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EDITORIAL

EPH-EPHRIN in human gastrointestinal cancers

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Abstract

Ever since its discovery two decades ago, the erythropoietin-producing hepatoma (EPH)-EPHRIN system has been shown to play multifaceted roles in human gastroenterological cancer as well as neurodevelopment. Overexpression, amplification and point mutations have been found in many human cancers and many investigators have shown correlations between these up-regulations and tumor angiogenesis. Thus, the genes in this family are considered to be potential targets of cancer therapy. On the other hand, the down-regulation of some members as a result of epigenetic changes has also been reported in some cancers. Furthermore, the correlation between altered expressions and clinical prognosis seems to be inconclusive. A huge amount of protein-protein interaction studies on the EPH-EPHRIN system have provided a basic scheme for signal transductions, especially bi-directional signaling involving EPH-ERPHRIN molecules at the cell membrane. This information also provides a manipulative strategy for harnessing the actions of these molecules. In this review, we summarize the known alterations of EPH-EPHRIN genes in human tumors of the esophagus, stomach, colorectum, liver and pancreas and present the perspective that the EPH-EPHRIN system could be a potential target of cancer therapy.

Key words: Erythropoietin-producing hepatoma; EPH-EPHRIN; Gastric cancer; Colorectal cancer; Methylation; Secreted form

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INTRODUCTION

Erythropoietin-producing hepatoma (EPH) amplified

sequence is an acronym for erythropoietin-producing hepatocellular carcinoma[1] from which the first member of the EPH family was isolated. The involvement of one gene in this family in human gastric cancer was reported in 1994[2] prior to the designation of this gene as EPHB2 according to a unified nomenclature system[3]. EPH and EPHRIN, receptor kinases EPH and their ligands EPHRIN (EFN), were classified according to the structures of the ligands, the EPHRINs. GPI-anchored-type ligands were called EPHRIN-As and transmembrane-type ligands were called EPHRIN-Bs. The corresponding receptors recognizing each ligand were called EPH-As and EPH-Bs. The relationships are mostly exclusive except for EPHA4-EPHRINB2 and EPHB4-EPHRINA2. Thus, we can say that the EPH-EPHRIN (or EPH-EFN) system has been recognized as a major player in human gastrointestinal carcinogenesis for more than 20 years[4]. In this paper, we review the accumulated data on alterations in EPH receptors in human gastrointestinal tract cancers by each category.

The mutations and a summary of the up-regulation and down-regulation of the EPH receptors are shown in Tables 1 and 2. Readers can access a database containing updates on alterations of genes of interest in specific organs[5] at the web site http://www.sanger.ac.uk/genetics/CGP/Studies/.

EPHA FAMILIES IN HUMAN GASTROINTESTINAL CANCERS

EPHA1

The original isolation paper described the over-expression and not the amplification of *EPHA1* in human colorectal cancer[1]. However, the significance of *EPHA1* in human cancers is far from being solved. Although *EPHA1* was first suspected to be an oncogene (growth factor receptor-like epidermal growth factor receptor), many investigators have recently focused on its down-regulation in human tumors and its possible clinical significance[6,7]. On the other hand, from the standpoint of the pro-angiogenic activity of EPHAs, Chen *et al*[8] reported that the silencing of *EPHA1* induces an anti-angiogenic effect in human hepatocellular carcinoma. Recently, down-regulation by epigenetic silencing was shown to be correlated with a poor survival outcome in patients with colorectal cancer[7,9]. Furthermore, Wang *et al*[10] extended their observation on colorectal cancer to gastric cancer; that is, they reported the correlation between *EPHA1* expression and gastric cancer metastasis and survival. Contrary to the situation reported by Dong *et al*[6] in colorectal cancer, *EPHA1* up-regulation was related to a poor survival outcome and the metastasis of gastric cancer.

EPHA1 expression is possibly regulated by environmental factors. Doleman *et al*[11] reported that *EPHA1* expression and *EPHB4* are influenced by n-3 fatty acid eicosapentaenoic acid (EPA). This observation may imply the important involvement of *EPH* pathways in the mechanism responsible for the presumed health benefits of polyunsaturated fatty acids (PUFA).

Table 1 Somatic mis-sense mutations of erythropoietin-producing hepatoma receptors in colon and stomach (http://www.sanger.ac.uk/genetics/CGP/Studies/, August 30, 2010)

	Amino acid substitutions		Organs
EPHA2	777G>S		Stomach
EPHA3	792S>P	806D>N	Colon
EPHA6	649R>S	813K>N	Stomach
EPHA7	768S>I		Colon
EPHA8	179R>C	873D>N	Colon, Stomach
EPHB1	719I>V	743R>Q	Stomach
EPHB4	889R>W	1030I>M	Colon,Stomach

EPH: Erythropoietin-producing hepatoma.

EPHA2

Most research published so far about the relationship between *EPHA2* expression and human gastrointestinal cancers has indicated that *EPHA2* up-regulation in tumor cells results in a more aggressive nature[12-14]. In addition, *EPHA2* has been extensively investigated from the standpoint of cell and vascular biology. The ligand for this receptor is EPHRINA1 (EFNA1), isolated as an acute phase reactant induced by TNF in endothelial cells[15]. This observation has tempted many investigators to study the expressions of *EFNA1* and its receptor *EPHA2* in tumor cells and their relation with tumor angiogenesis. In human cancers, Kataoka[12] demonstrated an increased microvessel density in *EPHA2* over-expressing colorectal cancers. The mechanisms by which the overexpression of EPHA2 contributes to the aggressive behavior of cancer cells have been widely debated. Fang *et al*[16] discussed the importance of receptor phosphorylation and the kinase activity of EPHA2 toward the aggressive and migratory nature of tumor cells. Miao *et al*[17] on the other hand, reported that the activation of *EPHA2* inhibits the Ras/MAPK pathway, that is, the activation of *EPHA2* may reduce the aggressive nature of tumor cells. The degradation of *EPHA2* is dependent on ligand inducible phosphorylation[18]; thus, the clinico-pathological effects of *EPHA2* activation should be assessed, including the complex situation of the genetic profile of the tumor cells themselves and their microenvironment.

