The long noncoding RNA of RMRP is downregulated by PERK, which induces apoptosis in hepatocellular carcinoma cells

Atsushi Yukimoto, Takao Watanabe, Kotaro Sunago, Yoshiko Nakamura, Takaaki Tanaka, Yohei Koizumi, Osamu Yoshida, Yoshio Tokumoto, Masashi Hirooka, Masanori Abe & Yoichi Hiasa

Endoplasmic reticulum (ER) stress plays an important role in hepatocyte degeneration, especially in patients with chronic liver injury. Protein kinase R-like endoplasmic reticulum kinase (PERK) is a key molecule in ER stress. PERK may contribute to apoptotic cell death in HCC, however the details of the mechanism are not clear. In this study, we identified PERK-associated molecules using transcriptome analysis. We modulated PERK expression using a plasmid, tunicamycin and siRNA against PERK, and then confirmed the target gene expression with real-time PCR and Northern blotting. We further analyzed the apoptotic function. Transcriptome analysis revealed that expression of the RNA component of mitochondrial RNA processing endoribonuclease (RMRP), which is a long noncoding RNA, was strongly correlated with the function of PERK. The expression of RMRP was correlated with the expression of PERK in experiments with the siRNA and PERK plasmid in both HCC cell lines and human HCC tissue. Furthermore, RMRP downregulation induced apoptotic cell death. RMRP is downregulated by PERK, which induces apoptosis in HCC. RMRP could be a new therapeutic target to regulate HCC in patients with chronic liver diseases associated with ER stress.

Hepatocellular carcinoma (HCC) is the third most common cause of cancer death in the world. Molecular targeted drugs such as tyrosine kinase inhibitors have therapeutic effects in HCC patients. However, treating patients with lower hepatic function reserve, and treatment effects are limited. New therapeutic targets for treating HCC are therefore required.

ER stress is triggered by inhibition of protein glycosylation, disruptions in calcium ion homeostasis, and hypoxia and induces the unfolded protein response (UPR). UPR is a phenomenon by which cells escape from damage and is involved in various diseases, such as cardiovascular disease, Alzheimer disease, and cancer. In hepatology, ER stress is a risk factor for chronic liver injury, especially in patients with NASH. In NASH model mice, ER stress in hepatocytes is increased by a high fat diet and leads to steatohepatitis. A previous study revealed that ER stress plays an important role in the pathogenesis of NASH. In HCC, induction of ER stress may lead to cell death. On the other hand, after adaptation to ER stress, the tumor size increases. ER stress is strongly related to cell survival and death in chronic liver injury and carcinogenesis.

PERK, inositol requiring enzyme 1, and activating transcription factor 6 are key regulators of ER stress. Among these molecules, we focused on PERK. PERK and PKR, which are both kinases that activate eukaryotic translation initiation factor 2-alpha (eIF2α), catalyze an early regulated step of protein synthesis initiation. PKR is a soluble molecule that exists in the cytosol, whereas PERK is expressed in the ER membrane. The factors that activate these two molecules are not the same. For example, PKR expression is stimulated by viral infections such as hepatitis C, whereas PERK expression is stimulated by ER stress. In a previous study, we reported that PKR is overexpressed and activated in HCC compared with surrounding non-HCC tissues. PKR is also related to cancer proliferation. PKR has high amino acid homology with PKR, suggesting a relationship between PERK and HCC.

Regarding the relationship between PERK expression and malignant diseases, PERK is an anti-proliferative molecule that retains a restriction point. Ectopic expression of PERK in tumors suppresses the growth of cancer.
cells in vivo, showing that PERK has tumor-suppressor function. However, adaption to ER stress induces upregulation of molecules that are needed for survival of cancer cells including HCC. To clarify the mechanism of ER stress in HCC, we need to identify another key downstream molecule that regulates HCC proliferation. We first identified a molecule known as RNA component of mitochondrial RNA processing endoribonuclease (RMRP), which is strongly linked to PERK. We evaluated the expression of RMRP in HCC and whether this molecule has critical effects for patients with HCC.

