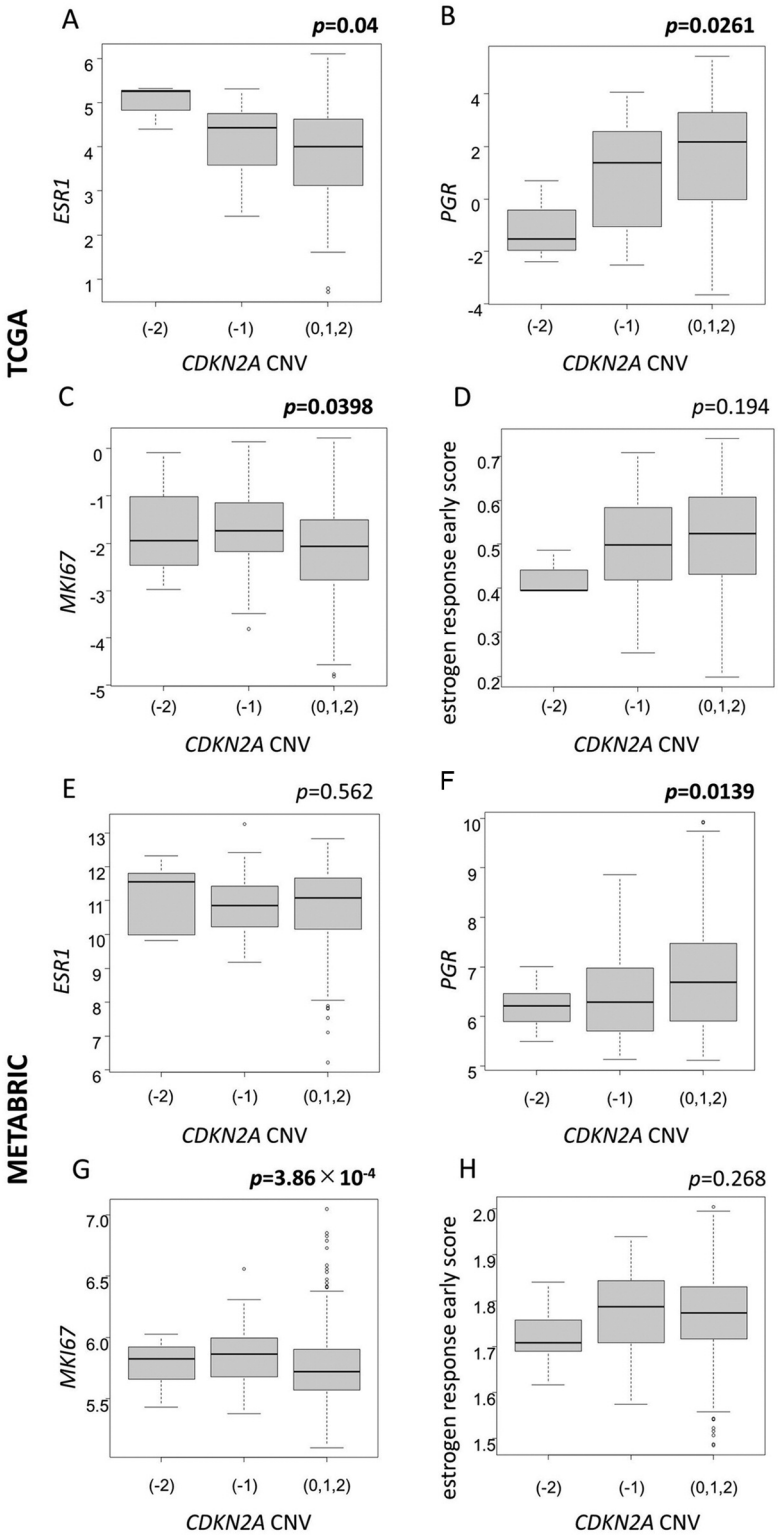

Figure 6. *Association between the CDKN2A CNV and the mRNA expression levels of ESR1, PGR, MKI67, and estrogen response early score. (A-C, E-G) Boxplots of the comparison of typical genes for luminal type (ESR1, PGR, MKI67) with the CDKN2A CNV [(-2) deep deletion, (-1) loss, (0, 1, 2) not deleted] in both TCGA and METABRIC cohorts. (D, H) Boxplots of the comparison of the estrogen response early score with CDKN2A CNV [(-2) deep deletion, (–1) loss, (0, 1, 2) not deleted] in both TCGA and METABRIC cohorts. p-Values were calculated by Kruskal–Wallis test and Mann–Whitney U-test. CNV: Copy number variation.*

