

Analyzing genetic and epigenetic *HORMAD* alterations in breast cancer resistance and metastatic events

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ABSTRACT: Epigenetic alterations in regulatory genes, genetic factors, and genomic instability, which cause breast cancer, can also contribute to disease resistance. *HORMAD*, which encode proteins containing HORMA domains and are involved in homologous recombination, have important roles in cancer emergence and progression. In this study, we uncovered putative breast cancer therapeutic targets by examining *HORMAD1* and *HORMAD2* genetic and epigenetic alterations. mRNA levels of *HORMAD1* and *HORMAD2* in breast cancer samples and normal breast tissues, as well as mRNA levels in normal, breast cancer, and metastatic breast cancer samples, were analyzed using TNMplot. Prognostic value, genetic alterations, epigenetic alterations, genetic variations, ROC plots, functional prediction, and immune infiltration of *HORMAD1* and *HORMAD2* were conducted with KMPlotter, cBioportal, methsurv, ClinVar, ROC Plotter, PredictSNP, PANTHER, and TIMER 2.0, respectively. Both *HORMAD1* and *HORMAD2* mRNA levels were lower in breast cancer samples, and lower in metastatic breast cancer samples. Patients expressing higher *HORMAD1* and *HORMAD2* levels had favorable overall survival (OS) rates than the opposite groups. *HORMAD1* and *HORMAD2* gene amplifications and deletions were also observed. Pathway enrichment analyses showed that Wnt signaling alterations contributed to cell proliferation. Increased DNA methylation levels were identified in *HORMAD2* when compared with *HORMAD1* in patients. Two 1021C>T (Q334) and 430A>G (T144A) variants of *HORMAD1* were shown to have clinical significance in patients. Also, functional prediction mutant analysis of *HORMAD1* confirmed that S287F exerted a deleterious effect on amino acid impact, however, further investigations are warranted. Receiver operating characteristic (ROC) plot data indicated a significant correlation between *HORMAD2* levels and anti-human epidermal growth factor receptor 2 (HER2) sensitivity. Genetic and epigenetic changes in *HORMAD1* and *HORMAD2* genes may be used as indicators and targets for overcoming breast cancer resistance and limiting metastasis in breast cancer cells via Wnt targeting. Further research is required to verify our findings.

KEYWORDS: *HORMAD*; breast cancer resistance; metastasis; bioinformatics; genetic and epigenetic alterations.

1. INTRODUCTION

Breast cancer is a major global health issue; it causes the highest number of deaths in women, and its incidence rates have steadily increased in recent decades [1]. One major cause of death is therapy failure, which emerges due to chemotherapy, targeted, and endocrine therapy resistance, which may ultimately lead to metastasis and death [1-5]. Similarly, epigenetic changes in regulatory genes, genetic factors, and genomic instability in breast cancer may also contribute to therapy resistance [6, 7]. Therefore, genetic and epigenetic modifications in regulatory genes, which cause genomic instability, resistance, and metastasis, must be investigated to develop new biomarker candidates and therapeutic targets.

Increased genomic instability is linked to *HORMAD* gene expression, a member of the cancer testis antigen family, which is normally expressed in germline cells but is also abnormally produced in cancer cells [8]. *HORMAD* genes encode proteins with HORMA domains and bind to DNA double-strand breaks formed during meiosis [9]. *HORMAD* proteins also promote synapse formation and initiate homologous

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recombination [9, 10]. The *HORMAD* family genes consist of *HORMAD1* and *HORMAD2* [10, 11]. Recent research has reported that *HORMAD* genes have important roles in cancer emergence and progression; approximately 60% of triple-negative breast cancer (TNBC) cell populations strongly expressed *HORMAD1* [12]. In other genomically unstable cancer models, such as lung adenocarcinoma, *HORMAD1* expression promoted homologous recombination [13]. In a Chinese Han genome wide association study, approximately 10% of lung cancer samples ectopically expressed *HORMAD2*, thus the novel cancer/testis *HORMAD2* gene was proposed as a new therapeutic for lung cancer [11]. In other tumor models, *HORMAD1* epigenetic activation, via hypomethylation in basal-like breast cancer, was implicated in lower susceptibility to rucaparib therapy, despite the fact that approximately 80% of such cancers expressed abnormally high *HORMAD1* levels [14]. *HORMAD2* hypomethylation also reduced cell growth and motility while boosting apoptosis via mRNA expression, whereas *HORMAD2* hypermethylation was putatively involved in thyroid cancer [15]. However, genetic and epigenetic *HORMAD* alterations in metastatic breast cancer and resistance remain unclear.

The Wnt/ β -catenin pathway is a cellular signaling cascade that plays a key role in DNA repair by preserving the integrity of the genome in cells and is intimately associated with the cancer genomes instability [16]. Activation of Wnt signaling in colorectal cancer cells is associated with genetic and epigenetic alterations in regulatory genes, including *ITF2* [17]. Hence, it is crucial to examine genetic and epigenetic modifications in DNA repair regulatory gene, such as *HORMAD*, in metastatic breast cancer and resistance. In this study, putative breast cancer therapeutic targets were examined by analyzing genetic and epigenetic *HORMAD1* and *HORMAD2* alterations. Investigating such alterations and associations with therapy resistance and metastasis may provide important clues underlying these key cancer mechanisms. Our bioinformatics approach may provide insights on individualized treatment approaches, drug response predictions, biomarker identification, and targeted medicines, with a view to enhancing therapeutic responses and metastatic control in breast cancer patients.

2. RESULTS

2.1. *HORMAD1* and *HORMAD2* mRNA levels in breast cancer samples

HORMAD1 ($p = 1.36 \times 10^{-01}$) and *HORMAD2* ($p = 2.82 \times 10^{-6}$) mRNA levels were significantly lower in breast cancer samples when compared with normal breast tissue (Figure 1A). Normal, breast cancer, and the metastatic breast cancer (MBC) samples were also analyzed using TNMPlot and showed decreased *HORMAD1* ($p = 5.08 \times 10^{-01}$) and *HORMAD2* ($p = 1.18 \times 10^{-16}$) mRNA levels in MBC samples when compared with normal and breast tumor tissues (Figure 1B).

2.2. Prognostic value

Analysis of prognostic value of *HORMAD1* and *HORMAD2* in breast cancer patients showed that patients with low levels of *HORMAD1* ($p = 0.6$) and *HORMAD2* ($p = 0.028$) have a better OS than those with low levels of mRNA (Figure 1C).

