RESEARCH COMMUNICATION

HGFK1 is Associated with a Better Prognostis and Reverses Inhibition by Gefitinib in NSCLC Cases

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Abstract

Purpose: Non small cell lung cancer (NSCLC) is the leading worldwide source of cancer-related deaths. Although some drugs targeting EGFR mutations have been developed, most advanced cases are still incurable. New targets for anticancer drugs are demanded. The kringle 1 domain of hepatocellular growth factor alpha chain (HGFK1) is a potent anti-angiogenesis factor. It has also emerged as a potential anticancer factor in hepatocellular carcinoma (HCC). The expression of HGFK1 protein in patients with NSCLC has not been reported to date. Method: Here, we assessed HGFK1 expression by Western blotting in 103 cases with advanced NSCLC to investigate the impact of HGFK1 on survival. Results: Results revealed 33 (30.1%) patients were classified as high expressors, this being significantly associated with less remote metastasis (P = 0.002) but not with lymph node metastasis (P = 0.062). There was also a significant association between HGFK1 expression and tumor size (P = 0.025) as well as clinical stage (P = 0.012). Kaplan-Meier survival analysis showed that both overall survival (OS) and progression free survival (PFS) of patients with HGFK1 expression were longer than those of patients without HGFK1 expression (P = 0.004 and P = 0.001 respectively). HGFK1 reversed gefitinib inhibition in the resistent NSCLC cell line A431/GR but did not inhibit the proliferation of NSCLC cells A431 and A431/GR directly. Reversion of gefitinib inhibition in A431/GR cells by HGFK1 was related to decreased phosphorylation of ERK and STAT5. Conclusions: HGFK1 may be a useful prognostic factor of advanced NSCLC patients and a potential drug for gefitinib resistant patients.

Keywords: HGFK1 expression - non-small cell lung cancer - gefitinib resistance

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Introduction

Non small cell lung cancer (NSCLC), especially advanced NSCLC is the leading worldwide source of cancer-related deaths (Chorostowska-Wynimko and Szpechcinski, 2007). Some drugs targeting on EGFR mutations, such as erotinib and gefitinib, have made a success in treating advanced NSCLC in past years (Gschwind et al., 2004; Hynes and Lane, 2005). Encouraging response to gefitinib is frequently observed in East Asian, female, adenocarcinoma histology, and non-smoking patients, and is closely associated with specific activating mutations in EGFR tyrosine kinase domain.

Since only 10-15% of unselected NSCLC patients have specific activating mutations and 20-30% of NSCLC patients have amplified wild-type EGFR (wtEGFR), more than 50% of patients with NSCLC are not suitable for erotinib and gefitinib treatment (Sharma et al., 2007; Ciardiello and Tortora, 2008). Furthmore, almost all patients had been sensistive to gefitinib treatment would become resistent to these drugs. New targets for anticancer drugs or new reagents for reversing inhibition by gefitinib on NSCLC cell are demanded (Pao et al., 2004; Bell et al., 2005; Cappuzzo et al., 2005).

HGFK1 is the kringle 1 domain of hepatocellular growth factor alpha chain (Xin et al., 2000). In vivo, HGFK1 inhibited tumor growth, decreased tumor microvessel density, and completely prevented intrahepatic, lung, and peritoneal metastasis. In vitro, HGFK1 exhibited both antiangiogenic and antitumor cell effects, inhibiting the proliferation of both murine microvascular endothelial cells (MEC) and tumor cells, and inducing apoptosis and G(0)-G(1) phase arrest in these cells. The inhibition effects on MECs and tumor cell worked mainly through EGF/EGFR signaling, with more minor contributions from VEGF/VEGFR and bFGF/bFGFR signaling. It may thus be considered as a novel therapeutic strategy for the treatment of HCC (Nie et al., 2008; Shen et al., 2008a; Shen et al., 2008b). Certainly, substantial evidence from clinical data is needed to for HGFK1 to be a potential

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target for anticancer drugs. The expression of HGFK1 and its function in NSCLC has not been reported to date. Here, we first assessed HGFK1 expression in 103 cases patients with advanced NSCLC to investigate the impact of HGFK1 on survival for the first time in our knowledge. Western blot was used to measure HGFK1 protein levels and classify tumors by HGFK1 expression in NSCLC patients. Then, we investigated the function of HGFK1 in NSCLC cell lines A431 and A431/GR. The possible pathway through which HGFK1 exterted its function was also investigated in this study.

