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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 ($p = 1.36x10^{-01}$) and *HORMAD2* ($p = 2.82x10^{-6}$) 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* ($p = 5.08x10^{-01}$) and *HORMAD2* ($p = 1.18x10^{-16}$) 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
HORMAD1	S287F	Missense_Mutation	SNP	A
HORMAD1	R303K	Missense_Mutation	SNP	T
HORMAD2	S140Kfs*5	Frame_Shift_Del	DEL	-

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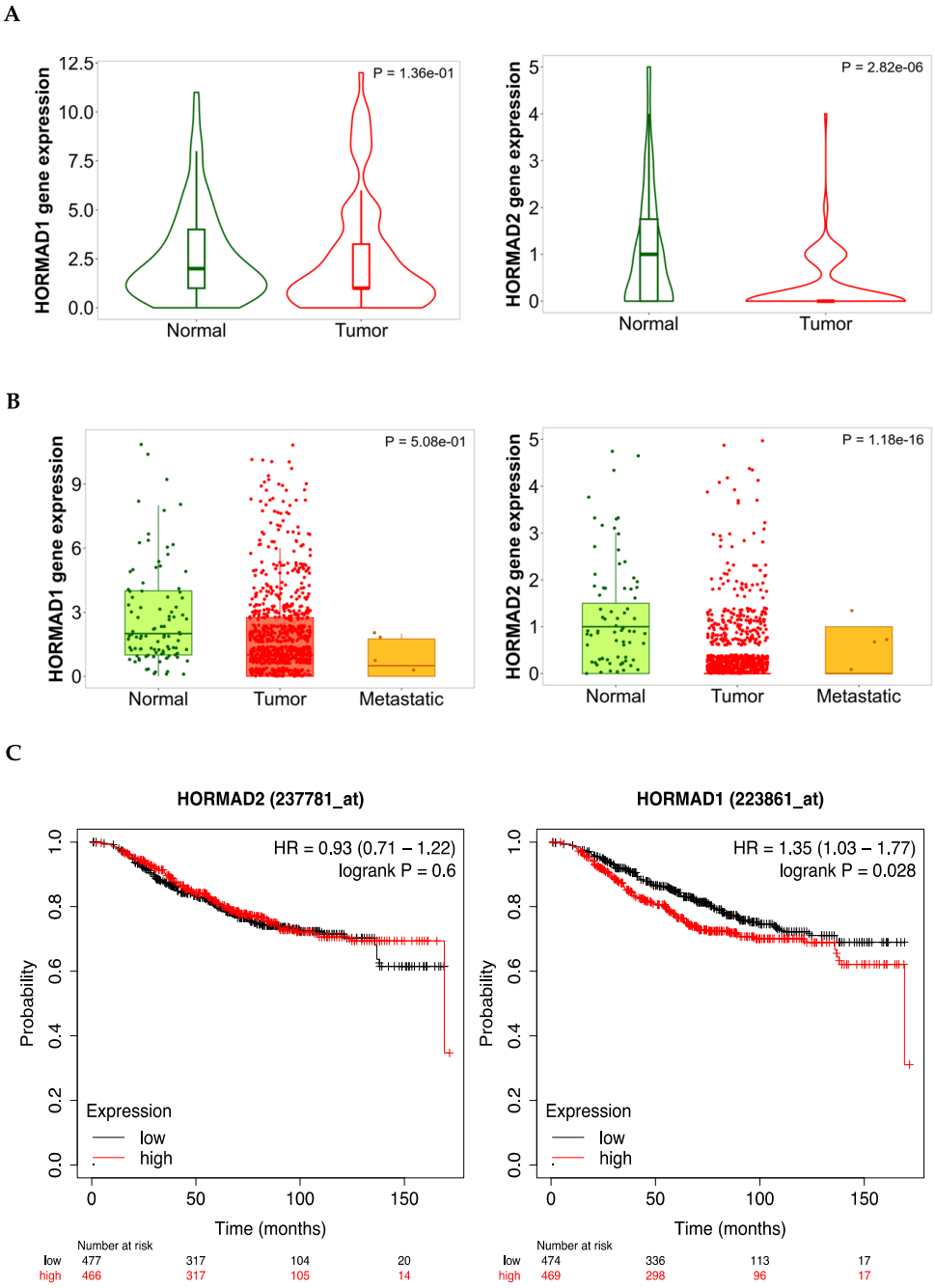

Figure 1. *HORMAD1* and *HORMAD2* mRNA levels in (A) normal and breast cancer samples, (B) normal, breast cancer, and metastatic breast cancer (MBC) samples (TNMPlot). (C). Overall survival data related to *HORMAD1* and *HORMAD2* mRNA levels in breast cancer samples (KMPlotter).

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).

