Hepatic metastasis of gastric cancer is associated with enhanced expression of ethanolamine kinase 2 via the p53–Bcl-2 intrinsic apoptosis pathway

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BACKGROUND: Gastric cancer (GC) with hepatic metastasis has a poor prognosis. Understanding the molecular mechanisms involved in hepatic metastasis may contribute to the development of sensitive diagnostic biomarkers and novel therapeutic strategies.

METHODS: We performed transcriptome analysis of surgically resected specimens from patients with advanced GC. One of the genes identified as specifically associated with hepatic metastasis was selected for detailed analysis. GC cell lines with knockout of the candidate gene were evaluated in vitro and in vivo. Expression of the candidate gene was analysed in GC tissues from 300 patients.

RESULTS: Ethanolamine kinase 2 (ETNK2) was differentially upregulated in GC patients with hepatic metastasis. ETNK2 expression was elevated in GC cell lines derived from haematogenous metastases. ETNK2 knockout significantly suppressed proliferation, invasion, and migration; increased apoptosis; reduced Bcl-2 protein expression; and increased phosphorylated p53 expression. In mouse xenograft models, ETNK2 knockout virtually abolished hepatic metastasis. Stratification of GC patients based on ETNK2 mRNA level revealed significant associations between high ETNK2 tumour expression and both hepatic recurrence and worse prognosis.

CONCLUSIONS: Upregulation of ETNK2 in GC enhances hepatic metastasis, possibly via dysregulation of p53–Bcl-2-associated apoptosis. ETNK2 expression may serve as a biomarker for predicting hepatic recurrence and a therapeutic target.

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METHODS
Clinical specimens and analysis
Resected GC tissues were obtained between 2006 and 2013 from 16 patients who underwent curative gastrectomy for pStage III GC followed by 5-1 adjuvant monotherapy and had no recurrences for >5 years (n = 4, group 1), liver-confined recurrences within 2 years after surgery (n = 4, group 2), peritoneal recurrences within 2 years after surgery (n = 4, group 3), and distant nodal recurrences within 2 years after surgery (n = 4, group 4). Before sequencing, those samples were satisfied by the following two quality checks: the optical density of extracted RNA was measured to confirm that the ratio of the absorbance at 260 and 280 nm ranged from 1.8 to 2.0, and RNA integrity number measured by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) was ≥8.0. RNA libraries were prepared by purification of amplicons with AMPure XP beads (Beckman Coulter, Brea, CA, USA), and transcriptome analysis was performed using the HiSeq platform (Illumina, San Diego, CA, USA).13 Samples were subjected to global expression profiling of 57,749 genes and significantly differentially expressed genes were selected based on log2-fold change and P value using the Cuffdiff package (Homo sapiens Ensemble GRC37, Ensemble reference 75).

For large-scale analysis of the selected gene expression, primary GC tissues were collected from 300 patients who underwent gastrectomy for GC at the Department of Gastroenterological Surgery, Nagoya University Hospital between 2001 and 2017. Uncommon histologic type, like hepatoid carcinoma, were excluded. Immediately after resection, fresh tissue samples were frozen in liquid nitrogen and stored at −80°C until analysis. Relevant clinical data were retrieved from a prospectively compiled departmental database. Written informed consent was obtained from all patients, as required by the Institutional Review Board of Nagoya University, Japan (approval no. 2014-0043).

Cell lines
GC cell lines, the differentiated type (AGS, IM95, MKN1, MKN7, MKN74 and N87) and the undifferentiated type (GCY, KATO-III, MKN45, NUGC2, NUGC3, NUGC4, OCUM1 and SC-6-JCK), were obtained from the Japanese Collection of Research Bio Resources Cell Bank (Osaka, Japan) or the American Type Culture Collection (ATCC, Manassas, VA, USA). A control, non-tumorigenic epithelial cell line (FHs 74) was purchased from the ATCC. The cells were cultured at 37 °C in RPMI medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% foetal bovine serum and bronectin, collagens I and IV, brinogen, and laminin I was added. GC cells were incubated in 6-well plates at 1 × 10^4 cells/well before use in experiments.

Quantitative reverse-transcription PCR (qRT-PCR) and PCR array analysis
ETNK2 mRNA levels were determined by qRT-PCR as previously described14 using the ABI StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as an endogenous control. Specific primer sequences are listed in Table S1. A PCR array analysis was performed to obtain the data that support the involvement of ETNK2 in epithelial–mesenchymal transition (EMT) by identifying cancer-related genes expressed simultaneously with ETNK2. We used the Human Epithelial to Mesenchymal Transition RT2 Profiler PCR Array (Qiagen, Hilden, Germany) to analyse 84 gene expression levels that encode proteins with the functions related to EMT in 14 GC cell lines and ETNK2 KO cell line. Genes that fulfilled the following criteria were considered as coordinately expressed genes with ETNK2: (1) expressing at levels with correlation coefficients ≥0.65 with ETNK2, and (2) down-regulated in the ETNK2 KO cells compared to the control MKN1 cells.