Materials and Methods

Patients

A total of 103 patients with advanced NSCLC (III and IV stage) were studied. All samples were obtained by biopsy. Clinical and radiological examination discovered the suspicious patients with NSCLC, and then the definitive pathological diagnosis of NSCLC had made carried out by the CT-guided puncture or incisional biopsy.

The 7th AJCC Staging system was used to categorize patients: those who were stage III at initial diagnosis and those who presented with stage IV NSCLC. Among patients with Stage III NSCLC, metastatic recurrence was characterized as limited metastatic (defined above) or extensive metastatic disease. All patients were assessed with diagnostic CT or PET/CT imaging to identify metastatic lesions. Brain metastases were assessed with MRI. In some patients bone metastases were identified with bone scan and/or MRI.

Collection of 103 admitted patients of NSCLC from Feb. 2006 to Sept. 2010 in Cancer Center, the Medical School, Hospital of Qingdao University and Department of Oncology, The Affiliated Sixth People's Hospital, Shanghai Jiaotong University. All patients were followed up after diagnosis. The basic demographics of this group and the pathological characteristics are shown in Table 1, respectively.

Western blot

For western blot, samples were homogenized in a solution of 10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin at pH 7.9. Protein extracts were then subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The polyclonal antibody against HGFK1 was purchased from Zhongshen Biotechnology Inc. (Shanghai, China). The membrane was incubated with primary and secondary antibodies, respectively, for 1 hour at room temperature. Signals were developed by ECL Kit (Amersham Pharmacia Biotech Inc., Buckinghamshire, UK). Recombined HGFK1 protein was used as positive control (Datasheet of sc-48386, Santa Cruz Biotechnology Inc.). Blot quantitation was done with a Molecular Dynamics Laser Densitometer (Model PSD) and the Image Quant Version 1 software (Mahata et al., 2011).

Cell lines and reagents

NSCLC cell lines A431 and acquired gefitinib resistant A431 (A431/GR) /GR were gifts from Dr. Carlos L. Arteaga (Vanderbilt-Ingram Cancer Center, Nashville, TN). A431/GR were cultured in the presence of 1 μM gefitinib as described previously (Engelman et al., 2007). Commercially available gefitinib and erlotinib were purchased from the pharmacy of affiliated Shanghai 6th People's Hospital of Shanghai Jiao Tong University for the experiments described in this study. Epidermal growth factor (EGF), chrysin, and benzoflavone were purchased from Sigma-Aldrich (St. Louis, MO). Since anti-human HGFK1 antibodies were not commercially available, we designed synthetic peptide: RSY KGT VSI TKS GIKC corresponding to the N-terminal amino acids of HGFK1 to produce rabbit polyclonal antisera against HGFK1. Anti-ERK, Anti-STAT5, Anti-pERK and Anti-pSTATs antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) was used for Western blot. Signals were developed by ECL Kit (Amersham Pharmacia Biotech Inc., Buckinghamshire, UK).

Table 1. Patient Demographics

Characteristic		No. of Patients (N = 103)	Percentage (%)
Age (years)	<62	50	48.5
	≥62	53	51.5
Sex	Male	62	60.2
	Female	41	39.8
Tumor size	<3 cm	39	37.9
	≥3 cm	64	62.1
Lympha node metatasis	No	37	35.9
	Yes	66	64.1
Remote Metastasis	No	44	42.7
	Yes	59	57.3
Histological type	Ad	50	48.6
	SCC	48	46.5
	Ad+SCC	5	4.9
Clinical stage	III	47	45.6
	IV	56	54.4
Smoke Status	Ever	61	59.1
	Never	42	40.9

Ad, adenocarinoma; SCC, squamous cell carcinoma; Ad+SCC, Adenosquamouscarcinoma

Statistical analysis

The statistical significance of the correlation between expression of HGFK1 and several clinicopathological parameters was assessed by Fisher's exact test, 2 test, or 2 test for trends as indicated. The probability of overall survival as a function of time was determined by the Kaplan-Meier method and the log-rank test. Multivariate survival analysis was performed using the Cox regression model. P values smaller than 0.05 were considered as significant. For the statistical evaluation, the SPSS software version 12.0 was used (SPSS, Inc., Chicago, IL).

