Prognostic significance of hypoxia-inducible factor-1α, TWIST1 and Snail expression in resectable non-small cell lung cancer

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ABSTRACT

Objective: Metastasis is the most common cause of disease failure and mortality for non-small cell lung cancer (NSCLC) after surgical resection. Both Snail and TWIST1 are epithelial-mesenchymal transition (EMT) regulators which induce metastasis. Intratumoral hypoxia followed by stabilization of hypoxia-inducible factor-1α (HIF-1α) promotes metastasis through regulation of certain EMT regulators. The aim of this study is to evaluate the prognostic value of HIF-1α, TWIST1 and Snail expression in resectable NSCLC patients.

Methods: A retrospective analysis of 87 patients with resectable NSCLC from Taipei Veterans General Hospital between 2003 and 2004 was performed using immunohistochemistry to analyze HIF-1α, TWIST1 and Snail expression. The association between HIF-1α, TWIST1 and Snail expression and patients' overall and recurrence-free survivals was investigated.

Results: Overexpression of HIF-1α, TWIST1 or Snail was shown in 32.2%, 36.8% and 55.2% of primary tumors, respectively. Overexpression of HIF-1α, TWIST1 or Snail in primary NSCLCs was associated with a shorter overall survival (P = 0.005, 0.026, 0.009, respectively), and overexpression of HIF-1α was associated with a shorter recurrence-free survival (P = 0.016). We categorized the patients into four groups according to the positivity of HIF-1α/TWIST1/Snail to investigate the accumulated effects of these markers on survival. Co-expression of more than two markers was an independent prognostic indicator for both recurrence-free survival and overall survival (P = 0.004 and < 0.001, respectively, by multivariate Cox proportional hazards model).

Conclusions: Co-expression of more than two markers from HIF-1α, TWIST1 and Snail is a significant prognostic predictor in NSCLC patients.

Introduction

Non-small cell lung cancer (NSCLC) is the leading cause of cancer death worldwide. Surgical resection is the treatment of choice for early-stage NSCLC.[1] Tumor recurrence and metastasis are the most common events encountered after resection that lead to mortality.[2-4] Chemotherapy and radiotherapy are common treatment modalities applied to recurrent lung cancer patients.[4,5] However, the combination modality did not significantly improve patients' survival. Many molecular markers were shown to predict prognosis and survival of NSCLC patients in the literature.[6-8] A constellation of 3 to 5 markers or more than 20 markers in NSCLC have been reported by different groups with little overlaping.[9-13] Since tumor metastasis is the main obstacle for long-term survival after surgical resection, identification of molecular markers related to metastasis may better reflect and predict the prognosis and survival in patients with NSCLC.

Epithelial-mesenchymal transition (EMT) is considered to be one of the major molecular mechanisms inducing tumor invasion and metastasis.[14,15] Repression of E-cadherin is a hallmark of EMT process.[15] Many EMT regulators including Snail, TWIST1, Slug, Zeb1, SIP1, and E47 were shown to induce EMT through the repression of E-cadherin expression.[16-19] Increased expression of Snail or TWIST1 was associated with tumor recurrence, metastasis and poor prognosis in different types of human cancers.[19-24] However, the roles of these two markers in NSCLC remain unknown. Intratumoral hypoxia, followed by activation of hypoxia-inducible factor-1 (HIF-1), is one of the most important mechanisms promoting tumor aggressiveness, metastasis and poor prognosis.[14,25] Hypoxic response is mainly mediated by a heterodimer complex (HIF-1), consisting of two basic helix-loop-helix (bHLH) transcription factors (HIF-1α and HIF-1β). HIF-1α is a cytoplasmic protein regulated by O_2 levels, whereas HIF-1β (also known as ARNT) is a constitutively expressed nuclear protein.[19,26] Increased HIF-1α expression correlates with metastasis, poor prognosis and resistance to therapy in a variety of tumors, including NSCLC.[9,27-29] HIF-1α stabilization was shown to induce the expression of certain EMT regulators.[19] However, the combined use of HIF-1α, TWIST1 and Snail as prognostic markers in NSCLC has not been investigated.

We have previously demonstrated that HIF-1α regulates the expression of *TWIST1* by binding directly to the hypoxia response element in the *TWIST1* proximal promoter.[30] Knockdown of TWIST1 or HIF-1α by short-interference RNA reverts EMT and metastatic phenotypes in lung cancer H1299 cells.[30] Co-expression of HIF-1α, TWIST1 and Snail in primary tumors of head and neck squamous cell carcinoma patients correlates with the highest percentage of metastasis and the worst prognosis.[30] In this report, we showed that increased HIF-1α, TWIST1, or Snail

expression was observed in a significant percentage of NSCLC patients using immunohistochemistry. Overexpression of HIF-1α, TWIST1 or Snail correlated with poor overall survival in NSCLC patients. Co-expression of any two or all markers from HIF-1α, TWIST1 and Snail in primary tumors of NSCLC patients correlated with a significantly worse prognosis. These results demonstrated the prognostic value of the three markers to predict the overall and recurrence-free survivals in resectable NSCLC patients.

Patients and Methods

Patients and treatment

From January 2003 to December 2004, 87 patients undergoing surgical resection for NSCLC at Taipei Veterans General Hospital were enrolled in this study. Informed consent was obtained in writing before patient enrollment. This study has been approved by the Institutional Review Board of Taipei Veterans General Hospital. The preoperative staging workup was performed as previously described.[31] Chest and upper abdomen computed tomography scans and bronchoscopy were routinely performed before operation. Whole-body bone scan and computed tomography scan of brain were utilized to exclude possible metastasis. Mediastinoscopy was performed only when enlarged mediastinal lymph nodes (diameter over 1.0 cm) were shown by computed tomography scan. Patients with suspected distant metastasis were excluded from operation procedures. All patients underwent complete resection of lung cancer with mediastinal lymph node dissection. The resected specimens and all dissected mediastinal lymph nodes were sent to pathologists for pathologic staging. TNM classification of the International Union Against Cancer was utilized for determination of disease stages.[32] The criteria of adjuvant therapy included advanced stage (chemotherapy) and better local disease control (radiotherapy). All patients were followed up at the outpatient department quarterly in the first two years and semi-annually thereafter.

