Prognostic and Clinical Implications of WNK Lysine Deficient Protein Kinase 1 Expression in Patients With Hepatocellular Carcinoma

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Abstract. *Background/Aim:* Hepatocellular carcinoma (HCC) is a particularly malignant form of cancer prevalent throughout the world; however, there is a pressing need for HCC biomarkers to facilitate prognosis and risk assessment. *Patients and Methods:* This paper reports on the potential prognostic value of WNK lysine deficient protein kinase 1 (WNK1) in cases of HCC. We analyzed the expression of WNK1 at the mRNA level using omics data from the UALCAN database. We then verified our findings through the immunohistochemical (IHC) staining of various human cancer tissue as well as 59 HCC samples paired with corresponding normal tissues. The prognostic value of mRNA or protein expression by WNK1 was evaluated using the Kaplan-Meier method. *Results:* Initial screening results revealed significantly higher WNK1 expression levels in HCC tissue compared to normal tissue. Verification using the paired HCC samples confirmed that the expression of WNK1

was indeed significantly higher in HCC tissue samples than in adjacent normal tissues. High WNK1 expression levels were significantly correlated with clinicopathological variables, including gender and histologic grade. Kaplan-Meier survival analysis revealed that high WNK1 expression levels were associated with poor HCC prognosis. Finally, univariate and multivariate analysis identified WNK1 as a prognostic factor for TNM stage in cases of HCC. *Conclusion:* In summary, WNK1 is overexpressed at the mRNA and protein levels, and correlated with poor prognosis. Thus, WNK1 expression could potentially be used as a biomarker in HCC prognosis.

Hepatocellular carcinoma (HCC) is responsible for an enormous number of cancer-related deaths, particularly in sub-Saharan Africa and Southeast Asia (1, 2). HCC is among the deadliest forms of cancer, and the treatment of HCC has been well demonstrated in multiple series, with an overall 5-year survival rate between 33% and 55% (3). The most common risk factors include infections with hepatitis B virus (HBV) or hepatitis C virus (HCV), chronic exposure to aflatoxin B (AFB1), excessive alcohol consumption (EAC), non-alcoholic steatohepatitis (NASH), and metabolic syndrome (METS). HCC commonly progresses from chronic liver inflammation and cirrhosis, which eventually trigger the formation of liver tumors (4, 5).

The fact that the early symptoms of HCC are not obvious means that at the time of diagnosis, most patients are in the middle or late stages of the disease (*i.e.*, beyond the window for surgery). Note that 50% of patients who undergo radical surgical

This article is freely accessible online.

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Key Words: Hepatocellular carcinoma, immunohistochemistry, WNK1, prognostic marker, survival.

resection experience tumor relapse and metastasis (6). At present, many of the details pertaining to hepatocarcinogenesis have yet to be elucidated. Researchers are actively seeking to identify diagnostic markers to enable the early detection and classification of these cancers and to guide medical interventions, such as gene targeted therapy (7, 8).

WNK lysine deficient protein kinase 1 (WNK1) is located on human chromosome 12 (12p13.33). It was first discovered in human kidneys. The WNK1 gene is a member of the WNK subfamily of serine/threonine protein kinases (9), which functions as an enzyme encoding a 230 kDa protein (2382 amino acids) (10). Mammals produce four WNK proteins, of which WNK1 is the largest (>2,000 residues) and most extensively expressed (11). WNK1 has the ability to dynamically regulate ion channels and cell volume. Note that WNK1 is activated under the pathological conditions of injury, inflammation, and hyperosmotic stress (12). The expression of SPS1-related proline/alanine-rich kinase (SPAK) and oxidative stress-responsive kinase 1 (OSR1) are regulated by WNK1 activation, which modulates the downstream expression of the sodium-potassium chloride cotransporter (NKCC1) and potassium-chloride cotransporter (KCC2) (13). NKCC1 and KCC2 levels have been shown to regulate the concentration of intracellular Cl^- , which plays a key role in hyperalgesia and allodynia in peripheral inflammation and nerve injury (13-15).