Results

Knock down of PERK expression with siRNA leads to an increase in RMRP. To investigate PERK-related genes, we transfected PERK siRNA into Huh7 and HLE cells. We confirmed that PERK mRNA expression and protein translation were knocked down, using RT-PCR (Fig. 1A) and Western blotting (Fig. 1B). Expression of phosphorylated eIF2α was also decreased. Thus, PERK signaling was inactivated by siRNA transfection in Huh7 and HLE cells.

We next conducted PERK overexpression experiments using a PERK expression plasmid. We transfected HCC cell lines with PERK expression or control plasmids, and then extracted RNA and protein. We verified that the RNA level of PERK was increased in PERK-overexpressing cells compared to the control group (Fig. 2A). Western blotting analysis showed that exogenous PERK expression appeared at 24 kDa, which was above the endogenous PERK protein band because of the green fluorescent protein sequence (Fig. 2B). Phosphorylated eIF2α expression was also increased, confirming that the PERK expression plasmid was functional. In PERK-overexpressing cells, the expression of RMRP was downregulated, as seen with RT-PCR (Fig. 2C) and Northern blotting (Fig. 2D).

ER stress also decreases RMRP expression. Because PERK is an ER stress molecule, we investigated the influence of ER stress on RMRP by adding tunicamycin dissolved in DMSO to Huh7 and HLE cells. As seen with RT-PCR, in the tunicamycin treatment group, PERK mRNA expression was increased (Fig. 3A). We also confirmed the upregulation of PERK protein with Western blotting (Fig. 3B). Increased expression of phosphorylated eIF2α indicated that the upregulated PERK was functional. In these cells, RMRP was decreased as seen with RT-PCR (Fig. 3C) and Northern blotting (Fig. 3D). Then, we transfected cells with PERK siRNA after treatment with tunicamycin. As a result, the RMRP expression level was increased significantly (Fig. 3E). These results indicate that PERK and RMRP function inversely in the same pathway that is involved in ER stress.

RMRP expression influences apoptosis. We next investigated the function of RMRP. To knock down RMRP expression, we used two different RMRP siRNAs. To confirm whether synthesized RMRP RNA was transfected into cells actually, we analyzed RMRP expression levels in RMRP RNA-transfected cells and control siRNA-transfected cells with real-time RT-PCR. RMRP RNA-transfected cells showed clearly increased RMRP expression (Fig. 4A).

As seen with flow cytometry analysis, annexin V-positive cells were defined as apoptotic cells. The percentages of apoptotic cells were: control siRNA, 8.1%; RMRP siRNA1, 18.5%; and RMRP siRNA2, 16.2% in HLE cells. In Huh7 cells, the percentages were: control siRNA, 7.1%; RMRP siRNA1, 17.9%; and RMRP siRNA2, 20.7%. The rates of apoptosis clearly increased after 24-h tunicamycin treatment. However, after simultaneous RMRP RNA transfection, the rates of apoptosis decreased compared with tunicamycin treatment alone (Fig. 4B, C).

TUNEL analysis also revealed that in the RMRP siRNA1 and RMRP siRNA2 groups, apoptotic cells were increased compared with the control siRNA group (Fig. 4D). The rate of apoptotic cells is also shown in Fig. 4E.
that this pathway mediates apoptosis induced by ER stress. p < 0.01) (Fig. 6). These results strongly support the hypothesis that RMRP is a PERK downstream molecule and correlation between PERK and RMRP expression in HCC specimens as seen with real-time RT-PCR (r = −0.44, p < 0.05) (Fig. 7). These results indicated that the PERK-RMRP apoptotic pathway includes miR-206 and Bcl-2, and moreover, suggested that apoptosis due to the PERK-RMRP axis is correlated not with the extrinsic pathway but with the intrinsic pathway (mitochondrial apoptosis).

The relationship between PERK and RMRP in clinical specimens. To investigate the clinical relationship between PERK and RMRP, we used HCC tissues collected during surgery. The clinical features of HCC patients are shown in Table 1. We found no difference in PERK expression according to the etiology of liver disease. We calculated the expression level of PERK RNA/GAPDH and RMRP RNA/GAPDH and found a negative correlation between PERK and RMRP expression in HCC specimens as seen with real-time RT-PCR (r = −0.44, p < 0.01) (Fig. 6). These results strongly support the hypothesis that RMRP is a PERK downstream molecule and that this pathway mediates apoptosis induced by ER stress.