Immunohistochemistry

The specimens processing and immunohistochemistry procedures were performed as previously described.[22,30,33] Tumor and neighboring normal tissue were cut into 6-μm sections from the NSCLC specimens for immunohistochemistry analysis. The samples were fixed in acetone, air-dried, and followed by bath in TBS solution (pH 7.6). The endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 5 minutes. For HIF-1α immunohistochemistry staining, a mouse monoclonal anti-HIF-1α antibody (catalog No. ab8366, Abcam Ltd) was used at the dilution of 1:50 and incubated at 4°C overnight. Tissue sections were also stained with a rabbit polyclonal antibody against TWIST1 (catalog No. ab50581, Abcam Ltd) or Snail (catalog No. ab17732, Abcam Ltd) at the dilution of 1:100 and incubated for 1 hour, respectively. Sections were again incubated with a biotinylated secondary antibody for 10 minutes. The sections were then visualized using streptavidin-horseradish peroxidase conjugate (DAKO LSAB kit; DAKO, Los Angles, California, USA), with 3-amino-9-ethylcarbazole as the chromogen. Finally, all slides were counterstained with hematoxylin.

Immunohistochemical Scoring

The interpretation of immunohistochemistry results for HIF-1α, TWIST1 and Snail

was performed independently by two pathologists, according to the criteria described previously.[22,30,33] The pathologists scoring the immunohistochemistry were blinded to the patients' outcome. The immunoreactivity of HIF-1α, TWIST1 and Snail was graded from 0 to 3+ (0, no staining; 1+, 1~25%; 2+, 26~50%; 3+, >50% nuclear staining) according to nuclear expression, and only 3+ (>50% nuclear staining) was considered as a positive immunohistochemistry result.[22,30,33]

Statistical analysis

The relationship between HIF-1α, TWIST1 and Snail expression and clinical-pathological characteristics was analyzed with $\chi 2$ test. The overall survival and disease-free survival were calculated by the Kaplan-Meier method. Univariate and multivariate analyses were performed by means of the Cox proportional hazards model using SPSS software (version 12.0; SPSS, Chicago, Illinois, USA). Variables with *p* value less than 0.1 after the univariate analysis were entered into multivariate analysis. The log-rank test and Cox proportional hazards model were used to make group comparisons for applying the HIF-1α/TWIST1/Snail prognostic model to predict the prognosis of early-stage NSCLC and outcome of patients receiving adjuvant chemotherapy. Statistical analysis was considered to be significant when the probability value was < 0.05.

Results

Overexpression of HIF-1α, TWIST1 and Snail and their correlation with clinicopathological factors in resectable NSCLC

The characteristics of these 87 NSCLC patients are listed in Table 1. Adjuvant therapies included chemotherapy alone for 22 patients, radiation alone for 5 patients, and a combination of chemotherapy and radiotherapy for 4 patients. With a median follow-up time of 43.2 months (95% confidence interval [CI], 37.1 ± 15.1), the 4-year overall survival rate was 74.0%. Tumor recurrence developed in 34 (39.1%) patients during follow-up. To determine the expression of HIF-1α, TWIST1 and Snail in NSCLC samples, immunohistochemical analysis of HIF-1α, TWIST1 and Snail expression was performed in 87 sets of NSCLC samples. A representative case of immunohistochemical staining of all three markers was shown in Fig. 1. Overexpression of HIF-1α, TWSIT1 and Snail (≥ 50% nuclear expression in tumor cells) was shown in 32.2%, 36.8% and 55.2% of lung tumor samples, respectively (Table 1).

Table 1. Characteristics and univariate analyses of 87 lung cancer patients

Variables	Case No. (%)	Recurrence-free survival			Overall survival		
		Median (months)	HR (95% CI)	*P* value	Median (months)	HR (95% CI)	*P* value
Age							
≤ 65 yr	25 (28.7)	36.3	-		__*	-	
> 65 yr	62 (71.3)	39.7	1.01 (0.50-2.01)	0.987	53.5	0.92 (0.38-2.25)	0.856
Gender							
Female	18 (20.7)	30.3	-		__*	-	
Male	69 (79.3)	39.7	0.86 (0.41-2.09)	0.857	53.5	1.73 (0.51-5.82)	0.377
TNM stage							
I	38 (43.7)	__*	-		__*	-	
II~IV	49 (56.3)	22.3	3.53 (1.72-7.25)	0.001	53.5	3.01 (1.11-8.14)	0.030
Histological type							
Adenocarcinoma	54 (62.1)	39.7	-		53.5	-	
Non-adenocarcinoma	33 (37.9)	35.7	1.13 (0.60-2.11)	0.707	__*	1.67 (0.74-3.80)	0.218
Extent of pulmonary resection							
Lobectomy or wedge resection	79 (90.8)	39.7	-		__*	-	
Pneumonectomy or bilobectomy	8 (9.2)	15.4	1.53 (0.54-4.29)	0.422	16.6	2.50 (0.84-7.46)	0.101
HIF-1α overexpression							
No	59 (67.8)	__*	-		__*	-	
Yes	28 (32.2)	15.0	2.18 (1.16-4.10)	0.016	__*	3.32 (1.43-7.70)	0.005
TWIST1 overexpression							
No	55 (63.2)	__*	-		__*	-	
Yes	32 (36.8)	28.8	1.26 (0.67-2.35)	0.479	__*	2.63 (1.12-6.15)	0.026
Snail overexpression							
No	39 (44.8)	__*	-		__*	-	
Yes	48 (55.2)	28.8	1.36 (0.73-2.53)	0.336	53.5	4.24 (1.43-12.54)	0.009

Abbreviation: HR, hazard ratio; CI, confidence interval. *Median survival was not reached.

