

Clinical characteristics of gastrointestinal stromal tumors with hypoglycemia

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Abstract. The development of tyrosine-kinase inhibitors has improved survival rates for patients with gastrointestinal stromal tumors (GISTs). Despite the progress, not all the patients can universally receive the benefit from treatment due to the individual underlying conditions in a real-world setting. The present study focused on the well-known but understudied condition of GIST with hypoglycemia. Hypoglycemia in GIST is characterized by hypoglycemic symptoms such as dizziness, sweating and confusion. It is caused by several factors such as multiple liver metastases, drug adverse effects, postoperative complications and paraneoplastic syndrome [non-islet cell tumor hypoglycemia (NICTH)]. Comprehensive analysis of this condition has been hindered due to its rarity, and has been mostly limited to case reports. In the present study, a single-institution retrospective analysis of GIST with hypoglycemia was conducted to investigate its prevalence and prognosis, and the cause of this condition. The present study identified that the prevalence of hypoglycemic episodes

of GIST was 4.1% in all patients with GIST, and recurrent hypoglycemic cases had a poor prognosis. The present study identified 1 case with recurrent hypoglycemia due to NICTH. Since NICTH is a rare hypoglycemic cause and requires further evaluation, an autopsy and genetic sequencing were performed using the available clinical materials. Through this histological and genetic investigation, the histological diversity of NICTH-GIST was revealed and insulin-like growth factor II (IGF-II) amplification was identified. Furthermore, a chronological analysis was performed using multiple resected archived samples from the same case, and revealed that diffuse IGF-II expression may have occurred in the early phase of tumor development. The present study catalogued the characteristics of GIST with hypoglycemia with a focus on NICTH-GIST.

Introduction

Recent development in cancer therapies has significantly improved the prognosis of patients for several years (1). Since patients enrolled in clinical trials represent only a selected demographic of cancer patients, it is important to translate these clinical trial results to the real-world settings. In real-world settings, the adverse effects and underlying conditions of each patient are taken into consideration for individualized treatment strategies (2,3). As one of these conditions, hypoglycemia is a rare but serious complication that is related to a high risk of mortality and poor prognosis (4,5). It is observed in several cancer types, not only in epithelial tumors such as insulinoma and hepatocellular carcinoma, but also in mesenchymal tumors such as gastrointestinal stromal tumors (GISTs). Hypoglycemia in GISTs is caused by several factors such as multiple liver metastases, drug adverse effect (diabetes treatment, and imatinib or sunitinib which is the treatment for GISTs) (6,7), postoperative complications (8), and paraneoplastic syndrome (9,10). Non-islet cell tumor hypoglycemia (NICTH) is a serious paraneoplastic syndrome caused by the secretion of an incompletely processed form of IGF-II which is called as 'big-IGF-II' (11,12). This complex precursor

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Abbreviations: AGC, automatic gain control; FFPE, formalin-fixed, paraffin-embedded; GISTs, gastrointestinal stromal tumors; HE, hypercellular epithelioid; IGF-II, insulin-like growth factor II; IHC, immunohistochemistry; NICTH, non-islet cell tumor hypoglycemia; SE, sclerosing epithelioid; SNVs, single-nucleotide variants; SS, sclerosing spindle; TMB, tumor mutation burden; WES, whole exome sequencing

Key words: GIST, hypoglycemia, IGF-II amplification, NICTH, paraneoplastic syndrome

of IGF-II secreted by the tumor functions in an insulin-like manner inducing hypoglycemia. The mechanism behind the inappropriate processing of IGF-II is suggested to involve protein-folding abnormalities, prohormone convertase abnormalities, imprinting of IGF-II, and genetic mutations (13-17).

Due to the limited number of cases of GIST with hypoglycemia, its characteristics remain understudied, and available data are mostly limited to single case reports. Understanding the characteristics and landscape of GISTs with hypoglycemia will provide further insights into the disease and will be informative for setting up effective treatment strategies with prognostic implications. Here, to gain further insight in this condition, we aimed to catalogue the prevalence, characteristics, prognosis, and cause of GISTs with hypoglycemia through a single-institution retrospective analysis.

