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In silico Evaluation of *WWC1* in Melanoma Using Bioinformatic Analyses

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ABSTRACT

Objective: It is suggested that *WWC1* has an active role in melanoma progression. Therefore, it was aimed to evaluate the *WWC1* gene expression profiles in melanoma, an aggressive malignant skin tumor.

Materials and Methods: Quantitative data from melanoma samples (n=592) were clinically evaluated using cBioPortal. Gene expression (GSE65904 and GSE22155) and gene methylation datasets (GSE120878) were retrieved from the Gene Expression Omnibus (GEO) database. Using the GeneMANIA database, the functions of given genes and pathways were evaluated. The STRING database achieved a protein-protein interaction (PPI) network was used to visualize it.

Results: Mutations in the *WWC1* were found in 6.7% of all melanoma samples, 8% of skin cutaneous melanoma, and 2.8% of metastatic melanoma. When the GeneMANIA platform was used to analyze gene interactions, it was determined that the *WWC1* gene shared common protein domains with three genes, was co-expressed with five genes, and interacted with 17 other genes. According to the function analysis results, the most effective of the ten functions of *WWC1* was Hippo signaling, with a coverage value of 0.16 (p=0.009). In addition, it then played a role in Notch signaling and organ growth. When the protein-protein interactions were examined, it was determined that it interacted with ten proteins and was co-expressed with nine.

Conclusion: The findings demonstrated the potential of *WWC1* to be effective in the progression of melanoma. Further research is needed to provide a more accurate analysis of *WWC1* expression and methylation.

Keywords: Melanoma, *WWC1*, KIBRA, Bioinformatic mining, GEO

INTRODUCTION

The skin is the body's biggest organ that serves as a protective barrier, regulating body temperature and preventing fluid loss. The epidermis, the outer layer of the skin, and the dermis underneath are the two main layers of the skin. It contains four main types of cells: keratinocytes (squamous cells, basal cells), Langerhans cells, Merkel cells, and melanocytes (1). The malignant form of melanocytes is called melanoma. Melanoma constitutes around 2% of all malignant skin cancers but is the deadliest (2). Studies show that melanoma incidence rises yearly as UV radiation exposure increases (3,4). Even though surgery may be the only curative option for the majority of patients with early-stage cutaneous melanoma, it is ineffective for those with metastatic melanoma (5). Most research is moving in this direction as preventive and predictive biomarkers, and drug targets are needed to improve the accuracy of melanoma diagnosis and treatment.

Differentially expressed genes are identified during the progression of melanoma (6). Genes that are variably expressed during melanoma progression are thought to be targets. As a result of the bioinformatics analyses applied to the microarray datasets taken from the Gene Ex-

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pression Omnibus (GEO) database, 142 differentially expressed genes were detected in melanoma by Xia et al (7). In addition, epigenetic regulation of genes expressed differently in melanoma was also obtained through bioinformatic analysis. Moreover, it was concluded that the expression change of interleukin 27 (IL-27) in melanoma may be effective on cytokine-based immune therapy (8). Li et al. (9) detected 266 miRNAs that expressed differently in melanoma and emphasized the possibility of miRNA and target genes as prognostic and therapeutic biomarkers. On the other hand, frequently mutated genes, such as *BRAF*, are therapeutic targets for melanoma.

The Hippo signaling pathway is an effective regulator of cell proliferation and differentiation (10). Dysregulation of the Hippo pathway can cause tumorigenesis (11,12). The mammalian Hippo pathway includes two major effectors containing WW domain such as Yes Associated Protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) (13). It has been reported that WW and C2 domain-containing (WWC) protein family (WWC1, WWC2, WWC3*)* regulate the Hippo pathway (14–17). Overexpression of WWC1, also called KIBRA (KIdney and BRAin) because of its high expression in kidney and brain, increases the phosphorylation of YAP/TAZ. It has been shown both *in vitro* and *in vivo* that the Hippo pathway supports melanoma invasion by increasing YAP activity (18,19). Considering the role of *WWC1* in the Hippo pathway, no research was found regarding its relationship with melanoma. This study aimed to evaluate *the WWC1*, one of the regulator of the Hippo pathway, in melanoma by *in silico* analysis methods.

MATERIALS AND METHODS

Datasets Used for Gene-based Identification and Analysis of Clinical Data

Quantitative data from a total of 592 melanoma samples (S), including metastatic melanoma (MM) (DFCI, Nature Medicine 2019; n=144) and skin cutaneous melanoma (SCM) (TCGA, Pan-Cancer Atlas; n=448), were evaluated by using cBioPortal for Cancer Genomics (https://www.cbioportal.org/) in the study. The GEO2R analysis tool provided by The National Center for Biotechnology Information (NCBI) was used to evaluate data sets [GSE65904 (10,11), GSE22155 (12), and GSE120878 (13)] from GEO database to determine whether *WWC1* was associated with melanoma carcinogenesis and progression.