WNK1 is similar to proteins of the mitogen-activated protein kinase (MAPK) pathway, as evidenced by the fact that it can be activated downstream of receptor tyrosine kinases. WNK1 can also be activated downstream of AKT in the epidermal growth factor receptor (EGFR) and insulin-like growth factor 1 (IGF1) signaling pathways, particularly in human embryonic kidney cells (16-18). WNK1 has been shown to regulate the decidualization, proliferation, and migration of endometrial stromal cells (18). Researchers have also noted the involvement of WNK1 in many processes associated with carcinogenesis. WNK1 has been shown to promote the proliferation of various cell types, such as prostate carcinoma and neural progenitor cells (19, 20). It also regulates cell migration and invasiveness in several tumor cell lines, including breast cancer, glioma, and pancreatic ductal adenocarcinoma. *In-vivo* analysis using mouse and zebrafish models has revealed that WNK1 promotes tumor metastasis in HCC and prostate adenocarcinoma, and plays a vital role in the angiogenesis of vascular remodeling and endothelial sprouting (20-27). Nonetheless, the actual role played by WNK1 in hepatocarcinogenesis has yet to be elucidated.

The primary objective in the current study was to investigate the expression patterns of WNK1 in HCC tumors and paracancerous tissue. We analyzed omics data obtained from the UALCAN database (28) to predict the expression status of WNK1 and its correlation with the survival of HCC

patients. We also performed IHC staining of HCC tumor tissues and normal tissues to confirm the above results. Finally, we assessed the prognostic and clinical implications of WNK1 expression.

Patients and Methods

Patients and tissue sections. Multiple organ normal tissue microarrays and multiple cancer tissue microarrays were purchased from Super Bio Chips Laboratories, South Korea (Catalog number: MBN4 and MB4) from the breast, liver, urinary bladder, ovary, pancreas and prostate tissues. Tissue microarrays of liver cancer (59 samples matched with microarrays of normal adjacent tissue) were also purchased from Super Bio Chips Laboratories (Catalog number: CSN5 and CS5). Each of the samples included clinical data, such as age, gender, diagnosis, TNM stage, tumor stage, and survival data. In accordance with guidelines outlined by the American Joint Committee on Cancer (AJCC), 7th edition (29), pathologists blinded to the patient data were tasked with classifying the tumor stages and tumor grades based on the TNM staging system guidelines. Written informed consent was provided by all patients and/or their families. The research method was based on Helsinki and HIPPA-approved protocols in accordance with the highest ethical standards and the Institutional Review Board (IRB).

Immunohistochemical (IHC) staining for tissue microarrays. The tissue microarray slides (4 μm) were dewaxed in histoclear and hydrated in an ethanol gradient (100%, 90%, 50%) and Milli-Q water for 10 min, respectively. Antigen retrieval was performed by heating tissue microarray slides to 100 °C with 10 mM citric acid buffer (pH 6.0) followed by blocking with 3% H_2O_2 for 10 min, respectively. The slides were then washed in 1× phosphate buffered saline with 0.1% Tween-20 (PBST) in three cycles of 10 min each. The slides were then blocked using 5% BSA at room temperature for 30 min prior to incubation with polyclonal rabbit anti-human WNK1 primary antibodies (Catalog number: GTX106197; 1:100 dilution; GeneTex Inc, CA, USA) at 4 °C overnight (30). Following incubation with a universal secondary biotinylated antibody at room temperature for 30 min, liquid diaminobenzidine (DAB) substrate was added for development using the EnVision™+Dual Link System (Dako, Carpinteria, CA, USA) with hematoxylin counterstaining. Finally, the slides were dehydrated, cleared, and mounted. We conducted a serial dilution test to assess the specificity of the primary WNK1 antibodies. The positive results obtained in the experiment were used as a positive control, and 1× phosphate buffered saline (PBS) was used as a negative control.

IHC staining results were evaluated by two independent pathologists who reviewed the slides in a double-blinded manner based on the extent of cytosolic and nuclear staining in accordance with previously reported semi-quantitative scoring methods (31). The slides were assigned three score levels based on the staining intensity of the WNK1 protein: 0 (negative), 1 (moderate staining), or 2 (strong staining), and the percentage of positively stained cells: 0 (<5%), 1 (5-50%), and 2 (>50%).

