Cytoplasmic CK1ε Protein Expression Is Correlated With Distant Metastasis and Survival in Patients With Melanoma

JENG-WEI LU[1*], SHU-HUI LIN[2,3*], CHUNG-MIN YEH[2,4], KUN-TU YEH[2,5],
LAN-RU HUANG[3], CHIA-YU CHEN[2] and YUEH-MIN LIN[2,5]

[1]Department of Biological Sciences, National University of Singapore, Singapore, Singapore;
[2]Department of Surgical Pathology, Changhua Christian Hospital, Changhua, Taiwan, R.O.C.;
[3]Department of Medical Laboratory Science and Biotechnology,
Central Taiwan University of Science and Technology, Taichung, Taiwan, R.O.C.;
[4]Department of Medical Technology, Jen-Teh Junior College of Medicine,
Nursing and Management, Miaoli, Taiwan, R.O.C.;
[5]School of Medicine, Chung Shan Medical University, Taichung, Taiwan, R.O.C.

Abstract. *Background/Aim: Casein kinase 1 epsilon (CK1ε) is a member of the casein kinase 1 family, which includes highly conserved and ubiquitous serine/threonine protein kinases. Recent research has revealed that CK1ε plays an important role in a variety of human cancer types; however, its role in human melanoma remains unclear. The aim of this study was to elucidate the clinical role of CK1ε in patients with melanoma. Patients and Methods: Samples from 34 patients with melanoma were analyzed by immunohistochemical staining. Formalin-fixed paraffin-embedded tissue microarrays were also examined by two histopathologists to assess CK1ε protein expression in humans. Results: Cytoplasmic CK1ε protein expression was significantly lower in tumor tissue than in normal tissue. Lack of cytoplasmic CK1ε protein was significantly correlated with distant metastasis (p=0.022) and poorer survival (p=0.030). However, Kaplan-Meier survival analysis revealed that elevated expression of cytoplasmic CK1ε protein was not significantly associated with the overall survival of patients with melanoma. Univariate and multivariate analyses demonstrated that lack of cytoplasmic CK1ε protein expression was related to distant metastasis (p<0.001 and p=0.004), showing that CK1ε was a prognostic factor.*

Conclusion: CK1ε protein expression might serve as a prognostic indicator in the treatment of patients with melanoma.

Skin cancers are the most prevalent type of human cancer (1), and melanoma is the most common form of malignant skin cancer (2). Melanoma is also the most aggressive skin neoplasm and is associated with low survival rates (3). During normal skin cell development, keratinocytes regulate melanocyte homeostasis through the production of growth factors and the establishment of intercellular interactions *via* adhesion molecules (4). A loss of these functional interactions can lead to the dysregulation of cell proliferation and can cause melanocytes to invade adjacent tissue, *i.e.*, tumor progression (5).

Enormous clinical advances have recently been made in the treatment of melanoma *via* surgery, radiotherapy, chemotherapy, and immunotherapy (6, 7). Nonetheless, the median survival duration of patients with melanoma is only 6-9 months following the first detection of distant metastasis, and the 5-year survival rate remains very poor for this disease (8). Even the development of targeted drug therapies for melanoma has had little effect on patient prognosis. Thus, there remains an urgent need for novel biomarkers that can be used for the accurate prognosis and effective treatment of melanoma (9, 10).

The casein kinase 1 (CK1) gene family comprises ubiquitous and highly conserved serine/threonine-specific protein kinases. At least seven isoforms (α, β, γ1, γ2, γ3, δ, and ε) of CK1 exist in mammals (11-14). Tissue-specific functions of CK1 isoforms are regulated by the differential expression of CK1 genes in tissues and by the activation of downstream targets by CK1 (15). CK1 kinases phosphorylate a variety of substrates which are involved in a number of physiological functions, including apoptosis, cytokinesis,

DNA repair, cell-cycle progression and differentiation, chromosome segregation, and circadian periodicity (11-13, 15-17). CK1ε is a protein product of the *CSNK1E* gene, a monomer in the cytoplasm that is associated with schizophrenia and Alzheimer's disease (18-20). In pancreatic, colonic adenocarcinoma, and salivary gland tumor cells, CK1ε has been shown to regulate cell division and tumor growth through the phosphorylation of key proteins in the Wnt signaling pathway (21-23). The loss of CK1ε has also been shown to induce apoptosis by activating β-catenin in glioblastoma (24).

