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Oncogenes and Tumor Suppressors

Molecular Cancer Research

Pattern-Specific Transcriptomics Identifies *ASGR2* as a Predictor of Hematogenous Recurrence of Gastric Cancer

Haruyoshi Tanaka[1], Mitsuro Kanda[1], Takashi Miwa[1], Chie Tanaka[1], Daisuke Kobayashi[1], Shinichi Umeda[1], Masahiro Shibata[2], Masaya Suenaga[1], Norifumi Hattori[1], Masamichi Hayashi[1], Naoki Iwata[1], Suguru Yamada[1], Goro Nakayama[1], Michitaka Fujiwara[1], and Yasuhiro Kodera[1]

Abstract

Hematogenous recurrence is a challenging clinical finding that often leads to fatalities of patients with gastric cancer. Therefore, the identification of specific biomarkers and potential therapeutic target molecules for hematogenous recurrence is required to improve the outcomes of these patients. Here, transcriptome and bioinformatics analyses were conducted to uncover candidate molecules differentially expressed in patients with hematogenous recurrence of gastric cancer. One potential candidate identified was asialoglycoprotein receptor 2 (*ASGR2*), and siRNA experiments were conducted to determine the effect of manipulating *ASGR2* expression has on cell phenotypes. *ASGR2* mRNA expression analysis using quantitative real-time reverse-transcription PCR was conducted with stage II/III gastric cancer clinical specimens ($n = 95$). Transcript levels were increased in gastric cancer cells as compared with a control nontumorigenic epithelial cell line. Knockdown of *ASGR2* decreased the adhesion and migration potential. Thus, although gastric cancer cell–invasive activity was significantly decreased by knockdown, forced expression of *ASGR2* promoted invasive activity. Using a mouse hepatic metastasis model, knockdown of *ASGR2* resulted in the absence of hepatic metastasis formation. High *ASGR2* expression in primary gastric cancer tissues was an independent predictor of shorter disease-free and overall survival. Finally, patients with high *ASGR2* expression were more likely to have a high cumulative rate of hematogenous recurrence but not peritoneal or nodal recurrence.

Implications: *ASGR2* expression is associated with the malignant phenotypes in gastric cancer and represents a specific biomarker of hematogenous recurrences after curative resection for gastric cancer. *Mol Cancer Res; 1–10. ©2018 AACR.*

Introduction

Gastric cancer, which is the fifth most common cancer after cancers of the lung, breast, colon, and prostate, often metastasizes or recurs, leading to fatalities (1). Although regimens involving the oral fluorinated pyrimidine S-1 improve the outcomes of patients with gastric cancer (2, 3), this is mainly attributed to the control of peritoneal metastasis (4). However, treatment of hematogenous metastasis must improve (5, 6). The identification of molecules that contribute to the generation of hematogenous metastasis will likely improve our understanding of the biology of metastatic gastric cancer and illuminate target molecules useful for diagnostic and therapeutic purposes.

During the multistep process of hematogenous metastasis, cancer cells may acquire diverse malignant phenotypes required to invade the circulation, survive within the bloodstream, evade the host immune system, and adapt to the environments of distant organs (7). During the last decade, bioinformatics analyses using next-generation sequencing, microarray platforms, or both have played key roles in identifying the molecular mechanisms underlying the hematogenous metastasis of cancer cells (8, 9). For example, numerous genes are associated with the metastatic potential of gastric cancer (10, 11). Therefore, determining the expression of these molecules and determining their contributions to the metastatic phenotype are required to develop useful biomarkers.

To this end, in the present study, we conducted transcriptome analysis to identify genes associated with hematogenous recurrence of gastric cancer and identified the asialoglycoprotein receptor 2 gene (*ASGR2*) as a candidate biomarker. *ASGR2* is a membrane-trafficking protein that was originally identified as a component of the Ashwell–Morell receptor (AMR) that mediates the clearance of circulating plasma asialoglycoproteins, asialoglycolipids, and platelets (12, 13). We are unaware of convincing data on the oncological roles of *ASGR2*. To validate the results of transcriptome analysis, we conducted expression and functional studies of *ASGR2* to evaluate its clinical implications. To the best of our knowledge, this is the first study to examine the role of *ASGR2* in gastrointestinal malignancies, including gastric cancer.

[1]Department of Gastroenterological Surgery (Surgery II), Nagoya University Graduate School of Medicine, Nagoya, Japan. [2]Department of Breast and Endocrine Surgery, Nagoya University Graduate School of Medicine, Nagoya, Japan.

Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

Corresponding Author: Mitsuro Kanda, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan. Phone: 81-52-744-2249; Fax: 81-52-744-2252; E-mail: m-kanda@med.nagoya-u.ac.jp

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Materials and Methods

Sample collection

The gastric cancer cell lines (AGS, GCIY, KATOIII, MKN1, MKN45, MKN74, N87, NUGC2, NUGC3, NUGC4, and SC-6-JCK) and the nontumorigenic tubular epithelial cell line FHs74 were obtained from the American Type Culture Collection or the Japanese Collection of Research Bio Resources Cell Bank or were established at our institute. All cell lines were tested for mycoplasma, analyzed using the short tandem repeat PCR method, and authenticated by the JCRB Cell Bank on June 30, 2015. Cell lines were cultured at 37°C in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FBS in an atmosphere containing 5% CO_2 (14).

Primary gastric cancer tissues were collected from 95 patients who underwent gastric resection for stage II/III gastric cancer without preoperative treatment at Nagoya University Hospital between 2001 and 2014. Since 2006, adjuvant S-1 monotherapy has been recommended to all patients with stage II/III gastric cancer, unless contraindicated. Two pathologists classified the primary gastric cancer tissue specimens according to the histology criteria of 7th edition of the Union for International Cancer Control (UICC) classification system and the Japanese Classification of Gastric Carcinoma, 3rd English edition (15, 16). Paired samples (primary gastric cancer tissues and noncancerous gastric mucosa, confirmed by hematoxylin–eosin staining) were separately collected, immediately frozen in liquid nitrogen, and stored at −80°C (17).

For transcriptome analysis, surgically resected specimens of 16 patients with stage III gastric cancer who underwent curative gastrectomy and adjuvant S-1 monotherapy were selected to provide an independent data set. These patients were categorized into four clusters (4 patients per cluster) according to clinical course as follows: cluster 1, no recurrences for longer than 5 years; cluster 2, hepatic-confined recurrences within 2 years after surgery; cluster 3, peritoneal recurrences within 2 years after surgery; and cluster 4, distant nodal recurrences within 2 years after surgery.