The cBioPortal was used to access the clinical information of patients. A total of 15 separate datasets were identified by the "melanoma" search in cBioPortal. As a result of the examination, two datasets were included in the study. Clinical data from 592 melanoma samples without any parameter distinction, including metastatic melanoma (DFCI, Nature Medicine 2019; n=144) and skin cutaneous melanoma (TCGA, Pan-Cancer Atlas; n=448), were analyzed using Graphpad 8.

Identifying RNA-seq Based Datasets

In the analyzes obtained from the all results of the study, repeated readings were combined in to single data, and their averages were included.

Analysis of Mutation Data

The sequence data of 592 patients collected through cBioPortal was compared to the reference genome. Two sets of melanoma (MM and SCM) data were examined for the mutation pattern of *WWC1*. Genomic alteration analysis was used to identify the genes that interact with *WWC1*.

Analysis of Expression Data

Firstly, the menu "resources" then the "gene and expressions" tab and the Gene Expression Omnibus database tab were switched on in NCBI. The keyword "melanoma" was searched in the GEO database. The results were scanned with "Expression profiling by array" and "Methylation profiling by array" sub-filters. GSE65904, GSE22155, and GSE120878 datasets were found suitable for the study. The raw file formats of GSE65904, GSE22155, and GSE120878 datasets were downloaded (10-13). The expression data were analyzed using GraphPad Prism 8. The *WWC1* expression data were analyzed along with all genes considered to be involved. Finally, the possible role of *WWC1* in the diagnosis was investigated due to the ROC curve analysis performed.

WWC1 Network Analysis

The *WWC1* was examined regarding gene interaction and functional relationships on the GeneMANIA platform (www.genemania.org), and proteins associated with the WWC1 (KIBRA) protein, and their degree of association were determined via the STRING database (www.string-db.org).

Statistical Analysis

The data obtained from all analyzes were accepted as significant in the 95% confidence interval, $p<0.05$ conditions. Normality, ANOVA, the Mann-Whitney U, the Wilcoxon Test and, Student t-test were used to compare numerical values of GSE65904, GSE22155, and GSE120878 datasets, respectively. To find statistical differences between categorical variables, the Chi-square test was utilized. The ROC curve analysis was used to analyze the expression coefficient. The Log-rank (Mantel-Cox) method was used to calculate the survival curves.

RESULTS

Results from cBioPortal

The study included two datasets containing 592 patients according to the determined keywords. Statistically significant results were obtained in 14 of 79 clinical parameters examined in patients with the *WWC1* mutations (n=39) of total melanoma samples (n=592). The samples with the *WWC1* mutations in mutation counts ($p<0.001$) and total mutations ($p=0.020$) were found to be significantly higher than samples without the *WWC1* mutations ($p<0.001$), (Figures 1a and 1b). When all samples were evaluated according to their histological classification, it showed that the LMM (Lentigo malignant melanoma) and NOS (Not otherwise specified) structures were high in samples with the *WWC1* mutations ($p<0.001$) by cBioPortal. The UV-induced mutations in patients with *WWC1* mutations were observed significantly more than in patients without *WWC1* mutations ($p=0.035$), (Figure 1c). The mutation that is present in a subset of tumor cells is defined as subclonal. The subclonal mutation development in samples with *WWC1* mutations was significantly high (p=0.010). It was determined that there was a significant difference in the early diagnosis of the disease in patients with *WWC1* mutations (p=0.036). When the expression correlation between genes that play an important role in melanoma progression was evaluated, it was determined that *WWC1* was significantly associated with *BRAF*. The relationship based on the amount of mutation was similar to that shown in the gene expression data (r=-0.12; p=0.012), (Figure 1d and Table 1).

Results from GEO2R

When the GSE22155 (including 18 patients with lymph node metastases and 38 patients with subcutaneous metastases) dataset, the *WWC1* expression was evaluated in terms of clinical parameters. These parameters were sex, age at metastases, type of metastases, age at primary diagnosis, localization of primary melanoma, Breslow, Clark, stage, *BRAF/NRAS*, *CDKN2A,* homo-

