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Expression of seven-in-absentia homologue 1 and hypoxia-inducible factor 1 alpha: Novel prognostic factors of nasopharyngeal carcinoma

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hypoxia-inducible factor 1 alpha (HIF1 $\alpha$ )

latent membrane protein 1 (LMP1)

Epstein-Barr virus (EBV)

nasopharyngeal carcinoma

## **ABSTRACT**

Nasopharyngeal carcinoma (NPC) is an EBV-associated cancer. We analysed Siah1 expression as well as LMP1 and HIF1 $\alpha$  expression by immuno-histochemical staining in 74 NPC biopsy specimens and found that the expression of Siah1 was significantly correlated with advanced tumour status and stage. Moreover, Siah1-positive and HIF1 $\alpha$ -positive cases had significantly worse prognoses. The expression score for LMP1 was remarkably correlated with that of Siah1, whereas there was little correlation between LMP1 expression and the other markers evaluated. This is the first study to evaluate the pattern and clinical significance of Siah1 and HIF1 $\alpha$  expression in NPC, and such an evaluation is valuable for identifying those patients at a high risk for a poor prognosis.

## 1. Introduction

Nasopharyngeal carcinoma (NPC) typically manifests with clinically invasive and metastatic features [1]. Despite recent advances in radiation techniques and chemotherapy, local failure and distant metastasis remain poor survival in patients with advanced NPC [2].

Tumour hypoxia is one of the most common phenomena in human solid cancers. Tumour hypoxia is known to contribute to resistance to chemotherapy and radiotherapy as well as the malignant tumour phenotype, involving increased invasiveness and poor prognosis [3, 4]. In particular, of the proteins associated with tumour hypoxia, hypoxia-inducible factor 1-alpha (HIF1 $\alpha$ ) is a key hypoxia regulatory molecule [5]. HIF1 $\alpha$  plays a major role in the development of the tumour phenotype and influences tumour growth rate, angiogenesis, invasiveness, and metastasis [6, 7]. Hui *et al.* showed that the overexpression of HIF1 $\alpha$  at the primary tumour was related to worse prognosis in patients with NPC [8]. Although the regulation of HIF1 $\alpha$  is complicated, Nakayama *et al.* identified a novel mechanism in which seven-in-absentia homologue (Siah)-family proteins contribute to the stabilisation of HIF1 $\alpha$  under hypoxic conditions [9]. Siah-family proteins are the human homologues of seven-in-absentia, a conserved RING finger E3 ubiquitin ligase and essential downstream component of the *Drosophila* Ras signaling pathway. Two homologues, Siah1 and Siah2, have been shown to be involved in the response to DNA damage, the hypoxic response,

inflammation, and several oncogenic signals including those propagated by Ras and epidermal growth factor receptor (EGFR) [10-13]. To date, few studies have addressed the role of Siah expression in cancer, and there has been no consensus as to the biologic significance of such expression [14, 15]. Thus, Siah expression and its role in the molecular pathogenesis of cancer, including NPC, remain unknown.

Recently, we demonstrated that Epstein-Barr virus (EBV)-associated latent membrane protein 1 (LMP1) increased the expression of HIF1 $\alpha$  via the induction of Siah1 in human nasopharyngeal epithelial cells [16]. Moreover, EBV is closely related to the carcinogenesis of NPC [1]. Among EBV proteins, LMP1 is a principal oncogenic protein that can transform resting B lymphocytes [17]. In addition, we have shown that the expression of LMP1 is responsible for the highly metastatic properties of NPC [17-19]. Therefore, we studied the expression of Siah1, HIF1 $\alpha$ , and LMP1 in NPC biopsy specimens. The objective of our current study was to evaluate the association between Siah1, HIF1 $\alpha$ , and LMP1 expression and the clinicopathological factors present in patients with NPC.

## **2. Materials and methods**

### *2.1. Patients*

We obtained 74 tumour specimens from patients with NPC who had been diagnosed at the Division of Otolaryngology at Kanazawa University Hospital as well as other branch hospitals between May 1998 and December 2009. The characteristics of these patients are shown in Table 1. Clinical status was determined according to the 1997 UICC/AJCC staging system [20].