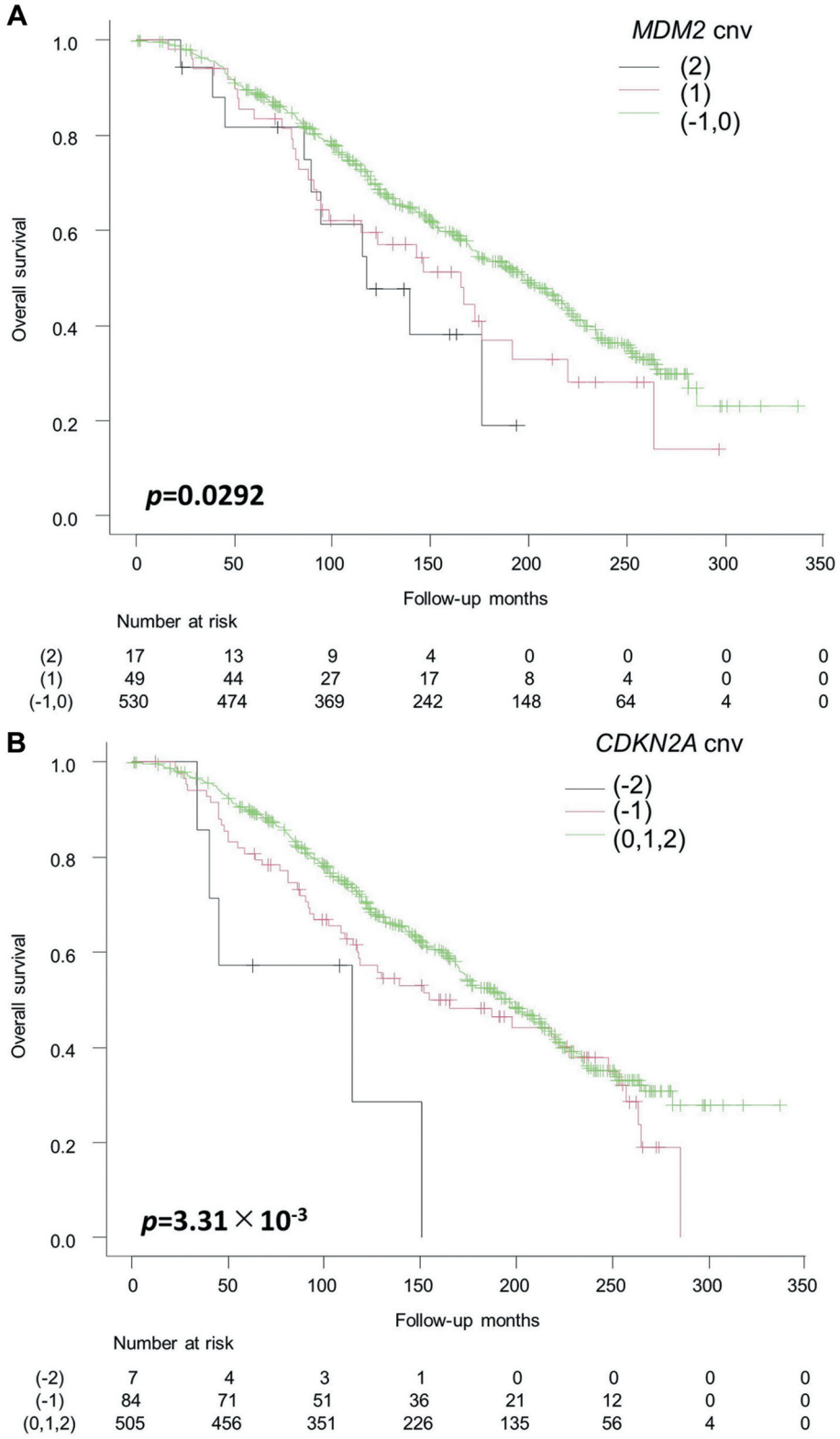

Figure 7. *The Kaplan–Meier survival curves of MDM2 and CDKN2A CNV in METABRIC cohort. (A) Kaplan–Meier survival curves for overall survival between MDM2 CN (2) amplified (black), (1) gain (red), and (-1,0) not amplified (green). (B) Kaplan–Meier survival curves for overall survival between CDKN2A CN (-2) deep deleted (black), (-1) loss (red), and (0, 1, 2) not deleted (green). p-Values were calculated using the log-rank test. CNV: Copy number variation; CN: copy number.*

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

Min Han: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing - original draft; Shigeo Yamaguchi: Conceptualization, Data curation, Formal analysis, Methodology, Software, Writing – review & editing; Mai Onishi: Formal analysis, Software, Writing – review & editing; Tomoaki Fujii: Methodology, Resource, Writing – review & editing; Masaki Hosoya: Formal analysis, Writing – review & editing; Xuan Wen: Writing – review & editing; Hidenori Kido: Writing – review & editing; Shunsuke Kato: Conceptualization, Project administration, Writing – review & editing.

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