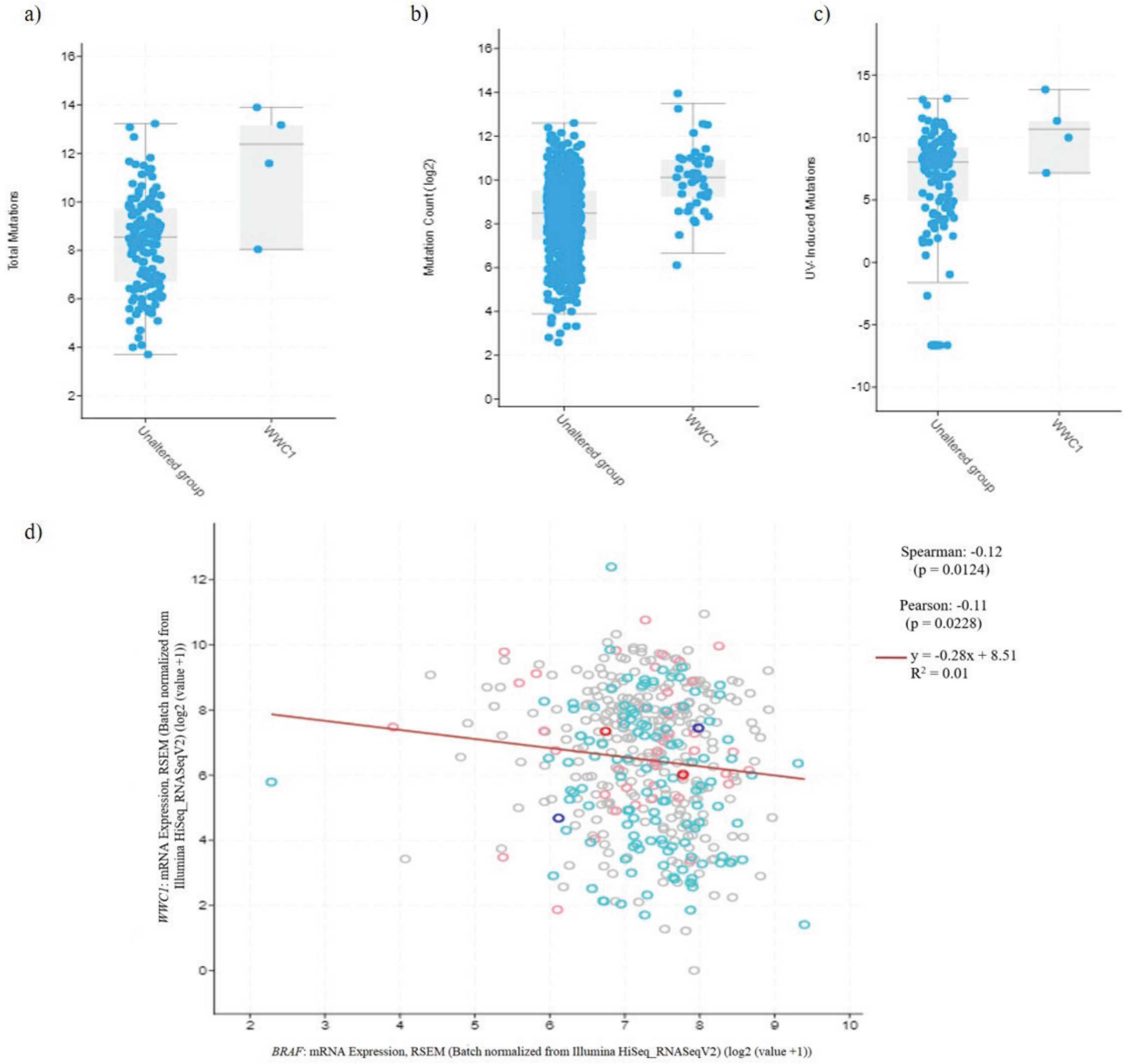

Figure 1. Alterations of *WWC1* determined with cBioPortal. a) Total mutations in all melanoma samples with *WWC1* mutations and unaltered group, b) Mutation count in all melanoma samples with *WWC1* mutations and unaltered group, c) UV-induced mutations in patients with *WWC1* mutations and unaltered group, d) Correlation analysis between *BRAF* and *WWC1*. Graphs through cBioPortal database.

Table 1. Clinical data were obtained from cBioPortal.

Clinical Attribute	**Attribute Type**	**Statistical Test**	**p-Value**
MUTATION STATUS			
Total mutations	• Sample	Wilcoxon Test	0.020
Mutation count	• Sample		<0.001
Tumor mutational burden, nonsynonymous	• Sample		<0.001
Mutation sub-clonal	• Sample		0.010
Mutation clonal	• Sample		0.003
UV-induced mutations	• Patients		0.035
CLASSIFICATION			
Histology	• Sample	Chi-squared Test	<0.001
International classification of diseases for oncology, Third Edition ICD-O-3 Histology code	• Patient		<0.001
Oncotree code	• Sample		0.026
Cancer type detailed	• Sample		0.026
DIAGNOSIS AND TREATMENT			
Immunotherapy	• Patient	Chi-squared Test	0.010
Prior diagnosis	• Patient		0.036