EPHA2 and its major ligand EFNA1 are perturbed by various metabolites including deoxycholic acid (DCA) and its derivative. Li *et al*[19] showed the up-regulation of EPHA2 by DCA in colorectal cancer cells. This may be another example of the involvement of EPH pathways and endogenous metabolites in addition to *EPHA1* and PUFA.

EPHA3

There have been few reports on the alteration of *EPHA3* in human tumors until a recent high throughput sequencing project identified a high prevalence of a somatic mutation in *EPHA3* in human cancers[20,21]. The somatic mutation in *EPHA3* resides in D806 where the residue is evolutionally conserved (Table 1). The prevalence does not seem to be high in any population; actually, no mutations of *EPHA3* were observed in follow-up studies of

Table 2 Up and down regulations of erythropoietin-producing hepatoma receptors in human cancers[3]

	Up-regulation		Down-regulation		
	Overexpression	Amplification	Promoter methylation	Loss of heterozygosity	Others or unknown
EPHA1	Stomach		Colon	Colon	Colon
EPHA2	Stomach, colon, esophagus			Colon	(Melanoma)
EPHA3			(Lung)		
EPHA4	Colon				
EPHA5					
EPHA6					
EPHA7	Stomach, colon		Stomach, colon		
EPHA8				Stomach, colon	
EPHB1					
EPHB2	Stomach, colon	Stomach	Colon	Colon	(Prostate)
EPHB3		Colon			
EPHB4		Colon	Colon	Colon	
EPHB5					
EPHB6	(Neuroblastoma)				

No published data in the blanks. Parenthesis indicate non-GI tract cancers. EPH: Erythropoietin-producing hepatoma.

Table 3 Receptors and ligands corresponding

	EFNA1	EFNA2	EFNA3	EFNA4	EFNA5	EFNB1	EFNB2	EFNB3
EPHA1	x							
EPHA2	x	x	x	x	x			
EPHA3	x	x	x	x	x			
EPHA4	x	x	x	x	x		x	x
EPHA5	x	x	x	x	x			
EPHA6		x						
EPHA7		x			x			
EPHA8	x	x	x	x	x			
EPHB1						x	x	x
EPHB2						x	x	x
EPHB3						x	x	x
EPHB4						x	x	x
EPHB5								
EPHB6						x	x	x

x indicates a known interaction. Each binding constant is shown in the reference Bowden *et al*[27]. EPH: Erythropoietin-producing hepatoma.

46 Japanese patients with colorectal cancer reported by Shao *et al*[22]. Cell signaling studies using a culture system disclosed a role of *EPHA3* in the formation of a cell's shape[23]. Thus, changes in *EPHA3* are likely to produce particular morphological and biological characteristics in the tumor cells carrying these changes, although no correlation between the *EPHA3* status and the clinico-pathological features of gastrointestinal cancers has yet been described. Although the clinical relevance is unknown, there is a report investigating the LINE-1 methylation pattern in the introns of *EPHA3* in tumor cells[24].

EPHA4

The over-expression of *EPHA4* has been reported in gastric and colorectal cancers[25,26]. In both cancers, the over-expression of *EPHA4* is an ominous sign with a shorter survival period and frequent liver metastasis respectively. *EPHA4* is the only type A receptor that binds a B family ligand, EPHRIN(EFN)B2, in addition to a type A ligand, EPHRIN(EFN)A2 (Table 3). A structural study has been conducted to reveal the stereoscopic interactions between several members of EPH receptors and EPHRIN(EFN)s[27]. The potential significance of *EPHA4* over-expression in clinical oncology and the possibility of its use as a therapeutic target remain unknown.

EPHA5

There is no information regarding alterations in *EPHA5* in human gastrointestinal cancers. *EPHA5* is not expressed in the intestine at any age, as reported by Islam *et al*[28].

EPHA6

Research on *EPHA6* in the gastrointestinal tract is sparse. *EPHA6* is commonly expressed in the testis and brain[29].

EPHA7

Since the first description of the down-regulation of *EPHA7* in colorectal cancer[30], several papers have assessed the expression of *EPHA7* in human gastrointesti-

nal cancers[31], human lung cancer[32] and prostate cancer[33]. The biological basis for these clinicopathological observations and their significance in oncology remain to be investigated. The promoter methylation of *EPHA7* was the first example of down-regulation by methylation in *EPH* receptors but a subsequent survey of other *EPH* receptors, including *EPHB* receptors in colon cancer, produced negative results[34]. Another topic concerning *EPHA7* is its secretory form. The secretory form of *EPHA7* contains only the extracellular part of the molecule and does not anchor at the cell membrane. Its biological and clinical significance remain unknown. A secretory form of *EPHA7* is known to exist in malignant lymphoma[35] and lung cancer[32] but no study has been conducted on the presence of the secretory form of *EPHA7* in clinical gastrointestinal cancer.

Although the clinical significance is still unclear, Kim *et al*[36] reported a single nucleotide polymorphism (SNP) at the *EPHA7* locus, rs2278107; this SNP was related to the chemoresponsiveness to fluoropyrimidine-based adjuvant chemotherapy for colorectal cancer[36].

EPHA8

EPHA8 was screened for mutation in Japanese colorectal cancer but no mutations were found[22], similar to other *EPHA* receptors such as *EPHA3* and *EPHA7*. The *EPHA8* receptor induces the sustained up-regulation of MAP kinase; thus, it is supposed to play a role in tumor cell growth and proliferation[37]. *EPHA8* is expressed during the fetal period of intestinal morphogenesis[28] and missense mutations in stomach cancer and colon cancer are known (Table 1).