Bioinformatics analysis to determine the effect of WNK1 mRNA expression on the prognosis of HCC patients. This study used bioinformatics generated from the UALCAN database web-portal (<http://ualcan.path.uab.edu>) for the analysis of omics data pertaining

Figure 1. Expression of WNK1 at the protein level in tumors and normal organ tissues. (A) IHC analysis of WNK1 protein expression in normal tissue samples from the breast, liver, urinary bladder, ovary, pancreas and prostate (above) and the corresponding tumors (below) using the same WNK1 antibodies. (B) Average score of 0, 1 or 2 of stained WNK1 in cells from all tumor tissues or normal control tissues of the breast, liver, urinary bladder, ovary, pancreas and prostate. *** $p < 0.001$. Scale bars=5 and 50 μm .

to the expression of WNK1, and the overall survival among 371 HCC patients on the Cancer Genome Atlas (TCGA) (28). Kaplan-Meier survival analysis was used to determine overall survival and the log-rank test was used to calculate the *p*-values.

Statistical analysis. IBM SPSS Statistics 12.0 Software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 8.0 software (GraphPad Software Inc, CA, USA) were used for statistical analysis. The differences between groups of cancer tissue samples and normal organ tissue samples were examined using the Student's *t*-test. All results are presented as the mean±SD. The Wilcoxon signed-rank test was used to determine the correlation between WNK1 protein scores in tumor and non-tumor tissues. The Chi-square test was used to determine the importance of the clinicopathological variables of WNK1 protein expression and HCC. The overall survival rates were derived using the Kaplan-Meier method and log-rank test. Univariate and multivariate analyses of the prognostic factors of HCC were performed using the Cox proportional hazard regression model (31, 32). The level of statistical significance was indicated by a *p*-value of *p*<0.05.

Results

WNK1 protein expression in tumor tissue. Immunohistochemical analysis was used to characterize WNK1 expression in tumors (breast, liver, urinary bladder, ovary, pancreas and prostate) and corresponding normal tissue. The samples were scored according to the staining results: 0, 1, or 2. Our results revealed significantly higher WNK1 protein levels in liver tumor tissues than corresponding normal tissues (*p*<0.001). We also observed weakly positive immunostaining in the cytoplasmic region in most of the tumors and normal tissue of the breast, urinary bladder, ovary, pancreas and prostate; however, the difference did not reach the level of statistical significance (*p*=0.145; *p*=0.363; *p*=0.196; *p*=0.773; *p*=0.483, respectively). Thus, we can conclude that WNK1 protein expression is characteristic of liver tumors, and that it should be detectable through initial screening (Figures 1A and B).

High mRNA expression of WNK1 is predictive of poor prognosis for HCC patients. TCGA data obtained from UALCAN revealed that, WNK1 expression levels were higher in samples from patients with HCC compared to normal tissue (*p*<0.001; Figure 2A). Kaplan-Meier survival analysis and the log-rank test were used to investigate the correlation between WNK1 overexpression (mRNA levels) and the prognosis for HCC patients. Overall survival analysis was based on data obtained from a cohort of 365 HCC patients on the UALCAN database (TCGA-liver hepatocellular carcinoma cohort) (28). Kaplan-Meier analysis revealed that the overall survival of patients with high WNK1 expression levels was shorter than that of patients with low/medium WNK1 expression levels (*p*=0.0037; Figure 2B).

Protein level expression of WNK1 in HCC tumor and adjacent normal tissues. We validated the results by

Figure 2. Correlation between WNK1 mRNA expression and survival rates of HCC patients. (A) WNK1 expression was higher in tissue samples from HCC patients than in normal tissue samples. (B) Kaplan-Meier survival curves for overall survival. Analysis was performed using TCGA-liver hepatocellular carcinoma samples from TCGA data obtained from UALCAN database by Kaplan-Meier survival analysis and log-rank test. High and low/medium WNK1 expression levels are respectively indicated in red and blue. **p<0.01; ***p<0.001.

performing IHC analysis on paired samples (liver cancer tissues vs. normal adjacent tissues) from 50 HCC patients. Based on IHC staining results, WNK1 protein expression levels were divided into three groups (Tumor-Nontumor), which were in-turn based on the paired tumor and adjacent normal tissue samples. Tumor-Nontumor values exceeding 0 (WNK1 classes I and II) indicated that WNK1 protein expression levels in the tumor tissue exceeded those in normal tissue, whereas Tumor-Nontumor values of 0 or less (WNK1 class III) indicated that the WNK1 protein expression level in the tumor tissue was higher than normal tissue. The WNK1 expression levels are presented in Figure

Figure 3. WNK1 expression at the protein level in HCC tumor samples and adjacent normal tissue. (A) IHC analysis of WNK1 protein expression in normal tissue (Above) and HCC tumor tissue (Below) by the same WNK1 antibody in terms of staining scores: 0 (<5%), 1 (5-50%), and 2 (>50%). (B) Summary of the frequency distribution of WNK1 protein staining scores in normal and HCC tissues. (C) Summary of the frequency distribution of WNK1 protein expression levels, calculated by normalizing the WNK1 expression scores of HCC tissues against those of normal tissues. Wilcoxon signed-rank test; *** $p < 0.001$. Scale bars=5 and 50 μ m.