This study conformed to the ethical guidelines of the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects. Written-informed consent was obtained from all patients for the use of clinical samples and data, as required by the Institutional Review Board of Nagoya University, Japan.

Global expression analysis

Total RNA preparations, which were isolated from samples as previously reported (18), passed a quality test using an Agilent 2100 Bioanalyzer (Agilent). RNA sequencing was performed using the paired-end method with a HiSeq sequencing system (Illumina). Each gene expression level was quantified as fragments per kilobase of transcript per million fragments sequenced value, applying the Cuffdiff package in Cufflinks according to annotations of GRCh37 (19). The differences among the expression levels of 57,749 genes between each recurrence-site cluster are expressed as the fold change ($\log_2$) and P value. The FDR was defined as 5%, and the Q value was calculated from multiple comparisons as a cutoff value of 0.05. Details are provided in the Supplementary Methods.

Additional data

To validate the data, cancer-wide and global cohort data were obtained from the Cancer Cell Line Encyclopedia (CCLE) and The Cancer Genome Atlas (TCGA) Research Network via the open-source c-BioPortal and GSE62254 download via the National Center for Biotechnology Information's Gene Expression Omnibus. CCLE microarray data for 1,019 cancer cell lines, TCGA RNA Seq V2 RSEM data of 295 gastric cancer cases, and GSE62254 microarray data of 300 gastric cancer cases were used to analyze *ASGR2* and coregulated genes' mRNA expression and gene set enrichment analysis. Gene set enrichment analysis was performed using GSEA software (ref. 20; Broad Institute, Cambridge, MA). GSE62254 clinical data of 283 gastric cancer patients and TCGA data of 105 patients with stages I–IIIA gastric cancer were used for prognostic analysis.

Analysis of *ASGR2* expression

mRNA expression levels were determined using quantitative real-time reverse-transcription qRT-PCR. Total RNA (10 μg per sample) was isolated from 12 cell lines and primary gastric cancer tissues to generate cDNAs according to published protocols (17).

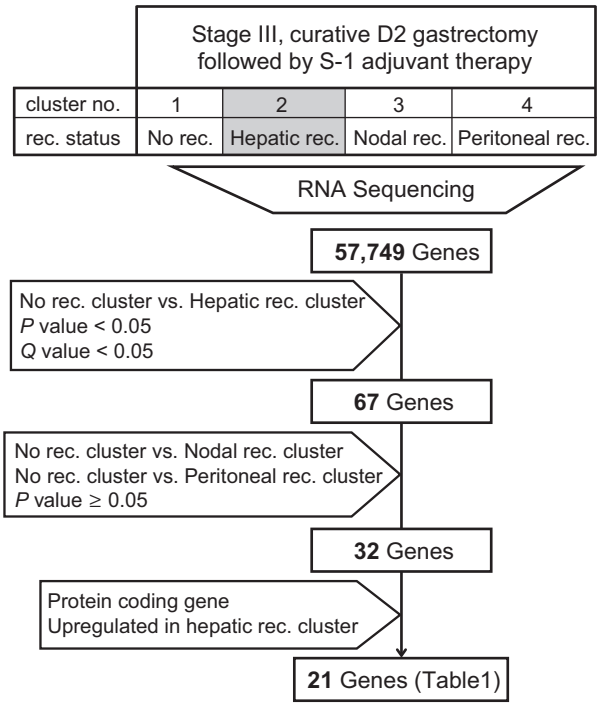

Figure 1.

Extraction diagram of candidate genes identified by global expression analysis. For transcriptome analysis, surgically resected specimens of 16 patients with stage III gastric cancer who underwent curative gastrectomy and adjuvant S-1 monotherapy were selected. These 16 patients were categorized into four clusters (4 patients per cluster) according to clinical courses as follows: cluster 1, no recurrences (rec.) for >5 years; cluster 2, hepatic-confined recurrences within 2 years after surgery; cluster 3, peritoneal recurrences within 2 years after surgery; and cluster 4, distant nodal recurrences within 2 years after surgery. Global expression profiling was conducted to compare the expression levels of 57,749 genes between each cluster. They were filtered to yield 67 genes associated with significant differences in expression ($P < 0.05$ and $Q < 0.05$) between clusters 1 and 2. We next filtered these 67 genes according to $P \geq 0.05$ for the no-recurrence group vs. the nodal-recurrence group and $P \geq 0.05$ for the no-recurrence group vs. the peritoneal-recurrence group to yield 32 genes. Among them, 32 genes that were upregulated in cluster 2 and comprised coding sequences were filtered to yield 21 genes, which we considered candidates drivers of hematogenous metastasis (Table 1).

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cDNAs were amplified using *ASGR2*-specific PCR primers (Supplementary Table S1). *ASGR2*-expression levels were normalized to those of glyceraldehyde-3-phosphate dehydrogenase. The RT^2 Profiler PCR Array Kit (Qiagen) was used to determine epithelial–mesenchymal transition (EMT)-related gene expression levels to identify cancer-related molecules expressed in association with *ASGR2* (21). Western blot analysis using mouse an anti-ASGR2 monoclonal antibody (LS-C196698; LifeSpan BioSciences Inc.), diluted 1:200 in 5% skim milk in 0.05% TBS-T, was performed as previously described (22). β-Actin served as an endogenous control.

Knockdown of *ASGR2* expression using siRNAs

ASGR2-specific siRNA sequences were designed using a web-based tool (Supplementary Table S1). *ASGR2*-specific or a control, nonspecific siRNAs (40 nmol/L of each; siControl; Bioneer) were used to transiently transfect MKN1 and N87 cells in the presence of LipoTrust EX Oligo (Hokkaido System Science) the day after splitting the cell cultures.

Forced expression of *ASGR2* transferring *ASGR2* cDNA vector

The *ASGR2* cDNA clones ligated as open reading frame sequences into a pFN21A with CMV vector were purchased (Promega). Using NEON (Thermo Fisher) system, 0.2 μg of the *ASGR2* vector was transfected into MKN1 cells (1×10^5).

Hepatic metastasis xenograft model assay

To investigate roles of *ASGR2 in vivo* hematogenous metastasis, mouse hepatic metastasis models were analyzed. siRNA specific for *ASGR2* or control nonspecific siRNAs were transiently transfected into MKN1 cell as described above. Two days after transfection, 5×10^5 cells were collected and diluted in 100 μL of PBS, which was injected into the portal vein of scid mice (CLEA Japan Inc.). Four mice were subjected in each group. Eight weeks after implantation, the mice were sacrificed.

