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Original Article

High expression of nuclear CCNE1 is associated with disease progression in nasopharyngeal carcinoma patients

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Abstract: This study examined the relevance between expression of nuclear cyclin E1 (CCNE1) and clinicopathologic data in patients with nasopharyngeal carcinoma (NPC), including patient survival. The expression of CCNE1 in NPC and normal nasopharyngeal (NP) tissues was assessed by immunohistochemistry. CCNE1 protein was expressed in the nucleus and cytoplasm, and had a significantly higher nuclear expression in NPC tissues than NP tissues. High expression of nuclear CCNE1 was positively associated with T classification (P=0.004), but not significantly correlated with other clinical features. Furthermore, the patients with NPC exhibiting high expression of nuclear CCNE1 had a poorer total survival ratio than patients with low nuclear expression of CCNE1. The survival time for stages T1-2 and clinical stages I-II NPC patients was inversely related to high expression of nuclear CCNE1. Our findings indicate that increased expression of nuclear CCNE1 is an underlying marker for the progression and poor prognosis of NPC patients.

Keywords: CCNE1, immunohistochemistry, nasopharyngeal carcinoma, prognosis

Introduction

Nasopharyngeal carcinoma (NPC) is an especially prevalent malignant tumor in southern China and Southeast Asia. The morbidity rate for NPC is 20-30/100,000 [1]. The pathogenesis underlying NPC is related to heredity (genetic susceptibility), Epstein-Barr virus (EBV) infection, environmental factors, dietary habits, and various tumorigenic factors. Current treatment for NPC is inadequate. Most NPC patients present with more advanced disease, including local invasion and early distant metastases, thus leading to a poor prognosis. Therefore, there is an urgent need to investigate the molecular events associated with NPC initiation, progression, and prognosis, which may facilitate an earlier diagnosis and prognostication, as well as implementing novel therapeutic strategies. Recent studies have shown that abnormal expression of key genes (EZH2, HDGF, and GAD1) and miRNAs (miR-BART17-5p, miR-15a, and miR-26a) deregulation contribute to the pathogenesis of NPC [2-6].

Cell cycle protein E1 (CCNE1) is composed of four exons and three introns which transcribe a 2.2 KB mRNA [7]. CCNE1 is expressed in the G1-S phase of the cell cycle by combining and activating cyclin-dependent kinase 2 (CDK2) to adjust mammalian cell division during the G1-S phase transition [7]. Substrate phosphorylation of the CCNE1-CDK2 complex plays an important role in triggering the synchronization of DNA replication, centrosome replication and regulation, chromosome refactoring, and biosynthesis of histones. Increased expression of CCNE1 is associated with disease progression in various malignancies and is associated clinically with worse outcomes and a poor prognosis in patients with breast, ovarian, bladder, endometrial, and colorectal cancers [8-12]. These reports indicate that CCNE1 is an important gene taking part in tumor pathogenesis;