Clustered, regularly interspaced, palindromic repeats-associated (CRISPR)/Cas9 editing and generation of stable ETNK2 KO cell lines
We employed genome editing using the CRISPR/Cas9 method to establish GC cell lines with stable ETNK2 KO as described previously.15,16 Briefly, a guide RNA (gRNA) complementing the sequences flanking ETNK2 exon 1 was designed using the Gene Art CRISPR gRNA Design Tool (Thermo Fisher Scientific) and synthesised using a Gene Art Precision gRNA Synthesis Kit (Thermo Fisher Scientific). The gRNA (240 ng) was incubated with 1 µg of Gene Art Platinum Cas9 nuclease (Thermo Fisher Scientific) at room temperature and then introduced into GC cells via electroporation using a Neon System (Thermo Fisher Scientific). After evaluating cleavage efficiencies according to the fragmentation patterns on agarose gel electrophoresis, single-cell clones were isolated using a standard limiting dilution method. Cells with stable ETNK2 KO were selected and KO was confirmed by DNA sequencing and western blot analysis. The sequences of the gRNA target and primers used to detect ETNK2 cleavage are described in Table S1.

Transient ETNK2 knockdown (KD) with small interfering RNA (siRNA)
GC cells were cultured in 24-well plates at 2.5 × 10^4 cells/well for 24 h and then transiently transfected by addition of 20 pmol ETNK2-specific siRNA or a control siRNA (Table S1) combined with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) or Lipofectamine RNAiMax (Invitrogen). The cells were cultured in serum-free RPMI medium for 72 h before use in experiments.

Transient ETNK2 overexpression
GC cells were incubated in 6-well plates at 1 × 10^4 cells/well and transfected by addition of 4 µg pCMV6-Entry ETNK2 expression vector (NM_018208; OriGene, Rockville, MD, USA) or a control pCMV6-Entry Tagged Cloning Vector with C-terminal Myc-DDK Tags (PS100001; OriGene) combined with Lipofectamine 2000 (Invitrogen). The cells were cultured at 37°C in RPMI medium for 48 h before use in experiments.

Cell proliferation, adhesion, invasion, and migration assays
Proliferation of parental GC cells or cells with ETNK2 KO, ETNK2 KD, or ETNK2 overexpression were analysed using the Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc., Kuma- to, Japan). Adhesion to the extracellular matrix proteins fibronectin, collagens I and IV, fibroogen, and laminin I was examined using a CytoSelect 48-Well Cell Adhesion Assay Kit (Cell Biolabs, Inc., San Diego, CA, USA). Cell invasion was assessed using BioCoat Matrigel invasion chambers (BD Biosciences, Bedford, MA, USA), and migration was assessed using a wound-healing assay as described previously.14

Cell cycle analysis
GC cell cycle analysis was performed using a Cell-Clock Cell Cycle Assay (Biocolor, Carrickfergus, UK) according to the manufacturer's protocol.16 Pixel colour detection and counting of labelled cells were quantified using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). To test the reproducibility of the cell croyck assay, we evaluated influences of ETNK2 KO on cell cycle regulation using the Muse Cell Cycle Kit (Merck Millipore, Billerica, MA, USA) under the manufacturer's protocol.

Apoptosis assays
Apopotic cells were detected by staining with annexin V-Alexa Fluor 568 conjugate (A13202, Thermo Fisher Scientific).19 Briefly, parental or stable ETNK2 KO GC cell lines (1 × 10^5 cells/ml) were mixed with 10 µl of annexin V conjugate and incubated for 15 min. Cells irradiated with ultraviolet light for 120 min served as a positive control. The cells were visualised by phase contrast and
Western blot analysis and Simple Western assays ETK2 and B cell lymphoma 2 (Bcl-2) were analysed by traditional western blot analysis. In brief, GC cells were lysed with RIPA buffer and protein concentrations were determined. Samples equivalent to 20 µg of total protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylenedifluoride membranes as previously described. Blots were probed with mouse anti-ETNK2 polyclonal antibody (LC-C1790607; LSBio, Seattle, WA, USA) diluted 1:100 or rabbit anti-Bcl-2 monoclonal antibody (ab32124; Abcam, Cambridge, UK) diluted 1:100 and then with anti-mouse or anti-rabbit IgG, horseradish peroxidase-linked antibody as secondary antibodies (#7076 and #7074; Cell Signaling Technology, Tokyo, Japan). Blots were developed with Can Get Signal Solution (NKB-101; TOYOBO, Osaka, Japan). B-Actin was used for an endogenous control.