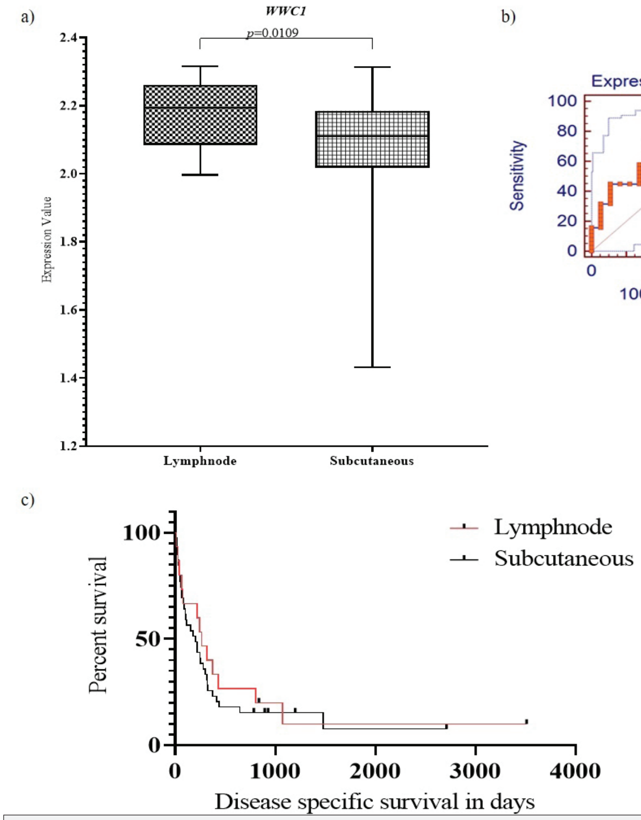

Figure 2. a) The statistical analysis of *WWC1* expression in lymph node and subcutaneous, b) ROC curve analysis of *WWC1* expression in lymph node and subcutaneous, c) *WWC1* expression effect on survival of the lymph node and subcutaneous metastasis groups. Values represent the mean ± SD, p<0.05. Graphs through GEO2R.

zygous deletion, germline, molecular subtype, *CD3*, *CD20*, and *Ki67*. A statistically significant difference was found only in the type of metastases parameter (p=0.010), (Figure 2a). As a result of the ROC curve analysis performed for the type of metastasis, the threshold value for *WWC1* expression of the subcutaneous group was found to be below 2.15 (73.68% sensitivity; 63.16% specificity), (p=0.004), (Figure 2b). The *WWC1* expression in the lymph node and subcutaneous metastasis groups did not have significant effect on survival (p=0.547), (Figure 2c).

As a result of the analysis of the GSE65904 dataset, a statistically significant difference was found between the *WWC1* expression of patients with regional lymph node metastases and the general patient group, including more than 50% of patients with metastases to internal organs (Figure 3a). It was determined that the *WWC1* expression of patients with regional lymph node metastases was less than 2.13 (sensitivity 47.8%, 90.7% specificity), and the general group was found to be more than 2.13 by ROC curve analysis (p<0.001), (Figure 3b). Because there were more than 50% of patients with internal organ metastases, the survival analysis revealed that the group labeled "General" had statistically significantly lower survival than the group with metastasis to the regional lymph node [Log-rank (Mantel-Cox) test], (p<0.001), (Figure 3c). A statistically significant difference was found in the *WWC1* expression between the cutaneous and lymph node locations of melanoma (p=0.023), (Figure 3d). The threshold value determined for *WWC1* expression at the tumor localization was determined as 2.08 (52.2% sensitivity; 72.7% specificity) (p=0.018), (Figure 3e). If the *WWC1* expression was higher than 2.08, the tumor was located in the cutaneous layer, and if it was below 2.08, lymph node metastasis was detected. No statistically significant effect of *WWC1* on survival was found between cutaneously located tumor samples and lymph node metastasis samples [Log-rank (Mantel-Cox)] test, (p=0.114), (Figure 3f).

As a result of the analysis of the GSE120878 dataset, it was determined that *WWC1* methylation expression was significantly different between invasive localized melanoma samples and nevus samples (p<0.001), (Figure 4a). As a result of the ROC curve analysis, it was determined that ≤ 0.618 *WWC1* methylation amount was the threshold value between invasive localized melanoma samples and nevus samples (84.93% sensitivity; 75.28% specificity), (p<0.001), (Figure 4b).