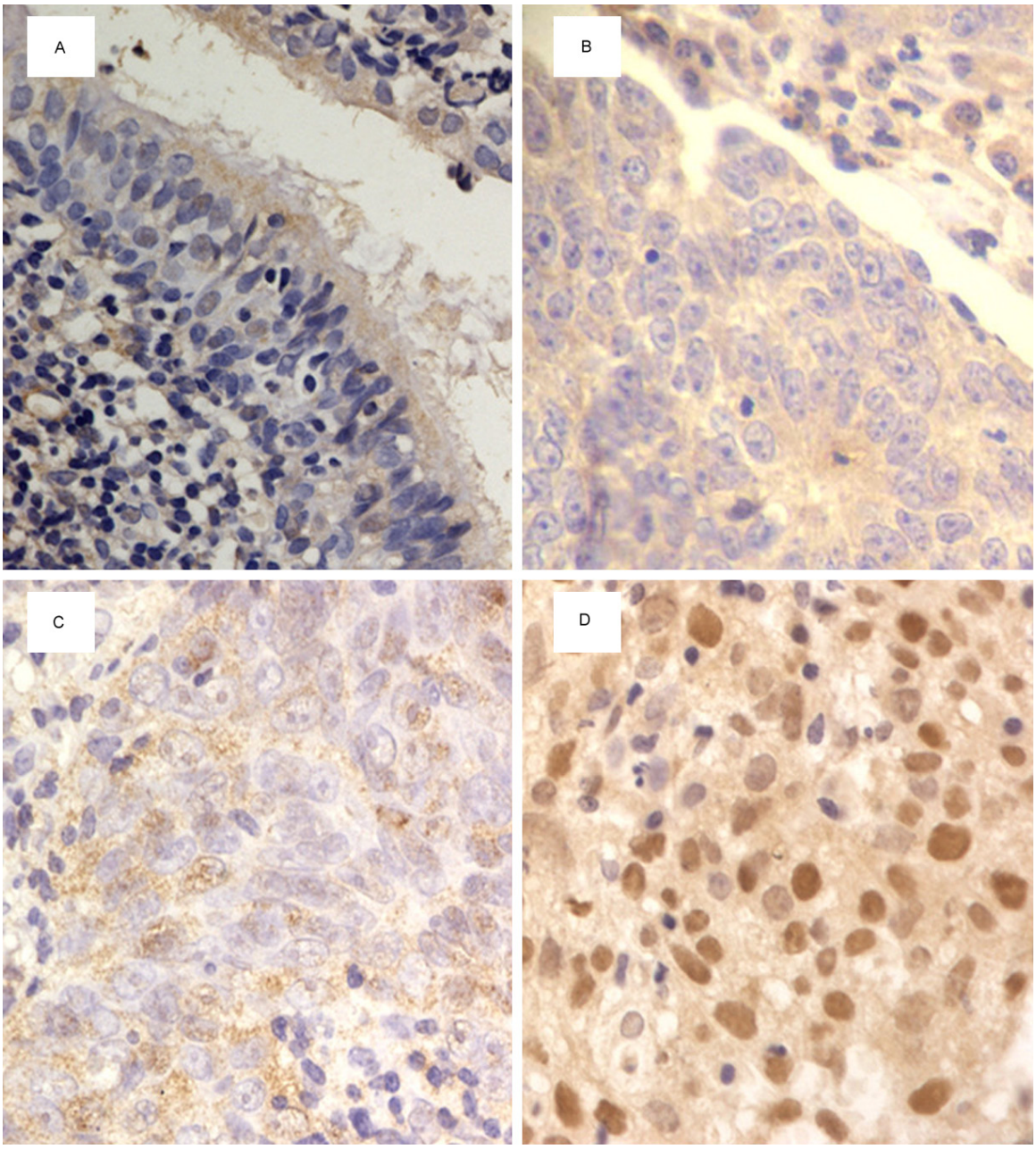

Figure 1. CCNE1 expression in nasopharyngeal carcinoma (NPC) samples (original magnification: ×400). CCNE1 protein expression in cytoplasm and nuclei of non-cancerous and malignant epithelial cells; A. CCNE1 protein expression in cytoplasm of normal nasopharynx tissues; B-D. CCNE1 protein expression in nuclei of NPC: B. Low expression, C, D. High expression.

Table 1. Increased nuclear expression of CCNE1 protein in NPC

Tissue	N	Nuclear CDK6 expression		*P* value
		High	Low	
NPC	154	74	80	0.000
Nasopharynx	35	8	27	

however, the role played by CCNE1 in NPC has not been determined. In this study we determined the expression of CCNE1 in NPC and healthy nasopharyngeal (NP) tissues by immunohistochemistry, as well as the correlation between CCNE1 nuclear expression and clinicopathologic features, including NPC patient survival.