Signal transducer and activator of transcription 3 (Stat3), Bcl-2 associated agonist of cell death (Bad), and p53 were analysed using Simple Western assays (ProteinSimple; San Jose, CA, USA) using a Jess Protein Normalisation Separation Module (ProteinSimple) according to the manufacturer’s protocol. Briefly, 6 µg of total protein was loaded and probed with the following primary antibodies at 1:50 dilution (all monoclonal antibodies; Cell Signaling Technology): rabbit anti-Stat3 (12640), rabbit anti-phosphorylated (p)-Stat3 (P-Tyr705) (#9145), rabbit anti-Bad (#9239), rabbit anti-p-Bad (P-Ser122) (#5284), rabbit anti-p53 (#2527), and rabbit anti-p-p53 (P-Ser15) (#9284). Protein expression levels were normalised to total protein and the data were evaluated using the Compass for Simple Western software (ProteinSimple).

Mouse xenograft model The Animal Research: Reporting of In Vivo Experiments guidelines were followed for all animal experiments, and the study was approved by The Animal Research Committee of Nagoya University (IRB no. 29329). Six-week-old male nude mice (BALB/cScl-nu/nu) were obtained from Chubu Kagaku Shizai (Nagoya, Japan) and mice were housed at least 1 week before experiments in temperature-controlled rooms with a free access to water supply. Parental or stable ETK2KO GC cell lines (5 × 10⁶ cells each) were resuspended in 50 µl of phosphate-buffered saline (PBS) plus 50 µl Matrigel (BD Biosciences) and subcutaneously injected into both flanks of the mice (n = 6/group). Tumour growth was measured every week, and the mice were sacrificed at 8 weeks after injection. Approximate tumour volumes (mm³) were measured. To visualise tumours, mice were injected with D-luciferin (150 mg/kg; Summit Pharmaceuticals International, Tokyo, Japan) intraperitoneally and luciferase activity was measured 15 min later using the IVIS. Living Image version 2.6 software (Xenogen) was used to acquire and analyse the data. As a second method to detect metastasis formation, we examined mice by magnetic resonance imaging (MRI; MRS 3000; MR solutions, Guildford, UK) at 12 weeks after GC cell injection, and the mice were then sacrificed. Mice were euthanised by CO₂ exposure for 5 min and were observed for 20 min after confirmation of respiration cease.

Statistical analysis Qualitative and quantitative variables were compared using Fisher’s exact test and Mann–Whitney test, respectively. To evaluate the paired bivariate correlation, we employed the Spearman’s rank correlation coefficient. We employed the KM method to evaluate the survival curves. Differences in survival, hazard ratios (HRs), and 95% confidence intervals (CIs) were calculated using the Cox proportional hazards models. Risk factors and odds ratios (ORs) for hepatic metastasis and recurrence were evaluated using logistic regression analysis. Cumulative recurrence rates were analysed using Grey test. All analyses were performed using the R software (The R Foundation for Statistical Computing, Vienna, Austria) and EZR software (Saitama Medical Center, Jichi Medical University, Saitama, Japan).

RESULTS ETK2 is overexpressed in primary GC tissues from patients with hepatic recurrence We performed transcriptome analysis of GC tissues from patients who underwent curative gastrectomy for pStage III GC and experienced no recurrence for ≥5 years or experienced hepatic, peritoneal, or distal nodal recurrence within 2 years of surgery. Among the 57,749 genes analysed, 23 molecules with high expression were identified exclusively in the hepatic recurrence
ETNK2 is highly expressed in GC cell lines derived from haematogenous metastases and correlates with genes associated with the EMT

We analysed the expression levels of ETNK2 mRNA in a panel of six differentiated and eight undifferentiated human GC cell lines (Fig. 1a). While the expression levels varied considerably, MKN1, MKN7, MKN74, N87, and NUGC3 cell lines expressed higher levels of ETNK2 than FHS74. To facilitate analysis of ETNK2 function in GC cells, we used the MKN1 cell line for genome editing, because it was originally derived from a liver metastasis lesion from a GC patient, expressed one of the highest levels of ETNK2 mRNA, had high abilities in cell migration and invasion in our previous studies, and is engrafted in nude mice for subcutaneous and in Nod-SCID mice for hepatic metastasis xenograft models. We generated two MKN1 cell lines with stable ETNK2 KO (KO ETNK2-1 and ETNK2-2) using the CRISPR-Cas9 method. Cleavage was confirmed by agarose gel electrophoresis (Fig. 1a) and DNA sequencing (Fig. 1b), which revealed a single base-pair deletion resulting in a frame shift in the ETNK2 coding sequence. Consistent with this, ETNK2 protein expression was undetectable by western blot analysis (Fig. 1b). When we determined the expression levels of 84 EMT-related genes, we found that the mRNAs encoding AHNAK nucleoprotein (AHNAK) and transforming growth factor beta 1 (TGFβ1) were expressed at levels that correlated significantly with those of ETNK2 mRNA (Fig. 1c). ETNK2 KO cell lines expressed lower levels of AHNAK and TGFβ1 than MKN1 cells (Fig. 1d).