Materials and methods

Clinicopathological characteristics of the patients. We extracted patient data from Keio University Hospital patients who were diagnosed with GISTs between April 1, 2011, and March 31, 2023. Additionally, we extracted data from patients with episodes of hypoglycemia (serum blood sugar level <70 mg/dl). Pathological samples for proteomic and genetic analyses were obtained from Keio University Hospital, and all procedures were performed in accordance with the protocol approved by the Keio University Hospital Institutional Ethics Committee (approval number: 20200347). The study protocol adhered to the Declaration of Helsinki and the Ethical Guidelines for Medical and Health Research Involving Human Subjects.

Serum IGF-I/IGF-II measurement, Immunohistochemistry and immunoreactivity. IGF-I was measured with electrochemiluminescence immunoassay (ECLIA method) by SRL Diagnostics (Tokyo, Japan) using Elecsys® kit. IGF-II was measured with radioimmunoassay (RIA) by Mayo Clinic Laboratories (Rochester, MN, USA) using their original assay kit created by Labcorp Inc.

Immunohistochemistry (IHC) was performed on formalin-fixed, paraffin-embedded (FFPE) sections using an automated Bond-Max stainer (Leica Biosystems, Melbourne, Australia) and primary antibodies against IGF-II (clone S1F2, 1:250; Merck Millipore, Darmstadt, Germany), Ki-67 (clone MIB-1, 1:200; DAKO, Santa Clara, CA, USA), and c-kit (clone A4502, 1:100; DAKO). The samples were incubated in citrate-based pH 6.0 epitope retrieval buffer 1 (Leica Biosystems, Wetzlar, Germany) for 20 min prior to the application of the primary antibodies for IGF-II and c-kit (30 min) and in ethylenediaminetetraacetic acid-based pH 9.0 epitope retrieval buffer 2 (Leica Biosystems) for 20 min prior to the application of the primary antibody for Ki-67 (30 min). All steps were performed according to the manufacturer's protocol, and the antigens were detected using the Bond Polymer Refine Detection Kit (Leica Biosystems, Newcastle Upon Tyne, United Kingdom). Placental tissue was used as the positive control for IGF-II.

IGF-II and c-kit staining were quantitatively evaluated using a BZ-X Analyzer (BZ-H4C; KEYENCE, Osaka, Japan). Five microphotographs per tissue area were randomly obtained (Nikon Digital Sight DS-R1i; Nikon Corporation, Tokyo, Japan)

at 400x magnification. Positive areas were automatically calculated using a hybrid cell count application on the BZ-X Analyzer (BZ-H4C). The number and cytoplasmic area of tumor cells in each tissue area were calculated using a BZ-X Analyzer (BZ-H4C). Quantitatively positive areas were corrected using the ratio of the mean number and cytoplasmic area of tumor cells between each area to eliminate the effects of these parameters. The Ki-67 index was expressed as the percentage of Ki-67-positive tumor cells among the total number of tumor cells, regardless of the immunostaining intensity. Counts were manually performed in five randomly selected fields in the hotspot area at 400x magnification. IHC staining of KIT and CD34 from different time points (4, 8, 14 years ago) were equivalent suggesting their maintained sample quality.

DNA extraction from FFPE tissues. FFPE tissues obtained from the autopsy specimens of liver metastatic lesions were used for DNA extraction. The tissues were collected and fixed in 10% neutral-buffered formalin for 48 h prior to genomic analysis. Next, 10-μm-width sections were obtained from FFPE tissue blocks, deparaffinized with xylene, and rehydrated in a graded series of ethanol. Genomic DNA was extracted from the FFPE sections using the GeneRead DNA FFPE Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Carlsbad, CA, USA) and Qubit 2.0 Fluorometer (Thermo Fisher Scientific) were used to quantify DNA, following the recommended protocols.