Results of *WWC1* Network Analysis

Using the GeneMANIA platform to analyze the gene interactions, it was determined that the gene shared protein domains with three genes and physically interacted with 17 other genes in addition to being co-expressed with five other genes. In light of these findings, the three genes with the highest co-expression relationship with *WWC1* are *RBM47* (RNA Binding Motif Protein 47), *SH3YL1* (SH3 domain-containing YSC84-like protein 1), and *CLDN7* (Claudin-7) respectively. When evaluating phys-

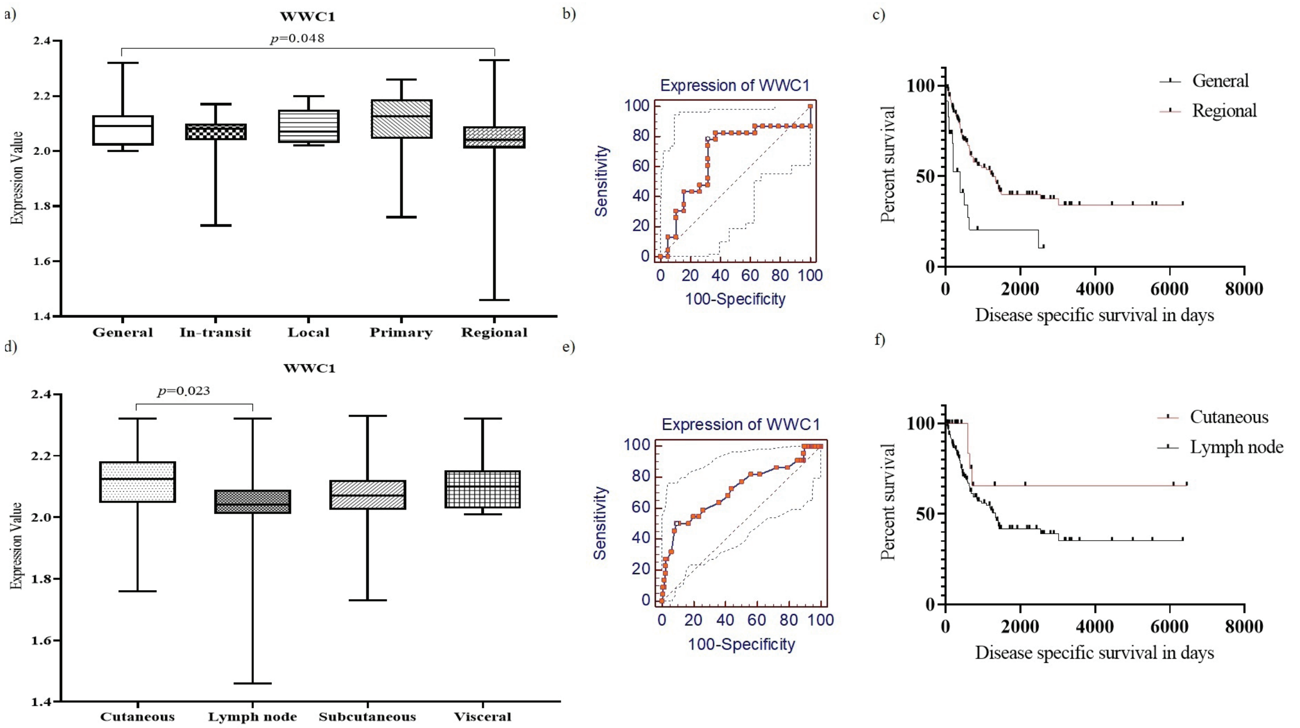

Figure 3. a) The statistical analysis of *WWC1* expression in tissues, b) ROC curves analysis of *WWC1* expression in tissues, c) *WWC1* expression effect on survival of the tissues. d) The statistical analysis of *WWC1* expression in tumor localization, e) ROC curve analysis of *WWC1* expression in tumor localization, f) *WWC1* expression effect on survival of the patients with different localized tumors. The graphs were performed through the GSE65904 dataset. Values represent the mean ± SD, p<0.05.