EPHB1

EPHB1 has been investigated in terms of signal transduction involved in the biological behavior of tumor cells[38,39], but little information is available on its status in human clinical cancer. An *EPHB1* mutation was recently identified in ovarian cancer and missense mutations have also been found in gastric cancer[40] (Table 1).

EPHB2

EPHB2 is the most extensively studied member of *EPH* receptors in the field of oncology. Kiyokawa *et al*[2] reported the overexpression of *EPHB2* in human gastric cancer and assigned it to the chromosomal locus at 1p36 which many investigators have assumed to be a tumor suppressor locus of human colon cancer because of the frequent loss of heterozygosity that has been documented[41]. Subsequently, Oba *et al*[42] demonstrated the loss of heterozygosity of the *EPHB2* locus in human colorectal cancer. Furthermore, Batlle *et al*[43] argued that *EPHB* receptor activity could suppress the progression of colorectal cancer and *EPHB2* is now viewed, at least in some contexts, as a tumor suppressor or a suppressor against tumor progression[26,44-48], although different aspects have also been discussed[49]. A group led by Hans Clevers put forward the comprehensive idea of *EPHB2-EPHRINB1* interplay at the bottom of human colon crypts[50,51]. They showed the clear territory of *EPHB2* and *EPHRINB1* in a human colorectal crypt, its important role in cell positioning and the ordered developmental migration of intestinal cells using *EphB2/EphB3* knockout mice[51]. This view is now prevalent[52] and they have further refined the concept of a stem cell unit in human gastrointestinal crypts[53,54]. Based on the mutually exclusive localization of *EPHB2* and *EPHRINB1*, Cortina suggested that tumor compartmentalization arising from the repulsive action of cells expressing *EPHB2* and *EPHRINB1* is a possible mechanistic basis for tumor suppression by the *EPHB2-EPHRINB1* system[55].

Then, what happened to the previous interpretation for the over-expression of *EPHB2* in human cancer[2,56,57]? Mao[58] reported *EPHB2* as a therapeutic antibody drug target for *EPHB2* over-expressing tumors. Mutation analyses in kinase genes have been very popular and somatic mutations of *EPHB2* have also been reported in many cancers[59,60], including GI tract cancers[61,62].

However, these mutations occur mostly in the microsatellite repeats of tumors with microsatellite instability or nonsense mutations causing RNA decay. No naturally occurring missense mutation that may positively or negatively influence the kinase activity of *EPHB2* has ever been reported. At this moment, we can only say that individual tumors may have an individual *EPHB2* status in an individual environment. The prevalence of methylation in the *EPHB2* promoter, on the other hand, is low compared with RASSF2 and O-6-methylguanine-DNA methyl transferase (MGMT) in early colorectal tumors[63].

There are reports investigating the possible contribution of germline *EPHB2* variants to rare polyposis syndrome[64,65]. The detailed mechanistic basis controlling the *EPHB2-EPHRIN* (*EFN*) *B1* system has also been investigated. Tanaka *et al*[66] reported that C-terminal *EFNB1* regulates matrix metalloproteinase secretion and that the phosphorylation of *EFNB1* regulates the dissemination of gastric cancer cells in an animal model[66]. He also showed the successful suppression of peritoneal dissemination in an animal model using an *EFNB1*-derived peptide[67]. The translational approaches using this method (use of EFNB1 peptide to suppress human cancer dissemination) have not yet been shown.

EPHB3

The localization and function of *EPHB3* partially overlaps with *EPHB2* in a Paneth cell compartment. *EPHB3* also has *EFNB1* as a ligand. Both are controlled by the beta-catenin/Tcf4 pathway[51].

Chiu reported that the over-expression of *EPHB3* enhanced cell-cell contact and suppressed tumor growth in HT-29 human colon cancer cells[68]. The defect in the positioning of Paneth cells is thought to arise from the disruption of the *EPHB2-EPHB3* system[69]. Clinicopathological information on *EPHB3* alone (not accompanied with *EPHB2*) in human gastrointestinal tract cancers remains limited. A clinical interpretation of the over-expression and/or amplification of *EPHB3* (Figure 1) in gastrointestinal cancer[70,71] awaits further investigations.

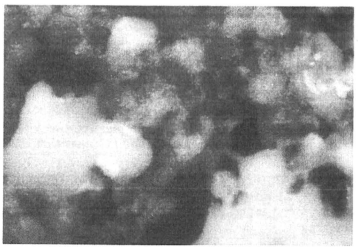

Figure 1 Fluorescence in situ hybridization of *EPHB3* (bacterial artificial chromosome RP11-328G15, red) in gastric cancer cells. Numerous red signals (more than 5) in a cell with two centromeres (green), indicating *EPHB3* amplification, are shown. The methodological details have been previously reported[88,78].

EPHB4

Kumar reported that *EPHB4* over-expression is more prevalent than *EPHB2* over-expression and the cyclic AMP-responsive element binding protein-binding protein (CBP) complex reciprocally regulates *EPHB2* and *EPHB4* (CBP complex suppresses *EPHB2* and induces *EPHB4* expression)[48]. *EPHB4* is thought to act in an *EPHB4-EPHB6* system[72] to regulate cancer cell invasiveness. The structure and dynamism on *EPHB4-EFNB2* was investigated[73,74] and the translational application of this basic knowledge awaits further investigation.