ETNK2 expression modulates the malignant behaviour of GC cell lines

Next, we examined the effects of ETNK2 KO on MKN1 cell proliferation, invasion, and migration in vitro. We found that three properties were significantly reduced compared with the unmanipulated parental MKN1 cell line (Fig. 1e–g). Similarly, ETNK2 KO MKN1 cells showed a slightly reduced ability to adhere to collagen I and collagen IV but not to the other matrix proteins tested, compared with the parental cell line (Fig. 1b). To confirm these findings, we transiently silenced or overexpressed ETNK2 in GC cells by transfection with ETNK2-targeting siRNA or an ETNK2 expression vector, respectively. We found that ETNK2 KD also decreased the proliferation and migration of MKN1 cells (Fig. 2a–c), consistent with the effects of stable ETNK2 KO. Moreover, forced expression of ETNK2 in NUGC4 and MKN45 cells, which expressed low ETNK2 mRNA levels (Fig. 1a), had the opposite effect and enhanced the proliferation of both cell lines (Fig. 2d–g).

ETNK2 KO induces apoptosis and cell cycle arrest in GC cell lines

To determine how ETNK2 KO inhibits cell proliferation, we first examined apoptosis using an annexin V assay. We found that the MKN1 cell lines with stable ETNK2 KO exhibited increased annexin V staining compared with parental MKN1 cells (Fig. 3a). ETNK2 KO also caused an increase in mitochondrial membrane potential depolarisation (Fig. 3b) and caspase activity (Fig. 3c), which are both consistent with induction of the intrinsic mitochondrial pathway of apoptosis. Moreover, western blot analysis revealed decreased expression of the anti-apoptotic protein Bcl-2 in ETNK2 KO MKN1 cells compared with parental cells (Fig. 3d), whereas Simple Western assays revealed no effect of ETNK2 KO on the expression of Bad, p-Bad (Ser122), Stat3, and p-Stat3 (Tyr705). (Fig. 3d). Notably, however, ETNK2 KO increased the expression of the phosphorylated form of p53 (Ser15) but not of total p53 (Fig. 3d). Accordingly, a decrease of the number of cells in G0/G1 phase and an increase of the number of cells in G2/M phase were exhibited in ETNK2 KO cells compared to the control MKN1 cells (Fig. 3e and Fig. S1c), indicative of cell cycle delay or arrest.

ETNK2 promotes the growth and hepatic metastasis of GC cells in a mouse xenograft model

To determine whether our in vitro findings on the behaviour of GC cells are also observed in vivo, we first examined the effects of ETNK2 KO on the growth of MKN1 cells after subcutaneous injection in BALB/c nude mice. Indeed, the ETNK2 KO cells exhibited significantly decreased growth compared with parental MKN1 cells (Fig. 4a). In IHC analysis, we found loss of ETNK2 expression in subcutaneous tumours from ETNK2 KO cells. Furthermore, we found increased expression of cleaved caspase-3 and cleaved PARP in subcutaneous tumours from ETNK2 KO cells compared to those from a control MKN1 cells. In contrast, no differences in HIF-1α expression were observed (Fig. 4b). To examine hepatic metastasis, we injected Nod-SCID mice with parental or ETNK2 KO MKN1 cells expressing a luciferase reporter and monitored the luminescence signals by whole-animal in vivo imaging. We observed that mice bearing ETNK2 KO tumours emitted significantly weaker luminescence signals compared with the parental tumours, and no hepatic metastasis could be detected by MRI imaging (Fig. 4c, d). At 12 weeks after cell injection, the macroscopic appearance of liver specimens from mice injected with parental MKN1 cells revealed multiple tumour nodules, whereas none were detected in the livers of mice implanted with the ETNK2 KO cell line (Fig. 4d).

High ETNK2 expression in GC tissues is associated with hepatic recurrence and poor prognosis