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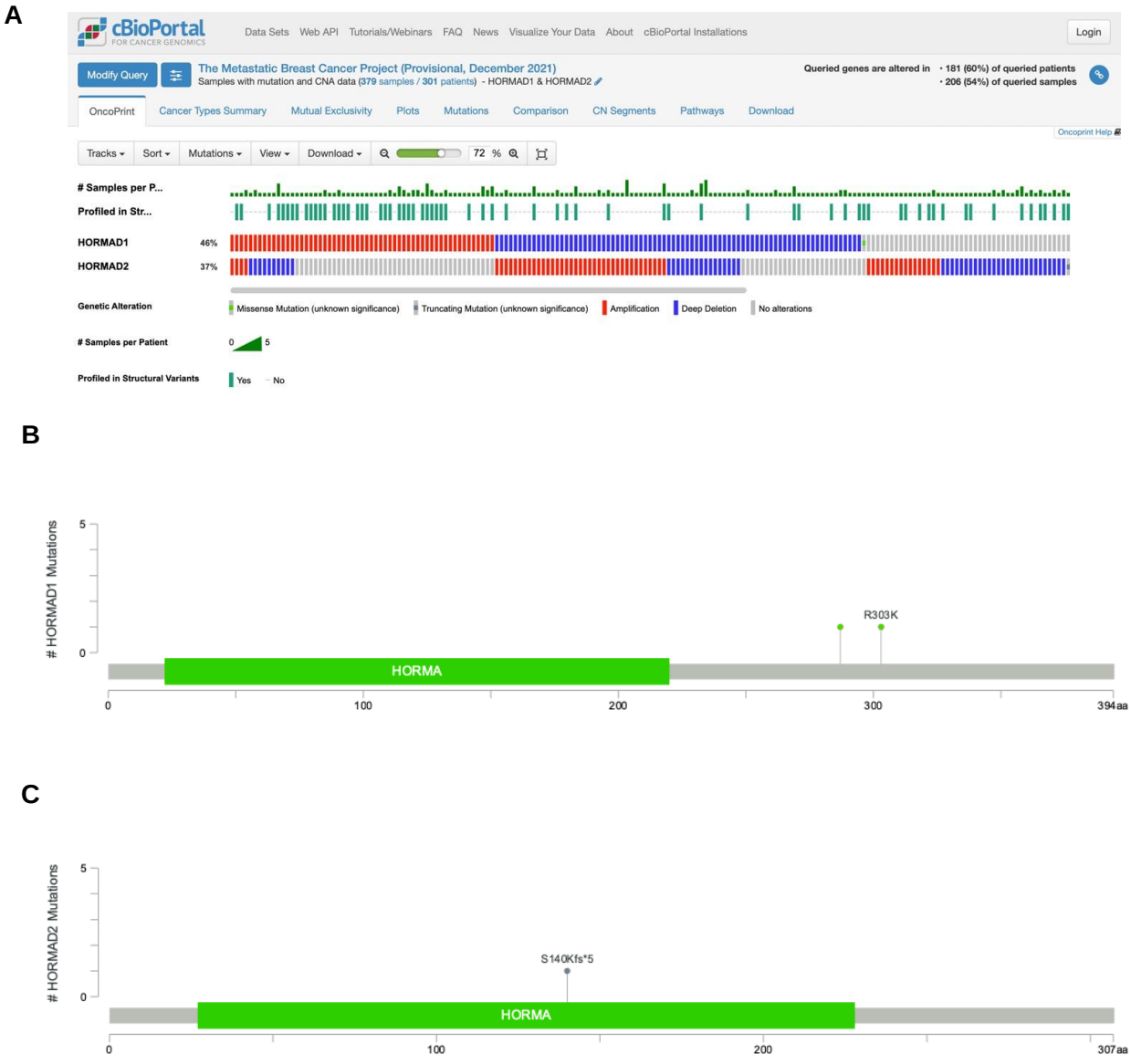

Figure 2. *HORMAD1* and *HORMAD2* genetic alterations (Metastatic Breast Cancer project (provisional, December 2021)). (A). Oncoprint analyses. (B) *HORMAD1* and (C) *HORMAD2* mutation profiles. Pathway enrichment analyses of *HORMAD1* and *HORMAD2* genetic alterations using (D) PathwayMapper and (E)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

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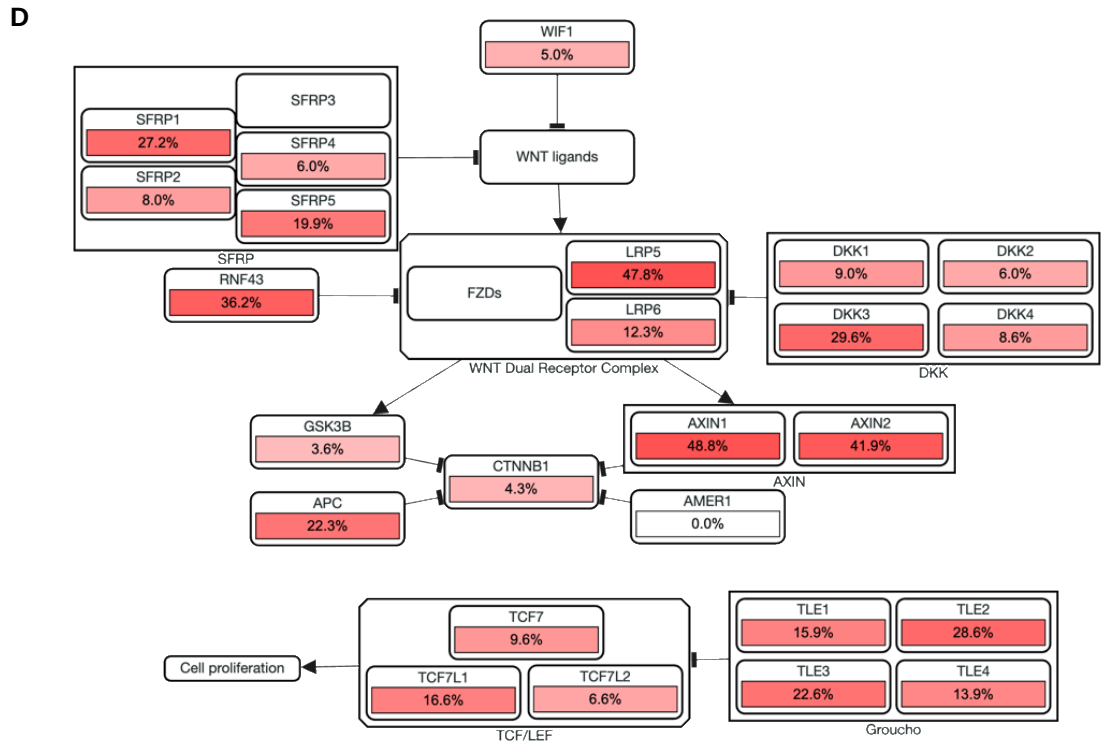