Discussion

The results of the present study revealed that inactivating the PERK pathway induced RMRP upregulation. We activated PERK by using overexpression plasmids and tunicamycin. As a result, ER stress led to a decrease in RMRP expression. PERK and RMRP were also inversely expressed in clinical specimens. We found that RMRP was related to apoptosis, and that RMRP downregulation led to apoptosis in HCC cell lines.

The functions of long noncoding RNAs (lncRNAs) are still unclear. LncRNAs are defined as RNAs that are longer than 200 bases and that are non-protein-encoding. RMRP is a lncRNA that consists of 267 nucleotides and is unique because it is transcribed by RNA polymerase III instead of RNA polymerase II. RMRP itself works as an endogenous siRNA with RNA-dependent RNA polymerase activity. RMRP is also an endonuclease that cleaves mitochondrial RNA. In addition, RMRP is associated with hTERT, and its RNA-dependent RNA polymerase activity produces double-stranded RNAs that can be processed into siRNA.

Mutations in the gene encoding RMRP are responsible for cartilage-hair hypoplasia, which is characterized by short stature, sparse hair, immune abnormalities, and a high incidence of malignancies. In several cancers, RMRP expression is increased, and RMRP downregulation leads to cell cycle arrest and apoptosis. For example, in multiple lymphoma, c-Myc promotes the transcription of RMRP, whereas in colon cancer, β-catenin enhances RMRP expression. On the other hand, RMRP is decreased in gastric cancer tissues and in dysplasia tissues compared to control tissues. The reason why RMRP is low in gastric cancer may be that ER stress affects RMRP expression via PERK. RMRP siRNA and RMRP overexpression have previously been used to change the expression of RMRP. However, how RMRP actually influences cancer progression is unclear, because examining RMRP plasmids that accurately transcribe RMRP RNA is difficult. Therefore, in this study, we used RMRP RNA and confirmed its expression with real-time RT-PCR (Fig. 4A).

Previous studies have reported the downstream molecules of RMRP. RMRP is not translated into protein, but it influences miRNA; it stimulates miR-613 and miR-206/TACR1 in HCC. In several cancers, miR-206 is known as an apoptosis-related molecule. Downregulated RMRP in our study increased miR-206, which targets Bcl-2 and consequently increases cleaved caspase 3 (Fig. 5), resulting in apoptosis. In contrast to downstream molecules that are upstream of RMRP have remained unidentified. In this study, we revealed that PERK, an ER transmembrane protein, is an important upstream molecule of RMRP, which in turn mediates the apoptosis pathway.

Apoptosis is an important mechanism for suppressing cancer progression. Apoptosis is induced by ER stress via UPR signaling when the stress is excessive and brief. Brief exposure to ER stress causes apoptosis of cancer cells. ER stress activates PERK, and PERK inhibits the proliferative effects on HCC. PERK arrests the cell cycle and initiates apoptosis. In this study, we used tunicamycin and PERK overexpression to activate transient ER stress, which induced apoptosis. Tunicamycin is useful as an ER stress inducer, because it inhibits GlcNac phosphotransferase to cause abnormal protein accumulation. Tunicamycin also activates mTORC1 and induces apoptosis. However, tunicamycin has not been used for cancer treatment because of its cytotoxicity. An RMRP-specific inhibitor could possibly be administered safely, and could be effective against HCC. The effect of tunicamycin, siRNA, and overexpressing plasmids is temporary, and thus, we need to confirm the continuous effects by downregulating RMRP in HCC in future work.
CHOP is a key molecule in the PERK apoptosis pathway\textsuperscript{37,38}. CHOP, which forms a heterodimer with CCAAT/enhancer-binding protein or liver activator protein\textsuperscript{39}, induces death by promoting protein synthesis and oxidation in the stressed ER\textsuperscript{40,41}. CHOP then releases calcium from the ER into the cytoplasm to trigger apoptosis. We transfected HCC cells with RMRP siRNA but CHOP did not appear to show different expression in the control siRNA group compared to the RMRP siRNA group as seen with Western blotting (Fig. S9). We therefore suggest that RMRP is not upstream of the CHOP pathway. On the other hand, miR-206 is downstream of RMRP. In HCC, ER stress activates PERK, downregulates RMRP, and influences miR-206. This pathway inhibits Bcl-2 and activates caspase 9 and caspase 3, and thus, the mitochondrial apoptosis pathway is activated. As a result, the cells undergo apoptosis. We think that this process may complement previously known pathways.