Table 2. Correlation between the clinicopathologic characteristics and expression of CCNE1 in nasopharyngeal carcinoma

Characteristics	N	CCNE1 expression		P*
		High	Low	
Age (year)				
<50	82	38 (46.3%)	44 (53.7%)	0.650
≥50	72	36 (50.0%)	36 (50.0%)	
Gender				
Male	105	46 (43.8%)	59 (56.2%)	0.120
Female	49	28 (57.1%)	21 (42.9%)	
Clinical stage				
I-II	56	23 (41.1%)	33 (58.9%)	0.190
III-VI	98	51 (52.0%)	47 (48.0%)	
T classification				
T1-T2	101	40 (39.6%)	61 (60.4%)	0.004
T3-T4	53	34 (64.2%)	19 (35.8%)	
N classification				
N0-N1	75	31 (41.3%)	44 (58.7%)	0.104
N2-N3	79	43 (54.4%)	36 (45.6%)	
Distant metastasis				
Yes	4	3 (75.0%)	1 (25.0%)	0.274
No	150	71 (47.3%)	79 (52.7%)	

Materials and methods

Sample collection

One hundred fifty-four NPC paraffin-embedded specimens with clinical and prognostic information were obtained from patients ranging in age from 21-83 years (median, 50.3 years) who received treatment at the People's Hospital of Zhongshan City. These clinical materials were used for research purposes with prior patient consent and approval from the hospital Ethics Committee. All samples had a pathologic diagnosis and were staged according to the 1997 NPC staging system of the UICC.

Immunohistochemistry

According to standard protocols, the NPC paraffin sections (3 μm) were deparaffinized in 100% xylene and rehydrated in a descending ethanol series (100%, 90%, 80%, and 70%). Heat-induced antigen retrieval was performed in 10 mM citrate buffer for 2 min at 100°C. A peroxidase blocking reagent containing 3% hydrogen peroxide and serum was used to block endogenous peroxidase activity and non-specific antigen, followed by incubation with rabbit polyclonal CCNE1 antibody (1:100; Origene, Rockville, MD, USA) overnight at 4°C. After washing, the sample was incubated with biotin-labelled antibody for 10 min at room temperature, and then incubated with streptavidin-conjugated horseradish peroxidase (HRP; Maixin, Inc., Fuzhou, China). A 3,3'-diaminobenzidine (DAB) chromogen solution in DAB buffer substrate was used to develop the peroxidase reaction. The tissue was counterstained with hematoxylin, mounted in neutral gum, and examined under a microscope.

Staining assessment

The stained tissue sections were independently reviewed by two pathologists blinded to the clinical parameters and evaluated for the presence of nuclear staining. Greater than or equal to 20% of nuclear-stained cells was considered high nuclear expression. Less than 20% staining was regarded as low nuclear expression.

Statistical analysis

SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA) was used to perform the statistical analysis. A Chi-square test was used to analyze the correlation between CCNE1 nuclear expression and clinicopathologic parameters in NPC tissue specimens. The Kaplan-Meier analysis with a log-rank test was used to examine the association between nuclear level of CCNE1 and survival. A P value <0.05 was considered statistically significant.

Results

Immunohistochemical analysis of CCNE1 protein expression in NPC tissues

We determined the level of nuclear expression and subcellular localization of CCNE1 protein in 154 archived paraffin-embedded NPC and 35 non-cancerous NP samples using immunohistochemical staining. Specific CCNE1 protein staining was detected in the nuclei and cytoplasm of non-cancerous and malignant epithelial cells (**Figure 1A-D**). Furthermore, we observed that the nuclear expression of CCNE1 was significantly increased in NPC tissues (74/154 [48.1%]) compared to NP tissues (8/35 [22.9%]; **Table 1**).

