RESEARCH COMMUNICATION

Role of Centromere Protein H and Ki67 in Relapse-free Survival of Patients after Primary Surgery for Hypopharyngeal Cancer

Jun-Xi Wang, Ying-Yao Zhang, Xue-Min Yu, Tong Jin, Xin-Liang Pan*

Abstract

Purpose: Centromere protein H (CENP-H) and Ki67 are overexpressed in some malignancies, but whether they are predictors of survival after primary resection for hypopharyngeal squamous cell carcinoma (HSCC) remains unknown. Methods: We assessed immunohistochemical expression of CENP-H and Ki67 in 112 HSCC specimens collected between March 2003 and March 2005 for analysis by clinical characteristics. The Kaplan-Meier method was used to analyze relapse-free survival and logistic multivariate regression to determine risk factors of relapse-free survival. Cholecystokinin octapeptide assays and flow cytometry were used to examine cell proliferation and apoptosis after siRNA inhibition of CENP-H in HSCC cells. Results: Overall, 50 (44.6%) HSCC specimens showed upregulated CENP-H expression and 69 (61.6%) upregulated Ki67. An increased CENP-H protein level was associated with advanced cancer stage and alcohol history (P=0.012 and P=0.048, respectively) but an increased Ki67 protein level only with advanced cancer stage (P=0.021). Increased CENP-H or Ki67 were associated with short relapse-free survival (P<0.001 or P=0.009, respectively) and were independent predictors of relapse-free survival (P=0.001 and P=0.018, respectively). siRNA knockdown of CENP-H mRNA inhibited cell proliferation and promoted cancer cell apoptosis *in vitro*. Conclusions: Upregulated CENP-H and Ki67 levels are significantly associated with short relapse-free survival in HSCC. These factors may be predictors of a relapsing phenotype in HSSC cases.

Keywords: Hypopharyngeal squamous cell carcinoma - centromere protein H - Ki67 - survival - cell proliferation

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Introduction

Hypopharyngeal squamous cell cancer (HSCC) is a rare malignancy accounting for approximately 0.5% of all human malignancies and representing about 3% to 5% of all head and neck cancers (Hoffman et al. 1998) (Cooper et al., 2009). Most patients with HSSC share a common history of tobacco and/or alcohol abuse. Despite improvements in surgical and chemoradiation approaches, most patients received an initial diagnosis in the advanced cancer stage, which results in high mortality.

Over the past decade, the 5-year survival rate has been about 30% (Cooper et al., 2009). Surgery or surgery with radiation is considered valid therapy (Hoffman et al., 1997; Gourin et al., 2004). Nevertheless, most patients experience swallowing or speech difficulties after curative resection. Even worse, some patients still experience cancer relapse after complete resection of primary tumors. Relapse often cannot be reliably and timely diagnosed for patients with malignancies or HSCC after primary treatment. Therefore, identifying molecular and immunohistochemical markers may help pinpoint relapse after primary curative resection.

Kinetochores play a fundamental role in accurate cell segregation. They have a role in chromosomes separating from each other and control the cell cycle during mitosis (Sugata et al., 1999). Kinetochores comprise facultative and constitutive kinetochore proteins. The constitutive kinetochore, centromere protein H (CENP-H), a coiled-coil structural and a nuclear signal protein, plays a crucial role in kinetochore organization and function throughout the whole cell cycle (Cleveland et al., 2003). Along with other members, CENP-H forms a functional complex required for faithful chromosome segregation (Sugata et al., 2000; Okada et al., 2006). Furthermore, knockdown of CENP-H led to severe mitotic phenotypes and reduced CENP-C level, which suggests that CENP-H plays an important role in the architecture and function of the kinetochore complex (Orthaus et al., 2006). The expression of CENP-H was found upregulated in malignant tumors such as colorectal cancer (Tomonaga et al., 2005). CENP-H may be relevant in tumorigenesis and a promising prognostic marker in non-small cell lung cancer, esophageal carcinoma, oral squamous cell car cinoma, and nasopharyngeal carcinoma (Shigeishi et al., 2006; Liao et al., 2007; 2009; Guo et al., 2008). However, the involvement of CENP-H as a relapse-associated biomarker in HSCC has not been clarified.