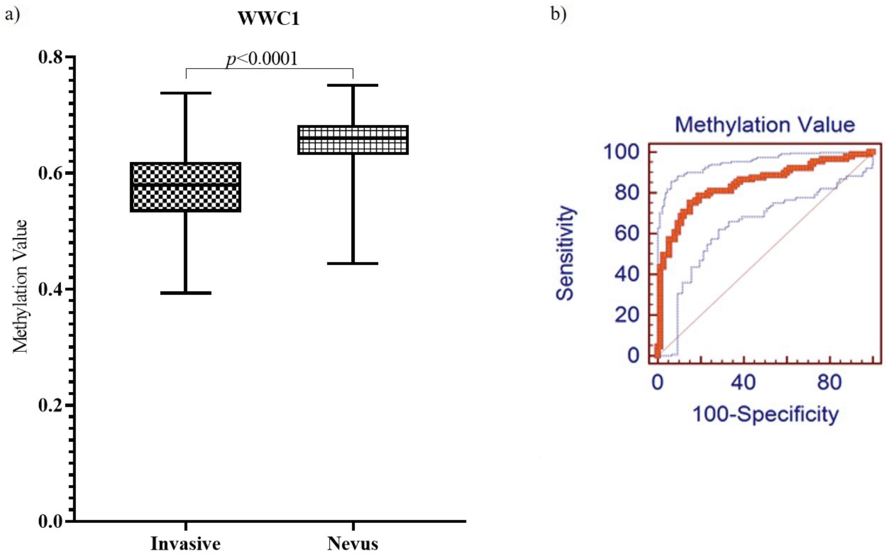

Figure 4. Analysis from GSE120878 dataset. a) The statistical analysis of *WWC1* expression in invasive tumor samples and nevus samples, b) ROC curve analysis of *WWC1* expression in invasive tumor samples and nevus samples. Values represent the mean ± SD*, p<0.0001.

ical interactions, it was determined that the highest were *NF2* (Moesin-Ezrin-Radixin Like (MERLIN) Tumor Suppressor), *SNX4* (Sorting nexin-4), and *DDR1* (Discoidin Domain Receptor Tyrosine Kinase 1) respectively. YAP1 (Yes1 Associated Transcriptional Regulator), WWC2 (WW and C2 domain containing 2) , and NEDD4 (NEDD4 E3 Ubiquitin Protein Ligase) are the three proteins that share the most protein domains with WWC1 (Figure 5 and Table 2).

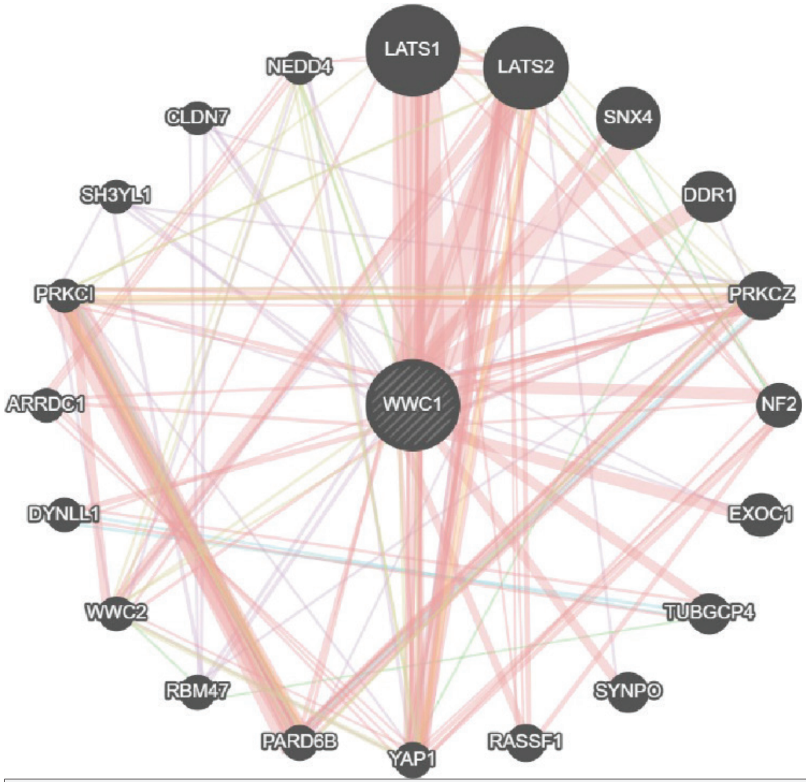

Figure 5. Gene interaction of *WWC1* diagram from GeneMANIA. *WWC1* most interacts with *LATS1* and *LATS2* in the diagram. The diagram shows physical interactions, co-expressions, genetic interactions, co-localizations, and shared of genes.

Table 2. Proteins that co-expressed with WWC1 that obtained from STRING (Clustering Method: Kmeans).