To assess the clinical significance of ETNK2 mRNA expression in GC, we first analysed its expression in data sets from institutional cohort, consisting of normal stomach tissues and GC specimens from patients with Stage I, II/III, or IV GC. ETNK2 mRNA was present at significantly higher levels in the more advanced stages of GC (II–IV) (Fig. 5a). We then performed receiver operating characteristic curve analysis to examine the ability of ETNK2 mRNA expression to predict disease recurrence within 5 years of curative gastrectomy in a 300-patient cohort from our institution, which gave a cut-off value of 0.006 for ETNK2 mRNA (Fig. 5d). Subsequently, we stratified the 300 patients into high (n = 87) and low (n = 213) ETNK2 mRNA expression groups based on the cut-off value. The clinicopathological characteristics of the two groups are shown in Table S2. High ETNK2 expression was significantly associated with vessel invasion, lymph node metastasis, and disease stages. Analysis of KM survival curves showed a significant association between high ETNK2 mRNA and significantly shorter overall survival in the full institutional cohort (n = 300, HR 1.58, 95% CI 1.07–2.33, P = 0.020; Fig. 5b). Disease-free survival in the Stage II/III GC patients subset tended to be short but not significant (n = 180, HR 1.43, 95% CI 0.83–1.43, P = 0.203; Fig. 5e). To validate our institutional data, we also analysed GC patient data sets from TCGA and KM plotter databases. High ETNK2 expression was significantly associated with worse overall survival in both the TCGA data set (HR 1.49, 95% CI 1.08–2.05,
| Symbol | H-rec/Non-rec | Full name | Function | Location | Localisation | P-rec/Non-rec | N-rec/Non-rec |
|--------|--------------|-----------|----------|----------|--------------|---------------|---------------|
| ETNK2  | 2.421 <0.001 | Ethanolamine kinase 2 | Ethanolamine phosphorylation | 1q32.1 | Cytosol | −0.606 | 1.390 |
| FABP3  | 3.774 <0.001 | Fatty acid-binding protein 3 | Fatty acid transporter | 1p35.2 | Extracellular space | 0.051 | 0.992 |
| TCF7L1 | 2.288 <0.001 | Transcription factor 7 like 1 | Transcription factor | 2p11.2 | Nucleus, cytosol | −0.260 | −0.386 |
| FRAS1  | 2.319 <0.001 | Frasier extracellular matrix complex subunit 1 | Cell adhesion | 4q21.21 | Extracellular space | 0.587 | 1.586 |
| RNF182 | 5.362 <0.001 | Ring finger protein 182 | E3 ubiquitin-protein ligase | 6p23 | Nucleus | −0.124 | 2.317 |
| CYP2W1 | 6.809 <0.001 | Cytochrome P450 family 2 subfamily W member 1 | Catalytic activity | 7p22.3 | Endoplasmic reticulum | 1.450 | 1.667 |
| PRS51  | 4.203 <0.001 | Protease, serine 1 | Serine protease | 7q34 | Extracellular space | 0.122 | 0.952 |
| RBP4   | 3.549 <0.001 | Retinol-binding protein 4 | Carrier for retinol | 10q23.33 | Extracellular space | −1.186 | 0.834 |
| GAL    | 4.278 <0.001 | Galanin and GMAP prepropeptide | Neuroendocrine peptide | 11q13.2 | Extracellular space | 2.076 | 1.340 |
| HMGA2  | 3.291 <0.001 | High mobility group AT-hook 2 | Transcriptional regulator | 1q14.3 | Nucleus | 0.421 | 0.612 |
| ASGR2  | 3.560 <0.001 | Asialoglycoprotein 2 | Mediator of endocytosis of plasma glycoproteins | 17p13.1 | Plasma membrane | −0.124 | 0.452 |
| SMN2    | 4.739 <0.001 | Smoothelin like 2 | Unknown | 17p13.2 | Nucleus | −0.879 | 1.108 |
| COMP   | 4.187 <0.001 | Cartilage oligomeric matrix protein | Extracellular matrix protein | 19p13.11 | Extracellular space | 0.760 | 1.173 |
| BCAM   | 2.123 <0.001 | Basal cell adhesion molecule | Laminin receptor | 19q13.32 | Plasma membrane | −0.554 | 0.042 |
| HIF3A  | 4.168 <0.001 | Hypoxia inducible factor 3 alpha subunit | Regulator of hypoxia-inducible genes | 19q13.32 | Nucleus, cytosol | 0.290 | −0.018 |
| TNN1   | 3.316 <0.001 | Troponin T1, slow skeletal type | Regulator of striated muscle contraction | 19q13.42 | Cytoskeleton, cytosol | 1.675 | −0.637 |
| GATA5  | 2.944 <0.001 | GATA-binding protein 5 | Transcription factor | 20q13.33 | Nucleus | −1.401 | −0.398 |
| HIC2   | 3.434 <0.001 | HIC ZBTB transcriptional repressor 2 | Transcriptional repressor | 22q11.21 | Nucleus | 0.523 | 0.843 |
| SUSD2  | 2.976 <0.001 | Sushi domain containing 2 | Cytokine receptor | 22q11.23 | Plasma membrane | 0.464 | 0.302 |
| MYO18B | 4.731 <0.001 | Myosin XVIII B | Regulator of muscle specific genes | 22q12.1 | Cytoskeleton | 4.325 | −0.660 |
| GPC3   | 2.990 <0.001 | Glypican 3 | Multifunction membrane protein | Xq26.2 | Plasma membrane | −0.997 | 0.465 |
| IGSS1  | 3.546 <0.001 | Immunoglobulin superfamily member 1 | Immunoglobulin | Xq26.2 | Plasma membrane | −0.899 | 0.601 |
| TKT1   | 6.109 <0.001 | Transketolase like 1 | Transketolase | Xq28 | Nucleus | −2.080 | −2.758 |

Log-FC log fold change, H-rec hepatic recurrence, Non-rec non-recurrence, P-rec peritoneal recurrence, N-rec nodal recurrence.
P = 0.015) and KM plotter data set (HR 1.86, 95% CI 1.56–2.23, P < 0.001) (Fig. 5b), and high expression was additionally associated with worse disease-free survival in the KM plotter data set (HR 1.59, 95% CI 1.21–2.10, P < 0.001) (Fig. S1e). Of note, the cumulative incidence of hepatic recurrence, but not of peritoneal recurrence, was also significantly higher in the high versus low ETNK2 expression group in the institutional data set (Fig. 5c). Finally, multivariable analysis identified high ETNK2 mRNA expression as an independent risk factor for hepatic metastasis and/or hepatic recurrence (Table S3).