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Ki67 is strongly expressed in proliferating cells and universally accepted as a proliferation marker, because it is present in all active phases but not the resting phase (G0) of the cell cycle during mitosis (Gerdes et al., 1983) (Scholzen et al., 2000). Ki67 has been widely studied and used in various fields. It has been used to predict survival and relapse in oral carcinoma (Wangsa et al., 2008; Faratzis et al., 2009).

We aimed to investigate the role of CENP-H and Ki67 in HSCC and an association with relapse-free survival after curative resection.

Materials and Methods

Patients and specimens

We enrolled 112 patients (mean±SD age 57.4 years [range 36-82 years]) with primary HSCC who underwent surgical resection as initial treatment at the Department of Otolaryngology-Head and Neck Surgery, Qilu Hospital, Shandong University, between March 2003 and March 2005. Patients were included if they had received a diagnosis of HSCC by postoperative pathologic examination and showed negative surgical margins after resection. Postoperative radiation was routinely recommended to reduce relapse risk. Patients with preoperative radiotherapy and/or chemotherapy were excluded. The study was approved by the Investigation and Ethical Committee of Qilu Hospital according to the Standards of the Declaration of Helsinki.

Cancer tissues were immersed in 10% neutral buffered formalin immediately after resection, then embedded in a paraffin block. As well, fresh cancer and matched normal epithelia specimens from HSCC patients were frozen in liquid nitrogen, then stored at -80 °C.

Postoperative follow-up

After resection, follow-up included history taking and physical examination, as well as electronic laryngoscopy every 3 months. Patients underwent CT or MRI every 8 months to identify relapse or not. Relapse-free survival was defined as the interval between the date of surgery and the date of diagnosis of relapse. Follow-up and face-to-face conversation with patients and/or relatives was over 15 min. The follow-up ended April 2010. The median follow-up was 48.5 months (range 2-73 months).

Antibodies

CENP-H and Ki67 antibodies were from Bethyl Laboratories (1:75 dilution, Montgomery, TX, USA) and Zhongshan Golden Bridge Biotechnology (Beijing; working solution), respectively.

Immunohistochemistry and evaluation

Immunohistochemistry of CENP-H and Ki67 expression involved tissue sections (4-μm) deparaffinized and rehydrated. Epitopes were retrieved by microwaving at 750W for 20 min in EDTA (pH 8.0) buffer to enhance immunoreactivity. After blocking the endogenous peroxidase activity with 3% H_2O_2 for 10 min and nonspecific antibody reaction with 5% normal goat serum for 20 min at room temperature, sections were incubated with primary antibodies overnight at 4 °C. Negative control sections were incubated with phosphate buffered saline (PBS) instead of primary antibodies. Antibody binding was detected with use of the avidin–biotin complex histofine universal kit (Zhongshan Golden Bridge Biotechenology) and visualized by the 3.3'-diaminobenzidine method for 5 min. Sections were then counterstained with Meyer's haematoxylin. Labeled nuclei were reported as percentage of total tumor cells counted and graded as 0 (negative staining); 1 + (low staining or < 20%); 2 + (intermediate staining or 20-50%); and 3 + (strong staining or > 50%). 0 and + were considered low and 2+ and 3+ high expression.

Cell lines and culture

The FaDu HSCC cell line (ATCC) and its derived lines (ATCC) were cultured as routine with RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2-humidified incubators with subculture every 3 days.

Knockdown of CENP-H in cells

Primer sequences for small interfering RNA (siRNA) for CENP-H were forward, 5'-GGUUGAUGCAAGUGAAGAATT-' and reverse, 5'UU-CUUCACUUGCAUCAACCTT3'. Negative scramble control sequences were forward, 5'-UUCUCCGAACGUGUCACGUTT-3' and reverse, 5'-ACGUGACACGUUCGGAGAATT-3 (Shanghai GenePharma). Transient transfection of siRNA in cells involved the Lipofectamine 2000 method (Invitrogen). Transfection efficiency was detected by western blotting.

