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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

Kazuya Shinmura[a], Hong Tao[a], Kiyoko Nagura[a], Masanori Goto[a], Shun Matsuura[a], Takahiro Mochizuki[b], Kazuya Suzuki[b], Masayuki Tanahashi[c], Hiroshi Niwa[c], Hiroshi Ogawa[d], Haruhiko Sugimura[a,*]

[a] 1st Department of Pathology, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi Ward, Hamamatsu 431-3192, Shizuoka, Japan
[b] 1st Department of Surgery, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi Ward, Hamamatsu 431-3192, Japan
[c] Division of Thoracic Surgery, Respiratory Disease Center, Seirei Mikatahara General Hospital, 3453 Mikatahara, Kita Ward, Hamamatsu 433-8558, Japan
[d] Division of Pathology, Seirei Mikatahara General Hospital, 3453 Mikatahara, Kita Ward, Hamamatsu 433-8558, Japan

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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

* Corresponding author. Tel.: +81 53 435 2220; fax: +81 53 435 2225.
E-mail address: hsugimur@hama-med.ac.jp (H. Sugimura).

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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;