Of the 74 patients, 59 patients received radiotherapy with cisplatin-based concurrent chemotherapy. Seven patients received radiotherapy due to increased age (>70 years) or renal dysfunction. According to the change of treatment protocol from 2005, 8 patients received alternating cisplatin-based chemo-radiotherapy, as described elsewhere [21]. The accumulated dose of radiation to the nasopharynx was 70-77 Gy in all cases, and the dose to the neck was 40-70 Gy. The median time of follow-up was 3.77 years (range 0.12-11.38 years). The survival period was calculated according to the date of initial treatment, and 29 of the patients studied were alive at the end of December 2009.

## *2.2. Immunohistochemical analysis of NPC tissues*

Primary NPC paraffin-embedded specimens were used for the immunohistochemical analysis of Siah1, HIF1 $\alpha$ , and LMP1 expression. Three-micrometre-thick sections were prepared from each block of tissue embedded in paraffin. Deparaffinised sections were treated with 3% hydrogen peroxide for 10 minutes to inactivate endogenous peroxidase activity. The sections were

incubated with protein blocker (Dako, Glostrup, Denmark) for 20 minutes and incubated at 4°C overnight with rabbit anti-human Siah1 antibody (TransGenic Inc., Kumamoto, Japan), rabbit anti-human HIF1 $\alpha$  antibody (H-206, Santa Cruz Biotechnology, Santa Cruz, CA), or mouse anti-LMP1 antibody (CS1-4, Dako) as the primary antibody. The sections were washed three times with phosphate-buffered saline (PBS, pH 7.2). After washing with PBS, the sections were exposed to Envision<sup>+</sup> secondary antibody (Dako) for 30 minutes. The reaction products were developed by immersing the sections in a 3'3-diamidobenzidine tetrahydrochloride (DAB) solution. The sections were counterstained with methyl green or haematoxylin.

### *2.3. In situ hybridization of EBV-encoded small RNA (EBER)*

In situ hybridization for the detection of EBV-encoded small RNA (EBER) was performed using EBER PNA probe/fluorescein and PNA ISH Detection kit (Dako) according to manufacturers' instruction. Sections were counterstained with haematoxylin.

### *2. 4. Evaluation of the specimens*

All slides were evaluated independently by two investigators (N. K. and S. K.) without previous knowledge of the clinical data and were then reviewed with the others. In each case, two arbitrary separate microscopic fields (200x) containing >200 tumour cells were evaluated. After

counting both immunoreactive cells and the total number of tumour cells, the average frequency of immunoreactive cells was calculated. The average percentage of immunoreactive cells was defined as the expression score and was used for statistical analysis. In addition, the percentages of immunoreactive cells demonstrated a wide range of staining expression, (from 0 to 100%), and one peak for Siah1, HIF1 $\alpha$ , and LMP1 expression demonstrated a frequency <10%. On the basis of these data, the cases were classified into negative and positive categories, as follows: negative, <10% immunoreactive cells; positive,  $\geq$ 10% immunoreactive cells. EBER expression was qualitatively classified into either positive or negative.

## *2. 5. Statistical Analysis*

IBM SPSS Statistics version 19 (IBM, Armonk, NY) was used for data analysis. The clinical characteristics of patients in relation to Siah1, HIF1 $\alpha$ , and LMP1 expression were analysed using Fisher's exact test and the chi-square test. The association between LMP1, Siah1, and HIF1 $\alpha$  expression and the overall patient survival rates was analysed using Kaplan-Meier estimates and the log-rank test. Univariate and multivariate analysis were performed to identify prognostic factors associated with overall survival using Cox regression analysis. The relationships between the expression scores of Siah1, HIF1 $\alpha$ , and LMP1 were analysed with Spearman's rank correlation coefficient. P values of < 0.05 were considered statistically significant.