Relationship between clinicopathologic characteristics and CCNE1 nuclear expression in NPC patients

We summarized the relationship between the clinicopathologic characteristics and CCNE1

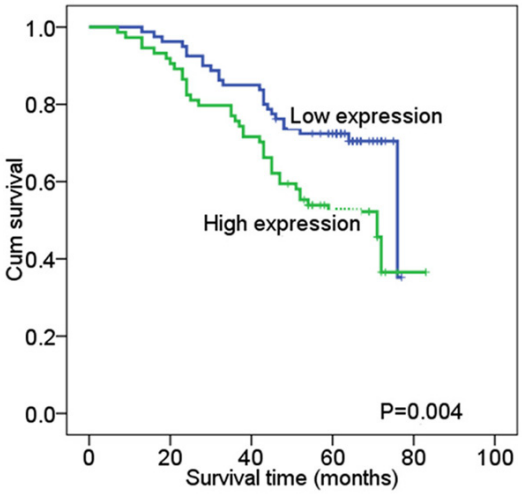

Figure 2. High expression of nuclear CCNE1 protein predicts nasopharyngeal carcinoma patient overall survival. Patients with high nuclear expression of CCNE1 had worse survival than patients with low nuclear expression of CCNE1.

nuclear expression in individuals with NPC in **Table 1**. A significant association existed between CCNE1 nuclear expression and patient age, gender, clinical stage, N classification, and distant metastasis (M classification) in the 154 NPC cases; however, high expression of nuclear CCNE1 was positively correlated with T classification (T1-T2 vs. T3-T4; P=0.004) in NPC patients (**Table 2**).

High expression of nuclear CCNE1 is associated with overall survival in NPC patients

Using Kaplan-Meier analysis with the log-rank test to determine the prognostic value of CCNE1 expression for NPC, we assessed the association between the levels of CCNE1 expression and patient survival. In 154 NPC cases with prognostic information, we observed that high nuclear expression of CCNE1 protein was correlated with overall survival. Patients with high nuclear expression had a worse prognosis than patients with low nuclear expression of CCNE1 (**Figure 2A**; P=0.004).

Increased CCNE1 nuclear expression is inversely associated with survival time of NPC patients with T1-2 stage and clinical stage I-II

We further analyzed the correlation between high expression of nuclear CCNE1 and prognosis of NPC patients by stratifying T classification, N classification, and clinical stage (**Figure 3**). High expression of nuclear CCNE1 protein was significantly associated with shorter survival time for NPC patients with T1-2 stage (P=0.030) and clinical stage I-II tumors (P=0.008). There was no association between CCNE1 nuclear expression and prognosis in NPC patients with T3-T4, N0-N1 or N2-N3 classifications, and clinical stage III-IV.

High nuclear expression of CCNE1 is not an independent prognosis factor for NPC patients

Univariate analyses showed that T, N, and M classifications, clinical stages, age, and CCNE1 nuclear expression were all significantly correlated with patient survival (P<0.001, P<0.001, P<0.001, P<0.001, P=0.008, and P=0.005, respectively). Multivariate analysis of CCNE1 nuclear expression adjusted for T classification, N classification, M classification, and clinical stages of NPC patients was performed to determine whether or not CCNE1 is an independent prognostic factor for NPC. These results indicated that the level of CCNE1 expression is not an independent prognostic factor for NPC (P=0.095; **Table 3**).

Discussion

Cell cycle control anomalies are thought to be a prerequisite for tumor development, and several studies have shown that constitutive expression of CCNE1 accelerates cell cycle entry into the S phase [13, 14]. CCNE1 can also induce chromosome instability by improper initiation of DNA replication and centrosome duplication [15, 16]. Furthermore, some investigations have demonstrated that in various malignancies, CCNE1 is consistently associated with disease progression and poor prognosis in breast, ovarian, bladder, endometrial, and colorectal cancer patients [8-12]. All of these previous reports have suggested that CCNE1 is a significant gene promoting NPC progression; however, the association between CCNE1 protein expression and clinical features and the prognosis of NPC was not examined.