CK1ε has been identified as a key regulator of the circadian rhythm; however, its functional role in tumor cell survival has only recently been elucidated. Indeed, recent studies, through pharmacological inhibition or shRNA-mediated ablation both *in-vitro* and *in-vivo,* have indicated that CK1ε impedes the growth and inhibits the survival of pancreatic, sarcoma, glioblastoma, breast, colorectal, ovarian, and leukemia cancer cells (24-32). In Huh7 and SK-Hep-1 hepatoma cells, the knockdown of CK1ε has been shown to promote the migration and invasion of hepatoma cells *via* siRNA (33). High CK1ε expression levels have been linked to worse prognosis (*i.e.* lower overall survival) in patients with ovarian cancer (29) but correlated with better prognosis in subsets of patients with breast cancer (32). A loss of cytoplasmic CK1ε expression has also been observed to have a strong association with poor prognosis (*i.e.* lower overall survival) in patients with oral squamous cell carcinoma or hepatocellular carcinoma (33, 34). Finally, *CSNK1E* gene polymorphism has been associated with clinicopathological characteristics of oral squamous cell carcinoma and appears to increase an individual's susceptibility to environmental carcinogens (35).

Nonetheless, the role of CK1ε in human melanoma remains unclear. In this study, we investigated the expression of CK1ε in melanoma tissue from patients in Taiwan with the aim of determining whether CK1ε can be used as a prognostic indicator for this disease. We also assessed the clinical implications of CK1ε expression in patients with melanoma.

Patients and Methods

Tissue microarrays and patients with melanoma. The study population included 34 patients with melanoma (17 males) with an average age of 75.4 (range=47-100) years. Note that 20 of these patients (58.8%) presented ulceration; two (5.9%) presented T1 melanomas; six (17.6%) presented T2; 11 (32.4%) presented T3; and 15 patients (44.1%) presented T4. In addition, seven of the patients (20.9%) presented lymph node metastasis, and 10 (29.4%) presented distance metastasis. Five of the patients (14.7%) were in tumor stage I; 13 (38.3%) were in stage II; eight (23.5%) were in stage III; and eight (23.5%) were in stage IV. Survival analysis identified 20 patients (58.8%) surviving to 2 years and 14 patients (41.2%) to greater than 2 years.

Tissue microarrays were derived from three cancer tissue cores and one non-cancerous tissue core. Cores were extracted from formalin-fixed paraffin-embedded tissue specimens, cut longitudinally, and arranged in

tissue microarray format (33, 34). This study was approved by the Institutional Review Board (IRB) of Changhua Christian Hospital at Changhua, Taiwan (IRB No. 141004; Nov 10, 2014). All melanoma tissue samples were obtained from the Changhua Christian Hospital and categorized in accordance with the seventh edition of the Cancer Staging Manual of the American Joint Committee on Cancer (36).

Immunohistochemical staining. Tissue microarray sections (6 μm) were dewaxed using absolute xylene, rehydrated using graded ethanol, and rinsed using Milli-Q water. Antigen retrieval was performed *via* microwave with pH 6.0 citrate buffer (10 mmol/l) for 10 min at high power followed by 10 min at medium-low power. The mixture was then cooled to room temperature over a period of 30 min. Endogenous peroxidase activity was deactivated using 3% hydrogen peroxide solution, whereupon nonspecific binding was blocked by incubating tissue in 1% bovine serum albumin (BSA) for 15 or 60 min at room temperature. The sections then underwent three 5-min rinse cycles using 1×phosphate buffered saline, followed by incubation with affinity-purified goat polyclonal primary antibodies against human CK1ε (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min at room temperature (33, 34).

The sections were subsequently incubated with horseradish peroxidase/Fab polymer conjugate and a substrate (3,3'-diaminobenzidine tetrahydrochloride) for 30 min to detect peroxidase activity. Following this, counterstaining using hematoxylin was performed (34). Positive and negative controls were run simultaneously on the same sections. Specifically, oral squamous cell carcinoma tissue known to express CK1ε was used as a positive control (34), and 1× phosphate-buffered saline (in place of primary antibody) was used as a negative control.

Evaluation of CK1ε expression via scoring. Stained sections were scored using a semiquantitative scoring system (34) by two independent histopathologists (Yueh-Min Lin and Kun-Tu Yeh) in a double-blinded setup. Stains were scored according to the percentage of immunoreactive cells (quantity score) and the estimated intensity of staining (intensity score). Staining intensity scores were classified into three levels: Score 0: no staining; score 1: weak staining; score 2: moderate staining; and score 3: strong staining. Staining intensity in the cytoplasmic membrane and nucleus were classified into two subgroups (whereby those with scores 0 and 1 were classified as negative, and those with score 2 and score 3 were classified as a positive).