Results

Invesitgation of HGFK1 expression in patients with NSCLC

The expression of HGFK1 in tumor samples of patients with NSCLC were investigated by Western blot (Figure 1). Tumor samples of 33 in 103 patients (30.1%) were

Table 2. Relationship of High HGFK1 Expression with Some Clinicopathological Factors in Patients with NSCLC

Characteristic	Cases	HGFK1 expression (%) High	low	P value
Age (years)				0.235
<62	53	18 (34.0)	35 (66.0)	
≥62	50	15 (30.0)	35 (70.0)	
Sex				0.102
Male	52	20 (38.5)	32 (61.5)	
Female	51	13 (25.5)	38 (74.5)	
Tumor size				0.025
< 3 cm	49	23 (46.9)	26 (53.1)	
≥3 cm	54	10 (18.5)	44 (81.5)	
Lympha node metatasis				0.062
No	67	24 (35.8)	43 (64.2)	
Yes	36	9 (25.0)	27 (75.0)	
Remote Metastasis				0.002
No	54	25 (46.3)	29 (53.7)	
Yes	49	8 (16.3)	41 (83.7)	
Histological type				0.052
Ad	42	11 (26.2)	31 (73.8)	
SCC	54	19 (35.2)	35 (64.8)	
Ad+SCC	7	3 (42.9)	4 (57.1)	
Clinical stage				0.012
III	49	22 (44.9)	27 (55.1)	
IV	54	11 (20.4)	43 (79.6)	
Smoke Status				
Ever	58	21 (36.2)	37 (63.8)	0.091
Never	45	12 (26.7)	33 (73.3)	

Ad, adenocarinoma; SCC, squamous cell carcinoma; Ad+SCC, Adenosquamouscarcinoma

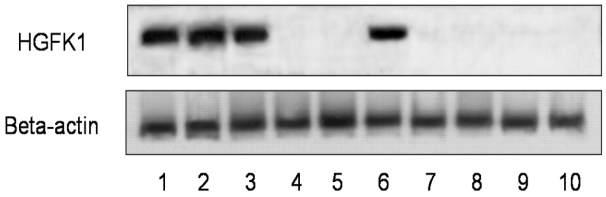

Figure 1. Status of HGFK1 Expression in NSCLC Tissue by Western Blot. HGFK1 expression in NSCLC tissues of all 103 patients were investigated by Western blot. Lane 1 in upper figure indicates the HGFK1 peptide as positive control. Lane 2-10 in upper figure indicates parts of 103 NSCLC tissues randomly. Lower figure indicates the expression of beta-actin. 100 μg total protein was added per lane

detected high HGFK1 expression. High expression of HGFK1 was significantly associated with less remote metastasis (P = 0.002) but did not associate with lymph node metastasis (P = 0.062). There was also a significant association between HGFK1 expression and tumor size (P = 0.025) as well as clinical stage (P = 0.012) was observed, while expression of HGFK1 was not correlated with gender, age, smoke status and histological type (Table 2).

High HGFK1 expression is a prognostic factor for overall survival (OS) and progression-free survival (PFS) in univariate Kaplan-Meier analysis

In Kaplan-Meier survival analysis, high expression of HGFK1 was a significant prognostic indicator for overall survival (P = 0.004) as well as progression free survival (P = 0.001) (Figure 2). In addition, the presence

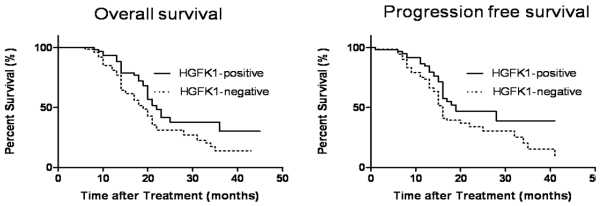

Figure 2. High HGFK1 Expression is a Prognostic Factor for Overall Survival (OS) and Progression Free Survival (PFS) in Univariate Kaplan-Meier Analysis