In the current study we first determined the expression of CCNE1 protein in NPC and NP tissues, as estimated by immunohistochemistry. CCNE1 expression was primarily located in the nucleus, with less CCNE1 expression in the cytoplasm of NPC tissues. Furthermore, the results indicated that nuclear CCNE1 protein

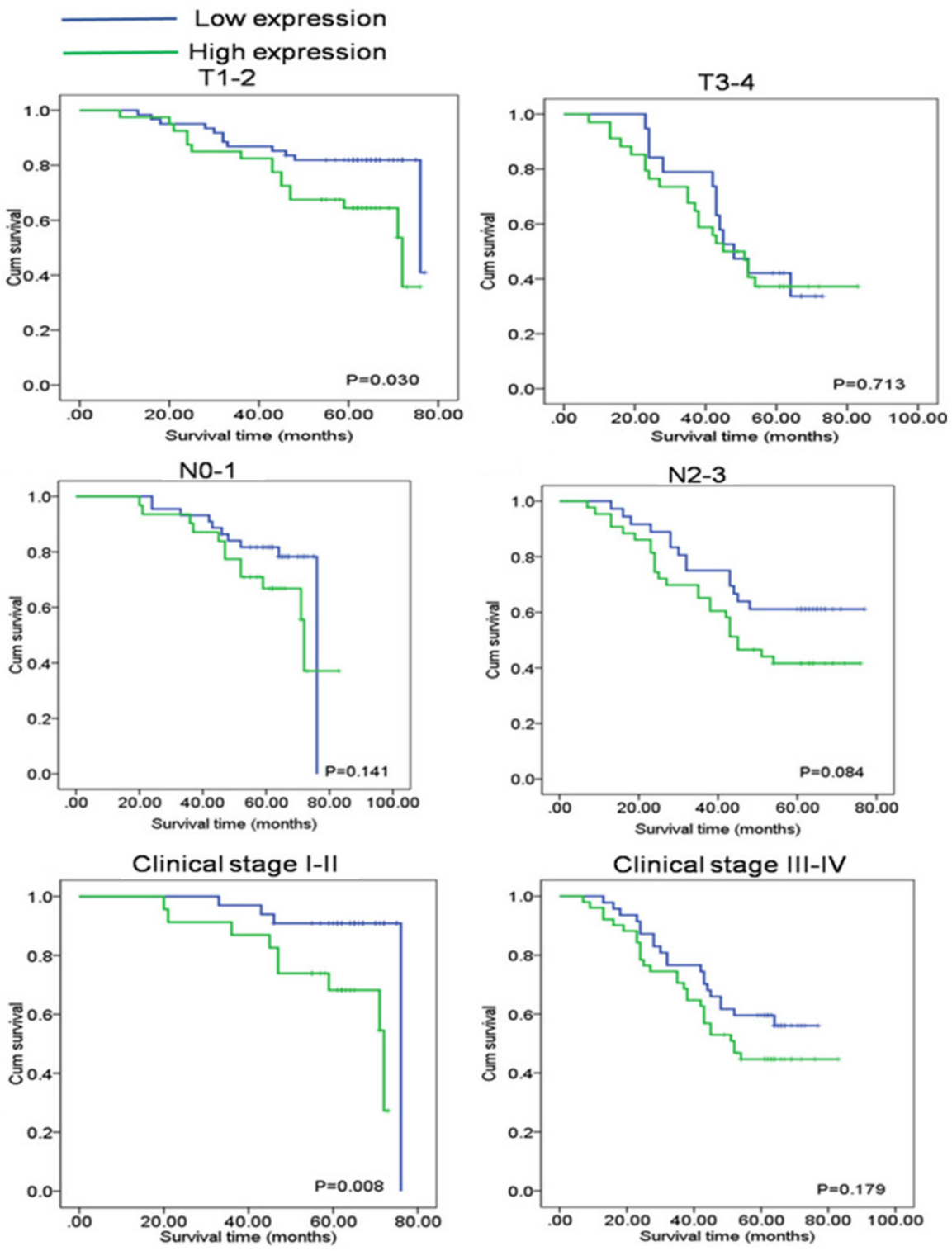

Figure 3. Association of CCNE1 expression with NPC patient survival time in strata analysis based on T classification, N classification, and clinical stage. High expression of CCNE1 protein was correlated significantly with shorter survival time for NPC patients with T1-T2 classifications and clinical stages I-II, but was not associated with the survival of patients with T3-T4, N0-N1, and N2-N3 classification or clinical stages III-IV.