EPHB6

EPHB6 is the oldest *EPH* family member to attract enthusiastic interest from cancer researchers, especially neuroblastoma researchers. *EPHB6* is unique in that there is no kinase activity. It is one of the major genes involved in the clinico-biological behaviors of neuroblastomas[75-77]. Unlike other *EPHBs*, a suppressor role of *EPHB6* has been pointed out from an early stage of research[78-80], although its over-expression has been identified in leukemic cells[81]. A functional enigma of *EPHB6*, a kinase defective receptor affecting tumor invasiveness, has been gradually clarified in the fields of lung cancer research[82] but the role of *EPHB6* in carcinogenesis in the human digestive tract is not clear, although its alteration such as promoter methylation in lung adenocarcinoma, has been recently reported[83]. Recently, some missense variants have been reported in familial colorectal cancer[84]. Somatic changes in colorectal cancers according to ethnic stratification have revealed *EPHB6* to be one of the most frequently deleted genes in African Americans[85].

EPH RECEPTORS AS THERAPEUTIC TARGETS

Choi *et al*[86] reported the discovery of *EPHB2* receptor kinase inhibitors. They also performed crystallographic analyses of *EPHA3* and *EPHA7* in complex with their inhibitors and discussed the possibility of generating new inhibitors using a structure-based design[86]. This discovery and other structural studies[27,73,87] should pave the way for the development of drugs that specifically inhibit tumor cells over-expressing these receptors.

EPHA2 has been considered as a target for anti-angiogenesis therapy for a long time[8,88-92]. The EPHA2-Fc receptor was used to inhibit an *EFNA1-EPHA2* forward signal and to reduce neovascularization in rodent retina[91].

CONCLUSION

Although the *EPH* family is well known to be involved in the development of neural and vascular systems, their pivotal contributions to cancer biology, especially in clinical settings, remain to be elucidated. Enthusiasm regarding the use of *EPHs* as cancer therapy targets remains less than that of expectations for other groups of kinase receptors such as *EGFR*, *HER2*, *MET* and *RAF*[40,93]. The unique biological nature of *EPHs* such as bidirectional signaling and the presence of a secreted form, however, may provide a possible clue to manipulating the regulation of *EPH-EPHRIN* systems for human gastrointestinal cancer therapy. Gastrointestinal cancers have a special niche in Asian diseases in terms of their heterogeneity and uniqueness in etiology, both genetic and environmental[94]. An extensive search of EPH-EPHRIN systems in Asian gastrointestinal cancer patients will provide an important tool for the clinical management of Asian gastrointestinal cancer patients.

The real scale of the involvement of those genes in carcinogenesis in the human gastrointestinal tract still remains unclear and several research groups including Asians continue the search for molecular alterations of the EPH-EPHRIN system that may be relevant to detection and treatment of gastrointestinal cancers. The information stated here will be updated every year in future.

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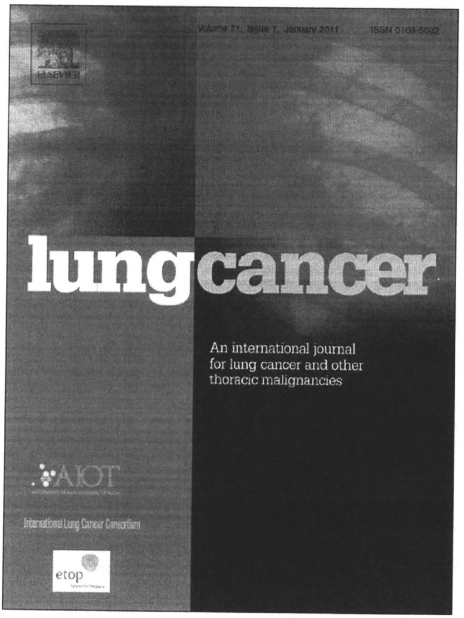

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Suppression of hydroxyurea-induced centrosome amplification by NORE1A and down-regulation of NORE1A mRNA expression in non-small cell lung carcinoma

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ABSTRACT

The candidate tumor suppressor NORE1A is a nucleocytoplasmic shuttling protein, and although a fraction of the NORE1A in cells is localized to their centrosomes, the role of centrosomal NORE1A has not been elucidated. In this study we investigated the role of NORE1A in the numerical integrity of centrosomes and chromosome stability in lung cancer cells. Exposure of *p53*-deficient H1299 lung cancer cell line to hydroxyurea (HU) resulted in abnormal centrosome amplification (to 3 or more centrosomes per cell) as determined by immunofluorescence analysis with anti-γ-tubulin antibody, and forced expression of wild-type NORE1A partially suppressed the centrosome amplification. The nuclear export signal (NES) mutant (L377A/L384A) of NORE1A did not localize to centrosomes and did not suppress the centrosome amplification induced by HU. Fluorescence *in situ* hybridization analyses with probes specific for chromosomes 2 and 16 showed that wild-type NORE1A, but not NES-mutant NORE1A, suppressed chromosome instability in HU-exposed H1299 cells that was likely to have resulted from centrosome amplification. We next examined the status of NORE1A mRNA expression in non-small cell lung carcinoma (NSCLC) and detected down-regulation of NORE1A mRNA expression in 25 (49%) of 51 primary NSCLCs by quantitative real-time-polymerase chain reaction analysis. These results suggest that NORE1A has activity that suppresses the centrosome amplification induced by HU and that NORE1A mRNA down-regulation is one of the common gene abnormalities in NSCLCs, both of which imply a key preventive role of NORE1A against the carcinogenesis of NSCLC.