Whole exome sequencing and mutation identification. Whole exome sequencing (WES) DNA libraries were prepared using the xGen Exome Research Panel v2 (Integrated DNA Technologies, Inc., Coralville, IA, USA) and sequenced using the NovaSeq 6000 system (Illumina, San Diego, CA, USA). Genome annotation and curation for sequencing data analysis were performed using an original bioinformatics pipeline called PleSSision (Mitsubishi Space Software, Tokyo, Japan) (18). In this pipeline, next-generation sequencing reads were mapped to the human reference genome (UCSC human genome 19) using the Illumina DRAGEN Bio-IT Platform v3.8. To identify single-nucleotide variants (SNVs), SAMtools (19) was used to accumulate the sequencing reads, and defective SNVs that showed conflicts between pairwise reads were discarded. We identified somatic mutations by comparing the number of mismatched bases in tumors with those in normal control samples using Fisher's exact test ($P < 0.001$). We identified cancer-specific somatic genomic alterations, such as SNVs, indels, and copy number alterations. The tumor mutation burden (TMB) was defined as the number of non-synonymous mutations in the entire region. All detected genomic alterations in 728 census genes (COSMIC v87) were annotated and curated using the COSMIC (<https://cancer.sanger.ac.uk/cosmic>), ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/>), CIViC (<https://civicdb.org/home>), SnpEff24 (20), and Clinical Knowledge Base (<https://ckb.jax.org/>) databases.

Protein sample preparation from FFPE tissue for liquid chromatography-mass spectrometry (LC-MS). FFPE tissues obtained from autopsy specimens of metastatic liver lesions used for DNA extraction were also used for protein extraction.

Briefly, 10- μ m-thick sections were obtained from FFPE tissue blocks, deparaffinized with xylene, and rehydrated in a graded series of ethanol. The dissected and collected tissue samples were incubated in protein extraction buffer [300 mM Tris-hydrochloride, 2% sodium dodecyl sulfate (SDS), and 4% β -mercaptoethanol] for 1 h at 90°C. Next, the samples were centrifuged for 10 min at 15,000 $\times$ g and 4°C, and the supernatant containing the extracted proteins and recombinant human IGF-II (Proteintech, Chicago, IL, USA) was subjected to SDS-polyacrylamide gel electrophoresis on 15% e-PAGEL mini gel (ATTO Co., Ltd., Tokyo, Japan). The gel was stained with EzStain Aqua (ATTO Co., Ltd.). Bands of 15 kDa in the extracted protein lane were excised and subjected to LC-MS (Kazusa Genome Technologies, Inc., Chiba, Japan).

In-gel digestion and LC-MS. Protein bands were excised and in-gel digestion was performed, as previously described (21). The digested peptides were directly injected into a 75 μ m $\times$ 12-cm PicoFrit emitter (New Objective) at 40°C and then separated using a 30-min gradient at a flow rate of 200 ml/min using an UltiMate 3000 RSLCnano LC system (Thermo Fisher Scientific). The peptides eluted from the column were analyzed by data-dependent acquisition-mass spectrometry using Q Exactive HF-X (Thermo Fisher Scientific). MS1 spectra were collected in the range of 380-1,240 m/z with 60,000 resolutions to hit an automatic gain control (AGC) target of 3×10^6 and a maximum injection time of 100 ms. The 30 most intense ions with charge states of 2+ to 5+ were dissociated in a data-dependent manner by collision-induced dissociation with step-normalized collision energies of 22, 26, and 30. Tandem mass spectra were acquired on an Orbitrap mass analyzer with 30,000 resolutions to set an AGC target of 1×10^5 and a maximum injection time of 55 ms. The MS files were searched against the human UniProt reference proteome (Proteome ID UP000005640, reviewed, canonical; 20,588 entries) using PEAKS Studio. The search parameters were as follows: precursor mass tolerance, 10 ppm; fragment ion mass tolerance, 0.015 Da; enzyme, trypsin; variable modifications; and oxidation (M). Peptide and protein identification was filtered so that both the peptide and protein false discovery rates were <1%.