Table 3. Summary of univariate and multivariate Cox regression analysis of overall survival duration

	Univariate analysis			Multivariate analysis		
Parameter	P	HR	95% CI	P	HR	95% CI
Age						
≥50 versus <50 years	0.008	1.722	1.153-2.570	0.007	1.752	1.167-2.630
Gender						
Male versus female	0.225	1.313	0.846-2.038			
Clinical stage						
I-II versus III-IV	0.000	2.383	1.479-3.840	0.558	0.796	0.350-1.813
T classification						
T1-T2 versus T3-T4	0.000	2.582	1.733-3.857	0.005	2.046	1.241-3.372
N classification						
N0-N1 versus N2-N3	0.000	2.246	1.477-3.416	0.035	2.003	1.048-3.826
M classification						
M0 versus M1	0.000	15.556	6.677-36.239	0.000	8.074	3.362-19.387
Expression of CCNE1						
High versus low expression	0.005	1.938	1.222-3.072	0.095	1.506	0.931-2.434

expression is significantly up-regulated in NPC tissues compared with non-cancerous NP tissues. This finding was consistent with previously reported findings in ovarian [9, 16] and breast cancer cell lines [8]. Indeed, these data suggest that CCNE1 is a significant gene participating in the pathogenesis of NPC.

Further, we analyzed the association between CCNE1 nuclear expression and clinical features of NPC patients. High expression of nuclear CCNE1 was not associated with patient age, gender, and lymph node or distant metastases, but high expression of nuclear CCNE1 was correlated positively with tumor size and clinical stage. This result was analogous to the finding from a study involving breast cancer [17], although these studies measured total CCNE1 protein. This result suggests that CCNE1 regulates the clinical progression of NPC.

In a subsequent study, we demonstrated that high nuclear expression of CCNE1 protein in NPC was inversely correlated with overall patient survival, thus indicating that high nuclear expression of CCNE1 is a significant biomarker for NPC prognosis. Our data were similar to the study conducted by Kuhling et al. [18] in node-negative patients. Kuhling et al. [18] found that patients having tumors with high expression of cyclin E had a shorter overall survival. Further, we evaluated the relationship between prognosis and T and N classification, and clinical stage using stratification. The results showed that high expression of nuclear CCNE1 was negatively associated with survival for stages T1-T2 NPC patients, but not stages T3-T4. An early study reported that high CCNE1 expression is a significant and independent predictor for prolonged overall survival in late stage ovarian cancer patients [16]. Due to the anatomic location and limited growth space, NPC growth may be restricted significantly in T3-4 tumors, despite high expression of CCNE1. We found that high expression of nuclear CCNE1 is inversely associated with survival time in patients with clinical stages I-II, suggesting that elevated nuclear CCNE1 expression may function in promoting cell proliferation and clinical progression in early-stage disease more than advanced-stage NPC.

In the end we assessed whether or not CCNE1 nuclear expression is an independent prognostic factor for NPC. Based on univariate analysis, overall survival was positively associated with T/N/M classification and clinical stage, but inversely correlated with CCNE1 nuclear expression. Regardless of patient disease status, multivariate analyses indicated that high nuclear expression of CCNE1 protein is not an independent predictor of prognosis for NPC patients. This result was contrary to a study of the OVCAD Consortium, which found that CCNE1 is an independent positive prognostic factor in advanced-stage serous ovarian cancer patients [16].

In summary, these results provide evidence that high expression of nuclear CCNE1 may be

involved in the clinical progression and poor prognosis of NPC patients; however, CCNE1 is not an independent prognostic factor for NPC.

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Disclosure of conflict of interest

None.

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