Cell proliferation, migration, invasion, migration, and adhesion assays

The proliferation of MKN1 and N87 cells was evaluated using the Premix WST-8 Cell Proliferation assay Kit (DOJINDO Inc.; ref. 23). The invasion of a Matrigel matrix by MKN1 cells was determined using BioCoat Matrigel invasion chambers (BD Biosciences). The migration of MKN1 and N87 cells was evaluated using a wound-healing assay as follows: Cells were seeded into culture inserts in a 60-mm diameter dish (ibidi GmbH) to establish wound gaps of a defined width. After 16 hours, the insert was removed, and the width of the wound was measured. The adherence of MKN1 cells to a matrix was evaluated using the CytoSelect48-Well Cell Adhesion Assay Kit (Cell Biolabs). Cells adhered to bottom of the wells were mechanically detached, stained with crystal violet, and the optical density at 560 nm was measured (24). More details are provided in the Supplementary Methods

Cell death assay

Cells (1×10^5/100 μL) were diluted in Annexin-binding buffer (10 mmol/L HEPES, 140 mmol/L NaCl, and 2.5 mmol/L $CaCl_2$, pH 7.4), and 10 μL of Annexin V conjugate (Thermo Fisher Scientific) was added, followed by incubation for 15 minutes at room temperature. The cells were mounted on slides after washing in buffer and then counted using a BZ9000 fluorescence microscope (Keyence; ×200 magnification, ≥100 cells were counted in five views). Cells irradiated with UV light for 8 hours were used as a positive control.

Statistical analysis

The significance of the difference between variables of two groups was assessed using the Mann–Whitney test for clinical valuables or the Student *t* test for two experimental variables, and the Kruskal–Wallis test followed by the Bonferroni test for three or more variables. The Fisher exact test was used to analyze categorical data of two groups. Goodness of fit was

Table 1. Candidate genes upregulated in gastric cancer tissues from patients with hepatic metastasis

	H-rec/ Non-rec					P-rec/ Non-rec	N-rec/ Non-rec
Symbol	Log_2	*P*	Full name	Location	Function	Log_2	Log_2
ASGR2	3.560	<0.001	Asialoglycoprotein 2	17p13.1	Mediator of endocytosis of plasma glycoproteins	−0.124	0.452
BCAM	2.123	<0.001	Basal cell adhesion molecule	19q13.32	Laminin receptor	−0.554	0.042
COMP	4.187	<0.001	Cartilage oligomeric matrix protein	19p13.11	Extracellular matrix protein	0.760	1.173
CYP2W1	6.809	<0.001	Cytochrome P450 Family 2 Subfamily W Member 1	7p22.3	Metabolic enzyme	1.450	1.667
FABP3	3.774	<0.001	Fatty acid binding protein 3	1p35.2	Metabolic enzyme	0.051	0.992
GAL	4.278	<0.001	Galanin and GMAP prepropeptide	11q13.2	Endocrine hormone of nervous systems	2.076	1.340
GATA5	2.944	<0.001	GATA binding protein 5	20q13.33	Transcriptional factor	−1.401	−0.398
GPC3	2.990	<0.001	Glypican 3	Xq26.2	Multifunction membrane protein	−0.997	0.465
HIC2	3.434	<0.001	HIC ZBTB transcriptional repressor 2	22q11.21	Transcriptional factor	0.523	0.843
HIF3A	4.168	<0.001	Hypoxia-inducible factor 3 alpha subunit	19q13.32	Regulator of hypoxia-inducible genes	0.290	−0.018
HMGA2	3.291	<0.001	High mobility group AT-Hook 2	12q14.3	Transcriptional factor	0.421	0.612
IGSF1	3.546	<0.001	Immunoglobulin superfamily member 1	Xq26.2	Immunoglobulin	−0.899	0.601
MYO18B	4.731	<0.001	Myosin XVIIIB	22q12.1	Regulator of muscle structure	4.325	−0.660
PRSS1	4.203	<0.001	Protease, serine 1	7q34	Serine protease	0.122	0.952
RBP4	3.549	<0.001	Retinol binding protein 4	10q23.33	Carrier for retinol	−1.186	0.834
RNF182	5.362	<0.001	Ring finger protein 182	6p23	Mediator of MHC-I antigen	−0.124	2.317
SMTNL2	4.739	<0.001	Smoothelin like 2	17p13.2	Unknown	−0.879	1.108
SUSD2	2.976	<0.001	Sushi domain containing 2	22q11.23	Cytokine receptor	0.464	0.302
TCF7L1	2.288	<0.001	Transcription factor 7 like 1	2p11.2	Regulator of cell cycle	−0.260	−0.386
TKTL1	6.109	<0.001	Transketolase like 1	Xq28	Metabolic enzyme	−2.080	−2.758
TNNT1	3.316	<0.001	Troponin T1, slow skeletal type	19q13.42	Regulator of muscle structure	1.675	−0.637

Abbreviations: H-rec, hepatic recurrence; N-rec, nodal recurrence; Non-rec, non-recurrence; P-rec, peritoneal recurrence.

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assessed by calculating the area under the curve of the receiver operating characteristic (ROC) curve to determine an optimal cutoff value. Overall survival (disease-specific) and disease-free survival rates were calculated using the Kaplan–Meier method, and the difference between survival rates was evaluated using the Wilcoxon test. A value of $P < 0.05$ was considered statistically significant. All statistical analyses were performed using R software (The R Foundation for Statistical Computing) on EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan). EZR is a graphical user interface for R that is a modified version of R commander designed to add frequently used biostatistical functions (25).

Results

Global expression profiling identifies *ASGR2* as a candidate biomarker of hematogenous recurrence of gastric cancer

Global expression profiling was conducted to compare the expression levels of 57,749 genes of each recurrence-site cluster. The quality of RNAs was sufficient, as indicated by the finding that follows: mean reads per sample = 21,830,000 pairs, yield data per sample = 4.38 Gb, mean rate ≥Q30 = 95.0%, mean quality score = 36.7, and mean total mapped-read rate = 94.2%.