Fig. 1 ETNK2 is upregulated in human GC cell lines and promotes malignant behaviours. a qRT-PCR analysis of ETNK2 mRNA levels in human GC cell lines. b CRISPR/Cas9-mediated knockout (KO) of ETNK2 in MKN1 cells. Upper panel: DNA sequencing of the PCR product encompassing the ETNK2 exon indicates successful base deletion in MKN1 KO cells. Lower panel: western blot analysis of ETNK2 protein expression in two MKN1 ETNK2 KO cell lines. c Spearman’s correlation analysis of 84 cancer-related genes with ETNK2 in GC cell lines. The levels of AHNAK and TGFB1 significantly correlate with that of ETNK2. d The fold changes of transcripts identified in PCR array analysis in ETNK2 KO cells. e Proliferation of parental and ETNK2 KO MKN1 cell lines. f Images (left) and quantification (right) of Matrigel invasion assays of parental and ETNK2 KO MKN1 cells. g Images (left) and quantification (right) of wound-healing migration assays of parental and ETNK2 KO cell lines. *P < 0.005. Data are presented as the mean ± standard deviation.
ETNK2 protein expression in GC tissues is associated with haematogenous recurrence

Finally, we also examined the relationship between ETNK2 protein expression and recurrence by IHC staining of GC tissues from our institutional cohort of 88 patients with Stage II/III GC. Although there were only a few cases with ETNK2 staining at the fundic gland region of the stomach, no stained cells were found in epithelial cells and stromal tissues. Figure 5d and Fig. S1f show the typical staining patterns for classification of negative, weak, or strong ETNK2 staining intensity. We found that a higher proportion of patients with haematogenous recurrence exhibited positive ETNK2 expression (weak or strong staining) in GC tissue compared with patients without haematogenous recurrence (Fig. 5d).

DISCUSSION

In this study, we conducted pattern-specific transcriptome analysis of GC tissues to identify molecules potentially involved in hepatic metastasis. One of the genes, ETNK2, was specifically upregulated in GC tissues from patients with hepatic recurrence after curative gastrectomy, suggesting a possible causative link. We established stable ETNK2 KO GC cell lines and demonstrated a role for ETNK2 in behaviours associated with metastasis, namely, enhanced proliferation, migration, and invasion and reduced apoptosis.

Compared to FHs74 cell, four out of five GC cell lines established from haematogenous metastatic tissues had higher expression levels of ETNK2, supporting our hypothesis that ETNK2 promotes hepatic metastasis. We also examined ETNK2 expression at the mRNA and protein levels in surgically resected GC specimens and identified significant positive associations between high expression and worse prognosis and hepatic recurrence. Thus ETNK2 expression in GC tissues may have potential utility as a biomarker for predicting hepatic recurrence.

The ETNK2 gene is located on human chromosome 1q32.1, and the gene product is ubiquitously expressed in human tissues. ETNK2 is a member of the choline/ethanolamine kinase family and catalyses the first step in the cytidine diphosphate ethanolamine pathway. This enzyme plays a role in the biosynthesis of phosphatidylethanolamine, a main constituent of cell membranes.1 Only a few previous reports of association between ETNK2 and malignancies and one report suggested that a higher level of Cpg methylation in the ETNK2 promoter was related to radiotherapy resistance in laryngeal squamous cell carcinoma.32 However, in general, little is known about the function of ETNK2 in GI tract cancers, including GC.

Phosphatidylethanolamine is abundant in mitochondria, and its depletion has been shown to induce apoptosis via changes in mitochondrial morphology and fragmentation in mammalian cells.33,34 Additionally, cell apoptosis is an important process to develop distant metastasis and regulated by multiple stimuli, for example, loss of adhesion to extracellular matrix by invasion and migration (anoikis), hypoxia in the circulation, and DNA damage by chemotherapy.35,36 Based on these previous reports, we investigated the involvement of ETNK2 in apoptosis. We hypothesised that ETNK2 may have anti-apoptotic effects and that ETNK2 KO would also affect the malignant phenotypes of GC cells. Consistent with this, we confirmed that ETNK2 KO promoted apoptosis and cell cycle arrest and attenuated the behaviours required for distant metastasis formation (proliferation, invasion, and migration). The extrinsic pathway of apoptosis is activated by binding of ligands to cell surface death receptors,38 whereas the intrinsic pathway is induced by mitochondrial membrane depolarisation resulting from opening of the mitochondrial permeability transition pore. Cytochrome c is released from the mitochondrial matrix into the cytosol, where it activates caspases, the 'executioners' of apoptosis.39,40 One of the consequences of caspase activation is the induction of DNA fragmentation, a
Hepatic metastasis of gastric cancer is associated with enhanced EMT is a process in which cells lose their epithelial properties and gain migration and invasive ability to become mesenchymal cells, which plays an important role in cancer metastasis. We conducted PCR array analysis to search for EMT-related genes whose expression is correlated with ETNK2. Consequently, we found that ETNK2 mRNA expression levels positively correlated to those of AHNAK and TGFB1. Activated TGFB1 phosphorylates Smad2 and Smad3 proteins. These Smad proteins activated by phosphorylation acts as transcription factors by assembling with Smad4 and regulates cell proliferation, migration, and differentiation. AHNAK has diverse role as oncogene or tumour-suppressor gene.51,52 AHNAK promotes EMT via TGFB/Smad signalling pathway and regulates cell migration and metastasis.53 Additionally, we revealed lower expression of AHNAK and TGFB1 in ETNK2 KO cell lines. Our results indicate that ETNK2 acted as an upstream mediator of AHNAK signalling and downstream target of TGFB1 in its signalling pathway.