### 3. Results

#### 3.1. Immunostaining for *Siah1*, *HIF1 $\alpha$* , and *LMP1*

The expression of *Siah1*, *HIF1 $\alpha$* , and *LMP1* was examined by immunohistochemical staining in NPC biopsy specimens (Fig. 1A-C). The expression of these proteins was detected as dark brown staining. The expression of *Siah1* revealed both cytoplasmic and nuclear localisation (Fig. 1A), whereas *HIF1 $\alpha$*  was detected in the nucleus of the tumour cells (Fig. 1B). The mean expression scores for *Siah1* and *HIF1 $\alpha$*  were  $12.23 \pm 9.43\%$  and  $6.73 \pm 5.74\%$ , respectively. *LMP1* expression was noted to be most prominent in the membrane and cytoplasm of the tumour cells (Fig. 1C). The mean *LMP1* expression score was  $12.46 \pm 12.01\%$ . Finally, of the 74 specimens, 30 cases (40.5%) were categorised as *Siah1*-positive, 27 cases (36.4%) were *HIF1 $\alpha$* -positive, and 35 cases (47.2%) were *LMP1*-positive.

#### 3.2. Expression of *EBER*

In situ hybridization of *EBER* is considered the gold standard technique for detecting and localizing latent EBV in tissue samples in laboratory test [22]. *EBER* were detected in 62 of 74 cases. Positive hybridization signals were restricted to the nucleus of the tumour cells and were not

observed in surrounding normal tissues or infiltrating lymphocytes (Fig. 1D). The expression of EBER was detected as blue staining. The expression of EBER was significantly correlated with histologic types. Of these 74 cases, 36 of 42 cases (85.7%) of non-keratinizing carcinoma (WHO type II) and all 26 cases (100%) of undifferentiated carcinoma (WHO type III) were EBER positive whereas 6 cases of squamous cell carcinoma (WHO type I) were negative ( $p = 0.02$ , Table 1).

### *3.3. Association between Siah1, HIF1 $\alpha$ , and LMP1 expression*

The relationship between Siah1, HIF1 $\alpha$ , and LMP1 expression was statistically evaluated. There was a remarkable correlation between the expression of Siah1 and LMP1 ( $r = 0.57$ ,  $p < 0.001$ , Fig. 2A), and a weak correlation between the expression of Siah1 and HIF1 $\alpha$  ( $r = 0.33$ ,  $p = 0.004$ , Fig. 2B). There was no significant relationship between the expression of HIF1 $\alpha$  and LMP1 ( $r = 0.20$ ,  $p = 0.09$ , Fig. 2C).

We also analysed relationship of Siah1, HIF1 $\alpha$ , and LMP1 expression in EBER-positive NPC cases to clarify the contribution of LMP1 to the regulation of Siah1 and HIF1 $\alpha$  was first found in EBV-expressing cell lines [16]. Of 62 EBER-positive cases, there was a remarkable correlation between the expression of Siah1 and LMP1 ( $r = 0.53$ ,  $p < 0.001$ , data not shown), and a weak correlation between the expression of Siah1 and HIF1 $\alpha$  ( $r = 0.38$ ,  $p = 0.001$ , data not shown). Similarly, there was a weak relationship between the expression of HIF1 $\alpha$  and LMP1 ( $r = 0.28$ ,  $p =$

0.02, data not shown).

### *3. 4. Relationship between clinicopathological features and the expression of Siah1*

The associations between the patients' clinicopathological features and the expression score for Siah1, HIF1 $\alpha$ , and LMP1 are shown in Table 1. Each TNM classification and stage grouping was divided into two categories [20], and the tumour stage was classified as "early" (T1 and 2) or "advanced" (T3 and 4). The lesional lymph node stages were classified as lymph node-negative (N0) or lymph node-positive (N1, N2, or N3). Cases of distant metastasis at the initial visit were excluded in this study. Clinical stage was divided into "early" (I, II) and "advanced" (III, IV) categories. The Siah1 expression score was significantly associated with the progression of the tumour and the clinical stage ( $p = 0.001$ ,  $p = 0.002$ , respectively). However, there was no significant relationship either the HIF1 $\alpha$  or LMP1 expression scores and the clinicopathological features of the patients.

### *3. 5. Patient prognosis and the expression of Siah1, HIF1 $\alpha$ , and LMP1*

Next, we evaluated the prognostic value of Siah1, HIF1 $\alpha$ , and LMP1 expression in patients with NPC. The patients with Siah1-positive tumours showed worse overall survival rates than those with Siah1-negative tumours ( $p = 0.017$ , Fig. 3A). Similarly, HIF1 $\alpha$ -positive cases had worse

prognosis than HIF1 $\alpha$ -negative cases (Fig. 3B,  $p = 0.015$ ). However, LMP1 expression had no influence on the prognosis (data not shown,  $p = 0.43$ ).