The 57,749 genes were filtered to yield 67, according to significant differences in expression ($P < 0.05$ and $Q < 0.05$) between cluster 1 (no recurrence) and cluster 2 (hepatic recurrence),

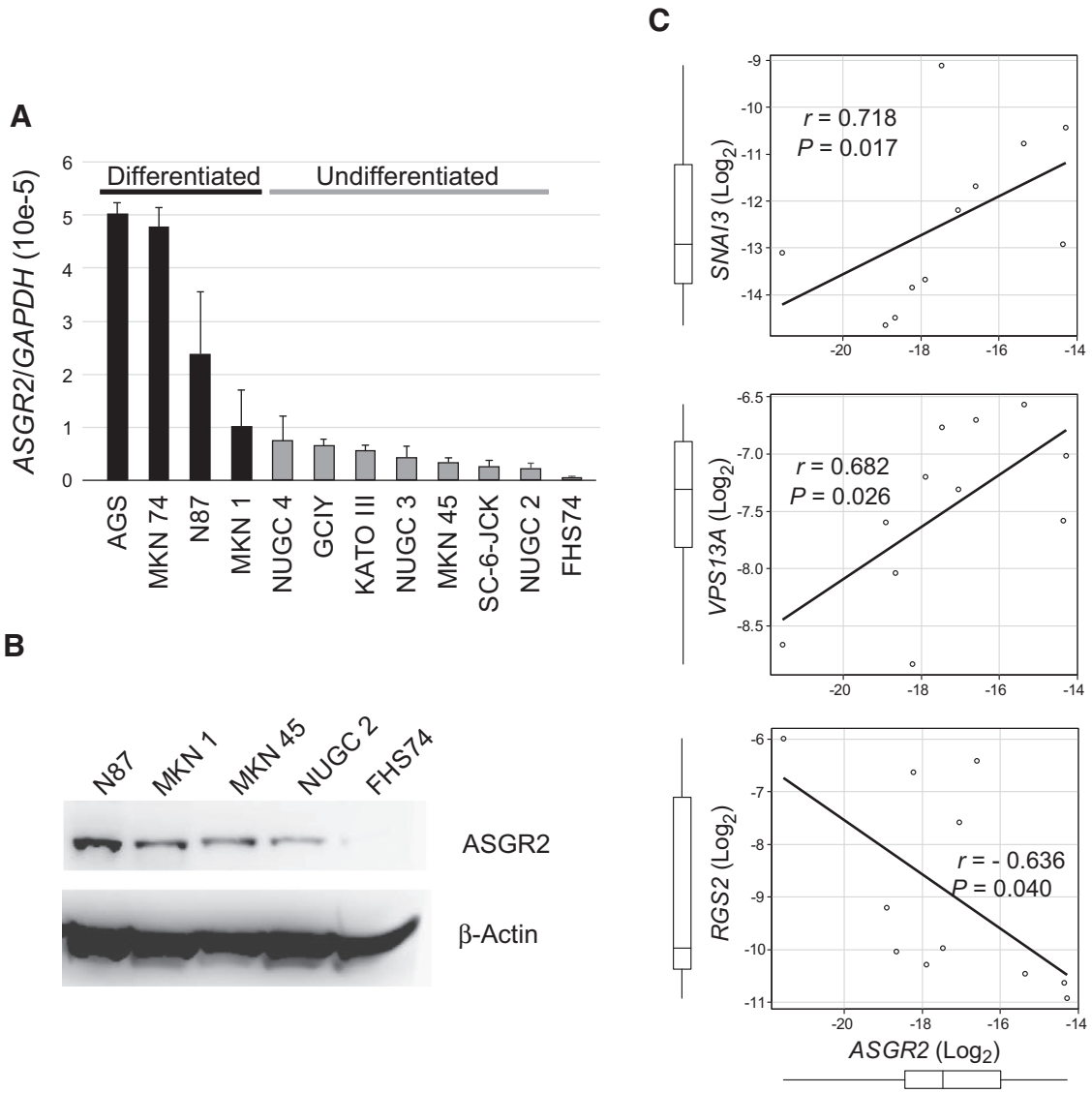

Figure 2.

ASGR2 expression of cell lines and coexpressed genes. **A,** *ASGR2* mRNA was more highly expressed in differentiated cells compared with undifferentiated cells. Its expression levels were below detectable values in FHs74 cells. **B,** Similarly, the levels of *ASGR2* were undetectable in FHs74 cells and highly expressed in differentiated, but not in undifferentiated, gastric cancer cells. **C,** ASGR2 protein was undetectable in FHs74 cells and highly expressed in differentiated compared with undifferentiated gastric cancer cells. *ASGR2*-expression levels were strongly positively correlated with *SNAI3* and *VPS13A*, and negatively correlated with *RGS2* expression.

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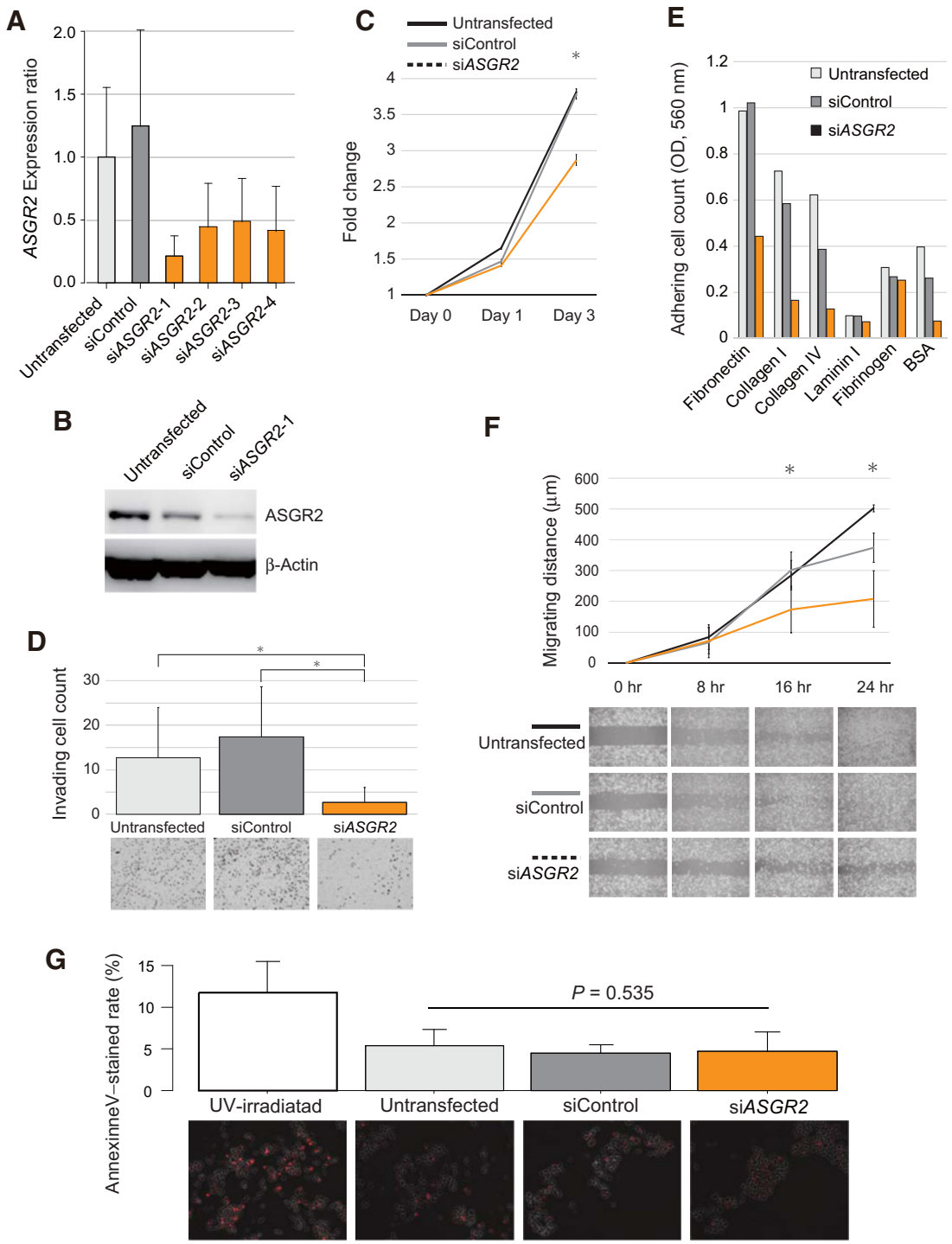

