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Elevated long noncoding RNA HAGLROS expression correlates with clinical progression and prognosis in osteosarcoma

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Abstract. – OBJECTIVE: Our study aimed to evaluate the expression pattern and prognostic value of long noncoding RNA HAGLROS (HAGLROS) in osteosarcoma.

PATIENTS AND METHODS: qRT-PCR was performed to detect the expression levels of HAGLROS in osteosarcoma tissues and matched normal bone tissues. The relationship between the expression of HAGLROS and the clinicopathological features was analyzed by chi-square test. The survival curves were calculated by the Kaplan-Meier method and the difference by the log-rank test. The Cox proportional hazards model for multivariate survival analysis was used to assess predictors related to survival.

RESULTS: Herein, we showed that HAGLROS was frequently upregulated in osteosarcoma tissue and cell lines compared to normal human bone tissues ($p < 0.01$). In addition, HAGLROS up-regulation more frequently occurred in osteosarcoma specimens with advanced TNM stage ($p = 0.023$), positively distant metastasis ($p = 0.002$) and poor differentiation ($p = 0.021$). Survival analysis showed that osteosarcoma patients with higher HAGLROS expression suffered poorer overall survival ($p = 0.012$) and disease-free survival ($p = 0.003$). In a multivariate Cox model, it was confirmed that HAGLROS up-regulation was an independent poor prognostic factor for both 5-year overall survival (HR=3.546, 95% CI: 1.273-5.326; $p = 0.002$) and 5-year disease-free survival (HR=3.854, 95% CI: 1.427-5.885; $p = 0.001$).

CONCLUSIONS: We showed that HAGLROS was an independent predictor of unfavorable prognosis in osteosarcoma patients and may serve as a potential target.

Key Words:
lncRNA, HAGLROS, Osteosarcoma, Prognosis.

Introduction

Osteosarcoma, arising from primitive mesenchymal bone-forming cells, is the most common primary malignant bone tumor in childhood and adolescence, comprising around 20% of all primary bone cancers[1,2]. This tumor can exist in any bone of human being, but the metaphyses of the long bones are the most commonly affected position[3]. With the development of new treatment protocols that combine chemotherapy, surgery and sometimes radiotherapy, the five-year overall survival rates have increased to 65% over the past ten year[4,5]. Unfortunately, the current therapies are not effective against the recurrent or metastatic osteosarcoma and the survival rate of osteosarcoma patients remains poor for those with relapse and metastasis[6,7]. Thus, there is an urgent need to identify diagnostic and prognostic markers for early detection and individualized treatment of osteosarcoma. Long non-coding RNAs (lncRNAs) is functionally defined as nonprotein coding transcripts >200 nt in length that are longer than 200 nucleotides without evident protein coding functions[8]. LncRNAs share similar characteristic with mRNAs and many of them are transcribed by RNA polymerase II and experience polyadenylation and splicing[9]. Though more and more dysregulated lncRNAs have been identified, only a small proportion of them have been functionally characterized in detail[10,11]. It has been confirmed that lncRNAs participate in multiple cellular biological processes, ranging from transcriptional and posttranscriptional regulation to apoptosis progress, cell differentiation and epigenetic regulation[12,13]. Emerging evidence shows that lncRNAs may play complex and extensive roles in regulation of the development and progression of tumors via acting as oncogenes or tumor suppressors[14-16]. LncRNA HAGLROS (HAGLROS) is located in chromosome 2q31.1 and has 1124 nucleotides in length. The expression pattern and function of HAGLROS in tumors remains largely unknown. In gastric cancer and

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Table I. The association between HAGLROS expression and characteristics of patients suffering from osteosarcoma.