3A. Most of tumors or adjacent normal tissues were scored 1, 2 (22.0%; 74.6%, respectively) and 0 or 1 (54.2%; 44.1%, respectively), and WNK1 were predominantly expressed in the membrane and cytoplasm of tumor cells (Figures 3A and B). The range of staining scores for WNK1 protein expression between tumor tissue and normal tissue (Tumor-Nontumor) was -2 to 2. Wilcoxon signed-rank test results confirmed that WNK1 protein expression in tumor samples was significantly higher than in normal tissue samples ($p < 0.001$) (Figure 3C).

WNK1 expression was positively correlated with clinicopathological variables in HCC patients. We used the Chi-square test to assess the function of WNK1 protein on clinicopathological variables typical of HCC. WNK1 staining results were positively correlated with gender ($p = 0.010$) and histologic grade ($p = 0.038$). However, we did not observe a positive correlation between WNK1 protein expression and age ($p = 0.317$), TNM stage ($p = 0.268$), T-class ($p = 0.120$), N-class ($p = 0.197$), or M-class ($p = 0.498$), as shown in Table I.

WNK1 protein overexpression was associated with poor prognosis of HCC patients. We evaluated the correlation between WNK1 protein expression and overall survival in tissues of 59 HCC patients by IHC staining; this was performed by Kaplan-Meier survival analysis and log-rank test. The HCC patients were first divided into three categories according to severity (WNK1 class I: $n = 26$; WNK1 class II: $n = 18$; and WNK1 class III: $n = 12$). We then analyzed the relationship between WNK1 expression levels and the overall survival of patients in each class. The overall survival rate of HCC patients with high WNK1 expression levels was significantly lower than among those with low expression levels (WNK1 class I vs. WNK1 class III; $p = 0.0093$). In terms of prognosis, we did not observe a significant difference between WNK1 class I patients and WNK1 class II patients ($p = 0.3241$) or between WNK1 class II and WNK1 class III patients ($p = 0.0846$). The median survival durations were as follows: WNK1 class I (18.5 months), WNK1 class II (52.0 months), and WNK1 class III (74.0 months). Clearly, high WNK1 protein expression levels are associated with a poor prognosis (Figure 4).

Table I. Clinical-pathological correlation of WNK1 expression with HCC patients.

Variables ^a	WNK1 scores (Nontumor)			Total (n=59)	p-Value ^c
	Score 0	Score 1	Score 2		
Age					
<60	22	17	1	40	0.757
≥60	10	9	0	19	
Gender					
Male	28	19	0	47	0.054
Female	4	7	1	12	
	WNK1 scores (Tumor-Nontumor) ^b				
Variables ^a	Class I	Class II	Class III		
Age					
<60	19	15	6	40	0.317
≥60	8	5	6	19	
Gender					
Male	25	16	6	47	0.010*
Female	2	4	6	12	
Histologic grade					
PD	15	5	1	21	0.038*
WD	2	0	2	4	
MD	9	10	7	26	
TNM stage					
I, II	13	10	9	32	0.268
III, IV	14	10	3	27	
T-class					
T1, T2	14	10	10	34	0.129
T3, T4	13	10	2	25	
N-class					
N0	18	15	9	42	0.197
N1	1	0	2	3	
N2	8	5	1	14	
M-class					
M0	21	15	11	47	0.498
M1	6	5	1	12	

T-class: Primary tumor; N-class: regional lymph nodes; M-class: distant metastasis. ^aCases with missing data were not included for analysis. ^bWNK1 score (Tumor-Nontumor): Class I, T-N=2; Class II, T-N=1; Class III, T-N=0. ^cp-Values were measured using the Chi-square test. * $p<0.05$.