2.3. Genetic alteration analyses

HORMAD1 and *HORMAD2* genetic alterations in patients from The MBC Project Provisional December 2021 showed that 46% of patients had genetic alterations in *HORMAD1* and 37% had genetic alterations in *HORMAD2*, with most alterations identified as amplifications and deletions (Figure 2A). Mutation profiling of *HORMAD1* showed two variants, which are R303K and S287F protein changes (Figure 2A-B and Table 1), which were considered missense mutations. A mutation in *HORMAD2*, specifically the *HORMA* domain, was identified as S140Kfs*5 (Figure 2C and Table 1) and was considered a frame shift deletion. We also performed pathway enrichment analyses on *HORMAD1* and *HORMAD2* genetic alterations, which showed that enriched Wnt signaling contributed to cell proliferation (Pathway Mapper, Figure 2D) and gene pathways involved in specific spermatogenic functions (NDEx database, Figure 2E).

Table 1. *HORMAD1* and *HORMAD2* genetic alteration profiles across breast cancer samples from the Metastatic Breast Cancer Project (Provisional 2021) (cBioportal).

Gene	Protein Change	Mutation Type	Variant Type	Var
<i>HORMAD1</i>	S287F	Missense_Mutation	SNP	A
<i>HORMAD1</i>	R303K	Missense_Mutation	SNP	T
<i>HORMAD2</i>	S140Kfs*5	Frame_Shift_Del	DEL	-

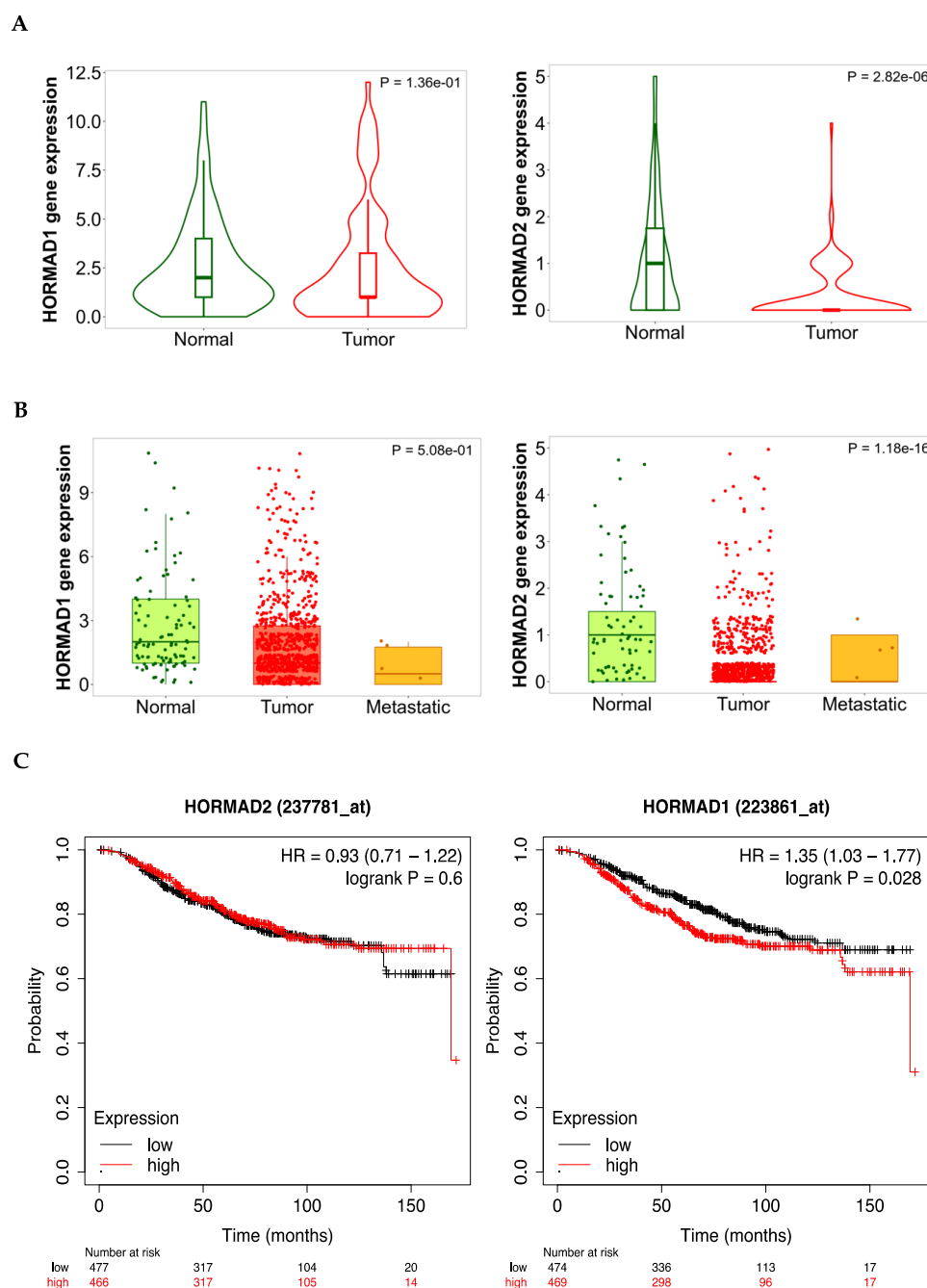


Figure 1. *HORMAD1* and *HORMAD2* mRNA levels in (A) normal and breast cancer samples, (B) normal, breast cancer, and metastatic breast cancer (MBC) samples (TNMPlot). (C). Overall survival data related to *HORMAD1* and *HORMAD2* mRNA levels in breast cancer samples (KMPlotter).

2.4. Epigenetic alteration analyses

HORMAD1 genetic alterations (Figure 3A and Table 2) showed six DNA methylation profiles in open sea areas at cg00935819, cg06893172, cg09034736, cg09059988, cg20767356, and cg22823121. We also identified twelve DNA methylation profiles in *HORMAD2*: cg01141459, cg04046669, cg13245431, cg14509403, cg15209808, cg16686158, cg17632937, cg21843594, cg21890667, and cg23268208 in island areas and cg10230314 and cg24211826 in open sea areas (Figure 3B and Table 2).