Statistical analysis. Fisher's exact test or the chi-squared test were used to analyze associations between CK1ε expression and clinicopathological variables of patients with melanoma. Univariate and multivariate analyses of overall survival was performed using the Kaplan-Meier method with the log-rank test and Cox proportional hazard regression model, respectively (33, 34, 37). All analyses were performed using SPSS software (SPSS, Version 15.0; SPSS Inc., Chicago, IL, USA), with the level of statistical significance set at $p < 0.05$.

Results

Correlation between cytoplasmic CK1ε expression levels and clinicopathological characteristics of melanoma patients in Taiwan. Twenty cases of benign nevi tissue were used as a negative control. As shown in Figure 1, the 34 melanoma patients were classified into two groups (negative or positive
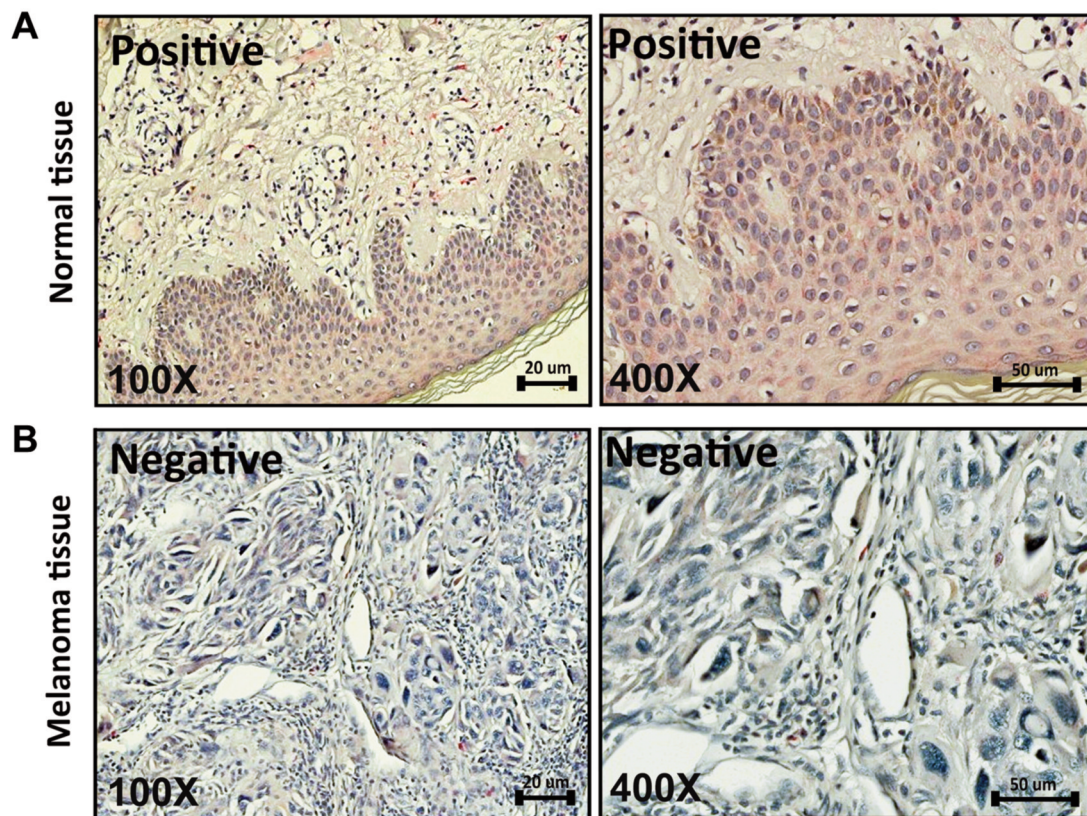
Figure 1. *Immunohistochemical analysis of cytoplasmic expression of casein kinase 1ε in normal tissue (positive) and melanoma tissue (negative).*

for cytoplasmic CK1ε expression) based on representative immunohistochemical staining results. Table I illustrates the degree of correlation between cytoplasmic CK1ε expression level and clinicopathological variables. We observed a statistically significant correlation between lack of cytoplasmic CK1ε expression and both distant metastasis (*p*=0.022) and poorer survival (*p*=0.030). No statistically significant correlations were observed between cytoplasmic CK1ε expression level and age, gender, ulceration, T status, lymph node metastasis, or tumor stage.