Figure 3.

Effects of *ASGR2* knockdown on the phenotypes of MKN1 cells. **A,** Inhibitory effect of the knockdown of *ASGR2* mRNA and (**B**) protein expression. **C,** The proliferation of cells transfected with si*ASGR2* was lower than that of untransfected and siControl cells according to the WST-8 assay. **D,** Cell counts of cells that invaded the Matrigel chamber were reduced in cells transfected with si*ASGR2*. **E,** Cell adherence to matrices was reduced by si*ASGR2*, particularly fibronectin, collagen I, and collagen IV. **F,** si*ASGR2* inhibited cell migration across the cell wound gap. **G,** However, si*ASGR2* did not appear to induce apoptosis. *, significant difference ($P < 0.05$).

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without a cutoff $\log_2$ value, which was not specifically determined (Fig. 1). We extracted 32 genes by filtering 67 genes associated with $P \geq 0.05$ for the no-recurrence group versus the nodal-recurrence group, and $P \geq 0.05$ for the no-recurrence group versus peritoneal-recurrence group. Among them, those expressed at relatively higher levels in cluster 2 (hepatic recurrence), which comprised coding sequences, 32 were filtered to yield 21 genes as candidate drivers of hematogenous metastasis (Table 1). Finally, according to the results of a pilot study of a small number ($n = 16$) of samples, we decided to focus on *ASGR2* as a candidate biomarker. Subsequently, we designed the present study as well as the validation of these results by testing a larger cohort.

ASGR2 expression and the identification of potentially interacting genes

Expression levels of *ASGR2* mRNA were heterogeneous among gastric cancer cell lines and below detectable values in the control cell line FHs74. *ASGR2* mRNA was highly expressed in all differentiated gastric cancer cells (Fig. 2A). Similarly, the levels of *ASGR2* were undetectable in FHs74 cells and highly expressed in differentiated, but not in undifferentiated, gastric cancer cells (Fig. 2B). When we analyzed the correlations between the expression levels of *ASGR2* and 84 cancer-related molecules among gastric cancer cell lines, we found that *ASGR2* expression positively correlated with those of *SNAI3* and *VSP13A* and inversely correlated with that of *RGS2* (Fig. 2C). To validate these findings, we evaluated the associations of the expression levels of *ASGR2* and *SNAI3* using our published data. Modest correlations between *ASGR2* and *SNAI3* expression levels were observed in the cancer cell lines of the CCLE data ($r = 0.224$; Supplementary Fig. S1A) and the clinical samples from the TCGA data ($r = 0.258$; Supplementary Fig. S1B).

A gene set enrichment analysis on GSE62254 provided some supportive data on involvement of *ASGR2* in the Janus kinase 2–signal transducer and activator of transcription-3 (JAK2–STAT3) pathway (Supplementary Fig. S2).

Effect of inhibiting or forced *ASGR2* expression on the malignant phenotypes of gastric cancer cells

Four *ASGR2*-specific siRNAs were designed to determine the effects of *ASGR2* knockdown on the phenotypes of gastric cancer cells. After confirming the knockdown effects of the four siRNAs (Fig. 3A and B; Supplementary Fig. S3A and S3B), we evaluated the proliferation, invasion, adhesion, migration, and apoptosis of gastric cancer cells. The proliferation of MKN1 and N87 cells transfected with *siASGR2* was marginally reduced compared with untransfected and siControl cells (Fig. 3C; Supplementary Fig. S3C). Invasion of MKN1 cells was significantly reduced following inhibition of *ASGR2* expression (Fig. 3D). N87 cells were unsuitable for the invasion assay, because they were unable to penetrate the Matrigel matrix. The adherence of si*ASGR2*-transfected MKN1 cells to fibronectin, collagen I, and collagen IV was decreased (Fig. 3E). Moreover, the migration of si*ASGR2*-transfected MKN1 and N87 cells was significantly inhibited (Fig. 3F; Supplementary Fig. S3D). In contrast, knockdown of *ASGR2* did not significantly affect apoptosis of either transfected cell line (Fig. 3G; Supplementary Fig. S3E).

On the other hand, forced expression of *ASGR2* significantly facilitates invasion activity of the MKN1 cell (Fig. 4A). Little influence on cell proliferation was observed by overexpression of *ASGR2* (Supplementary Fig. S4).