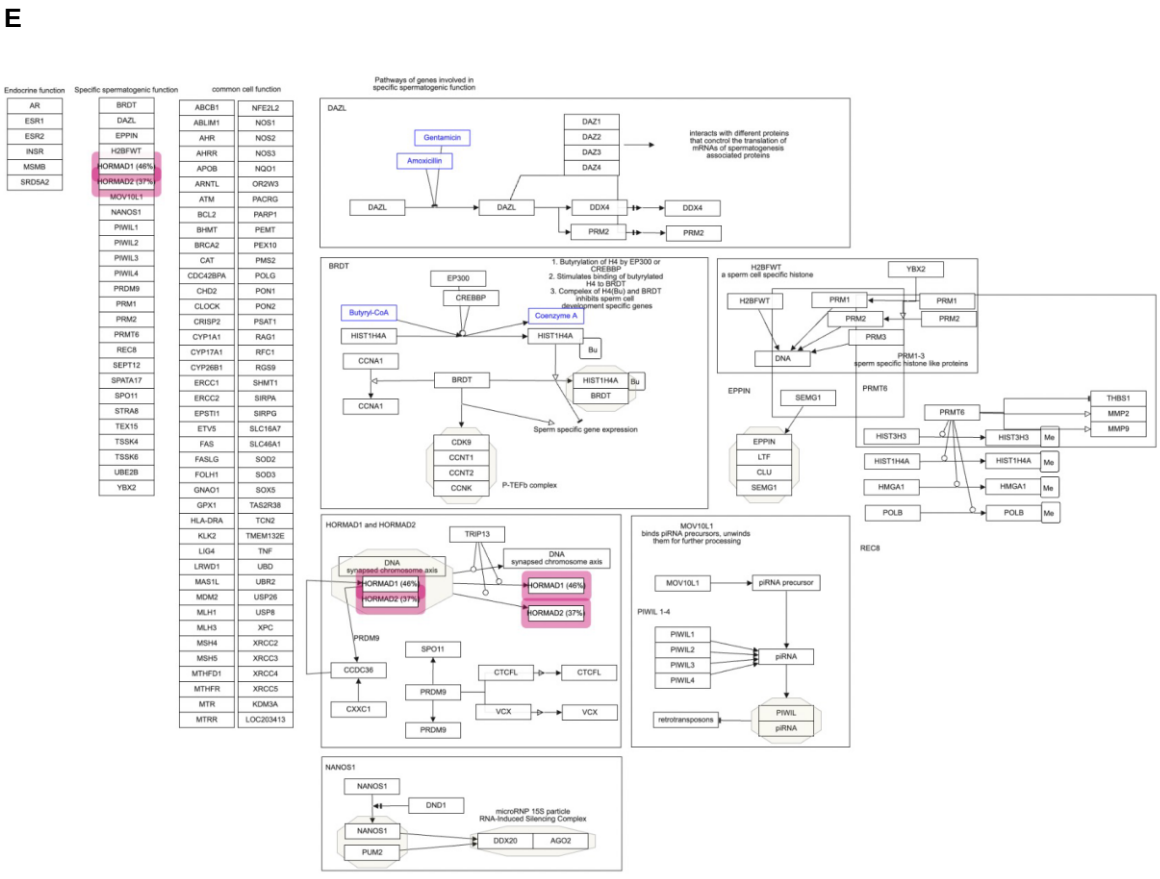

Figure 2. (continued)

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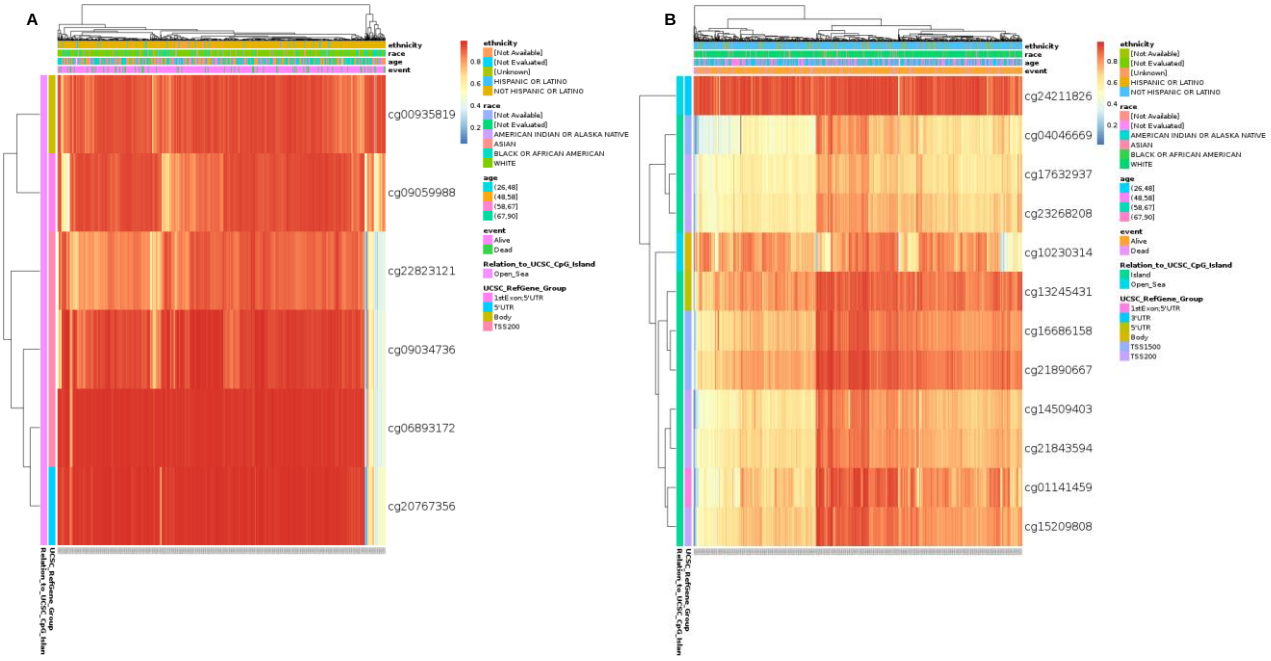