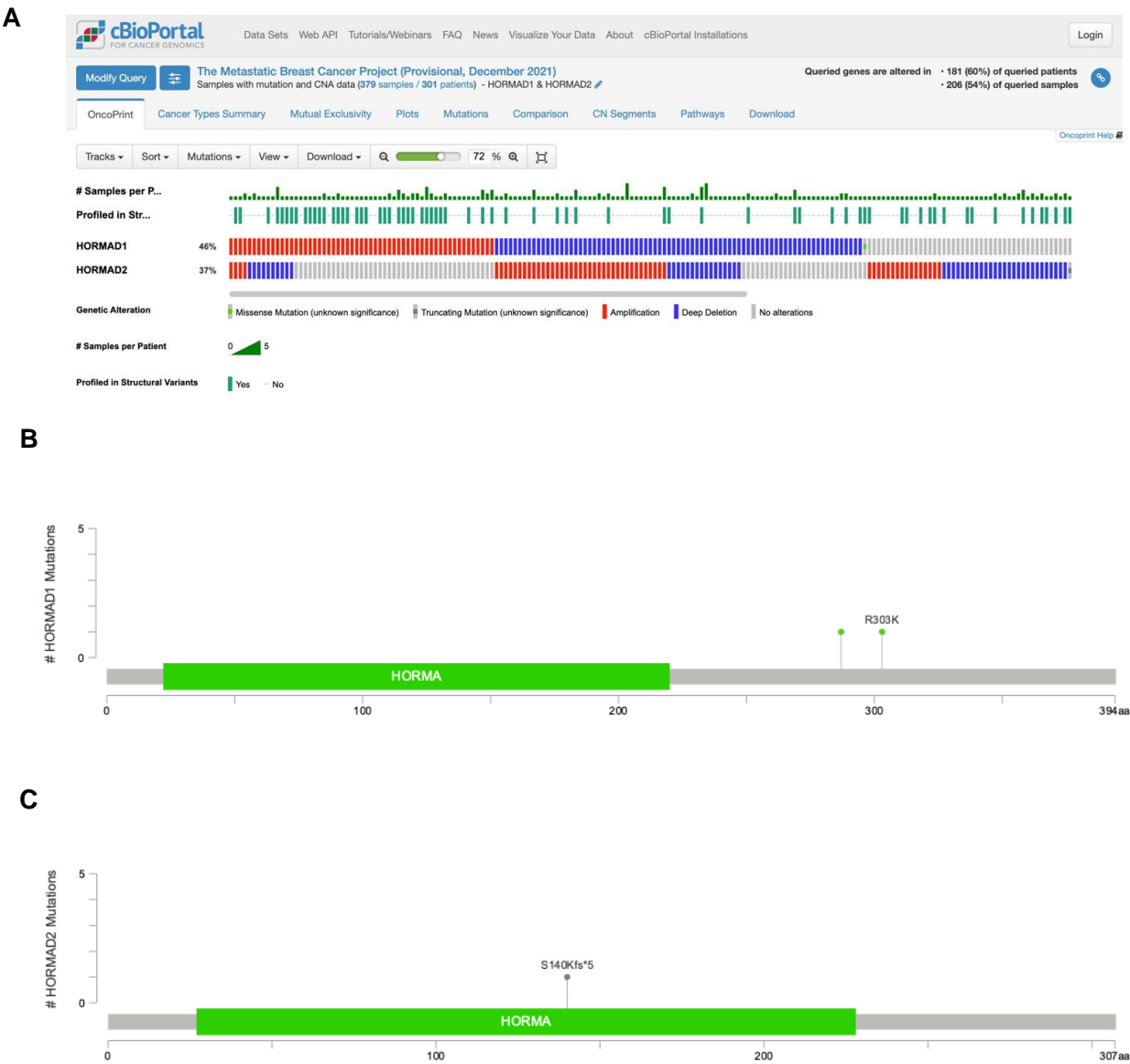


Figure 2. *HORMAD1* and *HORMAD2* genetic alterations (Metastatic Breast Cancer project (provisional, December 2021)). (A). Oncoprint analyses. (B) *HORMAD1* and (C) *HORMAD2* mutation profiles. Pathway enrichment analyses of *HORMAD1* and *HORMAD2* genetic alterations using (D) PathwayMapper and (E) NDEx databases.