1. Introduction

NORE1 (*RASSF5*) is a member of the RASSF gene family, and *NORE1A* is the longest and major splice isoform of the *NORE1* gene [1–3]. Its product, NORE1A, is a nucleocytoplasmic shuttling protein and has a growth-suppressive function [4–9]. Interestingly, a fraction of NORE1A is localized to centrosomes [6], but the role of centrosomal NORE1A has never been elucidated. Centrosomes are major microtubule-organizing centers, and at any given time during the cell cycle each cell contains one or two centrosomes [10–13]. When centrosome amplification (to 3 or more centrosomes per cell) occurs as a result of some mechanism, the amplification leads to aberrant mitotic spindle formation, merotelic kinetochore-microtubule attachment errors, lagging chromosome formation, and chromosome missegregation, all of which are thought to be possible causes of chromosome instability (CIN) [12–15]. One of the mechanisms underlying the induction of centrosome amplification is suggested by the fact that centrosomes in cells whose cell cycle has been arrested by exposure to a DNA synthesis inhibitor, *i.e.*, aphidicolin or hydroxyurea (HU), an S-phase entry inhibitor, *i.e.*, mimosine, or DNA damage have been shown to continue to duplicate, resulting in the generation of amplified centrosomes [13,16–20]. Efficient centrosome amplification in arrested cells has also been shown to occur when *p53* is either lost or mutationally inactivated [13,17].

Hypermethylation of the *NORE1A* promoter region has been detected in some types of cancers [2–5,21–23]. Hesson et al. and Irimia et al. found that the *NORE1A* promoter is hypermethylated in 24% and 28%, respectively, of primary non-small cell lung carcinomas (NSCLCs) in studies in which they used the methylation-specific-polymerase chain reaction (MS-PCR) method [22,23]. To our knowledge, however, there have been no report of studies that compared the level of NORE1A mRNA expression in the cancerous tissue of primary NSCLCs and corresponding

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non-cancerous tissue. Although promoter hypermethylation is a well-known mechanism underlying reductions of gene expression, other factors are also involved in the regulation of the expression level [24–26]. Accordingly, in the present study we investigated the level of NORE1A mRNA expression in primary NSCLCs and in NSCLC cell lines. In addition, since CIN is often observed in NSCLC [27], we hypothesized that centrosomal NORE1A has activity that controls the numerical integrity of centrosomes in NSCLC cells, and we tested this hypothesis in a *p53*-deficient H1299 NSCLC cell line.

2. Materials and methods

2.1. Cell lines, primary cancers, and reagents

An immortalized human airway epithelial cell line, 16HBE14o- (simian virus 40-transformed human bronchial epithelial cells) [28], 8 human adenocarcinoma of the lung cell lines, *i.e.*, cell lines A549, H358, H820, H2087, LC-2/ad, RERF-LC-MS, VMRC-LCD, and RERF-LC-KJ, 3 large cell carcinoma of the lung cell lines, *i.e.*, cell lines H460, H1299, and Lu65, and a squamous cell carcinoma of the lung cell line, cell line ABC-1, were used in this study. Cell line 16HBE14o- was a gift from Dr. D.C. Gruenert (California Pacific Medical Center Research Institute, San Francisco, CA) via Dr. T. Kaneko (Department of Internal Medicine, Yokohama City University, School of Medicine, Yokohama, Japan). Cell lines A549, LC-2/ad, RERF-LC-MS, VMRC-LCD, RERF-LC-KJ, H460, and ABC-1 were gifts from Dr. Niki (Jichi Medical University, Shimotsuke, Japan). Cell lines H358, H820, and H2087 were obtained from the American Type Culture Collection (Manassas, VA), and cell line Lu65 was obtained from the Health Science Research Resources Bank (Osaka, Japan). The cells were cultured and grown in RPMI1640 (Sigma–Aldrich, St. Louis, MO) medium (H820, H2087, LC-2/ad, RERF-LC-MS, VMRC-LCD, RERF-LC-KJ, H460, H1299, Lu65, and ABC-1) or DMEM (Sigma–Aldrich) medium (16HBE14o-, A549, and H358) supplemented with 10% fetal bovine serum (Equitech-Bio, Kerrville, TX), penicillin (100 units/ml), and streptomycin (100 μg/ml) under 5% CO_2 atmosphere at 37 °C. Lung cancer tissue and corresponding normal lung tissue from a total of 51 sporadic cases of primary NSCLC were obtained from Hamamatsu University Hospital (Japan) and Seirei Mikatahara General Hospital (Japan). The tissue sample obtained from surgically resected lung was frozen in liquid nitrogen and maintained at −80 °C until used. Patients were divided into smokers and non-smokers based on their smoking history, with "non-smoker" meaning a patient who had never smoked and "smoker" meaning all others. The study design was approved by the institutional review board of both Hamamatsu University School of Medicine and Seirei Mikatahara General Hospital. A stock HU solution was prepared by dissolving HU (Sigma–Aldrich) in phosphate-buffered saline (PBS) to a concentration of 1.0 M.

2.2. Quantitative real-time (QRT)-polymerase chain reaction (PCR)

Expression of the NORE1A mRNA transcript was measured by QRT-PCR with a LightCycler instrument (Roche, Palo Alto, CA). Total RNA was extracted with an RNeasy Plus Mini Kit (QIAGEN, Valencia, CA), and 3 μg of total RNA was converted to cDNA with a SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). PCR amplification of the NORE1A transcript and the transcript of the control housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPD*) was performed with the cDNA and a QuantiTect SYBR Green PCR kit (QIAGEN). The following PCR primers were used: 5′-GTG ACC TGT GCG GAC GAG AG-3′ and 5′-GGA TAA ACC CTC CTG CTG ACT GC-3′ for the NORE1A transcript and 5′-GCT CAG ACA CCA TGG GGA AG-3′ and 5′-TGT AGT TGA GGT CAA TGA AGG GG-3′ for the GAPD transcript. Although there are multiple transcripts in NORE1, the former set of PCR primers is NORE1A-specific. The relative amounts of NORE1A transcript were normalized to those of the GAPD transcript. T/N ratios were calculated by dividing the normalized transcript amounts in the cancerous tissue by the amounts in the non-cancerous tissue.