Figure 3. (A) *HORMAD1* and (B) *HORMAD2* DNA methylation profiles (MethSurv).

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

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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

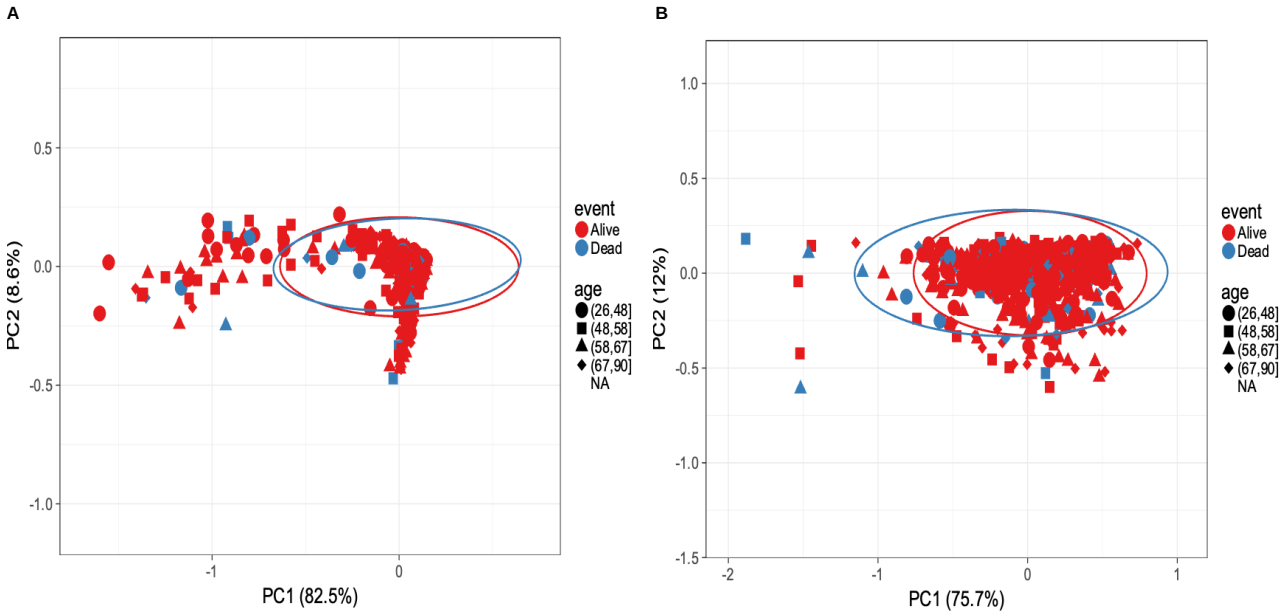

Figure 4. Principal component analysis of breast cancer patients samples from the TCGA (2017) study related to (A) *HORMAD1* and (B) *HORMAD2* expression.

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

Table 4. Mutant *HORMAD1* functional predictions.

	Amino Acid impact								Molecular mechanism (MutPred2)		Cancer driver mutations (FATHMM)	Analysis of Protein Stability	
Protein Change	PredictSNP	MAPP	PhD-SNP	Poly-Phen1	Poly-Phen2	SIFT	SNAP	Panther	Score	Affected functional Site		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 *HORMAD1* mRNA levels. *HORMAD1* had negative correlations with purity ($R=-0.124$, $p = 8.85\times10^{-5}$), CD8+ ($R=-0.065$, $p = 4.1\times10^{-2}$), macrophage ($R=-0.037$; $p = 2.41\times10^{-1}$), and cancer-associated fibroblast cells ($R=-0.066$; $p = 3.96\times10^{-2}$), but positive correlations with CD4+ ($R = 0.179$, $p = 1.26\times10^{-8}$), neutrophil ($R = 0.384$, $p = 3.20\times10^{-36}$), and dendritic cells ($R = 0.235$, $p = 6.83\times10^{-14}$) (Figure 6 and Table 5). *HORMAD2* had positive correlations with purity ($R = 0.063$, $p = 4.86\times10^{-2}$), CD4+ ($R = 0.096$, $p = 2.48\times10^{-3}$), neutrophil ($R = 0.121$, $p = 1.37\times10^{-4}$), and dendritic cells ($R = 0.115$, $p = 2.71\times10^{-4}$), and negative correlations with CD8+ ($R=-0.025$, 4.27×10^{-1}), macrophage ($R=-0.04$, $p = 2.05\times10^{-1}$), and cancer-associated fibroblast cells ($R = -0.04$, $p = 9\times10^{-1}$).