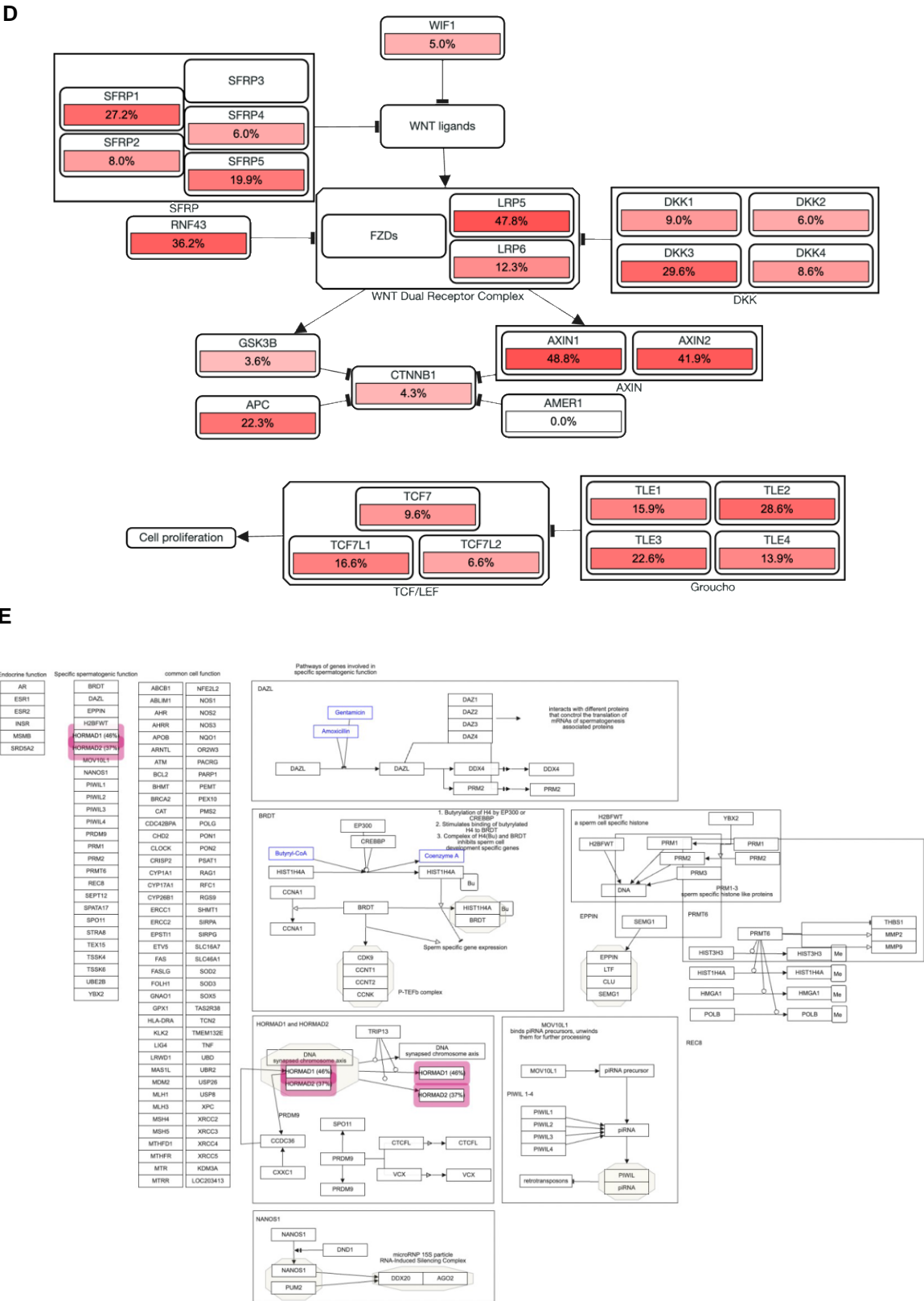


Figure 2. (continued)

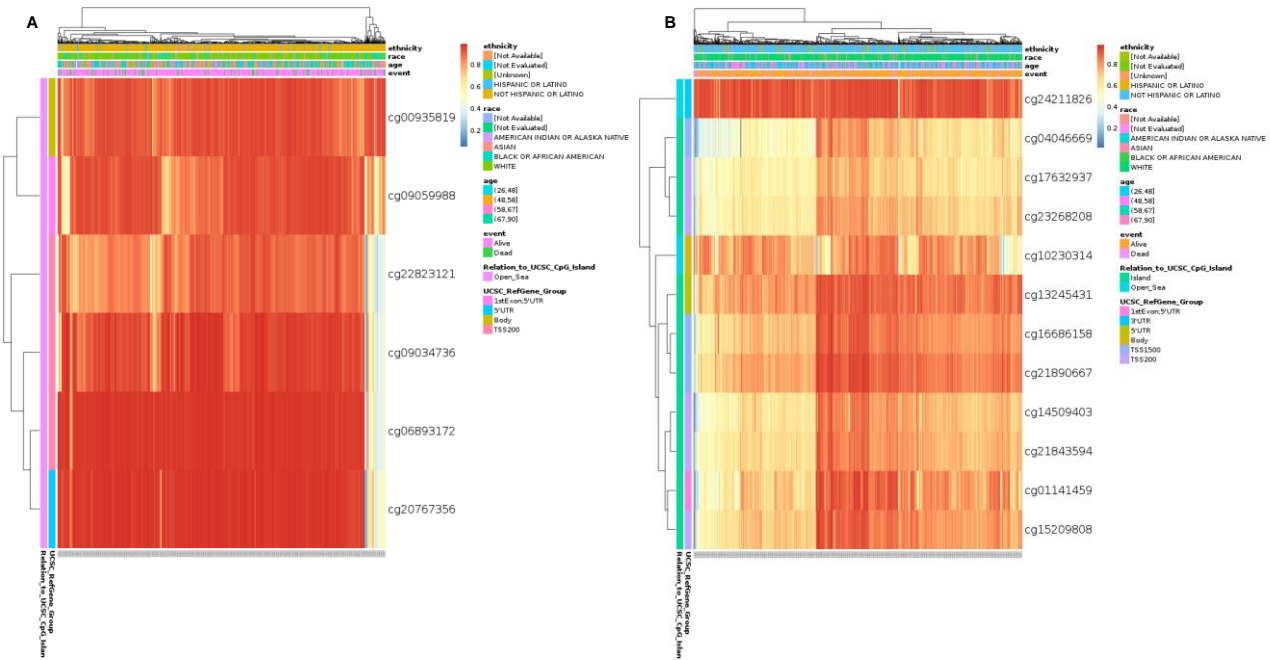


Figure 3. (A) *HORMAD1* and (B) *HORMAD2* DNA methylation profiles (MethSurv).

Table 2. *HORMAD1* and *HORMAD2* DNA methylation profiles across breast cancer samples from TCGA study (MethSurv).

HORMAD1		
UCSC_RefGene_Group	Relation_to_UCSC_CpG_Island	Position
cg00935819	Body	Open_Sea
cg06893172	TSS200	Open_Sea
cg09034736	TSS200	Open_Sea
cg09059988	1stExon;5'UTR	Open_Sea
cg20767356	5'UTR	Open_Sea
cg22823121	TSS200	Open_Sea
HORMAD2		
UCSC_RefGene_Group	Relation_to_UCSC_CpG_Island	Position
cg01141459	1stExon;5'UTR	Island
cg04046669	TSS1500	Island
cg10230314	Body	Open_Sea
cg13245431	5'UTR	Island
cg14509403	TSS200	Island
cg15209808	TSS200	Island
cg16686158	TSS1500	Island
cg17632937	TSS200	Island
cg21843594	TSS200	Island
cg21890667	TSS1500	Island
cg23268208	TSS200	Island
cg24211826	3'UTR	Open_Sea

2.5. Principal component analysis (PCA)

As we used breast invasive carcinoma TCGA 2017 study samples to analyze epigenetic alterations, we conducted PCA on *HORMAD1* and *HORMAD2* mRNA levels using ClustVis. In *HORMAD1* analyses, X and Y axes showed principal components 1 and 2, which explained 82.5% and 8.6% of total variance, respectively (Figure 4A). Similar *HORMAD2* analyses provided data that explained 75.7% and 12% of total variance, respectively (Figure 4B). Prediction ellipses are such that with probability 0.95, a new observation from the same group will fall inside the ellipse. N = 782 data points