Correlation between clinicopathological variables (age, gender, TNM stage, histological type and extent of pulmonary resection) and HIF-1α, TWIST1 and Snail expression was shown in Table 2. HIF-1α overexpression was marginally associated with TNM stage (stage II ~ IV) (P = 0.050). There was no correlation between TNM stage and TWIST1 or Snail overexpression (P = 0.364 and 0.377, respectively). TWIST1 overexpression was associated with the histological type of non-adenocarcinoma (P = 0.026). Snail overexpression was marginally associated with extent of pulmonary resection (P = 0.054). The association between HIF-1α, TWIST1 and Snail expression was demonstrated by Pearson $\chi 2$ test. Although overexpression of HIF-1α was not significantly associated with TWIST1 overexpression, it tended to correlate better with TWIST1 overexpression (P = 0.078) than with Snail overexpression (P = 0.474) (Table 3). Furthermore, there was no correlation between TWIST1 overexpression and Snail overexpression (P = 0.135) (Table 3).

Table 2. Association between the patterns of HIF-1α, TWIST1 and Snail expression and clinicopathological variables

	HIF-1α overexpression			TWIST1 overexpression			Snail overexpression			HIF-1α / TWIST1 / Snail overexpression		
Variables	No (%) (n=59)	Yes (%) (n=28)	*P* value	No (%) (n=55)	Yes (%) (n=32)	*P* value	No (%) (n=39)	Yes (%) (n=48)	*P* value	None or one (%) (n=53)	Two or three (%) (n=34)	*P* value
Age												
≤ 65 yr	15 (25.4)	10 (35.7)	0.322	16 (29.1)	9 (28.1)	0.924	9 (23.1)	16 (33.3)	0.293	14 (26.4)	11 (32.4)	0.550
> 65 yr	44 (74.6)	18 (64.3)		39 (70.9)	23 (71.9)		30 (76.9)	32 (66.7)		39 (73.6)	23 (67.6)	
Gender												
Male	49 (83.1)	20 (71.4)	0.211	42 (76.4)	27 (84.4)	0.374	31 (79.5)	38 (79.2)	0.971	43 (81.1)	26 (76.5)	0.600
Female	10 (16.9)	8 (28.6)		13 (23.6)	5 (15.6)		8 (20.5)	10 (20.8)		10 (18.9)	8 (23.5)	
TNM stage												
I	30 (50.8)	8 (28.6)	0.050	22 (40.0)	16 (50.0)	0.364	15 (38.5)	23 (47.9)	0.377	23 (43.4)	15 (44.1)	0.947
II~IV	29 (49.2)	20 (71.4)		33 (60.0)	16 (50.0)		24 (61.5)	25 (52.1)		30 (56.6)	19 (55.9)	
Histological type												
Adenocarcinoma	40 (67.8)	14 (50.0)	0.110	39 (70.9)	15 (46.9)	0.026	26 (66.7)	28 (58.3)	0.426	37 (69.8)	17 (50.0)	0.063
Non-adenocarcinoma	19 (32.2)	14 (50.0)		16 (29.1)	17 (53.1)		13 (33.3)	20 (41.7)		16 (30.2)	17 (50.0)	
Extent of pulmonary resection												
Lobectomy or wedge resection	55 (93.2)	24 (85.7)	0.258	51 (92.7)	28 (87.5)	0.416	38 (97.4)	41 (85.4)	0.054	50 (94.3)	29 (85.3)	0.154
Pneumonectomy or bilobectomy	4 (6.8)	4 (14.3)		4 (7.3)	4 (12.5)		1 (2.6)	7 (14.6)		3 (5.7)	5 (14.7)	

Table 3. Association between HIF-1α, TWIST1 and Snail expression in lung cancer patients

Variables	HIF-1α overexpression		*P* value	TWIST1 overexpression		*P* value
	No	Yes		No	Yes	
TWIST1 overexpression						-
No	41	14	0.078	-	-	
Yes	18	14		-	-	
Snail overexpression						
No	28	11	0.474	28	11	0.135
Yes	31	17		27	21	

Overexpression of HIF-1α, TWIST1 and Snail as prognostic factors in NSCLC patients

To investigate the prognostic impact of HIF-1α, TWIST1 and Snail overexpression in NSCLC, Kaplan-Meier survival analyses were carried out and the differences of survival between groups were examined. The results showed that overexpression of HIF-1α, TWIST1 or Snail alone in primary NSCLCs was associated with a shorter overall survival (P = 0.005, 0.026, 0.009, respectively) (Fig. 2A–2C). Considering the recurrence-free survival, overexpression of HIF-1α was associated with a shorter recurrence-free period (P = 0.016); whereas overexpression of TWIST1 or Snail did not influence recurrence-free survival (P = 0.479 and 0.336, respectively).