We confirmed our in vitro findings using a mouse xenograft model of GC. Both the tumorigenicity and ability to form hepatic metastases were strikingly reduced by ETNK2 KO; indeed, hepatic metastasis was virtually abolished. We also found increased expression of cleaved caspase-3 and cleaved PARP in ETNK2 KO with anti-caspase antibody. We revealed lower expression of AHNAK and TGFB1 in ETNK2 KO cell lines. Our results indicate that ETNK2 acted as an upstream mediator of AHNAK signalling and downstream target of TGFB1 in its signalling pathway.

We confirmed our in vitro findings using a mouse xenograft model of GC. Both the tumorigenicity and ability to form hepatic metastases were strikingly reduced by ETNK2 KO; indeed, hepatic metastasis was virtually abolished. We also found increased expression of cleaved caspase-3 and cleaved PARP in ETNK2 KO subcutaneous tumours by IHC analysis. In contrast, subcutaneous tumours formed by both parental MKN1 and ETNK2 KO cells have no differences in the expression of HIF-1α, which mediates the cellular response to hypoxia as transcriptome factor.54 Caspase-3 is an effector caspase that is cleaved and activated by initiator caspase. The activated caspase-3 induces apoptosis, as a result, PARP are cleaved by caspase-3 during apoptosis.55 These findings suggest the involvement of ETNK2 in cell apoptosis in vivo. Because hepatic metastasis was modelled here by directly injecting parental or ETNK2 KO GC cells into the portal vein of the mice, our results strongly support a role for ETNK2 in promoting hepatic metastasis formation, which is likely to be mediated by a reduction in apoptosis and/or enhancement of cell survival during portal vein reflux and/or invasion and growth within the liver microenvironment.

Fig. 3 ETNK2 knockout promotes cell cycle arrest and apoptosis of GC cells. a Fluorescence and phase contrast micrographs of untransfected and ETNK2 KO MKN1 cells stained with annexin V (red) to detect apoptotic cells. Irradiated cells served as a positive control. b Flow cytometric dot plots (upper) and quantification (lower) of untransfected and ETNK2 KO MKN1 cells stained to detect cells with mitochondrial membrane potential depolarisation. c Flow cytometric dot plots (upper) and quantification (lower) of untransfected and ETNK2 KO MKN1 cells stained with anti-caspase antibody. d Western blot analysis of apoptosis-related proteins in untransfected and ETNK2 KO MKN1 cells. β-Actin served as a loading control. e Light micrographs (left) and quantification (right) of untransfected and ETNK2 KO MKN1 cells stained with Redox dye to detect cells in G0/G1, S, and G2/M phases of the cell cycle. *P < 0.005. Data are presented as the mean ± standard deviation.

Cell cycle progression is regulated by a series of checkpoints, failure of which can lead to cell cycle arrest, inhibition of proliferation, and induction of apoptosis.67 We found that ETNK2 KO decreased the proportion of cells in the G0/G1 phase. Activated p53 induces transcription of p21, an inhibitor of the checkpoint regulatory protein cyclin-dependent kinase-1 and -2,68 suggesting another mechanism by which ETNK2 affects the GC cell cycle and proliferation. Taken together, our results suggest that ETNK2 may have anti-apoptotic effects in GC cells via direct or indirect regulation of p53 phosphorylation, leading to enhanced proliferation, invasion, and migration, culminating in hepatic metastasis formation.