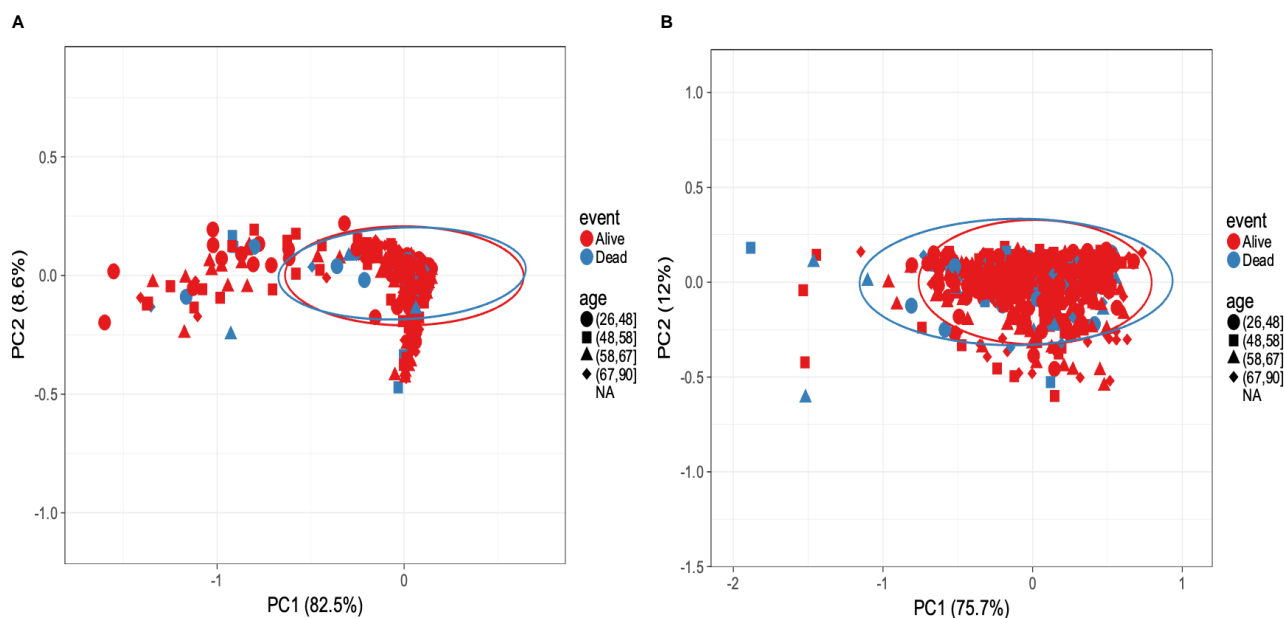


Figure 4. Principal component analysis of breast cancer patients samples from the TCGA (2017) study related to (A) *HORMAD1* and (B) *HORMAD2* expression.

2.6. Genetic variation analyses

We examined *HORMAD* genetic variants in ClinVar to examine links between *HORMAD1* and *HORMAD2* genotypes and medically important phenotypes. We identified eight genetic variations in *HORMAD1*, where in particular, 1021C>T (Q334) and 430A>G (T144A) variants exhibited pathogenic and benign clinical significance, respectively (Table 3). Moreover, five genetic variations in *HORMAD2* exhibited uncertain clinical significance (Table 3).

2.7. Functional predictions

Functional mutant *HORMAD* predictions were conducted using several database. The mutation effects of three variants were predicted; S287F and R303K in cBioportal and T144 in ClinVar. S287F was predicted to have deleterious effects on amino acid impact. No effect on affected functional site, have passenger on cancer driver mutations, decrease stability based on MuPro and increase stability based on I-Mu (Table 4). The other mutants (R303K and T144A) had no effects on amino acid impact on its functions.

2.8. ROC Plotter analyses

ROC plots identified connections between *HORMAD1* and *HORMAD2* levels and medication sensitivity, including endocrine, anti-HER2 therapy, and chemotherapy. No significant results were found for endocrine therapy sensitivity (Figure 5A). Significant correlative results were identified between *HORMAD2* levels and anti-HER2 sensitivity (Area under the Curve = 0.709 and P value = 5.8×10^{-3}) (Figure 5B). No significant correlative results were identified for chemotherapy sensitivity (Figure 5C).

Table 3. *HORMAD1* and *HORMAD2* genetic variations (ClinVar).

Name of variation	Gene	Protein change	Clinical significance (Last reviewed)
NM_032132.5(<i>HORMAD1</i>):c.896A>G (p.Asp299Gly)	<i>HORMAD1</i>	D299G, D292G	Uncertain significance (Last reviewed: Apr 7, 2022)
NM_032132.5(<i>HORMAD1</i>):c.718A>G (p.Ile240Val)	<i>HORMAD1</i>	I240V, I233V	Uncertain significance (Last reviewed: Dec 16, 2021)
NM_032132.5(<i>HORMAD1</i>):c.1168C>A (p.Pro390Thr)	<i>HORMAD1</i>	P390T, P383T	Uncertain significance (Last reviewed: Nov 8, 2022)
NM_032132.5(<i>HORMAD1</i>):c.1021C>T (p.Gln341Ter)	<i>HORMAD1</i>	Q334*, Q341*	Pathogenic (Last reviewed: Oct 18, 2022)
NM_032132.5(<i>HORMAD1</i>):c.920G>T (p.Ser307Ile)	<i>HORMAD1</i>	S300I, S307I	Uncertain significance (Last reviewed: Aug 1, 2022)
NM_032132.5(<i>HORMAD1</i>):c.1131T>G (p.Ser377Arg)	<i>HORMAD1</i>	S370R, S377R	Uncertain significance (Last reviewed: Aug 2, 2022)
NM_032132.5(<i>HORMAD1</i>):c.308C>T (p.Thr103Ile)	<i>HORMAD1</i>	T103I, T96I	Uncertain significance (Last reviewed: Apr 22, 2022)
NM_032132.5(<i>HORMAD1</i>):c.430A>G (p.Thr144Ala)	<i>HORMAD1</i>	T137A, T144A	Benign (Last reviewed: Jun 1, 2018)
NM_152510.4(<i>HORMAD2</i>):c.613A>T (p.Asn205Tyr)	<i>HORMAD2</i>	N205Y, N117Y	Uncertain significance (Last reviewed: Aug 23, 2021)
NM_152510.4(<i>HORMAD2</i>):c.118G>T (p.Ala40Ser)	<i>HORMAD2</i>	A40S	Uncertain significance (Last reviewed: Jan 18, 2022)
NM_152510.4(<i>HORMAD2</i>):c.841T>C (p.Cys281Arg)	<i>HORMAD2</i>	C193R, C281R	Uncertain significance (Last reviewed: Jun 28, 2022)
NM_152510.4(<i>HORMAD2</i>):c.781G>A (p.Gly261Ser)	<i>HORMAD2</i>	G261S, G173S	Uncertain significance (Last reviewed: Dec 19, 2022)
NM_152510.4(<i>HORMAD2</i>):c.892C>T (p.Pro298Ser)	<i>HORMAD2</i>	P210S, P298S	Uncertain significance (Last reviewed: Sep 1, 2021)

Table 4. Mutant *HORMAD1* functional predictions.