The molecules that influence PERK activity have been described\textsuperscript{42,43}. However, administration of these molecules is expected to have adverse effects, and thus, could not be applied in a clinical setting\textsuperscript{44}. On the other hand, PERK is further downstream of PERK, and no proteins are produced from RMRP because it is a non-coding RNA. Thus, an RMRP targeting strategy would be a novel therapeutic strategy that may not have serious adverse effects. Inhibiting RMRP may be beneficial for suppression of HCC growth by inducing apoptosis of HCC cells. However, to obtain sufficient effects of lncRNA, we need to produce or identify a suitable delivery system for HCC cells. As a next step, we need to use a delivery system and animal models, and then confirm the anti-tumor effect of antisense RMRP in HCC.

In conclusion, we revealed a relationship between PERK and RMRP in vitro and in vivo. RMRP is downregulated by PERK and induces apoptosis in HCC cells. RMRP could be a new therapeutic target to regulate HCC in patients with chronic liver diseases associated with ER stress.

Materials and methods

Cell culture. We used the HCC cell lines, HuH7 and HLE (Japanese Collection of Research Bioresources, Osaka, Japan). HuH7 and HLE cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific) and 1% penicillin. Cells were grown in a humidified atmosphere of 5% CO\textsubscript{2} and 95% air at 37°C. The medium was replaced three times per week.

RNA interference and tunicamycin assay. We used PERK specific siRNA1 (GAA GCU ACA UUG UCU AUA U, nt. 2424-2442), siRNA2 (UAG CAA AUC UUC UUC UGA A, nt. 2407-2426), and control siRNA (Dharmacon, Cambridge, UK). RMRP-specific siRNA1 (CCU AGG CUA CAC ACU GAG GAC UTT, nt. 22-44) and siRNA2 (GCC UGU AUC CUA GCC UAC ATT, nt. 14-33) were also obtained from Dharmacon. HuH7 and HLE cells were transfected with 50 pmol/L siRNA using RNAiMAX (Thermo Fisher Scientific). Twenty-four hours after transfection, RNA and protein were extracted from these cells. For the ER stress-induced assay, 1 μg/mL tunicamycin (Cayman Chemical Company, Ann Arbor, MI) was added to the cell culture, and the control was treated with dimethyl sulfoxide (DMSO). After 24 h, RNA and protein were extracted from the cells.

PERK plasmids. Plasmids encoding PERK were purchased from Origene (Rockville, MD) (RG214993). pCMV6 was used as a control plasmid. Plasmid (1 μg/mL) was transfected into HuH7 and HLE cells at 70% confluence using Lipofectamine 2000 (Thermo Fisher Scientific). After 24 h, RNA and protein were extracted from the cells.

RNA extraction, cDNA synthesis, and real-time RT-PCR. From HuH7 cells, HLE cells, and clinical specimens, total RNA was extracted with an RNaseq Plus Mini Kit (Qiagen, Venron, Netherlands). Extracted RNA was reverse-transcribed using TaqMan Reverse Transcription Reagents (Thermo Fisher Scientific). For PCR amplification, we used a PERK forward primer (5′-CCAGCCTTAAGAAACCAGA-3′) and reverse primer (5′-CTTGTGGTCCACTGGAAGAG-3′), total RMRP forward primer (5′-TGCGAAGGCCGTATCTT-3′) and reverse primer (5′-TGAGATGAGGCCCGGTGT-3′) and GAPDH primers (Hs.544577) (Thermo Fisher Scientific). Expression of each gene was normalized to GAPDH mRNA and is represented as fold changes compared to the control group. For the analysis of the relationship between PERK and RMRP, we used a Light Cycler...
PERK siRNA (n = 6). We used an Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA) for quality control. Library construction and sequencing with the Illumina HiSeq 2500 were outsourced to Eurofins Genomics K. K. (Tokyo, Japan). Fragmented mRNA was reverse transcribed to cDNA. cDNA libraries were prepared following adaptor ligation, size selection (200-bp inserts), and amplification of cDNA. Libraries were used for paired-end 2 × 100 bp sequencing (HiSeq 2500) with v4 chemistry (Illumina, San Diego, CA). The differences in RNA expression were analyzed.