2.3. Plasmid construction

An expression vector for NORE1A (pCMV5-Flag-NORE1A) [6] was kindly provided by Dr. A.V. Khokhlatchev (Department of Pathology, University of Virginia Health Science Center, VA). NORE1A cDNA was prepared by PCR amplification by using Pfu Turbo Hotstart DNA polymerase (Stratagene, La Jolla, CA), and pCMV5-Flag-NORE1A as a template, and inserted into pEGFP-C1 vector (Clontech, Palo Alto, CA) and pcDNA3 vector (Invitrogen) to construct GFP-NORE1A expression vector and NORE1A alone expression vector, respectively. Expression vectors for L290A/I292A-type, L344A/L346A-type, and L377A/L384A-type NORE1A were generated by site-directed mutagenesis with a QuikChange Site-Directed Mutagenesis kit (Stratagene). All of the plasmid vectors were confirmed by DNA sequencing with a BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Tokyo, Japan) and an ABI 3100 Genetic Analyzer (Applied Biosystems).

2.4. Cell culture, transfection, and puromycin selection

The H1299 cells were maintained at 37 °C in RPMI1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin under a 5% CO_2 atmosphere. A plasmid vector was transfected into the H1299 cells by using the LipofectAMINE 2000 reagent (Invitrogen) according to the supplier's recommendations. For the fluorescence *in situ* hybridization (FISH) analysis, cells were transfected with a pEGFP-C1 mammalian expression plasmid, containing or not containing NORE1A, together with a plasmid containing a puromycin resistance gene (pIRESpuro2, Clontech) at a 15:1 molar ratio. After incubation for 16 h, the medium was changed to medium containing puromycin (1.75 μg/ml). Successfully transfected cells enriched by exposure to puromycin for 40 h were then exposed to 2 mM HU for 40 h. After washing with medium, cells were cultured for an additional 72 h, and then used for the FISH analysis.

2.5. Western blot analysis

Western blot analysis using anti-NORE1A monoclonal antibody (clone 10F10, Upstate, Lake Placid, NY) or anti-β-tubulin monoclonal antibody (clone 2-28-33; Sigma–Aldrich) was performed as described previously [18].

2.6. Indirect immunofluorescence analysis

Cells were washed with PBS and fixed with methanol for 5 min at −20 °C. The cells were permeabilized with 1% Nonidet P-40 in PBS for 5 min, then incubated with 10% normal goat serum blocking solution (DAKO, Kyoto, Japan) for 30 min. The cells were then probed with mouse anti-γ-tubulin monoclonal antibody (clone GTU88; Sigma–Aldrich) at room temperature (RT) for 1 h. Indirect immunofluorescence labeling was performed by exposure to Alexa Fluor 594-conjugated goat anti-mouse IgG antibody (Molecular Probes, Eugene, OR) at RT for 1 h, and the nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma–Aldrich). The immunostained cells were examined under a fluorescence microscope (Olympus BX-51-FL; Olympus, Tokyo, Japan) equipped with epifluorescence filters and a photometric CCD camera (Sensicam;

PCO Company, Kelheim, Germany). The images captured were digitized and stored in the image analysis program (MetaMorph; Molecular Devices, Palo Alto, CA).

2.7. Cell proliferation assay

Cells were transfected with a pEGFP-C1 mammalian expression plasmid, containing or not containing NORE1A, together with a plasmid containing a puromycin resistance gene (pIRESpuro2, Clontech) at a 15:1 molar ratio. After puromycin selection, the cells were seeded, and the number of viable cells was counted after 24, 48, and 72 h by using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions.

2.8. FISH analysis

FISH analysis was performed as described previously [19,29]. In brief, trypsinized cells were treated with 0.075 M KCl hypotonic solution and incubated at RT for 15 min. The cells were fixed in Carnoy's fixative twice, and the fixed cell suspension was spread onto slides over a flame. Each Spectrum Orange-labeled centromere enumeration probe (CEP; Vysis, Des Plaines, IL) solution used to identify numbers of chromosome 2 or chromosome 16 was placed on a slide and covered with a coverslip. The slides with the hybridization mixture were denatured on a digital hot plate (HP-15; AS ONE Corp., Osaka, Japan) and then incubated overnight at 42 °C. After washing the slide in 50% formamide/2× SSC, mounting medium containing DAPI (Vector Laboratories, Burlingame, CA) was used for nuclear counterstaining. The slides were promptly examined under a fluorescence microscope (Olympus BX-51-FL; Olympus) equipped with epifluorescence filters and a photometric CCD camera (Sensicam; PCO Company). The images captured were digitized and stored in the image analysis program (MetaMorph; Molecular Devices). More than 200 cells were examined in each experiment.

2.9. Statistical analysis

Data shown in the graph are means ± standard error of three experiments, and the statistical analysis was performed by using the *t*-test, chi-square test, Wilcoxon matched pairs test and JMP version 7.0.1 software (SAS Institute, Cary, NC). *p*-Values less than 0.05 were considered statistically significant.

3. Results

3.1. Down-regulation of NORE1A expression in NSCLC cell lines

We first performed a QRT-PCR analysis for NORE1A mRNA transcripts in twelve NSCLC cell lines: A549, H358, H820, H2087, LC-2/ad, RERF-LC-MS, VMRC-LCD, RERF-LC-KJ, H460, H1299, Lu65, and ABC-1. The mRNA expression levels were markedly lower in all (12/12, 100%) of the NSCLC cell lines than in immortalized human airway epithelial cell line 16HBE14o- (Fig. 1A), indicating that NORE1A mRNA expression was down-regulated in the NSCLC cell lines. Since our previous study showed that in one of the above 12 cell lines, cell line H1299, under normal growth conditions, the percentage of cells containing 3 or more centrosomes is maintained at approximately 5% [19], we considered the H1299 cell line to be suitable to use to investigate the alterations of centrosome number. We investigated the level of NORE1A protein expression in cell line H1299. The fact that the level of NORE1A mRNA expression in H1299 cells is low suggested that the NORE1A protein level would also be low, and the results of our Western blot analysis showed that the level of NORE1A protein expression in H1299 cells was below the level of detection and much lower than the level of protein expression by the 16HBE14o- cell line and normal human lung tissue (Fig. 1B). A549 cells were used as a negative control in the analysis, because previous papers have reported a low level of NORE1A protein expression in A549 cells [4,6]. We therefore decided to use H1299 cells for the NORE1A transfection experiment to investigate the role of NORE1A in centrosomes.