Finally, we evaluated whether Siah1 or HIF1 $\alpha$  expression would represent an independent prognostic factor by Cox regression analysis. First, the association between the treatment protocol and patient prognosis was examined, and treatment differences were not shown to affect the prognosis of NPC patients, according to the results of a univariate analysis ( $p = 0.58$ , Table 2). Moreover, a univariate Cox regression analysis showed that age ( $\geq 50$ ), gender (female), advanced tumour status (T3 + T4), and the advanced stage (III + IV), expression of Siah1, and HIF1 $\alpha$  represented significant hazards (Table 2). Finally, a multivariate Cox regression analysis revealed that the expression of Siah1 and HIF1 $\alpha$ , age ( $\geq 50$ ), and advanced tumour stage (III + IV) were independent poor prognostic factors (Table 2).

#### **4. Discussion**

The current study showed that Siah1 expression was related to tumour progression in patients with NPC, whereas both HIF1 $\alpha$  and LMP1 expression did not demonstrate such an association. The expression of HIF1 $\alpha$  has previously been shown to be associated with a worse prognosis in nasopharyngeal carcinoma [8]. Here, we evaluated the prognostic role of Siah1

expression in addition to HIF1 $\alpha$  expression. A multivariate analysis revealed that Siah1 as well as HIF1 $\alpha$  expression as an independent poor prognostic factors for patients with NPC (Table 2). Upon these findings, we speculate that downstream factors of Siah1 other than HIF1 $\alpha$  may affect tumour progression. Siah was initially identified as a tumour suppressor gene [12], although recent studies have shown that knock-down of Siah by shRNA could significantly impede lung and pancreatic tumour growth *in vitro* and *in vivo*, indicating that expression of Siah itself promotes tumour growth [9, 13, 23]. Another study in breast cancer also showed that increased Siah expression was related to the aggressiveness of breast cancers [24]. Similarly, Siah1 nuclear expression was shown to be positively correlated with hepatocellular cancer progression [25]. These studies support our current results that Siah1 expression itself may influence the progression of NPC. Siah might serve a similar role as a downstream pathway component essential for oncogenic Ras signal as described in *Drosophila* [10, 26, 27]. Since Ras signal closely associated with cancer progression and transformation, such signal cascade may affect tumour progression by Siah1 as well as in human.

Furthermore, we previously demonstrated that the expression of LMP1 significantly correlated with the expression of Siah1; moreover, LMP1 could stabilised Siah1 expression, resulting in HIF1 $\alpha$  stabilisation *in vitro* [16]. In this study, the expression of LMP1 correlated with the expression of Siah1 in NPC biopsy specimens regardless of EBER (EBV) status. From these, Siah1 itself may play important role with tumour progression with or without EBV association.

On the other hand, neither LMP1 nor Siah1 expression correlated with HIF1 $\alpha$  expression in this study. Benders *et al.* reported a similar result and found that there was no significant relationship between the expression of LMP1 and HIF1 $\alpha$  in 18 NPC biopsy specimens, as analysed by immunohistochemistry. They concluded that the regulation of HIF1 $\alpha$  activity is dynamic and can change quickly due to variety of genetic/environmental stimuli. Thus, it is reasonable to speculate that, in addition to an LMP1-mediated pathway, HIF1 $\alpha$  stabilisation may also be influenced by the local tumour environment, including tumour oxygen status, which has been shown to be a potent regulator of HIF1 $\alpha$ . In our previous study, using cell culture system, HIF1 $\alpha$  is up-regulated by Siah1 through down-regulation of prolyl HIF-hydroxylase (PHD) 1 and 3 [16]. Thus, it is a matter of interest to examine the role of PHD in clinical NPC specimen. However, we could not have obtained proper antibodies for IHC. Thus, we would like to keep this issue as a future work.

We also have reported that vascular endothelial growth factor (VEGF) could be an important regulator of angiogenesis in NPC tissue [28, 29, 30]. In addition to these studies, we reported LMP1 induces VEGF through HIF1 $\alpha$  using epithelial cell lines [31]. Thus, the other factors related to hypoxic reaction may be of relevance to the gene examined in this study. The data presented here add more evidence that siah1, previously unexamined factor, is also involved in the malignant potential of NPC.