Cytoplasmic CK1ε expression was not correlated with short-term overall survival among patients with melanoma in Taiwan. We investigated the correlation between cytoplasmic CK1ε expression levels and overall survival among patients with melanoma. The results shown in Table I revealed a statistically significant correlation between the cytoplasmic expression of CK1ε and survival for 2 years or less. Among the 22 patients who survived for ≤2 years, cells from nine (45.0%) stained positively for cytoplasmic CK1ε expression. Note that Kaplan-Meier analysis with the log-rank test did not identify any statistically significant correlation between cytoplasmic CK1ε expression and overall survival (Figure 2).

Prognostic value of clinicopathological characteristics in cases of melanoma according to the Cox proportional hazard model. Cox proportional hazards models were used to perform univariate and multivariate analyses of clinicopathological parameters and clinical outcomes. In both univariate and multivariate analyses, distant metastasis was significantly associated with poorer overall survival (95% CI=1.986-14.866, *p*<0.001; and 95% CI=1.924-28.245, *p*=0.004, respectively) (Table II).

Discussion

The incidence of melanoma in North America and Europe is far higher than in East and Southeast Asian countries (38, 39). The high incidence of melanoma among light-skinned individuals can be attributed to excessive exposure to ultraviolet radiation from both natural (sunlight) and artificial (sunbed) light in conjunction with a failure to detect skin cancer in the early stages (40, 41). At present, malignant melanoma is rare in Taiwan; however, the incidence of this disease is increasing. In 2015, 266 cases of cutaneous malignant melanoma were diagnosed in Taiwan, which accounted for 0.25% of all malignant tumors and 0.25% of

Table I. *Correlation of cytoplasmic expression of casein kinase 1ε (CK1ε) protein with clinicopathological variables in Taiwanese patients with melanoma. Cases with missing data were not included for analysis.*

Variable	Category	No. of cases	Cytoplasmic CK1ε expression, n (%)		p-Value*
			Negative	Positive	
Age, years	Mean±SD	34	77.4±8.98	74.2±13.49	0.188
Gender	Female	17	8 (47.1%)	9 (52.9%)	0.290
	Male	17	5 (29.4%)	12 (70.6%)	
Ulcer	No	14	3 (21.4%)	11 (78.6%)	0.153
	Yes	20	10 (50.0%)	10 (50.0%)	
T Status	T1, T2, T3	18	4 (22.2%)	14 (77.8%)	0.076
	T4	16	9 (56.2%)	7 (43.8%)	
	T1, T2	8	2 (25.0%)	6 (75.0%)	0.444
	T3, T4	26	11 (42.3%)	15 (57.7%)	
Lymph node metastasis	No	27	8 (29.6%)	19 (70.4%)	0.079
	Yes	7	5 (71.4%)	2 (28.6%)	
Distance metastasis	No	24	6 (25.0%)	18 (75.0%)	**0.022**
	Yes	10	7 (70.0%)	3 (30.0%)	
Tumor stage	I, II	18	5 (27.8%)	13 (72.2%)	0.183
	III, IV	16	8 (50.0%)	8 (50.0%)	
Survival	≤2 Years	20	11 (55.0%)	9 (45.0%)	**0.030**
	>2 Years	14	4 (14.3%)	12 (85.7%)	

*Fisher's exact test or chi-squared test. Statistically significant p-values are shown in bold.

total cancer-related deaths (*n*=152). However, between 1996 and 2015, the age-adjusted incidence of melanoma in Taiwan increased by 57% among males and 44% among females (41). Note that in one study, among 59 Taiwanese patients diagnosed with stage III melanoma, the 5-year survival rate was 36% (42).

Several recent studies have reported that CK1ε functions as an oncogene or tumor-suppressor gene, depending on the type of cancer. For example, CKIε functions as an oncogene in ovarian cancer (29) and glioblastoma (24); however, it functions a tumor suppressor in hepatocellular carcinoma (33) and oral squamous cell carcinoma (34). Note that the function of CK1ε also appears to vary among cases of the same type of cancer (24, 27, 29, 31-34). Moreover, researchers have recently linked CK1ε expression to *MYC* oncogene activation in tumors of the colon, lung, and breast (43). In the current study, cytoplasmic CK1ε protein expression was lower in melanoma tumor tissue than in normal tissue (Figure 1). This is consistent with reports of reduced cytoplasmic CK1ε expression levels in tumor tissue from patients with hepatocellular carcinoma (33) and oral squamous cell carcinoma (34).