Generation of a prognostic prediction model for NSCLC patients using combination of HIF-1α/TWIST1/Snail staining results

To investigate the accumulative effects of HIF-1α, TWIST1 and Snail expression on prognosis of NSCLC, we divided these 87 patients into four groups according to the number of positive markers from HIF-1α, TWIST1 and Snail overexpression. The patients were scored according to the number of positive markers: 0 (none positive), 1 (one positive), 2 (two positive), and 3 (co-expression of all three markers). Kaplan-Meier overall survival curves were generated and differences between the four groups were examined. The results showed that patients with overexpression of any two of HIF-1α, TWIST1 and Snail (score 2) or all of the three markers (score 3) had a worse overall survival (Fig. 3A). We therefore divided the patients into the following two groups: score 0~1 vs. score 2~3. The result showed that patients with score 2~3 had a significantly shorter overall survival (Fig. 3B). A

similar result was shown in recurrence-free survival. Patients who scored 2~3 were correlated with a shorter recurrence-free survival as compared with those scored 0~1 (Fig. 3C).

Univariate analyses indicated that TNM stage had significant impact on overall survival (P = 0.030) and recurrence-free survival (P = 0.001) (Table 1). TNM stage and HIF-1α/TWIST1/Snail co-expression pattern were entered into multivariate analyses. To control potential confounders, age, gender, histological type, and extent of pulmonary resection were also entered into multivariate analyses. Multivariate analyses showed that TNM stage (P = 0.008) and HIF-1α/TWIST1/Snail co-expression pattern (P < 0.001) were independent prognostic markers for overall survival (Table 4). TNM stage (P < 0.001) and HIF-1α/TWIST1/Snail co-expression pattern (P = 0.004) were also significant independent predictors for recurrence-free survival (Table 4) (see supplementary Table 1 online for complete results of multivariate analyses). The prognostic effect of co-expression of more than two markers was confirmed by Cox proportional hazard model. It was an independent prognostic factor for overall survival as well as recurrence-free survival.

Table 4. Multivariate analyses for recurrence-free and overall survivals of 87 lung cancer patients

Variables	Hazard ratio (95% CI)	*P* value
Recurrence-free survival		
TNM stage		
I	-	
II~IV	4.53 (2.13-9.63)	< 0.001
HIF-1α/TWIST1/Snail co-expression pattern (IHC score)*		
0~1	-	
2~3	2.62 (1.35-5.09)	0.004
Overall survival		
TNM stage		
I	-	
II~IV	4.13 (1.46-11.69)	0.008
HIF-1α/TWIST1/Snail co-expression pattern (IHC score)*		
0~1	-	
2~3	7.16 (2.58-19.89)	< 0.001

Abbreviation: CI, confidence interval. *0, none positive; 1, one positive; 2, two positive; and 3, co-expression of all three markers.

Application of the HIF-1α/TWIST1/Snail prognostic model in predicting the prognosis of early-stage NSCLC and outcome of patients receiving postoperative adjuvant chemotherapy

The predictive value and application of the generated three-marker model in early and advanced NSCLC were examined respectively. For early-stage NSCLC (stage I and II, n=51) in our study, patients with score 2~3 (n=20) had significantly worse overall survival than those with score 0~1 (n=31) (hazard ratio [HR], 5.36, 95% CI, 1.08 to 26.57; *P* = 0.040). However, no difference was observed in recurrence-free survival (HR, 2.07; 95% CI, 0.78 to 5.54; *P* = 0.147). Among the 51 patients with early-stage NSCLC, 32 (62.7%) had adenocarcinoma. For early-stage (stage I and II) adenocarcinoma, patients with score 2~3 (n=10) had significantly worse overall survival than those with score 0~1 (n=22) (*P* = 0.045 by log-rank test). The difference was only marginally significant by Cox proportional hazards model (HR, 7.23; 95% CI, 0.75 to 69.59; *P* = 0.087). No difference was observed in recurrence-free survival (HR, 1.58; 95% CI, 0.39 to 6.34; *P* = 0.519). For stage I NSCLC (n=38), there is a trend toward worse overall survival in patients with score 2~3 (n=15) than those with score 0~1 (n=23) (*P* = 0.064 by log-rank test). However, there was no significant difference by Cox proportional hazards model (HR, 6.12; 95% CI, 0.68 to 54.78; *P* = 0.105). There was no difference in recurrence-free survival between the two groups (HR, 1.99; 95% CI, 0.53 to 7.42; *P* = 0.305). Of the 38 patients with stage I NSCLC, 23 (60.5%) had adenocarcinoma. At the last follow-up session, only one of the patients with stage I adenocarcinoma was dead. Three patients had tumor recurrence, including the patient who died. Since only one patient was dead, the difference of overall survival between patients with score 2~3 and those with score 0~1 was not calculated. There was no significant difference in recurrence-free survival (HR, 1.31; 95% CI, 0.12 to 14.46; *P* = 0.827) between stage I adenocarcinoma patients with score 2~3 (n=6) and those with score 0~1 (n=17).

In this cohort, 26 advanced NSCLC (i.e., stage III ~ IV) cases received adjuvant therapy after surgery. Compared with the cases without adjuvant therapy, patients undergoing adjuvant chemotherapy after surgical resection had a favorable overall survival than those without treatment (HR, 0.29; 95% CI, 0.09 to 0.92; *P* = 0.035). To test whether the generated three-marker model can predict the outcome of patients receiving adjuvant therapy, we examined the effect of HIF-1α/TWIST1/Snail co-expression pattern on overall and recurrence-free survivals in patients receiving adjuvant therapy. Patients with score 2~3 (n = 8) survived shorter than those with score 0~1 (n = 18) (HR, 7.89; 95% CI, 1.52 to 40.96; *P* = 0.014). However, recurrence-free survival between the two groups was similar (HR, 1.94; 95% CI, 0.72

to 5.27; P = 0.193). These results suggested that co-expression of more than two markers could be used as a predictor of poor prognosis in early-stage patients and poor outcome in patients receiving adjuvant therapy.