Clinicopathological Features	No. of cases	HAGLROS expression		*p*-value
		High	Low	
Gender				0.537
Male	116	55	61	
Female	77	40	37	
Age				0.129
≤ 40	94	41	53	
> 40	99	54	45	
Tumor diameter (cm)				0.130
≤ 5	124	56	68	
> 5	69	39	30	
Anatomical location				0.349
Tibia/femur	97	51	46	
Elsewhere	96	44	52	
Differentiation				0.021
Well and moderate	131	57	74	
Poor	62	38	24	
TNM stage				0.023
I + II	125	54	71	
III + IV	68	41	27	
Distant metastasis				0.002
Absent	147	63	84	
Present	46	32	14	
Response to chemotherapy				0.360
Good	128	60	68	
Poor	65	35	30	

colorectal cancer, HAGLROS was reported to be highly expressed and served as a tumor promoter *in vitro* and *in vivo*[17,18]. However, the exact expression, function, and mechanism of HAGLROS in osteosarcoma remain uncovered. In this study, HAGLROS expressions in tumor and adjacent normal bone tissues of 193 osteosarcoma patients were detected. The correlation between HAGLROS and the clinicopathological factors and overall survival was analyzed. Our data provided first evidence that HAGLROS was highly expressed in osteosarcoma and have potential to be a novel prognostic biomarker for osteosarcoma patients.

Patients and Methods

Patients and Tissue Samples

A total of 193 paired osteosarcoma tissues and adjacent normal bone tissues were obtained from the osteosarcoma patients with surgical treatment from The Affiliated Huaian NO.1 People's Hospital of Nanjing Medical University between January 2010 and May 2013. These osteosarcoma cases were from 116 males and 77 females, and none of the patients had received radiotherapy or chemotherapy before surgery. All of the samples were reassessed by two pathologists. All samples were frozen in liquid nitrogen, stored at -80°C and used for extraction of RNA. The clinicopathological features of all patients were showed in Table I. Informed consents were obtained from each patient to approve the use of their tissues for research purposes. The study protocol was approved by the Institute Research Ethics Committee at the Affiliated Huaian NO.1 People's Hospital of Nanjing Medical University (Nanjing, China).

Quantitative Real-Time Reverse Transcription-PCR

Total RNAs were extracted from frozen tumor tissues and matched normal bone tissues using TRIzol reagent as described by the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). The quantity and the quality of RNA were evaluated using a Nanodrop spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). RNA was reversed transcribed into cDNAs using the Primer-ScriptTM one step RT-PCR kit (TaKaRa, Otsu, Shiga, Japan). The PCR amplification was performed for 40 cycles at 94°C for 30 s, 60°C for

30 s, and 72°C for 30 s, on an Applied Biosystems 7900HT (Biosystems, Foster City, CA, USA). SDS2.3 software (Biosystems, Foster City, CA, USA) was used to analyze the collected data. The relative quantification values for lncRNA were calculated by the $2^{-\Delta\Delta Ct}$ method using GAPDH as an internal reference. The PCR primers used were as follow: 5'- AGCCACATCGCTCAGACAC-3' (sense) and 5'-GCCCAATACGACCAAATCC-3' (antisense) for GAPDH and 5'- TGTCACCCTTAAATACCGCTCT-3' (sense) and 5'- CTTCCTCCCACACAAATACTCC -3' (antisense) for HAGLROS.

Statistical Analysis

Statistical analysis was preceded by using SPSS statistical software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5.0 software (La Jolla, CA, USA). The comparison of the expression of HAGLROS between osteosarcoma tissues and adjacent normal bone tissues were performed using the two-sample Student's *t*-test. The chi-square test was applied to the examination of relationship between HAGLROS expression levels and clinicopathologic characteristics. Overall survival and disease-free survival rates were calculated by the Kaplan-Meier method with the log-rank test applied for comparison. COX regression model was used to analyze the influence of related factors on the survival time of patients with osteosarcoma. Differences were considered significant at *p*-value less than 0.05.

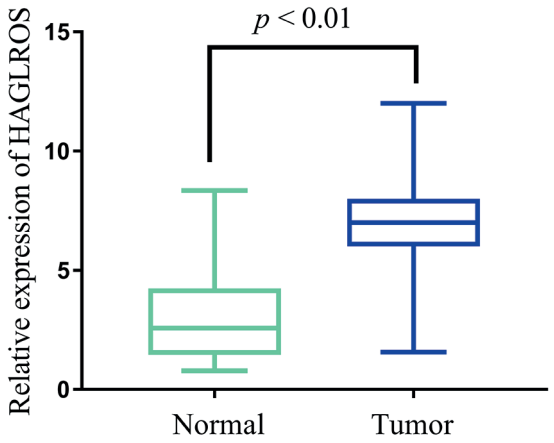

Figure 1. The relative expression levels of HAGLROS in osteosarcoma tissues and adjacent normal bone tissues. HAGLROS expression was significantly higher in osteosarcoma tissues than in the corresponding adjacent non-cancerous tissues ($p < 0.01$).