TNM stage as prognostic factor for HCC patients. The prognostic role of WNK1 protein between overall survival, WNK1 expression, and various clinicopathological variables (age, gender, histologic grade, TNM stage and WNK1 score) were further validated in the HCC patients by Cox proportional-hazards models. Univariate and multivariate analysis confirmed that TNM stage was significantly associated with the overall survival rate of HCC patients (Hazard ratio=0.349, 95% CI=0.199 to 0.612, $p<0.001$; Hazard ratio=0.373, 95% CI=0.201 to 0.691, $p=0.002$, respectively). The final model revealed that HCC patients in TNM stage faced an increased risk of early death (Table II).

Figure 4. Correlation between WNK1 protein expression and survival of HCC patients. Analysis was performed using 59 HCC patients and paired controls. Kaplan-Meier curves show the overall survival rates and WNK1 protein expression levels among the three classes of HCC patients: WNK1 class I (Tumor-Nontumor=2), WNK1 class II (Tumor-Nontumor=1), or WNK1 class III (Tumor-Nontumor=0). Class I, Class II, and Class III groups are indicated in red, blue, and green, respectively. ** $p<0.01$.

Discussion

HCC is a common malignant tumor of the liver with a very high mortality rate (33, 34). Due to the high malignancy, high invasive potential, and early metastasis onset of HCC, many patients are unable to receive radical resection in a timely manner (32, 35). The high mortality rate of patients is due mainly to tumor invasion, metastasis and recurrence after surgical resection (36, 37). Researchers are urgently exploring therapeutic targets to improve the diagnosis and treatment of HCC. Serum alpha-fetoprotein (AFP) levels are often used to identify and monitor HCC patients; however, many patients with advanced HCC present normal AFP levels (38). At present, based on genetic or epigenetic alterations, gene expression and genome-based candidate markers for HCC patients are under evaluation (39, 40).

Recent studies have implicated WNK1 in several important intracellular pathways associated with tumor development, including cell proliferation, invasion, metastasis and signal transduction (19, 21, 22, 24, 26, 27). Nonetheless, the expression and functional role of WNK1 in HCC patients have not been extensively studied. Various WNK1-related mutations are known to cause the human genetic disease referred to as pseudohypoaldosteronism type 2 (PHA2), which is characterized by hypertension and hyperkalemia. These findings support the assertion that WNK1 functions as a key regulator

Table II. Univariate and multivariate analysis of overall survival and clinical-pathological features of HCC patients.

Variables ^a	Univariate analysis	Total (n=59)	<i>p</i> -Value
	Overall survival; Hazard ratio (95% CI)		
Age			
<60		38	
≥60	0.946 (0.532 to 1.681)	18	0.850
Gender			
Male		46	
Female	0.907 (0.454 to 1.812)	10	0.782
Histologic grade ^a			
PD/WD		25	
MD	0.771 (0.918 to 0.519)	26	0.771
TNM stage			
I/II		31	
III/IV	0.349 (0.199 to 0.612)	25	<0.001***
WNK1 score			
I/II–III ^b	1.119 (0.588 to 2.128)	56	0.733
Multivariate analysis			
Variables ^a	Overall survival; Hazard ratio (95% CI)	Total (n=59)	<i>p</i> -Value
Age			
<60		38	
≥60	0.837 (0.413 to 1.698)	18	0.622
Gender			
Male		46	
Female	0.951 (0.451 to 2.004)	10	0.985
Histologic grade ^a			
PD/WD		25	
MD	0.923 (0.507 to 1.680)	26	0.793
TNM stage			
I/II		31	
III/IV	0.373 (0.201 to 0.691)	25	0.002**
WNK1 score			
I/II–III ^b	0.715 (0.431 to 1.781)	51	0.715

95% CI: 95% Confidence interval, PD: poorly-differentiated, WD: well-differentiated, MD: moderately-differentiated. ^aCases with missing data were not included for analysis. ^bWNK1 score (Tumor-Nontumor): Class I, T-N=2; Class II, T-N=1; Class III, T-N=≤0. *p<0.05; **p<0.01; ***p<0.001.