Western blot analysis

Harvested cells were lysed with ice-cold lysis buffer. Protein samples were seperated by electrophoresis in an 10% denature polyacrylamide gel, transferred to PVDF membranes (Immoblelon-p, Millipore, Bedford, USA), and were probed with antibodies. Proteins were visualized by enhanced chemiluminescence (ECL) western Blotting Detection Reagents (Millipore, Bedford, MA, USA).

Flow cytometry

At 48 hr after transfection, cells were harvested and centrifuged, then washed twice with PBS and stained with 50 μg propidium iodide and 100 μg annexin-V. Flow cytometry involved use of the Apoptosis Assay kit (Roche).

Cell proliferation assay

Cell growth was assessed by cholecystokinin octapeptide (CCK-8) assay. At 24, 48, and 72 hr after siRNA transfection, cells (5×10^3) were seeded into 96-well plates, and 10 μl CCK-8 (Cell Counting Kit; Bestbio, China) was added in each well for incubation at 37 °C for 1.5 hr. The optical density (OD) was measured at 450 nm for each well by use of a microplate reader (Bio-Rad Model 680, Richmond, CA, USA).

Statistical analysis

Statistical analysis involved use of SPSS v16.0

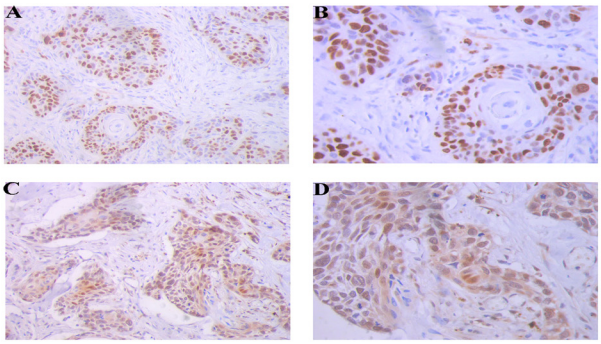

Figure 1. Representative CENP-H and Ki67 Immunohistochemical Staining in Hypopharyngeal Squamous Cell Carcinoma (HSCC). Ki67 (A, B) and CENP-H (C, D) immunostaining (original magnification ×200 and ×400)

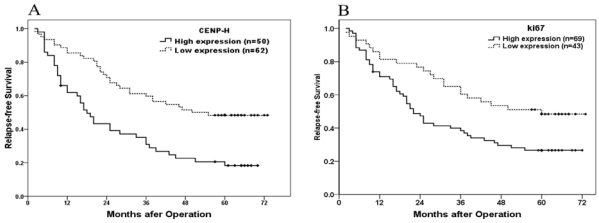

Figure 2. Kaplan-Meier Curves of Relapse-free Survival in HSCC Stratified by Low and High CENP-H (A) and Ki67 (B) Expression. (P<0.001 and P=0.009 respectively)

(SPSS Inc., Chicago, IL). Student's test was used to compare 2 groups, and chi-square test was used to analyze clinicopathologic variables by gene expression. The Kaplan-Meier method was used to analyze survival, with the log-rank test to compare relapse-free survival and survival. Cox regression multivariate analysis was used to determine predictors of survival. A $p < 0.05$ was considered statistically significant.

Results

CENP-H and Ki67 expression and their association with HSCC

CENP-H and Ki67 protein immunostaining was mainly observed in nuclei of HSCC cells of the 112 specimens (Figure 1): 50 cases (44.6%) showed upregulated CENP-H expression (62 specimens showed low CENP-H expression) and 69 cases (61.6%) showed upregulated Ki67 overexpression. CENP-H expression was associated with Ki67 expression (P= 0.005).

Association of CENP-H and Ki67 expression with clinicopathologic features

CENP-H overexpression was associated with the advanced cancer stage (P = 0.012) and alcohol consumption (P = 0.048) (Table 1), but Ki67 overexpression was associated with only advanced cancer stage (P=0.021).