Results

LncRNA HAGLROS was Up-Regulated in Osteosarcoma

To investigate the level of HAGLROS expression in human osteosarcoma, RT-PCR assays were performed in a total of 193 pair osteosarcoma tissues and matched normal bone tissues. As shown in Figure 1, we found the expression levels of HAGLROS be distinctly increased in osteosarcoma tissues compared to adjacent non-tumor tissues ($p < 0.01$). Our findings indicated that HAGLROS may contribute to the development of osteosarcoma.

HAGLROS Upregulation Associates with Aggressive Clinicopathological Parameters of Human Osteosarcoma

Then, we further explore the clinical significance of HAGLROS in osteosarcoma patients: a total of 193 osteosarcoma samples were divided into two groups on the basis of HAGLROS. The association between clinicopathological characteristics and HAGLROS expression was summarized in Table I. We found that high HAGLROS expression levels were closely correlated with differentiation ($p = 0.021$), advanced TNM stage ($p = 0.023$) and distant metastasis ($p = 0.002$). However, there were no significant correlations of miR-124 expression with other clinical features such as age, gender, tumor diameter, anatomical location, and response to chemotherapy ($p > 0.05$).

HAGLROS Up-Regulation Associates with Poor Prognosis of Osteosarcoma Patients

In order to further assess the prognostic significance of HAGLROS expression in osteosarcoma, survival curves were constructed by Kaplan-Meier method and compared by the log-rank test. As shown in Figure 2, we found that patients with high HAGLROS expression level would have a shorter five-year overall survival in comparison with those with low HAGLROS expression level (median survival, 36.0 *vs.* 58.0 months, $p = 0.0012$). In addition, we also found the similar trend in the disease-free survival analysis (median survival, 32.0 *vs.* 47.5 months, $p = 0.003$) (Figure 3). Then, we performed Cox proportional-hazards regression to explore the potential associations between HAGLROS expression and outcome. In multivariate analysis, HAGLROS expression level confirmed to be an independent prognostic factor for predicting the 5-year overall survival and

Table II. Multivariate survival analysis of overall survival and disease-free survival in patients with osteosarcoma.

Variables	Overall survival			Disease-free survival		
	RR	95% CI	*p*-value	RR	95% CI	*p*-value
Gender	1.332	0.561-1.893	0.315	1.532	0.744-2.137	0.169
Age	1.425	0.745-2.214	0.258	1.257	0.544-2.044	0.108
Tumor diameter	0.957	0.515-1.774	0.105	0.884	0.616-1.895	0.166
Anatomical location	1.237	0.789-1.899	0.123	1.341	0.654-2.214	0.119
Differentiation	2.785	1.345-4.326	0.015	2.994	1.568-4.654	0.009
TNM stage	2.564	1.547-4.775	0.007	2.676	1.633-5.121	0.013
Distant metastasis	3.215	1.256-5.342	0.004	3.652	1.548-5.887	0.001
Response to chemotherapy	1.232	0.665-1.894	0.321	1.447	0.857-2.231	0.114
HAGLROS expression	3.546	1.273-5.326	0.002	3.854	1.427-5.885	0.001

disease-free survival of osteosarcoma patients ($p < 0.05$, Table II).

Discussion

Osteosarcoma is the most common bone tumor occurring in childhood and adolescence, with high degree of high mortality[19]. Early diagnosis and predication of prognosis are very critical for the doctors for the design of individualized treatment, therefore improving the quality of life and prognosis of osteosarcoma patients[20,21]. Thus, the identification of promised biomarkers that can potentially contribute to the management of osteosarcoma is an attractive goal. Previously, various tumor-related, proteins and noncoding RNAs were reported to have great potential to act as cancer biomarkers[22, 23]. Recently, growing attention focused on the possibility of lncRNAs as a new biomarker for a predictor for diagnosis and prognosis of osteosarcoma patients due to its frequently dysregulated expressed and acting as regulators of tumors by functioning as tumor suppressors or oncogenes[24, 25].