3.2. Centrosomal localization of NORE1A in H1299 cells

We first tried to identify the subcellular localization of N-terminally GFP-tagged NORE1A. Immunofluorescence analysis of γ-tubulin, a major centrosomal protein [10,11], in H1299 cells transfected with GFP or GFP-NORE1A expression plasmid showed that in interphase some of the GFP-NORE1A was localized at the centrosomes as well as in the nuclei and cytoplasm (Fig. 1C, middle panels), and centrosomal localization of NORE1A was also detected in the mitotic phase (Fig. 1C, lower panels). When H1299 cells were examined for cell proliferation after transfection with GFP or GFP-NORE1A plasmid together with a plasmid containing a puromycin resistance gene and puromycin selection, only a slight reduction in growth was detected in the enriched GFP-NORE1A-transfected lung cancer cells compared with the GFP transfected cells (Fig. 1D), which is consistent with a previous report [6].

3.3. NORE1A suppresses the centrosome amplification induced by HU

When some cell lines have been exposed to the DNA synthesis inhibitor HU, the centrosomes continue to reduplicate, resulting in the generation of amplified centrosomes [13,16,30]. We therefore attempted to determine the effect of NORE1A on the regulation of centrosome number in cells exposed to HU. First, we exposed H1299 cells to HU for 40 h and counted the number of centrosomes they contained by immunofluorescence analysis with anti-γ-tubulin antibody. The results showed a significantly higher frequency of cells containing 3 or more centrosomes was among the HU-exposed cells than among the control cells (Fig. 2A), meaning that centrosome amplification had been induced in the cells exposed to HU. Next, we transfected H1299 cells with the GFP-NORE1A or control GFP expression plasmid and exposed them to HU for 40 h. Forced expression of NORE1A had no clear effect on the control cells, but resulted in a significant reduction in the frequency of HU-exposed cells containing 3 or more centrosomes (Fig. 2B), suggesting that NORE1A has activity that partially suppresses the centrosome amplification induced by HU.

3.4. Wild-type (Wt) NORE1A, but not NES-mutant NORE1A, suppresses the centrosome amplification induced by HU

Since NORE1A is a nucleocytoplasmic shuttling protein [8], the nuclear export signal (NES) may be present in NORE1A protein. Because the centrosomes are located in the cytoplasm [12,13], we investigated whether the NES mutation abrogates the ability of NORE1A to localize to centrosomes and control the numerical integrity of centrosomes. When the amino acid sequence of NORE1A was screened with the NES predictor program NetNES [31], three regions were suspected of a leucine-rich NES. We introduced point mutations to each putative NES and constructed GFP-NORE1A-L290A/I292A, -L344A/L346A, and -L377A/L384A expression plasmids, and Western blot analysis confirmed that all three GFP-NORE1A proteins were expressed at a similar molecular size (Fig. 2C). Then, their subcellular localization was investigated by examining the cells with a fluorescence microscope, and localization of NORE1A-wt, -L290A/I292A, and -L344A/L346A was observed in both the nucleus and cytoplasm, whereas NORE1A-L377A/L384A was localized in