Cluster Number	Cluster Color	Gene Count	Protein Name	Protein Identifier	Protein Description
1	Red	3	LATS1	ENSP00000437550	Serine/threonine-protein kinase LATS1 and LATS2; Negative regulator of YAP1 in the Hippo signaling pathway.
1	Red	3	LATS2	ENSP00000372035	
1	Red	3	NF2	ENSP00000344666	Along with WWC1 can function in the regulation of the Hippo/SWH (Sav/Wts/Hpo) signaling pathway.
2	Green	7	DDN	ENSP00000390590	Dendrin; Promotes apoptosis of kidney glomerular podocytes.
2	Green	7	FRMD6	ENSP00000343899	Ferm domain-containing protein 6; upstream regulator of the Hippo signaling.
2	Green	7	INADL	ENSP00000360200	InaD-like protein; Scaffolding protein that may bring different proteins into adjacent positions at the cell membrane.
2	Green	7	PTPN14	ENSP00000355923	Tyrosine-protein phosphatase non-receptor type 14; Acts as a negative regulator of the oncogenic property of YAP.
2	Green	7	SNX4	ENSP00000251775	Sorting nexin-4; May be involved in several stages of intracellular trafficking.
2	Green	7	SYNPO	ENSP00000377789	Synaptopodin; Actin-associated protein that may play a role in modulating actin-based shape and motility of dendritic spines.
2	Green	7	WWC1	ENSP00000427772	Protein KIBRA; Probable regulator of the Hippo/SWH signaling pathway.
3	Blue	1	FRMD1	ENSP00000283309	Ferm domain-containing protein 1; May be a regulator of hippo signaling.

Table 3. Functions of *WWC1* (Table from GeneMANIA).

Function	FDR (False Discovery Rate)		Genes in Network	Genes in Genome	Coverage
Organ growth	0.007	0.77%	4	58	0.07
Hippo signaling	0.009	0.99%	3	19	0.16
Notch signaling pathway	0.025	2.59%	4	103	0.04
Regulation of developmental growth	0.100	10.04%	4	156	0.03
Apical junction assembly	0.152	15.23%	3	68	0.04
Tight junction organization	0.152	15.23%	3	73	0.04
Regulation of Notch signaling pathway	0.152	15.23%	3	73	0.04
Tight junction assembly	0.152	15.23%	3	69	0.04
Regulation of protein localization to nucleus	0.205	20.55%	3	91	0.03
Intracellular steroid hormone receptor signaling pathway	0.205	20.55%	3	95	0.03

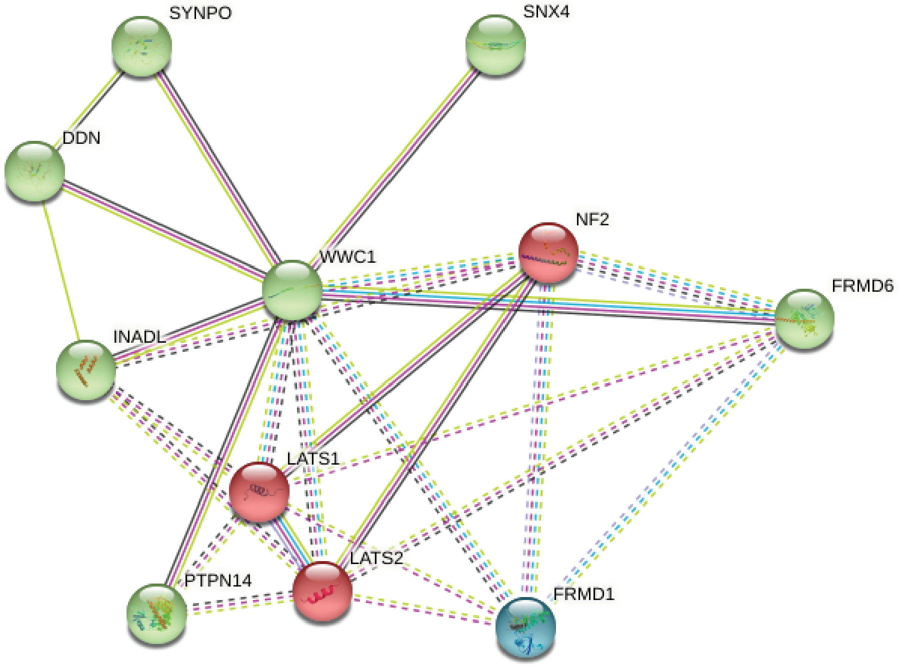

Figure 6. Protein interactions of WWC1 diagram from STRING. WWC1 interacted with ten proteins and co-expressed nine-tenths except for SNX4.

The *WWC1* had ten separate functions, and the function analysis results showed that it provided the most effective Hippo signaling, with a coverage value of 0.16 (FDR=0.009, Table 3). After that, it contributed to Notch signaling and organ development. It was discovered through protein-protein interactions that it interacted with ten proteins and co-expressed with nine of them (Figure 6).