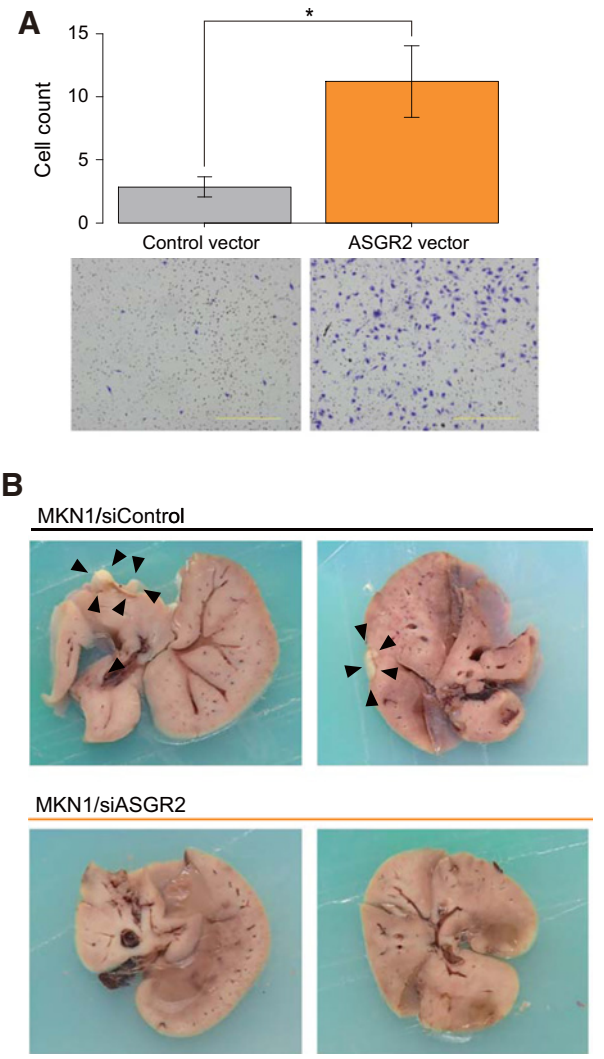

Figure 4.

A, Effect of *ASGR2* overexpression on the invasive activity of MKN1 cells. Invasion activity was promoted in MKN1 cells transfected with *ASGR2* vector than that of control vector. **B,** Knockdown of *ASGR2* assay on mouse hepatic metastasis model. Three of 4 mice-seeded MKN1 cells transfected by siControl progress hepatic metastasis, but any those of si*ASGR2*-transfected cell did not progress metastatic lesion.

Mouse hepatic metastasis model was established by injecting MKN1 cells to the portal vein systems. As a result, 3 of 4 mice-seeded MKN1 cells transfected by siControl progress hepatic metastasis, but any those of si*ASGR2*-transfected cell did not progress metastatic lesion (Fig. 4B).

Clinical implications of *ASGR2* expression in tumor tissues

To predict hematogenous recurrence within 2 years after surgery administered to 95 patients with stage II/III gastric cancer included in the present study, we used ROC curve analysis to determine the optimal cutoff value (0.003) of *ASGR2* mRNA expression in gastric cancer tissues (Fig. 5A). When we used this cutoff value to classify patients into high ($n = 54$) and low ($n = 41$) *ASGR2*-expression groups, we found that *ASGR2*

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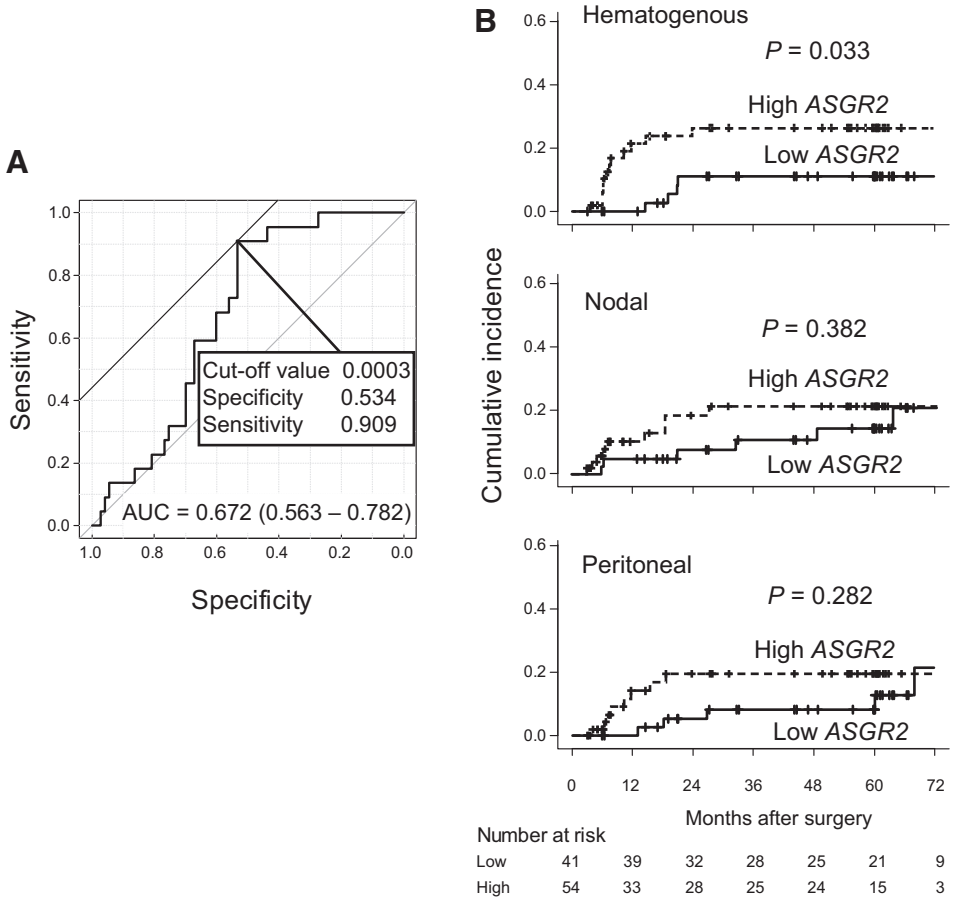

Figure 5.
Identification of the cutoff value and influence of *ASGR2* expression on hematogenous recurrence. **A,** ROC curve to predict hematogenous recurrence within 2 years. **B,** Cumulative hematogenous recurrence curve; the high *ASGR2*-expression group was more likely to have a higher recurrence rate than the low *ASGR2*-expression group.

expression was independent of all clinicopathologic variables (Supplementary Table S2).