In conclusion, the evaluation of Siah1 and HIF1 $\alpha$  expression by immunohistochemistry

may be valuable for identifying patients with NPC who are at a high risk for a poor outcome. Moreover, understanding the molecular mechanisms that regulate Siah expression will likely provide new insights into the pivotal function of Siah in cancer biology and should contribute to novel anti-cancer strategies [12, 13, 25].

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### Figure legends

Fig. 1. (A) Immunostaining for seven-in-absentia homologue (Siah1) is shown. Dark brown staining indicates nuclear and cytoplasmic expression of Siah1 in patients with NPC. (B) Immunostaining for hypoxia-inducible factor 1 alpha (HIF1 $\alpha$ ) is shown. Dark brown staining indicates nuclear expression of HIF1 $\alpha$ . (C) Immunostaining for latent membrane protein 1 (LMP1) is shown. LMP1 protein was detected at the membrane, cytoplasm, and nucleus of tumour cells. Dark brown staining indicates nuclear expression of LMP1. (D) In situ hybridization with oligonucleotides probe to EBER. Positive reaction is observed in almost tumour cells not in lymphocytes. Blue staining indicates expression of EBER. Original magnification, 400x.

Fig. 2. (A) Correlation between Siah1 and LMP1 expression in nasopharyngeal carcinoma. The Siah1 expression scores and the LMP1 expression scores for 74 NPC cases are plotted. Siah1 expression demonstrated a significant correlation with LMP1 expression according to the Spearman correlation coefficient ( $r = 0.53$ ,  $p < 0.001$ ). (B) Correlation between Siah1 and HIF1 $\alpha$  expression score in nasopharyngeal carcinoma. There was weak relationship between Siah1 and HIF1 $\alpha$  expression score ( $r = 0.33$ ,  $p = 0.004$ ). (C) Correlation between LMP1 and HIF1 $\alpha$  expression score in nasopharyngeal carcinoma. There was no significant relationship between LMP1 and HIF1 $\alpha$  expression score ( $r = 0.26$ ,  $p = 0.08$ ).

Fig. 3. Contribution of Siah1 (A) and HIF1 $\alpha$  (B) expression to the survival of 74 patients with NPC.

The relationship between Siah1 and HIF1 $\alpha$  expression and patient prognosis was examined by Kaplan-Meier analysis.

**Table 1**Relationship between the clinicopathological features of the patients and the expression of LMP1, Siah1, and HIF1 $\alpha$ .

Variables	Total number	LMP1 (+)		Siah1 (+)		HIF1 $\alpha$ (+)	
	( <i>n</i> = 74)	Number (%)	<i>P</i>	Number (%)	<i>P</i>	Number (%)	<i>P</i>
<b>Age (y)</b>							
<50 y	18	11 (61.1)		8 (44.4)		7 (38.8)	
≥50 y	56	24 (42.8)	0.17	22 (39.2)	0.78	20 (35.7)	0.99
<b>Gender</b>							
Female	17	10 (58.8)		8 (47.0)		5 (29.4)	
Male	57	25 (43.8)	0.41	22 (38.5)	0.58	22 (38.5)	0.57
<b>Histologic type (WHO)</b>							
Squamous cell carcinoma (I)	6	0 (0)		4 (66.6)		1 (16.6)	
Nonkeratinizing carcinoma (II)	42	22 (52.3)		17 (40.4)		18 (42.8)	
Undifferentiated carcinoma (III)	26	13 (50.0)	<b>0.02*</b>	9 (34.6)	0.35	8 (30.7)	0.28
<b>T classification</b>							
T1 + T2 (early)	29	10 (34.4)		5 (17.2)		7 (24.1)	
T3 + T4 (advanced)	45	25 (55.5)	0.06	25 (55.5)	<b>0.001*</b>	20 (44.4)	0.09
<b>N classification</b>							
N0 (negative)	19	10 (52.6)		11 (57.8)		3 (15.7)	
N1, 2, 3 (positive)	55	25 (45.4)	0.79	19 (34.5)	0.10	24 (43.6)	0.06
<b>Stage</b>							
I + II (early)	19	6 (31.5)		2 (10.5)		4 (21.0)	
III + IV (advanced)	55	29 (52.7)	0.11	28 (50.9)	<b>0.002*</b>	23 (41.8)	0.16
<b>Initial therapy</b>							
AL <sup>a</sup>	8	3 (37.5)		5 (62.5)		1 (12.5)	
CR <sup>b</sup>	59	29 (49.1)		22 (37.3)		23 (38.9)	
R <sup>c</sup>	7	3 (42.8)	0.60	3 (42.8)	0.41	3 (42.8)	0.33
<b>EBER expression</b>							
positive	62	35 (56.4)		25 (56.4)		23 (37.0)	
negative	12	0 (0)	<b>0.001*</b>	5 (0)	0.93	4 (33.3)	0.80