Next generation sequencing. We prepared Huh7 cells and transfected them with control siRNA or PERK siRNA (n = 6). We used an Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA) for quality control. Library construction and sequencing with the Illumina HiSeq 2500 were outsourced to Eurofins Genomics K. K. (Tokyo, Japan). Fragmented mRNA was reverse transcribed to cDNA. cDNA libraries were prepared following adaptor ligation, size selection (200-bp inserts), and amplification of cDNA. Libraries were used for paired-end 2 × 100 bp sequencing (HiSeq 2500) with v4 chemistry (Illumina, San Diego, CA). The differences in RNA expression were analyzed.

RMRP RNA. We created RMRP RNA using pBapo-CMV plasmids (Takara Bio Inc., Shiga, Japan). The RMRP sequence was inserted downstream of the T7 promotor sequence. RMRP RNA was synthesized using a MEGAscript kit (Thermo Fisher Scientific). Huh7 and HLE cells were transfected with 50 pmol/L RMRP RNA using RNAiMAX (Thermo Fisher Scientific). Twenty-four hours after transfection, RNA and protein were extracted from these cells.

Northern blotting. For Northern blotting, 1 μg RNA was mixed with 3 volumes of formaldehyde loading dye. Samples were applied to lanes of 2% agarose gels, run at 5 V/cm, and transferred onto positively charged nylon membrane (Thermo Fisher Scientific). Membranes were treated by crosslinking, and the RNA was immediately prehybridized. We created a digoxigenin (DIG)-labeled RNA probe (5′-DIG-UGU AGC CUA GGA UAC AG GCC-3′), and after prehybridization, membranes were hybridized overnight with this probe. We then incubated the membranes with an anti-DIG antibody, and signals were detected with CDP-STAR solution (Thermo Fisher Scientific). Membranes were treated by crosslinking, and the RNA was immediately prehybridized. We created a digoxigenin (DIG)-labeled RNA probe (5′-DIG-UGU AGC CUA GGA UAC AG GCC-3′), and after prehybridization, membranes were hybridized overnight with this probe. We then incubated the membranes with an anti-DIG antibody, and signals were detected with CDP-STAR solution (Thermo Fisher Scientific). Signals were visualized with an ImageQuant LAS 4000 system (GE Healthcare, Charles Coffin, NY).

Western blotting. Protein (20 μg) was applied to lanes of 4% to 12% Bis-Tris Gels (Thermo Fisher Scientific), separated by electrophoresis, and transferred onto Immobilon-P membranes (Millipore, Bedford, MA). Membranes were blocked with albumin and then incubated with the relevant antibodies: anti-PERK (product number: 3192), anti-eIF2α (5324), anti-phospho eIF2α (3398), anti-CCAAT/enhancer-binding protein homologous protein (CHOP) (5554), Bcl-2 (15071), cleaved caspase 3 (9664) (Cell Signaling, Danvers, MA), and anti-actin (1501) (Merck, Darmstadt, Germany) overnight at 4 °C. We used secondary antibodies from kits from GE Healthcare. Bands were labeled using the ECL Prime Kit (GE Healthcare) and visualized with ImageQuant LAS 4000 (GE Healthcare). The density of the bands was quantified by normalization to β-actin using ImageJ Software (National Institutes of Health, Bethesda, MD, USA).