Patients in the high *ASGR2*-expression group were more likely to have a high cumulative hematogenous recurrence rate compared with those in the low *ASGR2*-expression group (P = 0.033; Fig. 5B, top). In contrast, there was no difference in the cumulative rate of peritoneal or nodal recurrence between the high and low *ASGR2*-expression groups (Fig. 5B, bottom two plots). The high *ASGR2*-expression group experienced significant shorter disease-free survival compared with those in the low *ASGR2*-expression group [3-year survival rates, 51.0% (95% confidence interval, CI, 37.0%–63.5%) and 72.7% (95% CI, 56.1%–83.9%), respectively, P = 0.019; Fig. 6A]. Similarly, the high *ASGR2*-expression group had marginally shorter overall survival as compared with the low *ASGR2*-expression group [5-year survival rates, 62.3% (95% CI, 46.8%–74.4%) and 77.6% (95% CI, 59.9%–88.2%), respectively, P = 0.049; Fig. 6B]. To validate our institutional survival data, published data from TCGA and GSE62254 were evaluated for the prognostic significance of *ASGR2* expression in gastric cancer tissues as well. Using ROC curve analysis from the TCGA data, we determined the optimal cutoff value (0.146) of *ASGR2* expression to predict recurrence (Supplementary Fig. S5A). It was found that the high *ASGR2*-expression group experienced shorter disease-free survival compared with that of the low *ASGR2*-expression group (Fig. 6C). Result of the survival analysis using the GSE62254 data were consistent with those from our institutional data; high expression *ASGR2* was associated with poor overall survival (Supplementary Fig. S5B).

We next conducted subgroup analysis of disease-free survival according to UICC stage. We found that differences in survival were more apparent in patients with stage II gastric cancer compared with those with stage III gastric cancer. In patients with stage II gastric cancer, those in the high *ASGR2*-expression group experienced significantly shorter disease-free survival compared with those in the low *ASGR2*-expression group [3-year survival rates, 61.9% (95% CI, 38.1%–78.8%) and 93.3% (95% CI, 61.3%–99.0%), respectively, P = 0.021; Fig. 6D]. Similarly, the overall survival of patients with stage II gastric cancer was significantly shorter in the high *ASGR2*-expression group compared with those in the low *ASGR2*-expression group [5-year survival, 73.2% (95% CI, 46.7%–88.0%) and 100%, respectively, P = 0.033; Fig. 6E].

Discussion

Here, we developed a novel approach to global expression analysis, based on transcriptome analysis of specific recurrence patterns of stage III gastric cancer tissues, to identify molecules specifically associated with hematogenous metastasis of gastric cancer. We used this strategy to identify a possible role for *ASGR2* in determining the malignant phenotypes of gastric cancer cells. Specifically, *ASGR2*-expression levels in gastric cancer tissues predicted a higher risk of hematogenous recurrences in patients with stage II/III gastric cancer who underwent curative resection. Global expression analysis as RNA-seq, even if consisting of small number of

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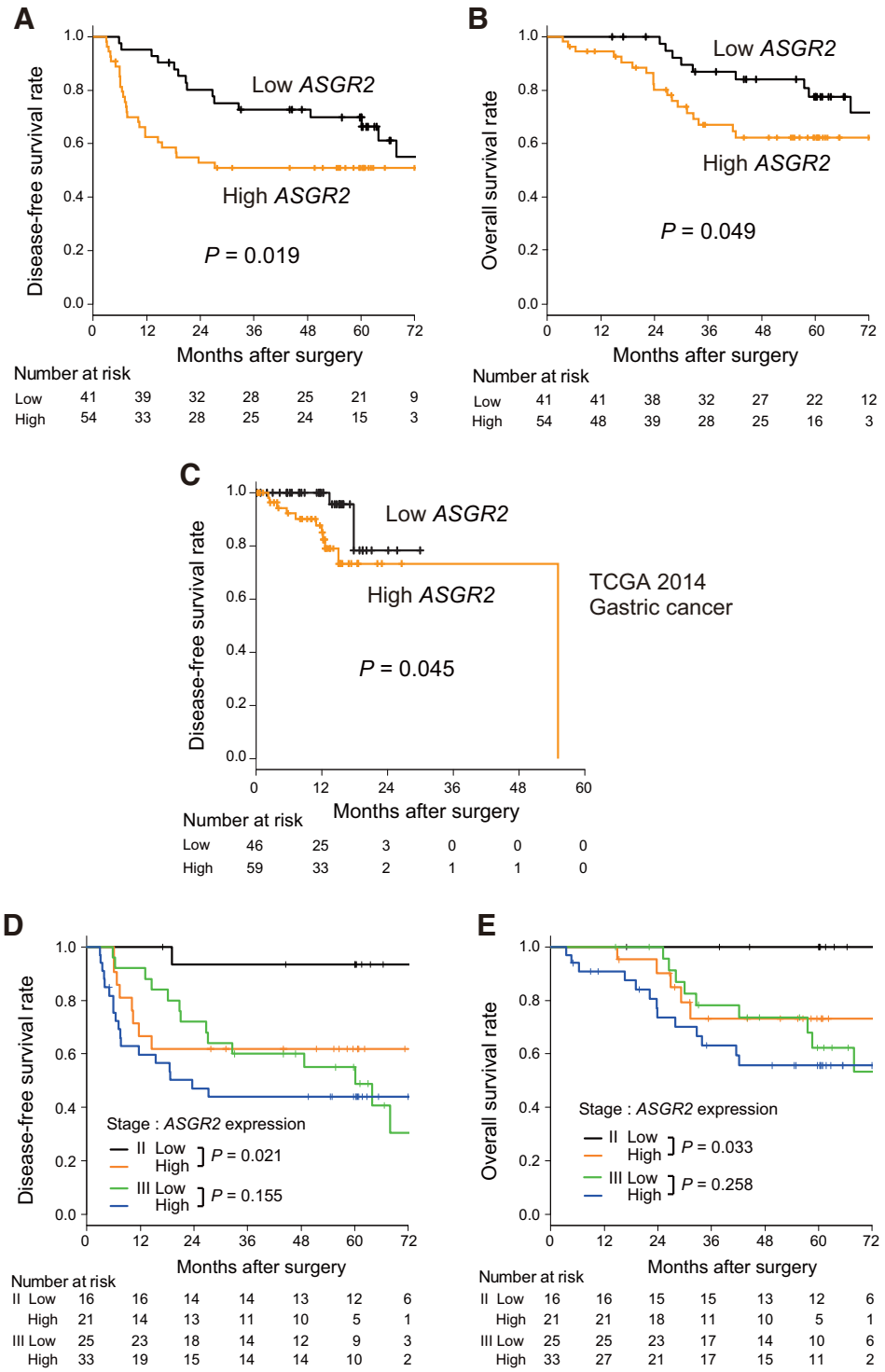

Figure 6.