The values represent the numbers of patients; *P* values were generated by comparisons between the two groups. Tumour (T), node (N) classification, and overall stage were classified on the basis of the UICC classification, 1997. Histologic types were classified on the basis of the WHO criteria. <sup>a</sup>Alternating cisplatin-based chemoradiotherapy; <sup>b</sup>Radiation with concurrent cisplatin-based chemotherapy; <sup>c</sup>Radiation only. \*Significant.

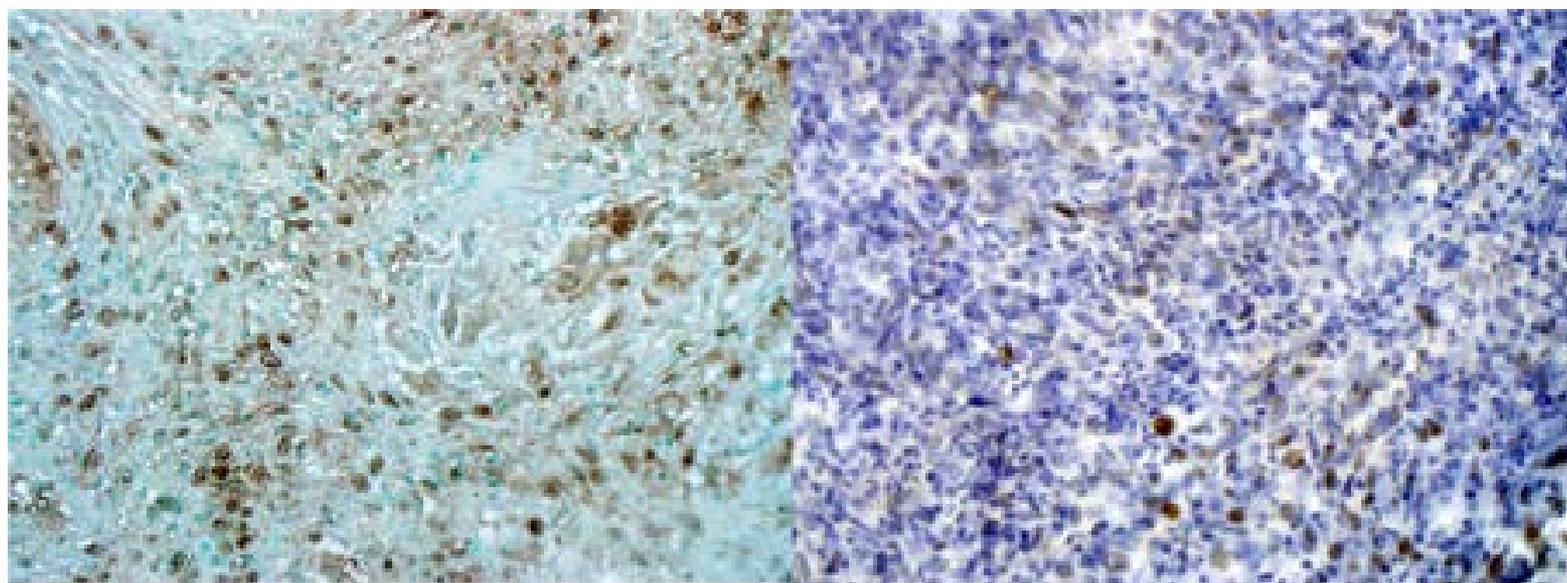
**Table 2**

Cox proportional hazard regression analysis of 74 nasopharyngeal carcinoma patients.