Discussion

This report investigated the prognostic role of HIF-1α, TWIST1 and Snail expression in resectable NSCLC patients. Our results showed that overexpression of HIF-1α, TWIST1 or Snail in primary NSCLCs was associated with a shorter overall survival. HIF-1α overexpression was associated with a shorter recurrence-free survival. TNM stage and HIF-1α/TWIST1/Snail co-expression pattern were significant independent prognostic indicators for both overall and recurrence-free survivals in multivariate analyses. Co-expression of any two or all of HIF-1α, TWIST1 and Snail correlated with a significantly worse overall and recurrence-free survivals in our study.

Many groups used different sets of markers to predict the prognosis and survival of NSCLC patients with some success.[6-13] However, there was little overlapping between the markers presented by different groups. Our study was the first demonstration to predict overall and recurrence-free survivals in NSCLC by utilizing a combination of metastasis-related markers. Our results showed that the expression of HIF-1α tended to correlate with TWIST1 expression but not with Snail expression. This observation was consistent with our previous results demonstrating the direct regulation of TWIST1 expression by HIF-1α.[30] In our previous study, we have also shown that knockdown of TWIST1 or HIF-1α by short-interference RNA reverts EMT and metastatic phenotypes in lung cancer H1299 cells.[30] In the case of head and neck cancer, co-expression of HIF-1α, TWIST1, and Snail correlates with metastasis and the worst outcome.[30] In this report, overexpression of HIF-1α was associated with worse overall and recurrence-free survivals, whereas overexpression of TWIST1 or Snail was only associated with a worse overall survival in NSCLC. By combination of HIF-1α, TWIST1 and Snail, we found that co-expression of any two or all of the three markers predicted worse overall and recurrence-free survivals in NSCLC patients. It is possible that different types of cancers use different signaling pathways to reach transformation and mediate metastasis. Our results provided the scenario that staining with two different markers will be suitable to reach prognostic significance in NSCLC.

Increasing evidences support the role of postoperative adjuvant chemotherapy in locally advanced NSCLC. However, the effect of adjuvant chemotherapy in early-stage NSCLC remains to be determined.[34,35] The lung cancer community is trying to sort out poor prognostic factors in stage I NSCLC for adjuvant therapy. In our cohort, we analyzed the predictive ability of the three-marker model in early-stage NSCLC as well as in those with advanced NSCLC receiving adjuvant chemotherapy. For early-stage (stage I and II) NSCLC or adenocarcinoma, the three-marker model demonstrated a difference in overall survival. For stage I NSCLC, there is a trend

toward worse overall survival in patients with score 2~3. Because only one of the patients with stage I adenocarcinoma was dead at the last follow-up session, the difference of overall survival between patients with score 2~3 and those with score 0~1 was not calculated. The differences of recurrence-free survival was not statistically significant in stage I adenocarcinma in our study (score 2~3 vs. score 0~1). However, the number of patients in this group is relatively small. Prospective multi-institutional studies with long-term follow-up are required to further validate our prognostic model in predicting the prognosis of stage I adenocarcinoma patients. For adenocarcinoma patients, our prognostic model could only be used to predict survival of stage I+II patients but not stage I patients (The summary of different published studies is shown in Table 5). However, our model has the advantage of using only three markers as compared with other studies predicting prognosis of adenocarcinoma patients with numerous markers (10 and 50 genes) (Table 5).[11,13] Our study also showed that co-expression of more than two markers was a predictor for poor outcome after adjuvant therapy in advanced NSCLC. These results suggest that the three-marker model may be able to sort out the poor-prognostic cases in early-stage NSCLC, as well as those with advanced disease receiving adjuvant therapy. Adjuvant therapy may be considered in early-stage NSCLC patients with co-expression of more than two markers after surgical resection. A more intensive treatment may also be indicated in patients of advanced diseases with co-expression of more than two markers.

In conclusion, our results showed that co-expression of any two or all of HIF-1α, TWIST1 and Snail is a significant prognostic marker to predict overall and recurrence-free survivals in resectable NSCLC patients. It is also a maker independent of TNM stage. The information generated will be valuable for the diagnosis, prognosis and management of NSCLC patients.

Table 5. Comparison of published reports with the current study on predictors of survival in early-stage NSCLC

Authors	Genes/Proteins	Histology	Stage	Survival Difference
Lau et al[9]	*STX1A, HIF1α, CCR7*	NSCLC	I	Overall survival
		NSCLC	II	Overall survival
Chen et al[10]	*DUSP6, MMD, STAT1, ERBB3, LCK*	NSCLC	I and II	Overall and relapse-free survival
Beer et al[11]	50 genes	Adenocarcinoma	I	Overall survival
Lu et al[12]	64 genes	NSCLC	I	Overall survival
Bianchi et al[13]	*E2F1, E2F4, HOXB7, HSPG2, MCM6, NUDCD1, RRM2, SERPINB5, SF3B1, SCGB3A1*	Adenocarcinoma	I	Overall survival
Hung et al (current study)	HIF-1α, TWIST1, Snail	NSCLC	I and II	Overall survival
		NSCLC	I*	Overall survival
		Adenocarcinoma	I and II†	Overall survival

Abbreviation: NSCLC, non-small cell lung cancer; *, indicates a prognostic trend which does not reach the statistical significance of $P < 0.05$. †, significance of $P < 0.05$ by log-rank test.

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Figure legends

Figure 1. Immunohistochemical staining of co-expression of HIF-1α, TWIST1 and Snail in corresponding normal tissue (N) and primary tumor (T) of a representative NSCLC case. The samples prepared for co-expression analysis were cut and examined at the same region. Black arrows indicate the nuclear expression of HIF-1α, TWIST1 and Snail. Photographs were taken at magnifications of 400x. Scale bars represent 100 μm.