Influence of *ASGR2* expression on survival. **A,** Disease-free survival curve as a function of *ASGR2* expression. The high *ASGR2*-expression group experienced shorter survival than the low *ASGR2*-expression group. **B,** The same findings were observed for overall survival. **C,** Validation analysis using TCGA data. The high *ASGR2*-expression group experienced shorter disease-free survival time than the low *ASGR2*-expression group. **D,** Subgroup analysis of survival stratified according to UICC staging. Disease-free survival curve. Higher *ASGR2* expression associated with worse prognosis of patients with stage II gastric cancer. **E,** Subgroup analysis of overall survival. The high *ASGR2*-expression group with stage II gastric cancer experienced worse prognosis than the low *ASGR2*-expression group.

patients, was followed by quantitative RT-PCR–based assay to be validated (26).

Gastric cancer cells must acquire an aggressive phenotype to form hematogenous metastasis (27, 28) and therefore may express molecules with activities that differ from those of cancer cells that invade the peritoneum and lymph nodes. To test this hypothesis, we designed a recurrence pattern-specific transcriptome analysis strategy to identify genes that were specifically upregulated in each recurrence pattern. Among 57,749 genes, 21 fulfilled our criteria, and we focused on *ASGR2* as an attractive candidate biomarker or target molecule, or both, because *ASGR2* is a component of a receptor that mediates proliferative signals, metabolizes drugs, or acts as an adhesion molecule in the parenchyma of other organs.

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Although *ASGR2* expression in hepatoma cell lines is documented (29, 30), to our knowledge, the expression of *ASGR2* has not been evaluated in malignant human tissues. *ASGR2* is a membrane-trafficking protein originally identified as a component of AMR, which is abundantly expressed on the sinusoidal surface of parenchymal cells of the liver and in blood monocytes (12, 31). AMR is an endocytic transport receptor that mediates the removal and degradation of circulating plasma asialoglycoproteins or asialoglycolipids (32, 33). Further, AMR mediates the clearance of aged, sialic acid–deprived platelets by stimulating the JAK2–STAT3 cascade, which regulates thrombopoietin mRNA expression (13, 34).

We found that *ASGR2* was highly expressed in differentiated gastric cancer cell lines. Generally speaking, differentiated gastric cancer metastasizes via the hematogenous route rather than to the peritoneum and lymph nodes (35). Moreover, we show that *ASGR2* was involved in the invasion, migration, and adherence of gastric cancer cells and that *ASGR2* expression was coregulated with *SNAI3*, which is a transcription factor involved in the EMT cascade (36–38). A gene set enrichment analysis using public database, *ASGR2* was suggested to be involved in the JAK2–STAT3 pathway. These findings suggest that *ASGR2* may interact with EMT cascade and JAK2–STAT3 pathway and contribute to promoting hematogenous metastasis.

Although correlation with vessel involvement pathologically on clinical samples was not detected, we believe that this finding illuminates the importance of *ASGR2* as a biomarker for hematogenous recurrence of gastric cancer. This is because it indicates that *ASGR2* expression is independent of established risk factors for hematogenous recurrences such as vessel involvement. High expression of *ASGR2* in primary gastric cancer tissues was associated with recurrence after curative gastrectomy, particularly hematogenous recurrence. The association with prognosis was more evident in patients with stage II gastric cancer. Our data and those published by others indicate that differentiated types of gastric cancer tend to metastasize to the liver, lung, and bone (hematogenous) with features similar to those of colorectal cancer, whereas undifferentiated tumors tend to expand to the peritoneal cavity (5, 39–41).

To translate our findings into clinical practice, the application of *ASGR2* as a potential biomarker should be discussed. First, if the utility of *ASGR2* expression as a biomarker to identify patients at high risk of hematogenous recurrence after gastrectomy is successfully validated by future prospective clinical trials, this can help physicians decide whether to implement perioperative chemotherapy. For example, in the Adjuvant Chemotherapy Trial of S-1 for Gastric Cancer (ACTS-GC) phase III clinical trial that evaluated the survival benefit of postoperative adjuvant S-1 monotherapy, the HR of peritoneal recurrence in the S-1 group was 0.687, whereas that of hematogenous recurrence was as high as 0.784 (4). In contrast, the CLASSIC phase III clinical trial that compared surgery alone with postoperative adjuvant capecitabine plus oxaliplatin reports a peritoneal recurrence HR in the capecitabine plus oxaliplatin group of 0.813, whereas that for hematogenous recurrence is 0.618 (41, 42). These findings show that the treatment efficacy for each recurrence pattern differed according to treatment regimen. Therefore, *ASGR2*-expression levels may serve as a determinant of postoperative adjuvant chemotherapy (S-1 monotherapy or capecitabine plus oxaliplatin) for patients with stage II/III gastric cancer. Moreover, *ASGR2* is suitable as a druggable target, because our *in vitro* experiments demonstrate an inhibitory effect of *ASGR2* knockdown on the malignant phenotype of gastric cancer cells.

The present study has several limitations. First, our mRNA expression data were obtained retrospectively, although they were verified using TCGA data. Second, the relatively small sample size, particularly for transcriptome analysis, limited statistical power. Therefore, the analysis of a larger cohort is required to evaluate clinical significance and establish optimal cutoff values. Third, we did not investigate the mechanisms involved in the development of hematogenous metastatic lesions, the role of *ASGR2* in this process, and downstream molecules associated with *ASGR2*. Although the members of the ASGR family coregulate each other, to our knowledge, other genes encoding upstream or downstream signaling components have not been reported in the field of oncology. Further investigations, including enforced-expression analysis and *in vivo* experiments using mouse xenograft models, are required to define the roles of *ASGR2* in hematogenous metastasis.

Together, our results strongly support the conclusion that *ASGR2* is associated with the malignant phenotypes of gastric cancer cells and may represent a specific biomarker for hematogenous recurrence after curative resection of gastric cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. Kanda, Y. Kodera
Development of methodology: H. Tanaka, M. Kanda
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Tanaka, M. Kanda, T. Miwa, S. Umeda, C. Tanaka, D. Kobayashi, M. Fujiwara, Y. Kodera
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Tanaka, M. Kanda
Writing, review, and/or revision of the manuscript: H. Tanaka, M. Kanda, T. Miwa, C. Tanaka, D. Kobayashi, S. Umeda, M. Shibata, M. Suenaga, N. Hattori, M. Hayashi, N. Iwata, S. Yamada, G. Nakayama, M. Fugiwara, Y. Kodera
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Kanda
Study supervision: Y. Kodera

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Haruyoshi Tanaka, Mitsuro Kanda, Takashi Miwa, et al.

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