Variables	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% CI)	<i>P</i>	Hazard ratio (95% CI)	<i>P</i>
Age ( $\geq 50$ )	3.79 (1.49-9.64)	<b>0.001*</b>	5.90 (2.17-16.03)	<b>0.001*</b>
Gender (female)	1.89 (1.00-3.64)	0.05*	1.38 (0.70-2.71)	0.34
Tumour (T3 + T4)	1.23 (1.01-1.53)	<b>0.05*</b>	0.844 (0.60-1.16)	0.30
Node (N positive)	0.89 (0.46-1.74)	0.75		NI
Stage (III + IV)	1.36 (1.03-1.78)	<b>0.02*</b>	1.54 (1.04-2.29)	<b>0.03*</b>
Histologic type (II + III)	0.96 (0.33-2.82)	0.43		NI
Therapy (AL <sup>a</sup> ; CR <sup>b</sup> ; R <sup>c</sup> )	0.77 (0.30-1.97)	0.35		NI
LMP1 expression	1.26 (0.69-2.28)	0.43		NI
Siah1 expression	2.04 (1.12-3.71)	<b>0.01*</b>	2.41 (1.26-4.62)	<b>0.008*</b>
HIF1 $\alpha$ expression	2.06 (1.13-3.74)	<b>0.01*</b>	1.99 (1.01-3.90)	<b>0.04*</b>
EBER expression	0.83 (0.38-1.81)	0.64		NI

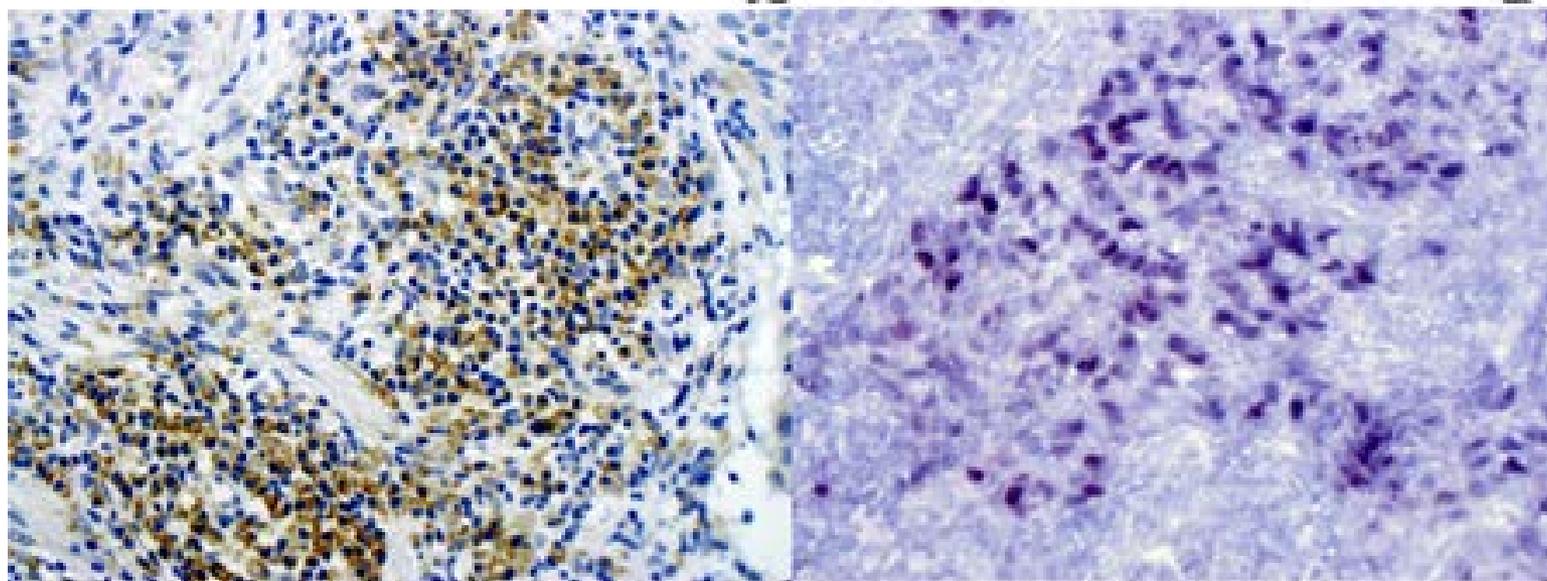
Factors with *P* values greater than 0.05 in the univariate models were not included (NI) in the multivariate analysis. <sup>a</sup>Alternating cisplatin-based chemoradiotherapy; <sup>b</sup>Radiation with concurrent cisplatin-based chemotherapy; <sup>c</sup>Radiation only.\*Significant.