Figure 2. Kaplan-Meier survival analysis in NSCLC patients with (A) HIF-1α (–) vs. HIF-1α (+), (B) TWIST1 (–) vs. TWIST1 (+), and (C) Snail (–) vs. Snail (+) in primary tumors. Overexpression of HIF-1α, TWIST1 or Snail in primary NSCLCs was associated with a shorter overall survival in the respective groups.

Figure 3. Kaplan-Meier survival analysis in NSCLC patients according to the number of markers including HIF-1α, TWIST1 and Snail which had increased expression. (A) The patients were divided into four groups: HIF-1α(-)/TWIST1(-)/Snail(-) (group1), any one of HIF-1α, TWIST1 or Snail overexpression (group 2), any two of HIF-1α, TWIST1 or Snail overexpression (group 3), and HIF-1α(+)/TWIST1(+)/Snail(+) (group 4). Any two of HIF-1α, TWIST1 or Snail overexpression (Group 3) and HIF-1α(+)/TWIST1(+)/Snail(+) (group 4) had a shorter overall survival when compared with the other groups. (B) The patients were re-divided into two groups: None or one of HIF-1α, TWIST1 or Snail overexpression (group 1), and any two or all of HIF-1α, TWIST1 or Snail overexpression (group 2). Co-expression of any two or all of HIF-1α, TWIST1 and Snail markers (Group 2) had a significantly worse overall survival. (C) The grouping method used in (B) was applied for recurrence-free survival analysis. Co-expression of any two or all of HIF-1α, TWIST1 and Snail markers (Group 2) had a significantly worse recurrence-free survival.

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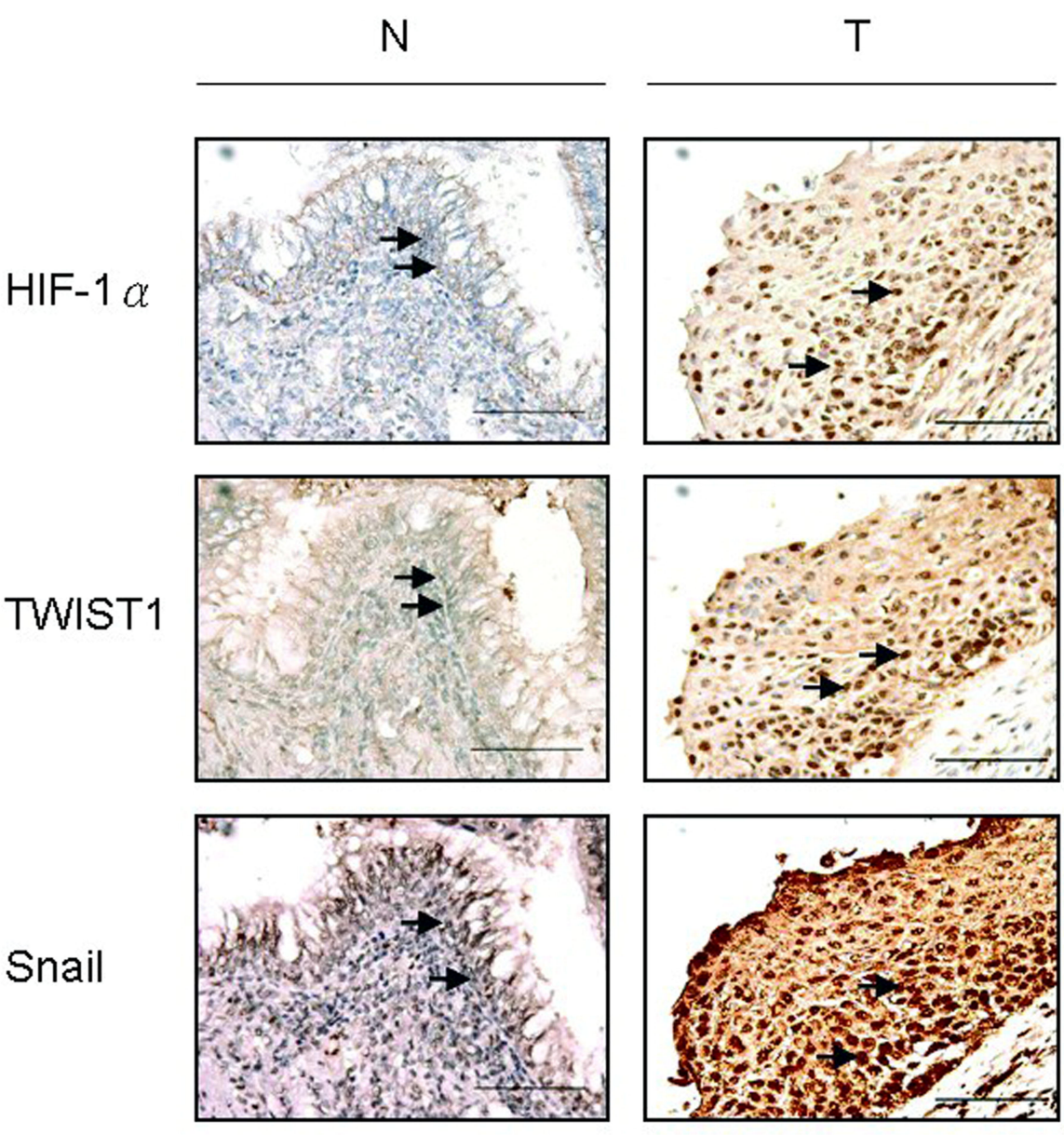
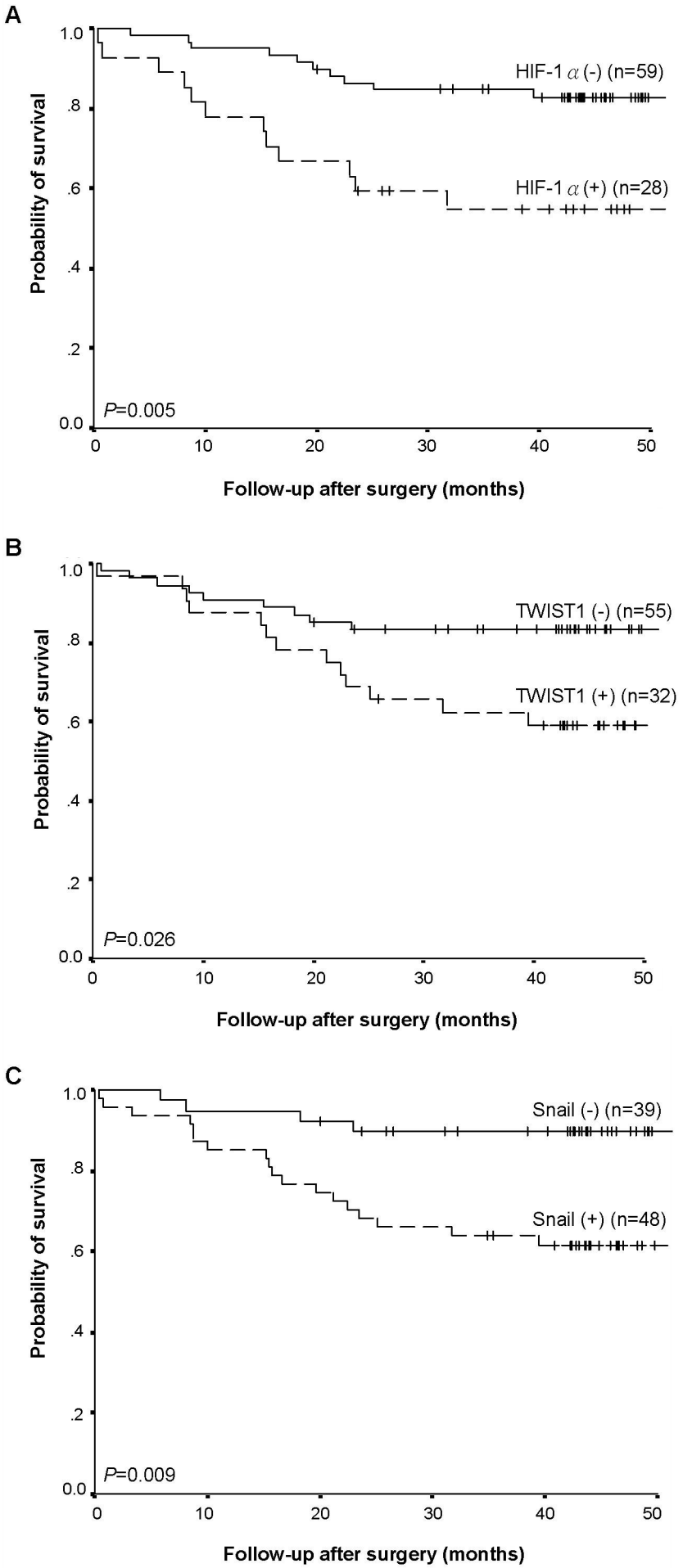
Supplementary Table 1. Multivariate analyses for recurrence-free and overall survivals of 87 lung cancer patients