EMT is a process in which cells lose their epithelial properties and gain migration and invasive ability to become mesenchymal cells, which plays an important role in cancer metastasis.69 We conducted PCR array analysis to search for EMT-related genes whose expression is correlated with ETNK2. Consequently, we found that ETNK2 mRNA expression levels positively correlated to...
We found that patients with high ETNK2 mRNA levels in clinical GC samples was significantly associated with vessel invasion, lymph node metastasis, and advanced disease stage with poor prognosis. Our results indicated that ETNK2 contributes, at least in part, to cancer progression via lymphatic systems. On the other hand, the cumulative incidence of hepatic recurrence was significantly higher in patients with high ETNK2 expression, whereas peritoneal recurrence was not influenced by ETNK2 mRNA expression. Moreover, high ETNK2 mRNA expression was also an independent risk factor for hepatic metastasis and hepatic recurrence, supporting our hypothesis that ETNK2 preferentially promotes hepatic metastasis in GC. Between hepatic metastasis and peritoneal dissemination, there are differences in the microenvironment around cancer cells, such as hetero aggregates containing and premetastatic niche in circulating tumour cell, lymphatic orifices on the peritoneal surface, and human peritoneal mesothelial cells altered by stimulation with a number of growth factors in peritoneal-free cancer cell. 56, 57 ETNK2 may promote hepatic metastasis by inducing anti-apoptotic effects and EMT in such a tumour microenvironment that is suitable specifically for hepatic metastasis formation. Similarly, detection of ETNK2 protein expression by IHC staining could also be helpful in predicting hepatic recurrence after curative gastrectomy. Of note, IHC is a simple and frequently used procedure in clinical settings. Patients identified to have high tumour expression of ETNK2 could undergo aggressive postoperative surveillance using enhanced

Fig. 4  ETNK2 knockout reduces the growth and hepatic metastasis of GC cells in a mouse xenograft model. a Images of mice and excised tumours (upper) and quantification of tumour volumes (lower) after subcutaneous injection of mice with untransfected or ETNK2 KO MKN1 cells. b Results of immunohistochemical analysis of ETNK2, cleaved caspase-3, cleaved PARP, and HIF-1α in subcutaneous tumours formed by parental MKN1 cells and ETNK2 KO cells. c In vivo bioluminescent imaging of hepatic metastases (upper) and quantification of the bioluminescence signal in mice injected with untransfected or ETNK2 KO MKN1 cells (lower). d MRI and macroscopic image of the liver in mice injected with untransfected or ETNK2 KO MKN1 cells. *P < 0.005. Data are presented as the mean ± standard deviation.
MRI or ultrasonography to ensure early detection of hepatic recurrence.

Current evidence supports the importance of multimodal therapy for advanced GC. Although S-1 monotherapy as post-operative adjuvant chemotherapy for advanced GC has shown little success in suppressing haematogenous recurrence, more aggressive adjuvant doublet chemotherapy has been beneficial.60–62 However, aggressive chemotherapy can have serious adverse effects. Therefore, using ETNK2 expression as a biomarker for hepatic recurrence may enable more individualised selection of appropriate adjuvant chemotherapy regimens for patients undergoing curative resection for GC.

Our study has several limitations. First, p53–Bcl-2-mediated apoptosis and malignant phenotypes are required for metastasis to sites other than the liver, including the peritoneal cavity, and we cannot conclude that ETNK2 specifically promotes hepatic metastasis. In this regard, useful information could be obtained from experiments with co-cultured tumour cells and hepatic sinusoidal endothelial cells/peritoneal mesothelial cells and/or evaluation of orthotopic mouse xenograft models. Second, we identified ETNK2 by transcriptome analysis of patients with hepatic recurrence who underwent curative gastrectomy for pStage III GC followed by S-1 adjuvant monotherapy. Because many anti-cancer drugs induce apoptosis, it is possible that ETNK2 is associated with drug resistance. Although such data were not available for this study, they will contribute to a better understanding of the role of ETNK2 in GC. Finally, assays to detect ETNK2 expression in serum samples would greatly advance the possible clinical applications of our findings.

In conclusion, this study demonstrated that ETNK2 promotes hepatic metastasis formation of GC, possibly via dysregulation of the p53–Bcl-2-associated intrinsic apoptosis pathway and enhancement of malignant phenotypes. ETNK2 expression in GC tissues may have utility as a biomarker for predicting hepatic recurrence. ETNK2 and associated signalling pathways may also serve as targets for the development of new therapeutic strategies for the suppression of hepatic recurrence and improvement of the prognosis of patients with advanced GC.

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AUTHOR CONTRIBUTIONS
M. Kanda, Y.K., and T.M. made substantial contributions to conception and design. T.M., M. Koike, S.U., K.S., and H.T. made substantial contributions to acquisition of data. D.S., C.T., N.H., M.H., S.Y., and G.N. made substantial contributions to statistical analysis and interpretation of data. T.M. wrote the draft of manuscript. All authors agreed to be accountable for all aspects of the work and approved the final version of the manuscript.

ADDITIONAL INFORMATION
Ethics approval and consent to participate This study conforms with the ethical guidelines of the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects (2013). The Institutional Review Board of Nagoya University approved this study (approval no. 2014-0043). Written informed consent was obtained from all patients. The Animal Research Committee of Nagoya University approved the experiments using animals (approval no. 28210).

Consent to publish Not applicable.

Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests The authors declare no competing interests.

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