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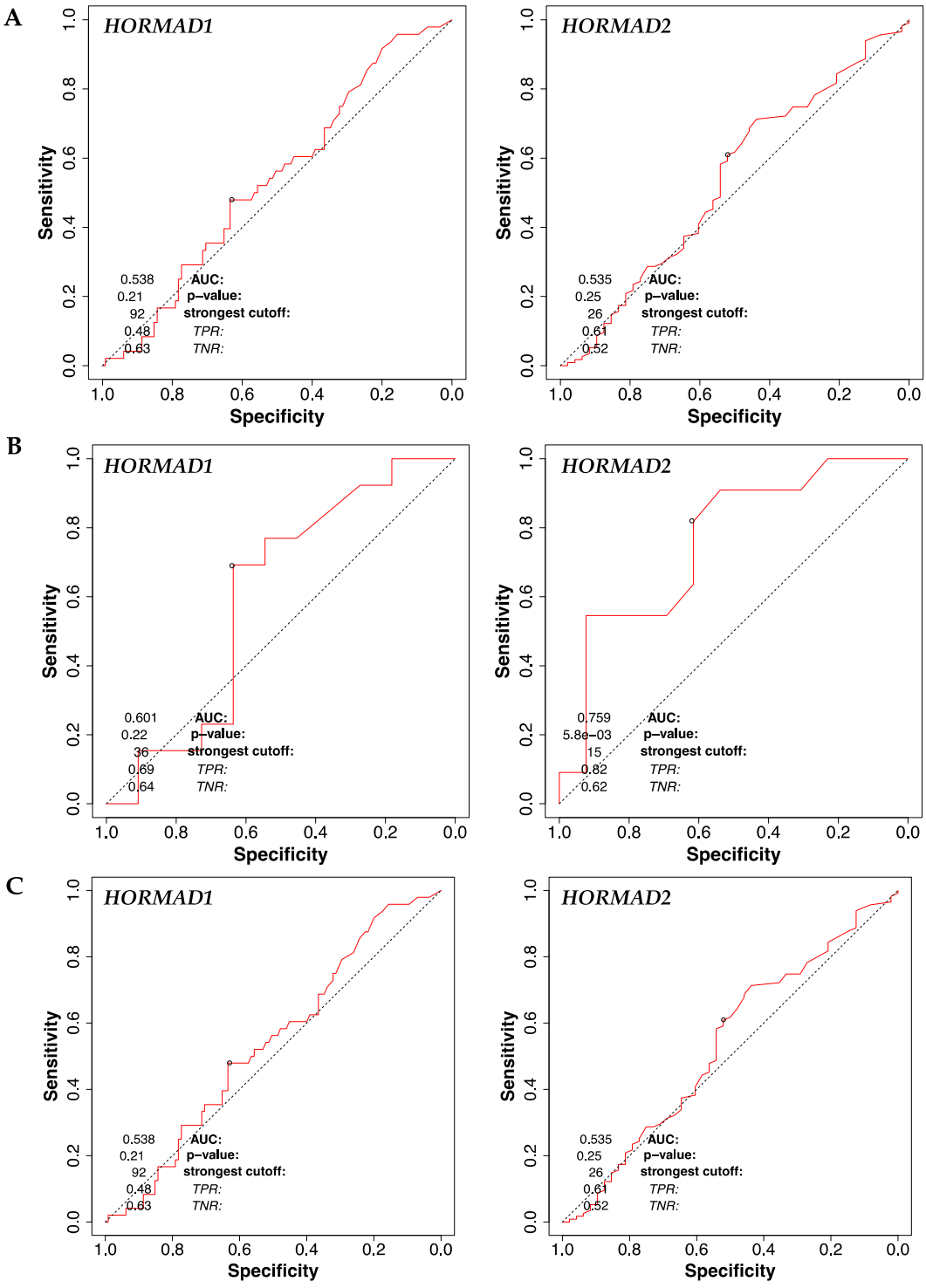

Figure 5. Receiver operating characteristic plotter showing *HORMAD1* and *HORMAD2* levels related to (A) endocrine therapy (B) anti-HER2 therapy, and (C) chemotherapy sensitivity.