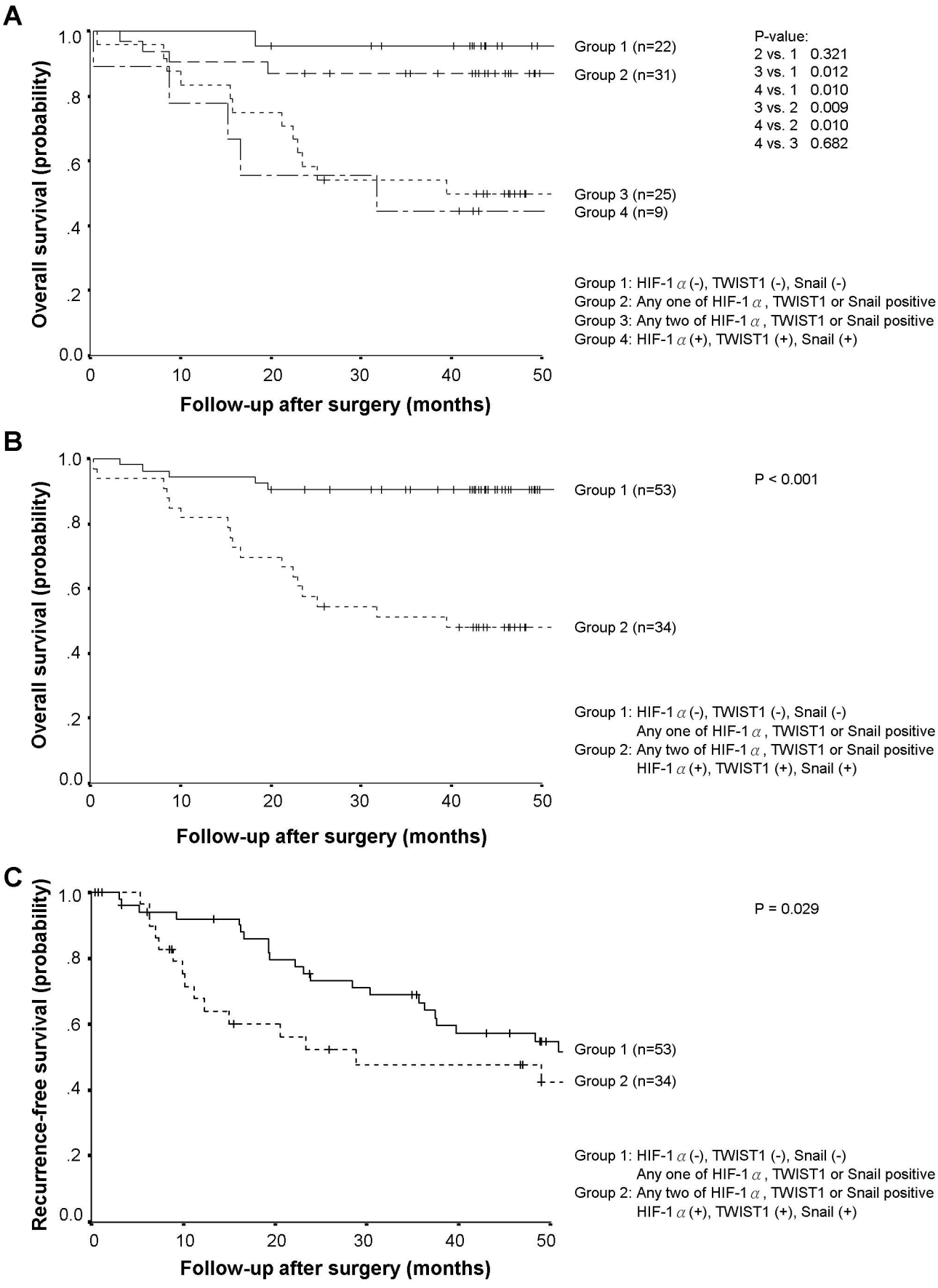
Variables	Hazard ratio (95% CI)	*P* value
Recurrence-free survival		
TNM stage		
I	-	
II~IV	4.53 (2.13-9.63)	< 0.001
HIF-1α/TWIST1/Snail co-expression pattern (IHC score)*		
0~1	-	
2~3	2.62 (1.35-5.09)	0.004
Age		
≤ 65 yr	-	
> 65 yr	1.14 (0.57-2.34)	0.724
Gender		
Female	-	
Male	1.15 (0.50-2.67)	0.737
Histological type		
Adenocarcinoma	-	
Non-adenocarcinoma	1.08 (0.56-2.08)	0.810
Extent of pulmonary resection		
Lobectomy or wedge resection	-	
Pneumonectomy or bilobectomy	1.29 (0.44-3.80)	0.649
Overall survival		
TNM stage		
I	-	