Association of CENP-H and Ki67 expression with relapse-free survival

Patients were stratified by low and high protein expression to determine whether CENP-H and Ki67 expression were related to patient relapse-free survival. By Kaplan–Meier survival analysis, relapse-free survival was shorter for patients with than without CENP-H and Ki67 overexpression (P<0.001 and P = 0.009, respectively (Figure 2). Multivariate analysis confirmed CENP-H and Ki67 overexpression as independent predictors of relapse-free survival (P = 0.001 and P = 0.018, respectively) (Table 2).

Table 1. Clinicopathologic Variables Associated with Expression Patterns of or Ki67 or CENP-H

Variables	n	Ki67 High	Ki67 Low	P	CENP-H High	CENP-H Low	P value
Sex							
Male	93	56	37	0.503	43	50	0.453
Female	19	13	6		7	12	
Age, years							
≤60	58	38	20	0.378	24	34	0.472
>60	54	31	23		26	28	
TNM stage[a]							
I+II	26	11	15	0.021	6	20	0.012
III+IV	86	58	28		44	42	
Histologic grade							
Well or moderate	44	24	20	0.216	17	27	0.304
Poor	68	45	23		33	35	
Smoking history							
Current and former	87	55	32	0.513	41	46	0.324
Never	25	14	11		9	16	
Alcohol history							
Current and former	79	50	29	0.571	40	39	0.048
Never	33	19	14		10	23	

[a]TNM, Tumor-Node-Metastasis

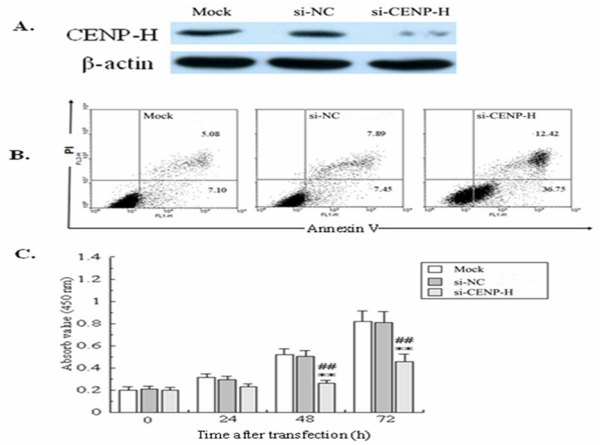

Figure 3. Knock-down of CENP-H Affects Apoptosis, Proliferation of HSCC Cells. A, Western blot analysis of CENP-H expression in FaDu cells transfected with control siRNA (si-NC) or CENP-H siRNA (si-CENP-H) for 48 hr. Similar results were obtained in 3 independent experiments. B, FaDu cells were treated as in (A). Cells were stained with Annexin V and PI, and then analyzed by flow cytometry. Similar results were obtained in 3 independent experiments. C, FaDu cells were treated as in (A) for indicated times. Cholecystokinin octapeptide assay was performed to detect cell growth. Data are mean ± SD (n=3) of 1 representative experiment (**P<0.01)

Role of CENP-H in HSCC apoptosis

CNPH protein was markedly downregulated after CENP-H siRNA transfection in HSCC cells at 48 hr (Figure 3A). We determined whether siRNA knockdown of CENP-H induced cell apoptosis by FACS analysis. siRNA knockdown of CENP-H protein level greatly increased apoptosis as compared with controls (P<0.01)

Table 2. Variables in the Equation for the Multivariate Logistic Regression Model

Risk factors	B	SE	Wald	df	P value	Exp(B)
Cancer stage	0.474	0.248	3.637	1	0.057	1.606
CENP-H	0.877	0.257	11.659	1	0.001	2.403
Ki67	0.644	0.273	5.582	1	0.018	1.905

SE, standard error

(Figure 3B), which indicates that CENP-H overexpression in HSCC might promote cancer cell proliferation by inhibiting cell apoptosis.

Role of CENP-H in HSCC cell proliferation

CCK-8 assay was used to measure HSCC cellular proliferation after siRNA knockdown of CENP-H. Cellular proliferation was decreased with CENP-H siRNA knockdown in HSCC cells at 48 and 72 hr ($P<0.01$) (Figure 3C), so CENP -H overexpression in HSCC may be involved in cancer cell proliferation.