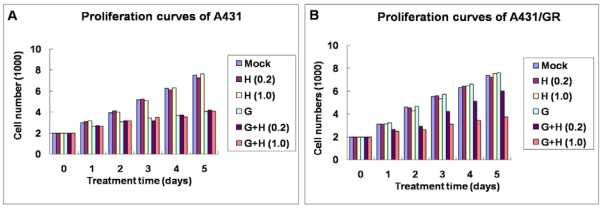

Figure 3. Effects of HGFK1 on Gefitinib Treated A431/GR and A431 Cells. A. Proliferation curves of NSCLC cell line A431 treated with gefitinib alone or with gefitinib combined with HGFK1 (0.2 μg/ml and 1.0 μg/ml respectively). B. Proliferation curves of NSCLC cell line A431/GR treated with gefitinib alone or with gefitinib combined with HGFK1 (0.2 μg/ml and 1.0 μg/ml respectively). Mock: Administration of PBS as control; H (0.2): treated with 0.2 μg/ml HGFK1; H (1.0): treated with 1.0 μg/ml HGFK1; G: treated with 2 μM gefitinib; G+H (0.2): treated with 0.2 μg/ml HGFK1 and 2 μM gefitinib, G+H (1.0): treated with 1.0 μg/ml HGFK1 and 2 μM gefitinib

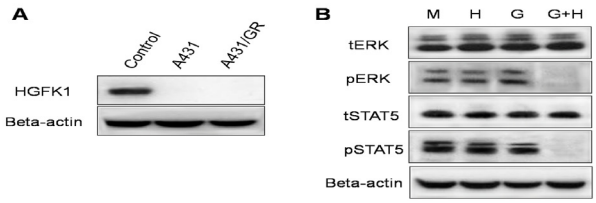

Figure 4. The Possible Mechanism Through Which HGFK1 Exterted its Function on Gefitinib Treated A431/GR and A431 Cells. A. Investigation of status of HGFK1 expression in NSCLC cell lines A431 and A431/GR by Western blot. B. Investigation of the expressions of total ERK (tERK), phosphorylated ERK (pERK), total STAT5 (tSTAT5) and phosphorylated STAT5 (pSTAT5) in A431/GR cell. M: mock, H: treated with 1 μg/ml HGFK1, G: treated with 2 μM gefitinib, G+H: treated with 1 μg/ml HGFK1 and 2 μM gefitinib. 100 μg total protein was added per lane

of metastases and histological grade were significant prognostic indicators for both overall survival and progression free survival. Lymph node metastasis, remote metastasis, clinical stage and smoke status were also significant prognostic indicators for overall survival and progression-free survival.

HGFK1 reversed inhibition by gefitinib on A431/GR cell and did not inhance inhibition by gifitinib in A431 cell.

Since HGFK1 was previously demostrated to exert inhibition effect on liver cancer cell through EGFR signaling pathway, we wondered whether HGFK1 exerts inhibition effect on NSCLC cells. Results showed that no inhibition effect on A431 and A431/GR by treatment of HGFK1 alone was found.

We then treated A431 and A431/GR by gefitinib combined with HGFK1. Results showed that HGFK1 reversed inhibition by gefitinib on gefitinib resistence

NSCLC cell A431/GR. However, HGFK1 did not inhance inhibition by gifitinib on A431 cell (Figure 3).

Reversion of gefitinib inhibition on A431/GR cell by HGFK1 was related to ERK-STAT5 signaling

HGFK1 expression was investigated in NSCLC cell lines A431 and A431/GR. Both A431 and A431/GR did not express HGFK1 (Figure 4A). When treated A431 and A431/GR by gefitinib combined with HGFK1, phosphorylated ERK1/2 (p-EKK1/2) and phosphorylated STAT5 (p-STAT5) expression were down-regulated, while the expression of total ERK1/2 and STAT5 did not changed (Figure 4B). These results suggested that HGFK1 reversed the inhibition by gefitinib relating to phosphorylation status of ERK1/2 and STAT5. Within the administration of HGFK1, gefitinib decreased the phosphorylation status of ERK1/2 and STAT5 and inhibited the proliferation of A431/GR cell.