Apoptosis assay. Huh7 and HLE cells were transfected with 50 pmol/L control siRNA or RMRP siRNA. The cells were alternatively treated with 1 μg/mL tunicamycin (CAYMAN) dissolved in DMSO or an equivalent volume of DMSO alone and cultured for 24 h. The attached cells were collected and assayed to evaluate apoptosis with annexin V-PE and 7-amino-actinomycin D (Tonbo Biosciences, San Diego, CA). To visualize stained cells, we used a FACScalibur system (Becton Dickinson, Franklin Lakes, NJ). The data were analyzed with FlowJo software (TreeStar Corporation, Ashland, OR). A Megstain apoptosis TUNEL kit II (MBL, Nagoya, Japan) was used to detect oligonucleosomal DNA breaks in Huh7 and HLE cells.

Caspase assay. Huh7 and HLE cells were seeded in 96-well plates. After transfection with control siRNA or RMRP siRNA, Caspase Glo 3/7, Caspase Glo 8, or Caspase 9 Glo reagent (Promega, Southampton, UK) was added to the attached cells, which were cultured for 1 h. Caspase activity was analyzed with a luminometer (GloMax-96 microplate luminometer, Promega).

Patients and HCC specimens. We obtained HCC samples from patients who underwent surgery at our hospital between 2007 and 2018. The clinical features of the 32 specimens we examined are detailed in Table 1. Rapidly after resection, specimen samples were incubated with RNA later (QIAGEN) overnight at 4 °C and then frozen and stored at − 80 °C. Remaining samples were fresh frozen and stored at − 80 °C until they were used for protein extraction.
Ethics statement. The experimental procedures were conducted in accordance with the approved guidelines. The study used clinical samples was approved by the Institutional Review Board (approval ID: 1411010) and was performed in accordance with the ethical principles of the Declaration of Helsinki. Written informed consent was obtained from all patients.

Statistical analysis. Statistical analyses were performed using JMP version 13.00 (SAS Institute, Cary, NC). Quantitative variables were expressed as means with standard deviations. Statistical differences were determined using the Student’s t-test or a one-way analysis of variance. Correlation coefficients were calculated by Pearson’s correlation coefficient. Differences were considered significant at p < 0.05.
Data availability
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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| General parameters (n = 32) |
|-----------------------------|
| **Age (years); median (range)** | 70 (63–76) |
| **Sex, male/female (n)** | 25/7 |
| **Etiology, HCV/HBV/NBNC (n)** | 12/9/11 |
| **Aspartate aminotransferase (IU/L); median (range)** | 46 (31–69) |
| **Alanine aminotransferase (IU/L); median (range)** | 44 (25–68) |
| **Platelets (10^4 cells/μL); median (range)** | 14.9 (10.2–21.0) |
| **Albumin (g/dL); median (range)** | 3.8 (3.4–4.0) |
| **Bilirubin (mg/dL); median (range)** | 0.7 (0.6–1.0) |
| **Prothrombin Time (%); median (range)** | 86.4 (78.9–97.7) |
| **Alpha-fetoprotein (ng/mL); median (range)** | 23 (6–131) |
| **DCP (mAU/mL); median (range)** | 378 (64–2171) |
| **Child Pugh class A/B (n)** | 30/2 |
| **Tumor size (cm), median (range)** | 3.6 (2.5–6.8) |
| **Tumor differentiation (well/moderate/poor)** | 8/21/3 |
| **Tumor multiplicity (solitary/multiple)** | 27/5 |

**Figure 6.** Expression of PERK and RMRP in human HCC tissues. RNA expression of PERK and RMRP was analyzed and compared, and the correlation coefficient was calculated by Pearson’s correlation coefficient. We found a negative correlation between PERK and RMRP expression in HCC specimens with real-time RT-PCR.
13. Vandewynckel, Y. P. A.Y. performed the experiments, analyzed the data, and wrote the manuscript; T.W. designed and supervised the experiments and revised the manuscript. K.S., Y.N., T.T., O.Y., Y.T., M.H., and M.A. provided technical assistance.

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Author contributions

A.Y. performed the experiments, analyzed the data, and wrote the manuscript; T.W. designed and supervised the experiments and revised the manuscript. K.S., Y.N., T.T., O.Y., Y.T., M.H., and M.A. provided technical assistance.
advice on the molecular and histological experiments and data analysis. Y.H. designed and performed the experiments, analyzed the data, and supervised the experiments and critically revised the manuscript.

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**Competing interests**
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**Correspondence** and requests for materials should be addressed to Y.H.

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