Note, however, that CK1ε expression has not previously been clinically implicated in melanoma. In this retrospective analysis of 34 patients with melanoma in Taiwan, we observed significant correlations between CK1ε cytoplasmic protein expression and a number of pathological variables. Specifically, low cytoplasmic CK1ε protein expression levels were significantly correlated with distant metastasis and
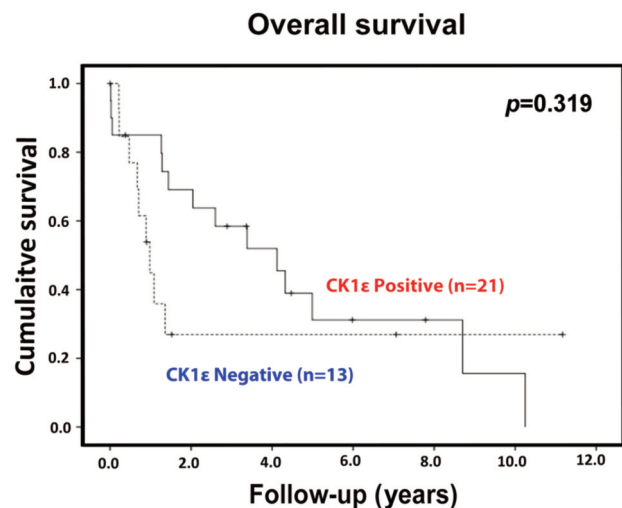

Figure 2. *Kaplan-Meier analysis of the correlation between cytoplasmic casein kinase 1ε (CK1ε) expression levels and overall survival of patients with melanoma. Analysis was performed using 34 melanoma samples, and survival curves were compared with the log-rank test.*

poorer survival (Table I). Univariate and multivariate analyses further indicated that lack of cytoplasmic CK1ε protein expression was also related to distant metastasis (Table II). These results are consistent with previous data on hepatocellular carcinoma and oral squamous cell carcinoma (33, 34). However, we did not detect a significant correlation

Table II. *Hazard ratios (HR) for the effect of clinicopathological variables on overall survival in Taiwanese patients with melanoma.*

Variable		Univariate analysis			Multivariate analysis		
		HR	95% CI	*p*-Value	HR	95% CI	*p*-Value
Expression of CK1ε	Negative	1.0			1.0		
	Positive	1.5	0.652-3.672	0.322	1.3	0.412-3.102	0.812
T Status	T1, T2	1.3			1.0		
	T3, T3	1.0	0.319-2.366	0.783	1.6	0.402-6.357	0.506
Lymph node metastasis	No	1.0			1.0		
	Yes	2.1	0.787-5.473	0.140	1.9	0.645-5.703	0.242
Distant metastasis	No	1.0			1.0		
	Yes	5.4	1.986-14.866	**0.001**	7.3	0.1924-28.245	**0.004**
Tumor stage	I, II	1.0			1.4		
	III, IV	1.5	0.650-3.610	0.330	1.0	0.177-2.777	0.612

CK1ε: Casein kinase 1ε; CI: confidence interval. Statistically significant *p*-values are shown in bold.

between cytoplasmic CK1ε expression and the overall survival of patients with melanoma (Figure 2). This may be due to the small sample size employed by our study. Further analysis with a larger sample will be required to confirm these results.

CK1ε has been identified as an important positive regulator of the canonical Wnt/β-catenin (13, 44-46) and noncanonical Wnt pathways (47, 48). The noncanonical CK1ε/β-catenin signaling network has also been linked to tumorigenesis (24). CK1 has also been shown to inhibit activity of forkhead O transcription factor (FOXO) (49), and this leads to tumorigenesis in glioblastoma, rhabdomyosarcoma, leukemia, breast cancer, and prostate cancer (50). Furthermore, in an *in-vivo* analysis of metastatic melanoma cells, CK1ε expression was found to be down-regulated. However, the inhibition of CK1ε expression/activity does not appear to significantly affect cell growth or survival in metastatic melanoma *via* specific inhibitors or knockdown with siRNA. Conversely, one study reported that the overexpression of CK1δ or CK1ε in melanoma cells failed to induce cell death and cell-cycle arrest, despite the fact that p53 signaling was activated (51). At this point, the mechanism underlying the occurrence of CK1ε in melanoma remains unclear. Further studies are thus needed to elucidate this important issue.

CK1ε plays a key role as tumor suppressor or oncogene during tumorigenesis, and CK1ε has also been shown to trigger a number of responses which vary according to the genetic composition of the target cell and/or its environment. Results of the current study indicate that, in addition to acting as a tumor-suppressor, CK1ε might also serve as a clinical prognostic indicator and pharmacological target in the treatment of patients with melanoma.

Conflicts of Interest

The Authors have no conflicts of interest.

Authors' Contributions

JWL, SHL and YML analyzed and drafted the article. CMY, KTY, LRH and CYC assisted with data interpretation. All Authors critically revised the article and approved the final version.

Acknowledgements

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