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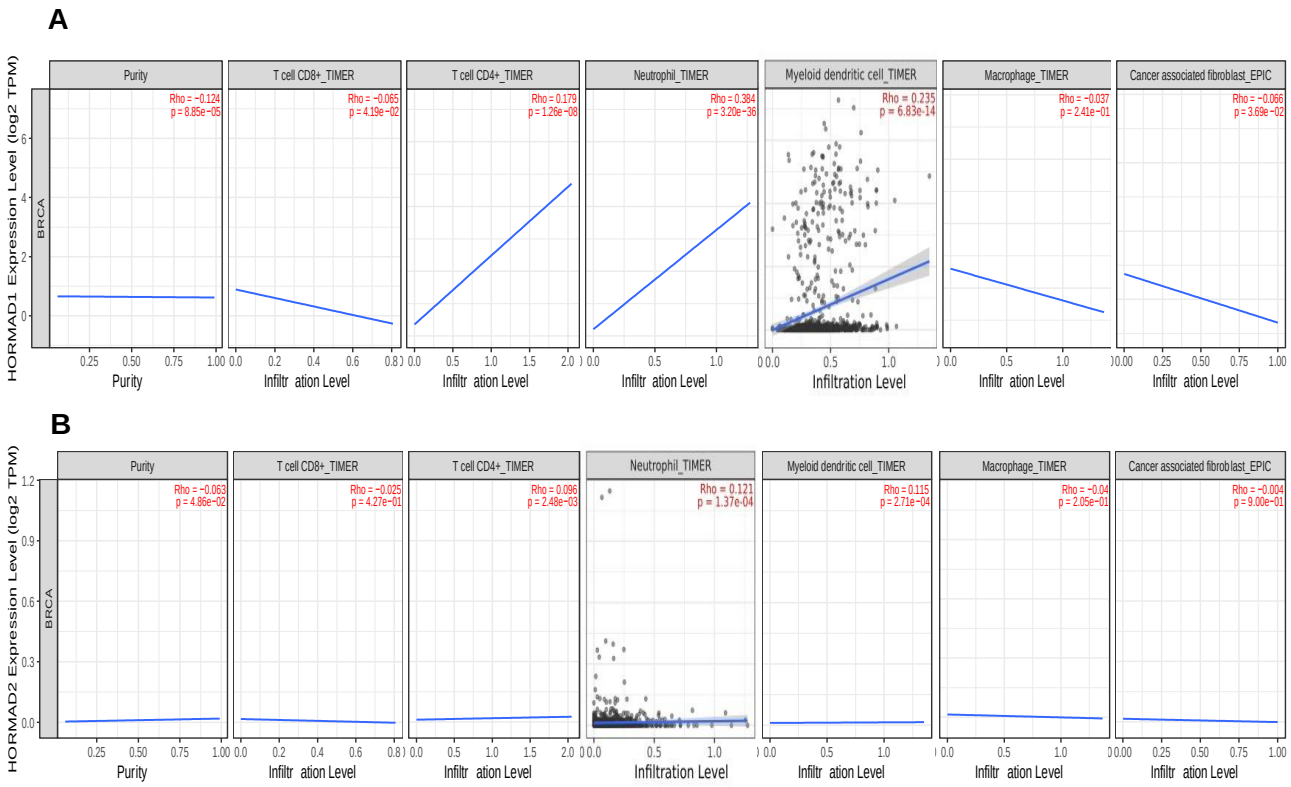

Figure 6. Correlations between (A) *HORMAD1* and (B) *HORMAD2* mRNA levels and immune cell infiltration, including purity, CD8+, CD4+, neutrophil, dendritic, macrophage, and cancer-associated fibroblast cells (TIMER2.0).

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

Gne	Purity		CD8+		CD4+		Neutrophil	
	R	p	R	p	R	p	R	p
HORMAD1	-0.124	**$8.85x10^{-5}$**	-0.065	**$4.1x10^{-2}$**	0.179	**$1.26x10^{-8}$**	0.384	**$3.20x10^{-36}$**
HORMAD2	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
HORMAD1	0.235	**$6.83x10^{-14}$**	-0.037	$2.41x10^{-1}$	-0.066	**$3.96x10^{-2}$**
HORMAD2	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 caner cells [21]. This observation was supported by a previous study where *HORMAD1* increased Akt and GSK3β 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 results 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 *HORMAD1* mRNA level and immune filtration in patients we re 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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REFERENCES

[1] Wilkinson L, Gathani T. Understanding breast cancer as a global health concern. Br J Radiol. 2022; 95(1130): 20211033. https://doi.org./10.1259/bjr.20211033

[2] Hartkopf AD, Grischke EM, Brucker SY. Endocrine-resistant breast cancer: Mechanisms and treatment. Breast Care (Basel). 2020; 15(4): 347-354. https://doi.org./10.1159/000508675

[3] Ramos A, Sadeghi S, Tabatabaeian H. Battling chemoresistance in cancer: Root causes and strategies to uproot them. Int J Mol Sci. 2021; 22(17):9451. https://doi.org./10.3390/ijms22179451.

[4] Riggio AI, Varley KE, Welm AL. The lingering mysteries of metastatic recurrence in breast cancer. Br J Cancer. 2021; 124(1): 13-26. https://doi.org./10.1038/s41416-020-01161-4

[5] Rimawi MF, De Angelis C, Schiff R. Resistance to Anti-HER2 Therapies in Breast Cancer. Am Soc Clin Oncol Educ Book. 2015 (35): e157-e164. https://doi.org./10.14694/EdBook_AM.2015.35.e157

[6] Fath MK, Azargoonjahromi A, Kiani A, Jalalifar F, Osati P, Oryani MA, Shakeri F, Nasirzadeh F, Khalesi B, Nabi-Afjadi M, Zalpoor H, Mard-Soltani M, Zahra Payandeh Z. The role of epigenetic modifications in drug resistance and treatment of breast cancer. Cell Mol Biol Lett. 2022; 27(1): 52. https://doi.org./10.1186/s11658-022-00344-6.

[7] Li A, Schleicher SM, Andre F, Mitri ZI. Genomic alteration in metastatic breast cancer and ıts treatment. Am Soc Clin Oncol Educ Book. 2020 (40): 30-43. https://doi.org./10.1200/EDBK_280463

[8] Liu K, Wang Y, Zhu Q, Li P, Chen J, Tang Z, Shen Y, Cheng X, Lu LY, Liu Y. Aberrantly expressed HORMAD1 disrupts nuclear localization of MCM8-MCM9 complex and compromises DNA mismatch repair in cancer cells. Cell Death Dis. 2020 ;11(7): 519. https://doi.org./10.1038/s41419-020-2736-1.