of renal ion transport (41, 42). Researchers have also obtained evidence that WNK1 mutations are a driver in chronic lymphocytic leukemia (43). Previous studies have reported high expression of WNK1 mRNA and/or protein levels in human renal cancer tissue, indicating that WNK1 promotes the progression of renal tumors through the activation of the TRPC6-NFAT pathway (44). In the current study, WNK1 mRNA expression levels were higher among HCC patients than among normal controls (Figure 2A), and WNK1 protein was also significantly overexpressed in 79.7% of HCC tissue samples (Figure 2B). In statistical analysis of 59 real-world

samples, we found that WNK1 expression was related to clinicopathological variables typical of HCC patients, including gender and histological grade, but not to age, TNM stage, T-class, N-class, or M-class (Table I). Nonetheless, WNK1 overexpression is clearly related to the development and progression of HCC. Note that HCC is related to overall survival and TNM stage, but not to age, gender, histological grade, T-class, N-class, or M-class (Table II). Our results are consistent with previous studies indicating that high WNK1 expression levels are closely related to tumor progression (Figures 2 and 3). To the best of our knowledge, no previous study has investigated the expression of WNK1 in HCC patients or sought to determine its clinical role. This is the first study to report a correlation between WNK1 mRNA and protein expression levels and HCC. This is also the first study to combine clinical-pathological characteristics with WNK1 expression levels in formulating the prognosis for HCC patients.

Patient survival rates are crucial to the formulation of treatment plans. The standard mortality rate for liver cancer is 5.9 per 100,000, (45, 46). Note that the overall survival rate of liver cancer patients in Asian countries, Europe and North America are 34.8%, 19% and 18.1%, respectively (47). The main factors affecting the long-term survival of HCC patients are early diagnosis and effective therapy to combat tumor metastasis and tumor recurrence (8, 48). Kaplan-Meier survival data obtained in this study revealed that the presence of WNK1 is closely related to a poor prognosis (Figures 2 and 4). This is consistent with the results from Kaplan-Meier Plotter (kmplot.com); HCC patients with high expression of WNK1 have a lower overall survival rate (*p*=0.011). We also analyzed breast, bladder, ovarian and pancreatic ductal cancers, which showed high expression of WNK1 has lower overall survival rate in patients with bladder (*p*=0.0076) and ovarian cancers (*p*=0.00097) (Data not shown). These results are different from our WNK1 protein expression profiles in multiple human cancers and we need to increase the number of patients for further analysis (Figure 1). Univariate and multivariate analysis both identified TNM stage as an important independent factor affecting the overall survival of HCC patients (Table II). Our results indicate that WNK1 expression can be used as a prognostic biomarker for HCC patients, and may also be a useful target for chemotherapy.

Angiogenesis is crucial to tumor growth, invasion, and metastasis (49). WNK1 has recently been implicated in the angiogenesis of gliomas and gastrointestinal stromal tumors in humans and in *in-vivo* experiments (25-27, 50, 51). Silencing of the WNK1 gene was shown to reduce the invasiveness of breast cancer cells in experiments focusing on siRNA. Those results indicated that the role of miRNA-93 could be attributed to a reduction in WNK1 expression (21). miRNA-524-5p agomir treatment was also shown to inhibit the angiogenesis of colon cancer through the down-

regulation of WNK1 expression (52). It appears that 3-phosphoinositide-dependent protein kinase 1 (PDK1)-WNK1 signaling could be a potential therapeutic target in the treatment of HBV-related liver cancer (53). In the current study, we limited our investigation to WNK1 expression and the prognostic indicators of HCC patients. Determining the utility and prognostic value of WNK1 as a drug target will require research on the underlying molecular mechanisms as well as *in-vitro* and *in-vivo* analysis.

Conclusion

In summary, this study revealed that WNK1 was aberrantly overexpressed in tissues from HCC patients, and positively correlated with gender, and histological grade. We also observed a correlation between WNK1 expression levels and the overall survival of HCC patients. Our findings indicate that WNK1 may be a positive regulator of HCC progression, thereby raising the possibility of it being used as a biomarker in predicting the prognosis of HCC patients.

Conflicts of Interest

The Authors have no conflicts of interest.

Authors' Contributions

YJH, JC and KTY analyzed and drafted the article. YJH, JC, KTY, ZG, YML and JWL assisted with data interpretation. YJH, YML and JWL wrote the manuscript. All Authors critically revised the manuscript and approved the final version.

Acknowledgements

This work was supported by grants from Ministry of Science and Technology (MOST 108-2320-B-016-011-MY2 and MOST 108-3111-Y-016-010), Ministry of National Defense-Medical Affairs Bureau (MAB-109-040), and Ministry of Health and Welfare (MOHW109-TDU-B-212-134014) at Taiwan, ROC.

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Received May 20, 2020

Revised June 4, 2020

Accepted June 5, 2020