II~IV	4.13 (1.46-11.69)	0.008
HIF-1α/TWIST1/Snail co-expression pattern (IHC score)*		
0~1	-	
2~3	7.16 (2.58-19.89)	< 0.001
Age		
≤ 65 yr	-	
> 65 yr	1.16 (0.45-3.01)	0.757
Gender		
Female	-	
Male	2.21 (0.64-7.55)	0.208
Histological type		
Adenocarcinoma	-	
Non-adenocarcinoma	1.28 (0.55-3.02)	0.570
Extent of pulmonary resection		
Lobectomy or wedge resection	-	
Pneumonectomy or bilobectomy	2.09 (0.64-6.82)	0.224

Abbreviation: CI, confidence interval. *0, none positive; 1, one positive; 2, two positive; and 3, co-expression of all three markers.


N
T
HIF-1 α
TWIST1
Snail


A
1.0
.8
.6
.4
.2
0.0
Probability of survival
HIF-1 α (-) (n=59)
HIF-1 α (+) (n=28)
P=0.005
0
10
20
30
40
50
Follow-up after surgery (months)
B
1.0
.8
.6
.4
.2
0.0
Probability of survival
TWIST1 (-) (n=55)
TWIST1 (+) (n=32)
P=0.026
0
10
20
30
40
50
Follow-up after surgery (months)
C
1.0
.8
.6
.4
.2
0.0
Probability of survival
Snail (-) (n=39)
Snail (+) (n=48)
P=0.009
0
10
20
30
40
50
Follow-up after surgery (months)


A
Overall survival (probability)
1.0
.8
.6
.4
.2
0.0
0
10
20
30
40
50
Follow-up after surgery (months)
Group 1 (n=22)
Group 2 (n=31)
Group 3 (n=25)
Group 4 (n=9)
P-value:
2 vs. 1 0.321
3 vs. 1 0.012
4 vs. 1 0.010
3 vs. 2 0.009
4 vs. 2 0.010
4 vs. 3 0.682
Group 1: HIF-1α(-), TWIST1 (-), Snail (-)
Group 2: Any one of HIF-1α, TWIST1 or Snail positive
Group 3: Any two of HIF-1α, TWIST1 or Snail positive
Group 4: HIF-1α(+), TWIST1 (+), Snail (+)
B
Overall survival (probability)
1.0
.8
.6
.4
.2
0.0
0
10
20
30
40
50
Follow-up after surgery (months)
Group 1 (n=53)
Group 2 (n=34)
P < 0.001
Group 1: HIF-1α(-), TWIST1 (-), Snail (-)
Any one of HIF-1α, TWIST1 or Snail positive
Group 2: Any two of HIF-1α, TWIST1 or Snail positive
HIF-1α(+), TWIST1 (+), Snail (+)
C
Recurrence-free survival (probability)
1.0
.8
.6
.4
.2
0.0
0
10
20
30
40
50
Follow-up after surgery (months)
Group 1 (n=53)
Group 2 (n=34)
P = 0.029
Group 1: HIF-1α(-), TWIST1 (-), Snail (-)
Any one of HIF-1α, TWIST1 or Snail positive
Group 2: Any two of HIF-1α, TWIST1 or Snail positive
HIF-1α(+), TWIST1 (+), Snail (+)