Protein Change	Amino Acid impact								Molecular mechanism (MutPred2)	Affected functional Site	Cancer driver mutations (FATHMM)	Analysis of Protein Stability	
	PredictSNP	MAPP	PhD-SNP	Poly-Phen1	Poly-Phen2	SIFT	SNAP	Panther				MuPro	I-Mu
S287F	Neu	Neu	Neu	Neu	Neu	Neu	Neu	Del	0.064	none	Pass/Other	Dec	Inc
R303K	Neu	Neu	Neu	Neu	Neu	Neu	Neu	Neu	0.043	none	Pass/Other	Dec	Inc
T144A	Neu	Neu	Neu	Neu	Neu	Neu	Neu	Neu	0.021	none	Pass/Other	Dec	Inc

Neu: Neu, Del: Deleterious, Pas: Passenger, Dec: Decrease stability, Inc: Increase stability

2.9. Immune infiltration

TIMER2.0 was used to analyze immune infiltration data in relation to *HORMAD1* and *HORMAD2* mRNA levels. *HORMAD1* had negative correlations with purity ($R = -0.124$, $p = 8.85 \times 10^{-5}$), CD8+ ($R = -0.065$, $p = 4.1 \times 10^{-2}$), macrophage ($R = -0.037$; $p = 2.41 \times 10^{-1}$), and cancer-associated fibroblast cells ($R = -0.066$; $p = 3.96 \times 10^{-2}$), but positive correlations with CD4+ ($R = 0.179$, $p = 1.26 \times 10^{-8}$), neutrophil ($R = 0.384$, $p = 3.20 \times 10^{-36}$), and dendritic cells ($R = 0.235$, $p = 6.83 \times 10^{-14}$) (Figure 6 and Table 5). *HORMAD2* had positive correlations with purity ($R = 0.063$, $p = 4.86 \times 10^{-2}$), CD4+ ($R = 0.096$, $p = 2.48 \times 10^{-3}$), neutrophil ($R = 0.121$, $p = 1.37 \times 10^{-4}$), and dendritic cells ($R = 0.115$, $p = 2.71 \times 10^{-4}$), and negative correlations with CD8+ ($R = -0.025$, 4.27×10^{-1}), macrophage ($R = -0.04$, $p = 2.05 \times 10^{-1}$), and cancer-associated fibroblast cells ($R = -0.04$, $p = 9 \times 10^{-1}$).

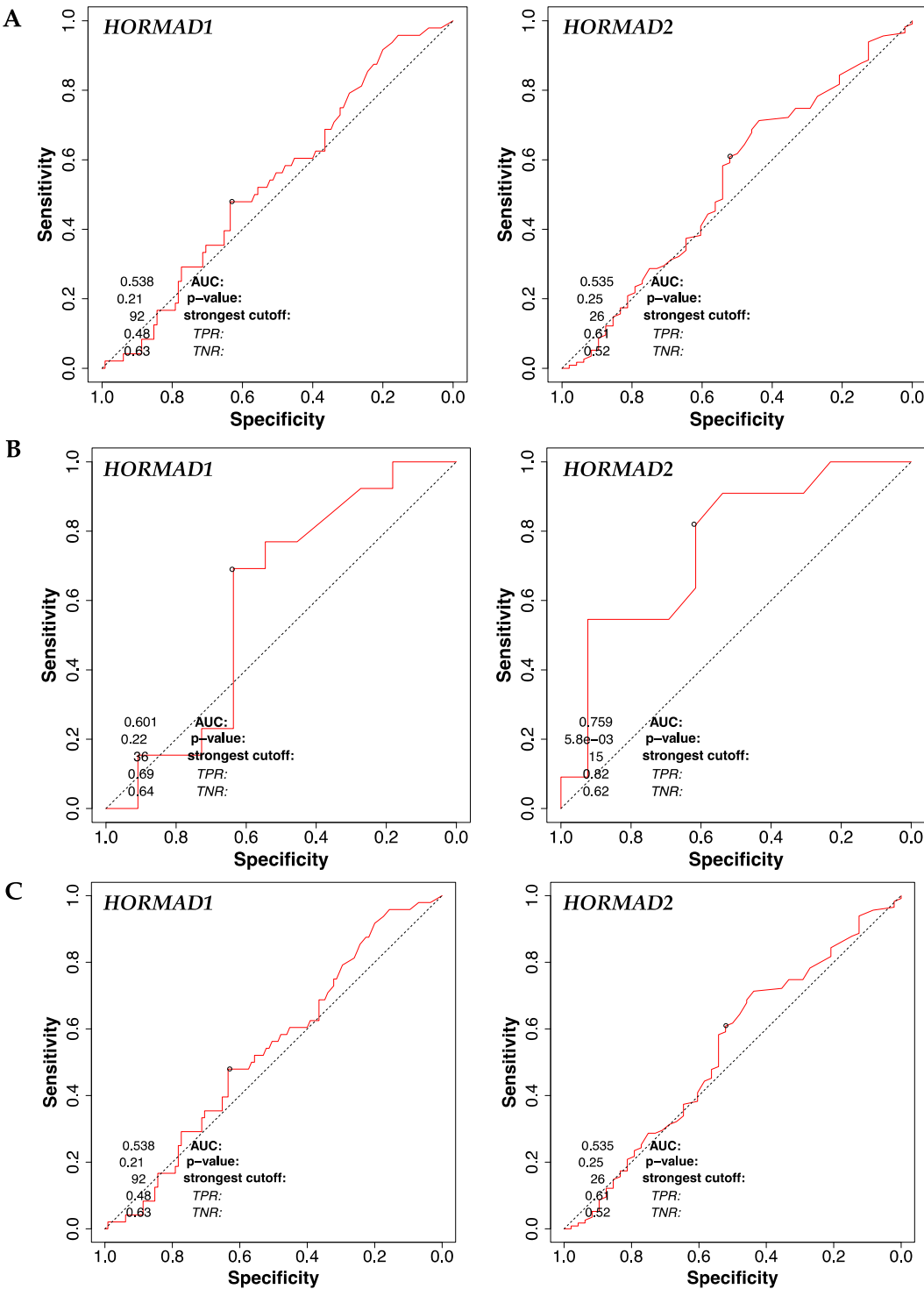


Figure 5. Receiver operating characteristic plotter showing *HORMAD1* and *HORMAD2* levels related to (A) endocrine therapy (B) anti-HER2 therapy, and (C) chemotherapy sensitivity.