[9] Shin YH, Choi Y, Erdin SU, Yatsenko SA, Kloc M, Yang F, Wang JP, Meistrich ML, Rajkovic A. Hormad1 mutation disrupts synaptonemal complex formation, recombination, and chromosome segregation in mammalian meiosis. PLoS Genet. 2010; 6(11): e1001190. https://doi.org./10.1371/journal.pgen.1001190

[10] Daniel K, Lange J, Hached K, Fu J, Anastassiadis K, Roig I, Cooke HJ, Stewart AF, Wassmann K, Jasin M, Keeney S, Toth A. Meiotic homologue alignment and its quality surveillance are controlled by mouse HORMAD1. Nat Cell Biol. 2011; 13(5): 599-610. https://doi.org./10.1038/ncb2213

[11] Liu M, Chen J, Hu L, Shi X, Zhou Z, Hu Z, Sha J. HORMAD2/CT46.2, a novel cancer/testis gene, is ectopically expressed in lung cancer tissues. Mol Hum Reprod. 2012; 18(12): 599-604. https://doi.org./10.1093/molehr/gas033

[12] Watkins J, Weekes D, Shah V, Gazinska P, Joshi S, Sidhu B, Gillet C, Pinder S, Vanoli F, Jasin M, Mayrhofer M, Isaksson A, Cheang MCU, Mirza H, Frankum J, Lord CJ, Ashworth A, Vinayak S, Ford JM, Telli ML, Grigoriadis A, Tutt ANJ. Genomic complexity profiling reveals that HORMAD1 overexpression contributes to homologous recombination deficiency in triple-negative breast cancers. Cancer Discov. 2015; 5(5): 488-505. https://doi.org./10.1158/2159-8290.Cd-14-1092

[13] Chen B, Tang H, Chen X, Zhang G, Wang Y, Xie X, Liao N. Transcriptomic analyses identify key differentially expressed genes and clinical outcomes between triple-negative and non-triple-negative breast cancer. Cancer Manag Res. 2019; 11: 179-190. https://doi.org./10.2147/cmar.S187151

[14] Wang X, Tan Y, Cao X, Kim JA, Chen T, Hu Y, Wexler M, Wang X. Epigenetic activation of HORMAD1 in basal-like breast cancer: role in Rucaparib sensitivity. Oncotarget. 2018; 9(53): 30115-30227. https://doi.org./10.18632/oncotarget.25728

[15] Lin Q, Hou S, Guan F, Lin C. HORMAD2 methylation-mediated epigenetic regulation of gene expression in thyroid cancer. J Cell Mol Med. 2018; 22(10): 4640-4652. https://doi.org/10.1111/jcmm.13680

[16] Zhang X, Yu X. Crosstalk between Wnt/β-catenin signaling pathway and DNA damage response in cancer: a new direction for overcoming therapy resistance. Front Pharmacol. 2023; 14:1230822 https://doi.org./10.3389/fphar.2023.1230822

[17] Savio AJ, Daftary D, Dicks E, Buchanan DD, Parfrey PS, Young JP, Weisenberger D, Green RC, Gallinger S, McLaughlin JR, Knight JA, Bapat B. Promoter methylation of ITF2, but not APC, is associated with microsatellite instability in two populations of colorectal cancer patients. BMC Cancer. 2016; 16: 113. https://doi.org./10.1186/s12885-016-2149-9

[18] Zong B, Sun L, Peng Y, Wang Y, Yu Y, Lei J, Zhang Y, Guo S, Li K, Liu S. HORMAD1 promotes docetaxel resistance in triple negative breast cancer by enhancing DNA damage tolerance. Oncol Rep. 2021; 46(1):138. https://doi.org./10.3892/or.2021.8089

[19] El-Botty R, Vacher S, Mainguené J, Briaux A, Ibadioune S, Dahmani A, Montaudon E, Nemati F, Huguet L, Sourd L, Morriset L, Château-Joubert S, Dubois T, Maire V, Lidereau R, Rapinat A, Gentien D, Coussy F, Bièche I, Marangoni E. HORMAD1 overexpression predicts response to anthracycline-cyclophosphamide and survival in triple-negative breast cancers. Mol Oncol. 2023;17(10):2017-2028. https://doi.org./10.1002/1878-0261.13412

[20] Lin Q, Hou S, Guan F, Lin C. HORMAD2 methylation-mediated epigenetic regulation of gene expression in thyroid cancer. J Cell Mol Med. 2018; 22(10): 4640-4652. https://doi.org./10.1111/jcmm.13680.

[21] Pohl SG, Brook N, Agostino M, Arfuso F, Kumar AP, Dharmarajan A. Wnt signaling in triple-negative breast cancer. Oncogenesis. 2017; 6(4): e310. https://doi.org./10.1038/oncsis.2017.14