Discussion

Centromere and kinetochore proteins play a pivotal role in accurate chromosome segregation. However, the underlying mechanism is not fully understood. Centromere fission can result in chromosomal instability and generate aneuploid because of defects (Martinez et al., 2011). Aneuploidy may induce tumorigenesis (Duesberg et al., 2000; Duesberg et al., 2003; Bharadwaj et al., 2004). Previous studies found abnormal phenotypes in chicken DT40 cells after CENP-H knockdown (Fukagawa et al., 2000; Mikami et al., 2005). Deletion of CENP-H in zebra fish could upregulate components of the intrinsic apoptotic pathway, thus reducing tumorigenesis (Zhao et al., 2010). However, no study has examined CENP-H expression or its association with relapse-free interval in patients undergoing curative resection for HSCC. Previous study found CENP-H overexpressed in primary colorectal cancer at both protein and mRNA levels (Tomonaga et al., 2005). In this study, we found CENP-H protein level upregulated in 112 specimens of HSCC and associated with advanced cancer stage and alcohol consumption. Thus, CENP-H overexpression may be involved in HSCC progression and play an important role in tumorigenesis. Previous studies showed CENP-A and CENP-F associated with epithelium-originated malignancies (Guardia et al., 2001; Tomonaga et al., 2003; O'Brien et al., 2007). Kinetochore protein activity may be altered in some epithelial malignancies. The overexpression of CENP-H in HSCC prompted us to evaluate the relationship between its protein expression and relapse-free survival. We found an association of CENP-H overexpression associated with poor relapse-free survival in HSCC, which suggests that CENP-H may play a critical role in increasing the risk of tumor relapse.

Compared to CENP-H, Ki67 was more commonly expressed in our HSCC samples. We found a significant association of Ki67 expression and cancer stage but not other factors. These results are not consistent with previous studies (Faratzis et al., 2009) and may relate to differences in epithelial malignancy and patient population. In agreement with previous findings (Shigeishi et al., 2006; Liao et al., 2009), we found a significant association of increased CENP-H and Ki67 protein expression, probably in part because of the role of CENP-H in promoting tumor cell proliferation. We also found increased Ki67 protein expression associated with poor relapse-free survival in patients with HSCC. Furthermore, increased CENP-H and Ki67 protein expression were independent prognostic factors of survival. In our study, patients with HSCC showed high risk of relapse after primary surgery or surgery and radiation. However, the current technology cannot completely determine early occult relapse without histopathological evaluation, so some patients cannot be treated because of delay in diagnosis. Immunohistochemical tumor markers, as well as CENP-H and Ki67 expression analysis, may help predict the risk of relapse after treatment.

Knockdown of CENP-H in HSCC cells induced cell apoptosis and impaired cell proliferation, which suggests that CENP-H may have an important role in HSCC cell proliferation and may explain the association of increased CENP-H expression with high relapse rate after curative resection. Limitless replicative potential is an important feature of malignant tumors and the most important biological mechanisms in oncogenesis (van Diest et al., 1998; Hanahan et al., 2000). Tumor growth strongly depends on the balance between proliferation and apoptosis. Our findings can point to new therapeutic strategies based on regulating CENP-H or Ki67 activity, because surgery and/or radiation therapy often fail to prevent tumor relapse.

The strengths of our study are that we excluded patients with preoperative radiotherapy and/or chemotherapy and the edges of resection were confirmed to be negative by routine histologic examination. Also, our study featured adequate follow-up and face-to-face conversation with patients and/or relatives to obtain detailed information. However, our sample size was small, and the results need to be confirmed in a larger popoulation.

In conclusion, our study demonstrated that increased CENP-H protein expression was common in HSCC and significantly associated with increased Ki67 protein expression. In addition, both CENP-H and Ki67 protein overexprssion was significantly associated with short relapse-free survival. CENP-H and Ki67 protein expression may have clinical potential for adjuvant therapy as indicators of cancer-associated survival after resection. Although our results are promising, future study is required to investigate the mechanism underlying the role of CENP-H in promoting tumor growth and in prognosis.

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