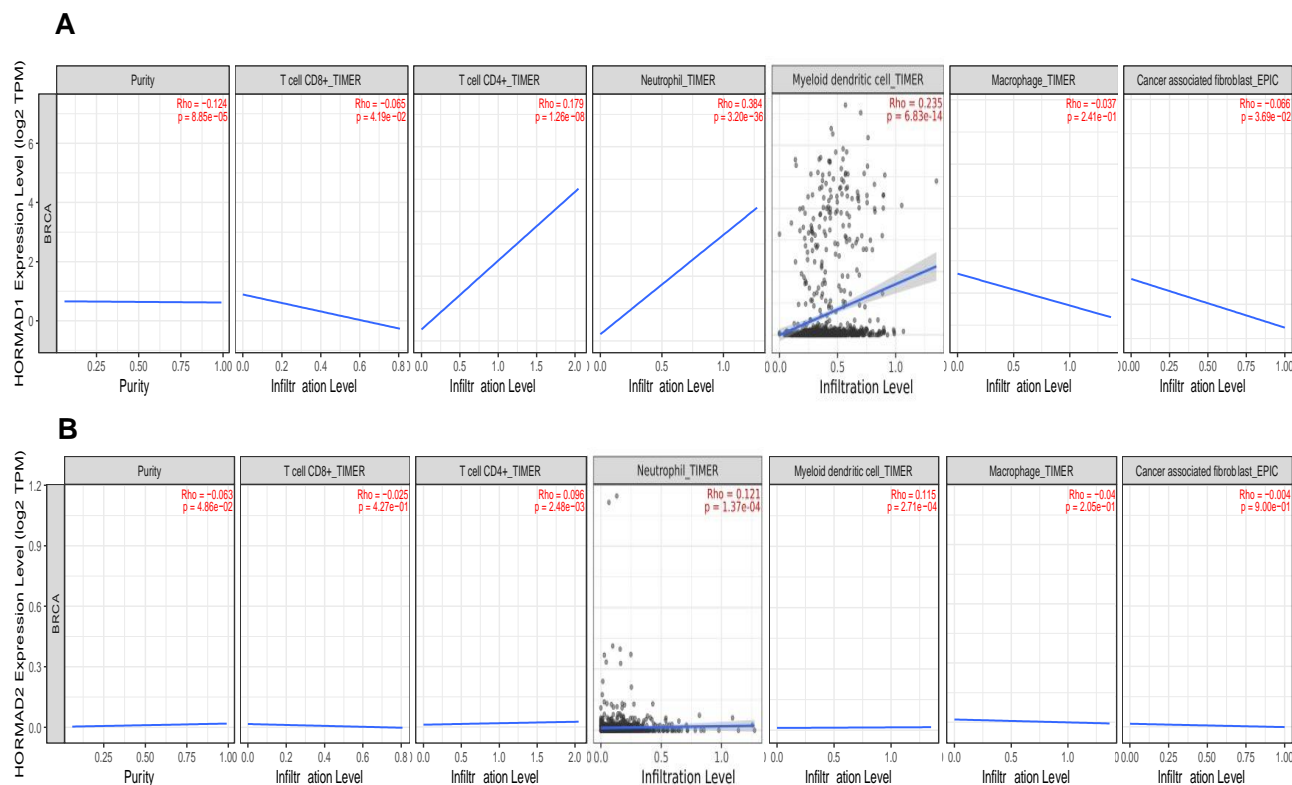


Figure 6. Correlations between (A) *HORMAD1* and (B) *HORMAD2* mRNA levels and immune cell infiltration, including purity, CD8+, CD4+, neutrophil, dendritic, macrophage, and cancer-associated fibroblast cells (TIMER2.0).

Table 5. *HORMAD1* and *HORMAD2* correlations with immune cell infiltration (TIMER2.0).

Gene	Purity		CD8+		CD4+		Neutrophil	
	R	p	R	p	R	p	R	p
<i>HORMAD1</i>	-0.124	8.85x10⁻⁵	-0.065	4.1x10⁻²	0.179	1.26x10⁻⁸	0.384	3.20x10⁻³⁶
<i>HORMAD2</i>	0.063	4.86x10⁻²	-0.025	4.27x10 ⁻¹	0.096	2.48x10⁻³	0.121	1.37x10⁻⁴
Gene	Dendritic cells		Macrophage		Cancer associated fibroblast			
	R	p	R	p	R	p		
<i>HORMAD1</i>	0.235	6.83x10⁻¹⁴	-0.037	2.41x10 ⁻¹	-0.066	3.96x10⁻²		
<i>HORMAD2</i>	0.115	2.71x10⁻⁴	-0.04	2.05x10 ⁻¹	-0.04	9x10 ⁻¹		

3. DISCUSSION

We investigated *HORMAD1* and *HORMAD2* genetic and epigenetic alterations in breast cancer resistance and metastasis mechanisms. *HORMAD1* and *HORMAD2* mRNA levels were lower in breast cancer samples, but even lower in MBC samples. Patients with higher *HORMAD1* and *HORMAD2* mRNA levels had favorable OS rates than the opposite groups. These data contrasted with a previous study which showed that *HORMAD1* was highly expressed in TNBC, the most aggressive and MBC, and thus targeting *HORMAD1* leads to impairing homologous recombination, makes breast cancer cells more susceptible to cisplatin [12]. Another study showed that the primary cause of docetaxel resistance was possibly *HORMAD1* actions in DNA damage tolerance in tumor cells; thus, *HORMAD1* may be a crucial therapeutic target for TNBC [18]. In patients with breast cancer, high *HORMAD1* expression levels were associated with improved responses to anthracycline-cyclophosphamide and longer metastasis-free survival rates [19].

However, for *HORMAD2*, our results were supported by a previous study where *HORMAD2* overexpression induced apoptosis and inhibited cell proliferation in thyroid cancer cells [20].

In breast cancer patients from the MBC 2021 study, *HORMAD1* and *HORMAD2* gene amplifications and deletions were observed. Two mutations, including R303K and S287F in *HORMAD1*, were considered missense mutations, whereas S140Kfs*5 in *HORMAD2* was considered a deletion/frameshift deletion. Pathway enrichment analyses identified alterations in Wnt signaling which contributed to cell proliferation. Wnt signaling also regulates chemoresistance in breast cancer cells [21]. This observation was supported by a previous study where *HORMAD1* increased Akt and GSK3 β phosphorylation, which led to decreased β -catenin phosphorylation and increased epithelial to mesenchymal transition, metastasis, and progression in lung cancer cells [22].

We identified more DNA methylation levels in *HORMAD2* when compared with *HORMAD1* in patients with breast cancer. This observation was supported by a previous study where hypomethylated *HORMAD1* in basal-like breast cancer possibly decreased sensitivity to rucaparib therapy [14]. Also, invasive melanoma was shown to include four differentially methylated *HORMAD2* fragments [23]. Moreover, *HORMAD2* hypermethylation was linked to thyroid carcinoma, whereas *HORMAD2* hypomethylation decreased cell growth and motility, and facilitated apoptosis by boosting *HORMAD2* mRNA levels [20]. The 1021C>T (Q334) and 430A>G (T144A) variants were also shown to have clinical significance in patients with breast cancer. Additionally, functional predictions of the *HORMAD1* variant S287F confirmed deleterious effects on its amino acid functional impact, however, further studies are required to corroborate this.