[22] Liu K, Cheng L, Zhu K, Wang J, Shu Q. The cancer/testis antigen HORMAD1 mediates epithelial–mesenchymal transition to promote tumor growth and metastasis by activating the Wnt/β-catenin signaling pathway in lung cancer. Cell Death Discov. 2022; 8(1): 136. https://doi.org./10.1038/s41420-022-00946-1
[23] Motwani J, Rodger EJ, Stockwell PA, Baguley BC, Macaulay EC, Eccles MR. Genome-wide DNA methylation and RNA expression differences correlate with invasiveness in melanoma cell lines. Epigenomics. 2021; 13(8): 577-598. https://doi.org./10.2217/epi-2020-0440
[24] Bartha Á, Győrffy B. TNMplot.com: A web tool for the comparison of gene expression in normal, tumor and metastatic tissues. Int J Mol Sci. 2021; 22(5):2622. https://doi.org./10.3390/ijms22052622
[25] Győrffy B. Survival analysis across the entire transcriptome identifies biomarkers with the highest prognostic power in breast cancer. Comput Struct Biotechnol J. 2021; 19: 4101-4109. https://doi.org/10.1016/j.csbj.2021.07.014
[26] Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Anders Jacobsen, Caitlin J Byrne, Michael L Heuer, Erik Larsson, Yevgeniy Antipin, Boris Reva, Arthur P Goldberg, Chris Sander, Nikolaus Schultz. The cbio cancer genomics portal: An open platform for exploring multidimensional cancer genomics data. Cancer Discovery. 2012; 2(5): 401-404. https://doi.org./10.1158/2159-8290.Cd-12-0095
[27] Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun Y, Jacobsen A, Sinha R, Larsson E, Cerami E, Sander C, Schultz N. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal. 2013; 6(269): pl1. https://doi.org./10.1126/scisignal.2004088
[28] Modhukur V, Iljasenko T, Metsalu T, Lokk K, Laisk-Podar T, Vilo J. MethSurv: A web tool to perform multivariable survival analysis using DNA methylation data. Epigenomics. 2018; 10(3): 277-288. https://doi.org./10.2217/epi-2017-0118
[29] Metsalu T, Vilo J. ClustVis: a web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap. Nucleic Acids Res. 2015; 43(W1): W566-W570. https://doi.org./10.1093/nar/gkv468
[30] Landrum MJ, Lee JM, Benson M, Brown GR, Chao C, Chitipiralla S, Gu B, Hart J, Hoffman D, Jang W, Karapetyan K, Katz K, Liu C, Maddipatla Z, Malheiro A, McDaniel K, Ovetsky M, Riley G, Zhou G, Holmes JB, Kattman BL, Maglott DR. ClinVar: Improving access to variant interpretations and supporting evidence. Nucleic Acids Res. 2018; 46(D1): D1062-D1067. https://doi.org./10.1093/nar/gkx1153
[31] Fekete JT, Győrffy B. ROCplot.org: Validating predictive biomarkers of chemotherapy/hormonal therapy/anti-HER2 therapy using transcriptomic data of 3,104 breast cancer patients. Int J Cancer. 2019; 145(11): 3140-3151. https://doi.org./10.1002/ijc.32369
[32] Bendl J, Stourac J, Salanda O, Pavelka A, Wieben ED, Zendulka J, Brezovsky J, Damborsky J. PredictSNP: Robust and accurate consensus classifier for prediction of disease-related mutations. PLoS Comput Biol. 2014; 10(1): e1003440. https://doi.org./10.1371/journal.pcbi.1003440
[33] Stone EA, Sidow A. Physicochemical constraint violation by missense substitutions mediates impairment of protein function and disease severity. Genom Res. 2005; 15(7): 978-986. https://doi.org./10.1101/gr.3804205
[34] Capriotti E, Fariselli P, Calabrese R, Casadio R. Predicting protein stability changes from sequences using support vector machines. Bioinformatics. 2005; 21 (Suppl 2):ii54-58. https://doi.org./10.1093/bioinformatics/bti1109
[35] Ramensky V, Bork P, Sunyaev S. Human non- synonymous SNPs: server and survey. Nucleic Acids Res. 2002; 30(17): 3894-3900. https://doi.org./10.1093/nar/gkf493
[36] Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS, Sunyaev SR. A method and server for predicting damaging missense mutations. Nat Methods. 2010; 7(4): 248-249. https://doi.org./10.1038/nmeth0410-248
[37] Sim NL, Kumar P, Hu J, Henikoff S, Schneider G, Ng PC. SIFT web server: predicting effects of amino acid substitutions on proteins. Nucleic Acids Res. 2012; 40(Web Server issue): W452-457. https://doi.org./10.1093/nar/gks539
[38] Bromberg Y, Rost B. SNAP: predict effect of non-synonymous polymorphisms on function. Nucleic Acids Res. 2007; 35(11): 3823-3835. https://doi.org./10.1093/nar/gkm238
[39] Tang H, Thomas PD. PANTHER-PSEP: predicting disease-causing genetic variants using position-specific evolutionary preservation. Bioinformatics. 2016; 32(14): 2230-2232. https://doi.org./10.1093/bioinformatics/btw222
[40] Pejaver V, Mooney SD, Radivojac P. Missense variant pathogenicity predictors generalize well across a range of function-specific prediction challenges. Hum Mutat. 2017; 38(9): 1092-1108. https://doi.org./10.1002/humu.23258
[41] Shihab HA, Gough J, Cooper DN, Stenson PD, Barker GL, Edwards KJ, Day INM, Gaunt TR. Predicting the functional, molecular, and phenotypic consequences of amino acid substitutions using hidden Markov models. Hum Mutat. 2013; 34(1): 57-65. https://doi.org./10.1002/humu.22225
[42] Cheng J, Randall A, Baldi P. Prediction of protein stability changes for single-site mutations using support vector machines. Proteins. 2006; 62(4): 1125-1132. https://doi.org./10.1002/prot.20810
[43] Capriotti E, Calabrese R, Casadio R. Predicting the insurgence of human genetic diseases associated to single point protein mutations with support vector machines and evolutionary information. Bioinformatics. 2006; 22(22): 2729-2734. https://doi.org./10.1093/bioinformatics/btl423
[44] Li T, Fu J, Zeng Z, Cohen D, Li J, Chen Q, Li B, Liu XS. TIMER2.0 for analysis of tumor-infiltrating immune cells. Nucleic Acids Res. 2020; 48(W1): W509-w514. https://doi.org./10.1093/nar/gkaa407