DISCUSSION

The incidence of melanoma increases each year with increased exposure to sunlight. Following the increasing incidence, melanoma studies interested in melanoma etiology, pathogenesis, new diagnostic techniques, and potential therapeutic approaches are developing rapidly. However, the addition of unpredictable environmental factors that increase oncogenic activity factors such as tumor heterogeneity and drug resistance limit treatment options, shorten patient survival, and adversely affect the stability of treatment. Oncogenomics has advanced thanks to recent developments in high-throughput genome analysis tools, including next-generation sequencing (NGS) and microarray-based techniques (20). These resources are essential for the growth of cancer genomic projects including the International Cancer Genome Consortium (ICGC; https://icgc.org/) and The Cancer Genome Atlas (TCGA; http://cancergenome.nih.gov/). These projects have made it possible to evaluate the genetic, epigenetic, and omic information of cancer patients from around the world. The main aims of the programs are to advance personalized treatment, better understand the molecular pathways of complex diseases like cancer and communicate the consequences on clinical phenotypes as datasets available to all researchers (21). Several oncogenic websites have been developed to help access the numerous cancer datasets in response to this aim. The cBioPortal website includes genomic information for several cancer types, such as copy number variations, mRNA and microRNA expression, DNA methylation, and protein.

The analysis performed on the cBioPortal platform revealed a positive correlation between the accumulation of mutations and the *WWC1* mutation. Knight et al. reported that 5q deletion on tumor development and metastatic progression were significantly affected by KIBRA (22). As a result of our analysis, the *WWC1* mutations showed a statistically significant increase in LMM, one of the histological subclasses of melanoma. There are molecular differences between melanoma and histological subclasses, and the molecular differences are consistent with our findings (23). Tumor heterogeneity, as with all malignancies, is one of the limitations of treatments. One of the significant sources of heterogeneity, sub-clonal mutation, causes the intercellular genomic sequence to vary from one another.

Additionally, *WWC1* mutations were seen, particularly during subclonal development. It is assumed that *WWC1* may facilitate subclonal formation (24). The changes that affect gene expression such as transcript levels and protein expression may accompany *WWC1* mutations. A study revealed a correlation between the *BRAF* expressions and *WWC1,* which actively contributed to the growth of melanoma (23). Changes in the expression of many genes occurred in the onset and progression of melanoma (6). Detecting these changes will benefit both early diagnosis and narrow the treatment options according to the needs of the patient (25,26). Results obtained from the GSE65904 dataset showed that the expression of the *WWC1* gene helped to know whether the tumor was located cutaneously or in the regional lymph node. Patients who have metastasized to internal organs were approximately 50% of the general patient population as described by the GSE65904 dataset. Compared to the general patient population, patients with regional lymph node metastases displayed higher levels of the *WWC1* gene, demonstrated a tumor suppressor characteristic in melanoma. *WWC1* has also been a tumor suppressor in triple-negative breast cancer, clear cell renal cell carcinoma, and hepatocellular carcinoma studies (17,22,27).

Interestingly, when the GSE22155 dataset was analyzed, it was found that the *WWC1* expression was increased in melanoma patients with stage 4 lymph node metastases when compared to those with subcutaneous metastases. In previous studies, conflicting results regarding the tumor suppressor property of *WWC1* expression were presented, and that it could exhibit different behaviors in different cancer types (17,28,29). In addition, it was observed that its epigenetic regulation played an active role in cancer progression. Studies showed that the tumor suppressor property of *WWC1* was inhibited by silencing through methylation in the promoter region. Therefore, *in vitro* and *in vivo* analyzes are needed to determine its characteristic feature in melanoma.

KIBRA (WWC1), one of the proteins of the Hippo pathway, which has an important role in tumorigenesis, was reported to interact with both genes and proteins in cancers (30). As a result of the analysis using GeneMANIA, the *WWC1* was co-expressed with genes that supported the migration, proliferation, and development of cancer cells (31,32). In addition, it was found that *WWC1* in melanoma exhibited co-expression with *CLDN-7*, the seventh member of the claudin family, in which expression dysregulation was associated with cell migration (33,34). Murray et al. (35) showed that increased merlin (*NF2*) expression has a suppressive role in the development of melanoma both *in vitro* and *in vivo*. It was determined that increased *NF2* expression suppressed proliferation, migration and invasion in melanoma cells, and tumor volume and invasion in the *in vivo* melanoma model (35). In the study, it was determined that there was a physical interaction between *WWC1* and *NF2*, which played a role in activating the Hippo pathway. The significant suppression of melanoma cell proliferation by a *DDR* tyrosine kinase inhibitor (DDR1-IN-1) *in vitro*, *ex vivo*, and in tumor xenografts highlighted the potential of *DDR1* inhibition in melanoma. The interaction of WWC1 with the protein products of the same genes was determined by the STRING database. Other genes (*DDR1*, *YAP1*, and *NEDD4*), which were found to interact physically with *WWC1* in our study, and were also shown to be the genes involved in the development of melanoma in previous studies (18,36,37).

CONCLUSION

It is the first study to show that *WWC1* may have an impact on the progression of melanoma. The effects of changes in the *WWC1* and *WWC1*-related genes in melanoma are predicted to become clear in the future.

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