ROC plot data identified significant correlations between mRNA *HORMAD2* levels and anti-HER2 sensitivity, and this result is consistent with previous studies showing *HORMAD* correlations with breast cancer agent sensitivity. *HORMAD1* overexpression was correlated with platinum-based chemotherapy chemosensitivity in TNBC [12]. High *HORMAD1* expression was also associated with improved responses to anthracycline-cyclophosphamide and longer metastasis-free survival rates in patients with breast cancer [19]. Importantly, ours is the first study to identify correlations between *HORMAD* genes and anti-HER2 therapy, therefore future studies must explore the HER2 signaling/*HORMAD2* axis. While our immune infiltration analyses were significant, Spearman's correlation coefficients were relatively low, except for *HORMAD1*-neutrophils ($r = 0.34$) and *HORMAD1*-dendritic cells ($r = 0.235$), therefore, these findings require more in-depth exploration.

Our study had several limitations. We used several databases which exhibited some data variations, therefore some discrepancies and inconsistencies cannot be ruled out. Also, our findings were predictive and did not describe the functional significance of genetic and epigenetic alterations, so future experimental validation is required. Our examination of *HORMAD1* and *HORMAD2* genetic and epigenetic alterations may identify new therapeutic targets which facilitate new drug development, which may improve patient outcomes by mediating resistance and metastasis actions. Future studies should investigate the downstream consequences of *HORMAD* mutations on different signaling pathways and identifying new drugs which inhibit these pathways.

4. CONCLUSION

The study results showed that *HORMAD1* and *HORMAD2* genetic and epigenetic alterations may function as potential biomarkers and targets for breast cancer resistance and metastasis by targeting Wnt signaling. Further studies are required to validate and expand our findings, with a view to exploring *HORMAD* gene correlations with anti-HER2 therapy and immune cell infiltration.

5. MATERIALS AND METHODS

5.1. *HORMAD1* and *HORMAD2* mRNA levels in breast cancer samples

HORMAD1 and *HORMAD2* mRNA levels in breast cancer, normal breast, and metastatic breast cancer tissue were analyzed using TNMPlot (<https://tnmplot.com/analysis/>) [24]. Gene symbols were submitted to the database and several parameters selected. For normal vs. breast cancer tissue mRNA analyses, selection parameters included RNA-Seq data and paired tumor and adjacent normal tissues. Statistical analyses were performed using Mann Whitney tests. To analyze normal, breast cancer, and metastatic breast cancer mRNA levels, selection criteria included RNA-Seq data and paired tumor and adjacent normal tissues. Statistical analyses were performed using Kruskal Wallis tests.

5.2. Prognostic value

HORMAD1 and *HORMAD2* prognostic values in patients were analyzed using KMPlotter (<https://kmplot.com/analysis/>) [25]. *HORMAD1* and *HORMAD2* genes symbols were submitted to KMPlotter using mRNA gene chip data, and several parameters such as: split patients by median, and overall survival (OS).

5.3. Genetic alteration analyses

HORMAD1 and *HORMAD2* genetic alterations in patients were analyzed using cBioportal (<https://www.cbioportal.org/>) [26, 27]. *HORMAD1* and *HORMAD2* gene symbols were submitted to the cBioPortal and The metastatic breast cancer (MBC) project 2021 was selected. Further analyses were conducted for instances Oncoprint, mutation analysis, and pathway alterations.

5.4. Epigenetic alterations

HORMAD1 and *HORMAD2* epigenetic alterations in patients were analyzed using methsurv (<https://biit.cs.ut.ee/methsurv/>) [28]. *HORMAD1* and *HORMAD2* gene symbols were submitted to the site, and selected parameters included such as The Cancer Genome Atlas Program (TCGA) cancer datasets of breast invasive carcinoma TCGA 2017, gene visualization, and further DNA methylation was shown as heatmap.

5.5. Principal component analysis (PCA)

PCA of *HORMAD1* and *HORMAD2* mRNA levels in the breast invasive carcinoma TCGA 2017 study were conducted using ClustVis (<https://biit.cs.ut.ee/clustvis/>) [29].

5.6. Genetic variation analyses

To understand associations between *HORMAD1* and *HORMAD2* genotypes and medically significant phenotypes, we used the ClinVar database (<https://www.ncbi.nlm.nih.gov/clinvar/>) [30].

5.7. ROC plotter

ROC plots relating to *HORMAD1* and *HORMAD2* mRNA levels and drug sensitivity (endocrine, anti-HER2 therapy, and chemotherapy) were generated using ROC Plotter (<https://www.rocplot.org/>) [31].

5.8. Functional predictions

Functional *HORMAD* mutant predictions were conducted using several databases. The amino acid impact on its activity was analyzed using PredictSNP (<http://loschmidt.chemi.muni.cz/predictsnp>) [32], which incorporated six prediction tools, including Multivariate Analysis of Protein Polymorphisms (MAPP) [33], predictor of human deleterious single nucleotide polymorphisms (PhD-SNP) [34], PolyPhen-1 [35], PolyPhen-2 [36], Sorting Intolerant from Tolerant (SIFT) [37], Single Nucleotide Amplified Polymorphisms (SNAP) [38], and Protein Analysis Through Evolutionary Relationships (PANTHER) [39]. Molecular mechanisms underlying mutant *HORMAD1* amino acids were predicted using MutPred2 (<http://mutpred.mutdb.org/>) [40]. *HORMAD1* cancer driver mutations were predicted using Functional Analysis through Hidden Markov Models (v2.3) (FATHMM) (<http://fathmm.biocompute.org.uk/>) [41]. Protein stability was predicted using MuPro (<https://mupro.proteomics.ics.uci.edu/>) [42] and I-Mu (<http://gpcr2.biocomp.unibo.it/cgi/predictors/I-Mutant3.0/I-Mutant3.0.cgi>) [43].

5.9. Immune infiltration analyses

Analyses relating to *HORMAD1* and *HORMAD2* mRNA level and immune filtration in patients were conducted using TIMER2.0 (<http://timer.cistrome.org/>) [44]. *HORMAD1* and *HORMAD2* gene symbols were submitted and immune cells selected, including purity, CD8+, CD4+, neutrophil, dendritic, macrophage, and cancer-associated fibroblast cells. Spearman's correlation coefficients between *HORMAD1* or *HORMAD2* and immune cell abundance were then calculated.

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