Fig. 1.



**A**

**B**

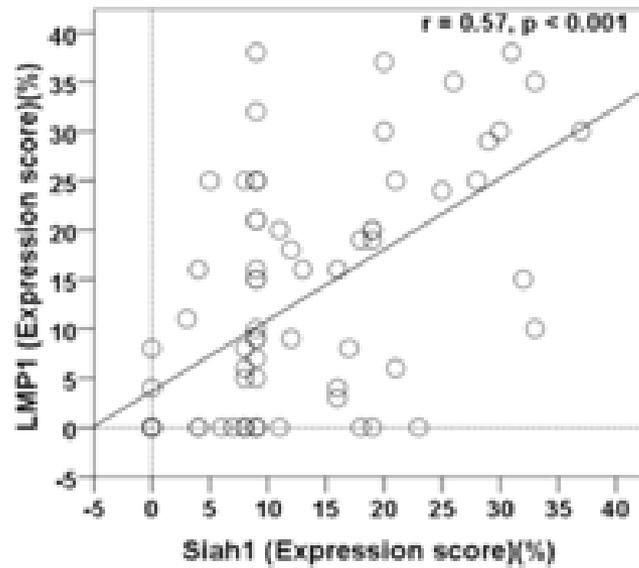


**C**

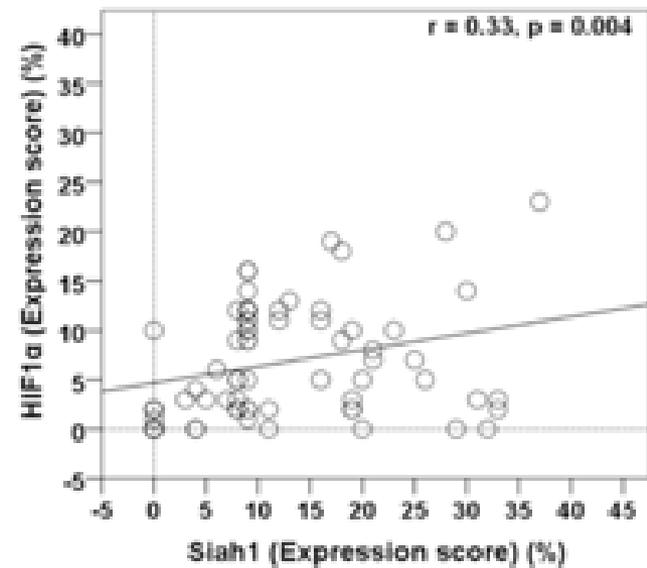
**D**

Fig. 2.

A



B



C

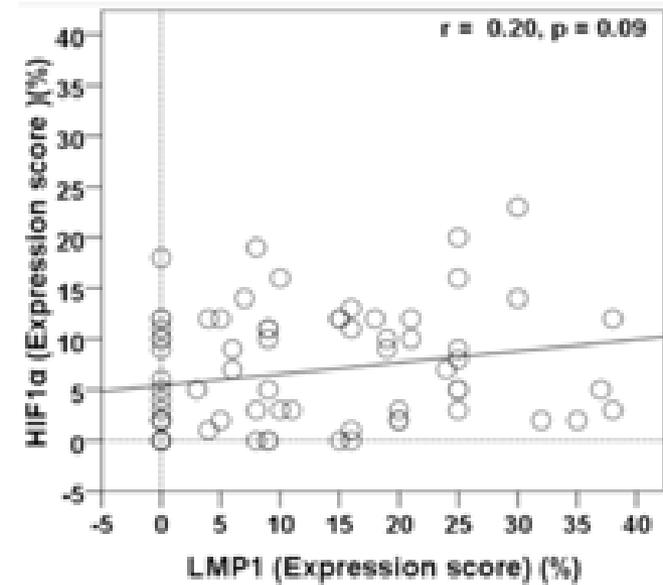


Fig. 3.