Discussion

Here we present evidence for the first time in our knowledge that the expression levels HGFK1 has prognostic significance in patients with advanced NSCLC. Specifically, individuals whose tumors had relatively higher HGFK1 expression survive longer as compared to patients with relatively lower levels (Figure 2). We also investigate the correlation between high expression of HGFK1 and clinicopathological factors of patients with advanced NSCLC (Table 2).

EGFR tyrosine kinase is reported overexpressed in a variety of solid tumors. Since it plays important roles in cancer aetiology and progression, EGFR tyrosine kinase is a rational target for cancer therapies. In the past decade, some small molecular inhibitors of EGFR tyrosine kinase (EGFR TKIs) have shown promising clinical activity. Moreover, clinical studies reported that treatment of selective EGFR TKIs as monotherapy, including gefitinib (ZD1839, Iressa) and erlotinib (OSI-774, Tarceva), leads to tumor regression in 12-27% of advanced NSCLC patients (Fukuoka et al., 2003; Kris et al., 2003; Perez-Soler et al., 2004). Encouraging response to gefitinib is closely associated with specific activating mutations in EGFR tyrosine kinase domain in patients of East Asian, female, adenocarcinoma histology, and non-smoking (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004). Nevertheless, the sensitivity to EGFR TKIs may not be determined only by these EGFR activating mutations. 20-30% of NSCLC patients with amplified wild-type EGFR (wtEGFR) still demonstrated significant survival benefits from gefitinib and erlotinib treatment (Tsao et al., 2005). Moreover, approximately 10-20% of gefitinib-responders were also found to have no identifiable EGFR mutations (Kim et al., 2008; Nishimura et al., 2008).

Most patients treated with these agents, however, had progressive disease even after showing an initial dramatic response (Guo et al., 2011). Then, the strategy of treatment in the next step is of importance. Currently, there is no clear evidence suggesting an optimal treatment strategy for patients with acquired TKI failure. Some trials are investigating the role of anticancer therapies in the third- or fourth-line setting after EGFR TKI failure. An irreversible TKI targeting the EGFR, HER2, and vascular endothelial growth factor receptor was evaluated in the third- and fourth-line settings after EGFR TKI failure (Moyer et al., 1997). A small molecule Met inhibitor combined with an EGFR TKI has proven to be a reasonable strategy to overcome erlotinib-resistant T790M NSCLC (Li et al., 2008). Combining an insulin-like growth factor inhibitor with erlotinib to reverse resistance to erlotinib is also under investigation (Tang et al., 2008; Kosaka et al., 2011). However, most of these treatments are not clinically available.

Before this study, although its cellular function is still elusive, HGFK1 emerged to play a role as a tumor suppressor. No clinical evidence about the relationship between HGFK1 gene and tumor cell proliferation in the NSCLC is found up to date. Our data demonstrated that the high expression of HGFK1 was significantly associated with tumor size and remote metastasis but did not associate with lymph node metastasis. When HGFK1 expression is low, the tumor of NSCLC patients tends to grow faster and more easily metastasis to lymph node. There was also a significant association between HGFK1 expression and clinical stages observed.

We demonstrated a survival benefit for HGFK1 expression in patients without adenocarinoma histology and this observation confirms the effect of adenocarinoma histology for prognostic stratification of NSCLC patients. Although we do not yet know the mechanistic basis for this subgroup bias, we do acknowledge that such stratification might be relevant for future targeted therapy and/or early predictions of survival of patients with adenocarinoma.

One interesting result of this study is that HGFK1 reverses the inhibition by gefitinib in gefitinib resistent NSCLC cell line A431/GR. Since TKI resistance becomes increasingly important in the treatment of NSCLC, our result provide promising insight for the use of HGFK1 in future investigations. Combined with the administration of HGFK1, gefitinib could be re-considered for those NSCLC patients who had already benefited from prior gefitinib treatment.

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