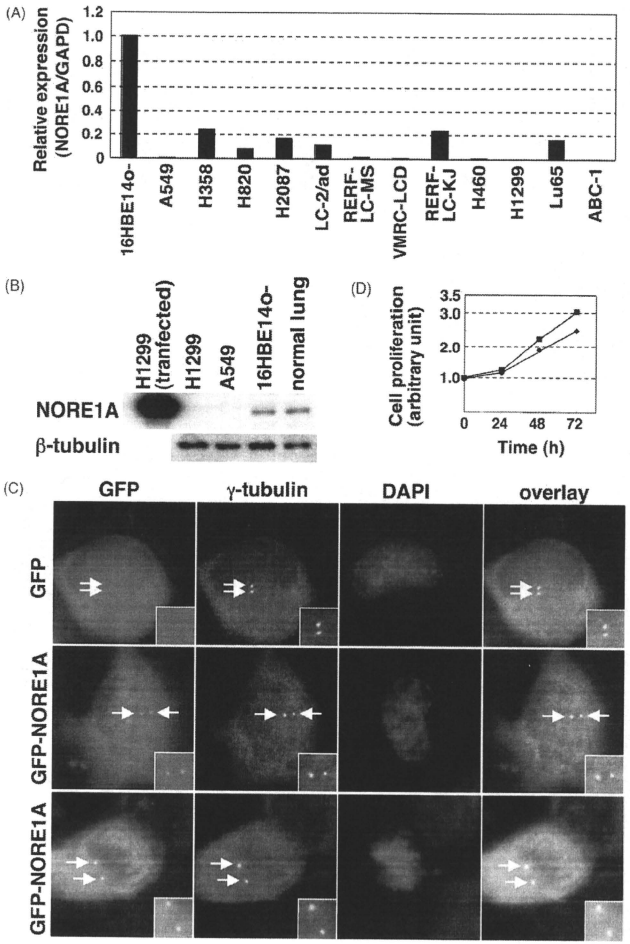

Fig. 1. Down-regulation of NORE1A expression in non-small cell lung carcinoma (NSCLC) cell lines and centrosomal localization of NORE1A. (A) Measurement of the level of expression of NORE1A transcripts in NSCLC cell lines by quantitative real-time-polymerase chain reaction with a LightCycler instrument. The amounts of NORE1A transcripts normalized to the amount of transcripts of a housekeeping gene, *GAPD*, in 12 NSCLC cell lines are shown in the graph. The NORE1A expression level of human airway epithelial cell line 16HBE14o- was measured as a control and set equal to 1.0. (B) Measurement of the level of NORE1A protein expression in cell lines H1299 and A549 NSCLC by Western blot analysis with anti-NORE1A monoclonal antibody. H1299 cells transiently transfected with NORE1A expression vector, human airway epithelial cell line 16HBE14o-, and human non-cancerous lung tissue were also used. A smaller amount of total cell extract was applied in the NORE1A-transfected H1299 cell lane for SDS-PAGE than in the other lanes. Expression of β-tubulin protein was analyzed as an internal control. (C) Detection of the subcellular localization of NORE1A in H1299 cells. H1299 cells transiently transfected with GFP or GFP-NORE1A expression vector (green) and then immunostained with mouse anti-γ-tubulin monoclonal antibody (red). Nuclei were stained with DAPI (blue). Representative immunostaining images are shown. Arrows point to the positions of centrosomes. The upper and middle panels show interphase cells, and the lower panels show mitotic phase cells. (D) Proliferation of H1299 cells transfected with GFP vector (squares) or GFP-NORE1A expression vector (diamonds). Cell numbers were counted by using a Cell Counting Kit-8.

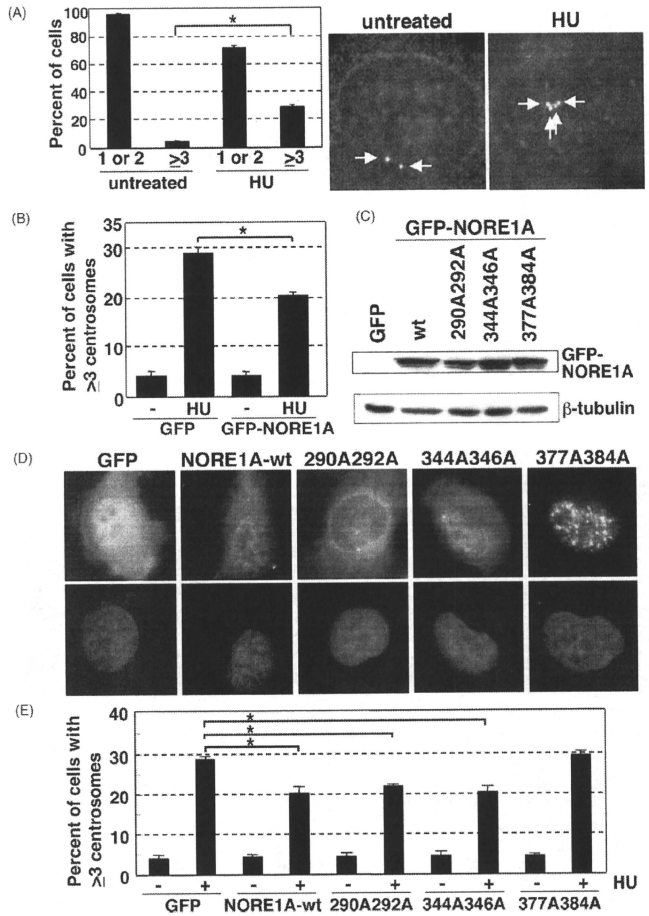

Fig. 2. Wild-type (wt) NORE1A, but not nuclear export signal (NES)-mutant NORE1A, suppresses centrosome amplification induced by hydroxyurea (HU). (A) Induction of centrosome amplification in H1299 cells as a result of HU exposure. H1299 cells were exposed or not exposed to HU for 40 h, and then immunostained with mouse anti-γ-tubulin monoclonal antibody (red). Nuclei were stained with DAPI (blue). The number of centrosomes per cell was counted, and the counts are shown in the left panel. A *t*-test was performed for statistical analysis and an asterisk in the graph indicates a statistically significant difference. Representative immunostaining images are shown in the right panels. Arrows indicate the centrosomes. (B) NORE1A suppresses centrosome amplification induced by HU. H1299 cells were transiently transfected with GFP-NORE1A or GFP expression plasmid, and 18 h later, the cells were exposed or not exposed to HU for 40 h, and then immunostained with mouse anti-γ-tubulin antibody. The percentages of cells containing 3 or more centrosomes were calculated. A *t*-test was performed for statistical analysis and an asterisk in the graph indicates a statistically significant difference. (C) Expression of the wt form and putative NES mutant forms of GFP-NORE1A proteins detected by Western blot analysis with anti-NORE1A monoclonal antibody. H1299 cells were transiently transfected with GFP-NORE1A expression vectors and examined for NORE1A expression. Expression of β-tubulin protein was analyzed as an internal control. (D) Subcellular localization of the wt form and putative NES mutant forms of GFP-NORE1A proteins in H1299 cells. H1299 cells were transiently transfected with GFP or GFP-NORE1A expression vectors (green), and then examined for their localization under a fluorescence microscope. Nuclei were stained with DAPI (blue). Representative immunostaining images are shown. (E) NORE1A-L377A/L384A does not suppress centrosome amplification induced by HU. H1299 cells were transiently transfected with the wt form and putative NES mutant forms of GFP-NORE1A or GFP expression plasmid, and 18 h after transfection, the cells were exposed to HU for 40 h or not exposed, and then immunostained with mouse anti-γ-tubulin antibody. The percentages of cells containing 3 or more centrosomes were determined. A t-test was performed for statistical analysis and an asterisk in the graph indicates a statistically significant difference.