Recently, the expression pattern and biological function of HAGLROS have been reported in several tumors. For instance, Zheng et al[17] reported that HAGLROS expression was increased in colorectal cancer and could be associated with advanced clinical stages. Functional investigation of *in vitro* and *in vivo* confirmed that suppression of HAGLROS may induce apoptosis and inhibit autophagy in colorectal cancer cells by modulation of miR-100/ATG5 axis. Chen et al[18] found that HAGLROS, modulated by STAT3, an important transcription factor, was highly expressed in gas-

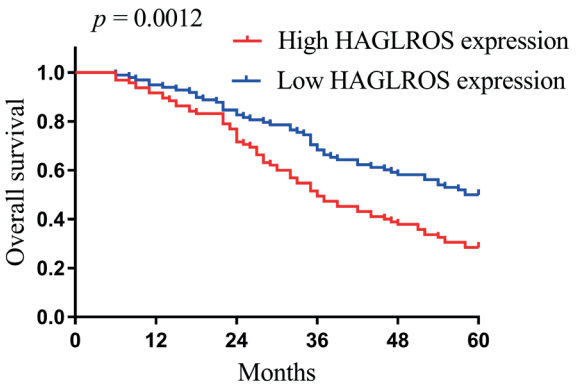

Figure 2. Overall survival curves for two groups defined by low and high expression of HAGLROS in osteosarcoma patients. The patients with high HAGLROS expression had a significantly shorter overall survival than those with high HAGLROS expression ($p = 0.0012$).

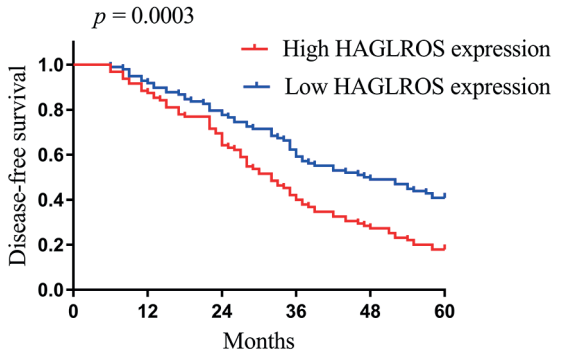

Figure 3. Disease-free survival curves for two groups defined by low and high expression of HAGLROS in osteosarcoma patients. The patients with high HAGLROS expression had a significantly shorter disease-free survival than those with high HAGLROS expression ($p = 0.0003$).

tric cancer tissues and cell lines and associated with shorter overall survival of the patients with this tumor. Further lost-functional assays indicated that knockdown of HAGLROS suppressed the proliferation, migration and invasion by mTOR signal-mediated inhibition of autophagy. These results highlighted the tumor-promotive roles of HAGLROS in above two cancers. However, whether HAGLROS was abnormally expressed in osteosarcoma, its potential significance has not been investigated. In this study, we firstly detected the expression levels of HAGLROS in osteosarcoma patients by RT-PCR and the results showed that HAGLROS expression was significantly upregulated in osteosarcoma tissues compared to matched normal bone tissues, which was consistent with the expression trend of HAGLROS in gastric cancer and colorectal cancer. Then, we further explored the association between HAGLROS expression and clinicopathological characteristics in osteosarcoma, finding that high expression of HAGLROS was distinctly associated with poor differentiation, advanced TNM stage and positively distant metastasis, suggesting that HAGLROS may be involved in the modulation of clinical progress of this malignancy. Moreover, we collected the follow-up data of five years and performed Kaplan-Meier assays to further explore the prognostic value of HAGLROS in osteosarcoma patients, finding that osteosarcoma patients with high HAGLROS levels tended to have shorter overall survival and disease-free survival than patients with lower levels. More importantly, multivariate analysis showed that high HAGLROS expression was an independent prognostic factor of overall survival and disease-free survival of osteosarcoma patients.

Conclusions

We firstly reported that HAGLROS expression was significantly increased in osteosarcoma clinical samples and positively correlated with the malignant status of osteosarcoma. In addition, higher HAGLROS expression level was associated with poor prognosis in osteosarcoma patients. These data highlight the significance of HAGLROS in osteosarcoma progression, indicating that HAGLROS may be a critical cancer biomarker for osteosarcoma poor prognosis and a potential therapeutic target. However, the molecular mechanisms of HAGLROS involved in osteosarcoma need to be further studied.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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