Statistical analysis. Overall survival was assessed using the log-rank test and summarized using Kaplan-Meier methods. Hazard ratios were calculated using Cox proportional hazards regression models. All p-values were based on two-tailed hypotheses, and $P < 0.05$ was considered statistically significant. Statistical analyses were performed using the JMP version 15.1.0 software (SAS Institute, NC, USA). IGF-II positive area and Ki67 index were compared as follows: Differences between two groups were evaluated using two-sided paired Student's t-tests. Comparisons of more than two groups were performed with repeated-measures ANOVA followed by Bonferroni's multiple comparison test. Statistical analyses were performed using Graph-Pad Prism software version 9.0 g (GraphPad software Inc. CA, USA).

Results

Recurrent hypoglycemia is a poor prognostic factor in GISTs. Data of patients who were diagnosed with GISTs (all stages)

within the past 12 years (between April 1, 2011, and March 31, 2023) were extracted from the single-institution database (N=195) (Table SI). To identify patients with GISTs who experienced an episode of hypoglycemia during their clinical course, we defined hypoglycemia as plasma blood sugar level <70 mg/dl according to the current criteria (22). Eight patients out of 195 patients met this criteria (4.1%). The causes of hypoglycemia were diverse, including tumor growth, adverse drug effects, post-operation, liver failure, and unknown (Table SI). Although hypoglycemic episodes are generally inferred to cause poor prognosis, several confounding factors derived from the underlying fatal disease make it difficult to manifest the prognostic effect of hypoglycemic episodes in cancer patients. To avoid these confounding factors, we focused on patients with unresectable or metastatic GISTs (N=35). Among these 35 patients, five patients experienced an episode of hypoglycemia, suggesting a prevalence of 14.2% (5/35) in patients with unresectable or metastatic GISTs. To minimize the confounding of individual treatment effect, we also excluded the patients who were currently under active chemotherapy (N=8) for our survival analysis. We defined 'survival' as the prognosis after the final administration of chemotherapy and analyzed the data of 27 patients (Fig. 1A). The clinical characteristics of the study population are summarized in Table I. The survival curve between the two groups (episodes with/without hypoglycemic episodes) did not show any statistically significant survival differences ($P=0.55$) (Fig. 1B). Based on this observation, we hypothesized that transient hypoglycemic episodes would have a marginal effect on survival compared with recurrent hypoglycemic episodes. Thus, we divided the five patients who experienced hypoglycemic episodes into two groups: transient and recurrent episodes of hypoglycemia. Transient hypoglycemia was defined as a single episode of hypoglycemia, and recurrent hypoglycemia was defined as a hypoglycemic episode more than twice at least one week apart or persistent hypoglycemia. To confirm our hypothesis, we compared the survival between these two groups. The prevalence of recurrent hypoglycemic episodes in patients with unresectable or metastatic GISTs was 5.7% (2/35). Although the number of patients was small, those with recurrent hypoglycemia had shorter survival rates than those with transient hypoglycemia (Fig. 1C). Overall, patients with recurrent hypoglycemia showed poorer survival than those without recurrent episodes of unresectable or metastatic GISTs ($P=0.007$) (Figs. 1D and S1A).

Clinical course and retrospective diagnosis of patients with metastatic GISTs with recurrent hypoglycemia. From the survival analysis, we found that patients with GISTs with recurrent hypoglycemic episodes had a poorer prognosis than those with no or transient episodes of hypoglycemia. Although the number of patients who suffered from recurrent hypoglycemic episodes was limited, we considered that the identification of the cause of hypoglycemia would improve the comprehensiveness of our catalogue. We performed the initial screening for the cause of hypoglycemia by focusing on the metastatic sites (such as pancreas, brain, adrenal gland), and history of surgical resection (such as gastrectomy, small intestine) but none of them made a reasonable explanation as the cause of hypoglycemia (Table I). We also evaluated the

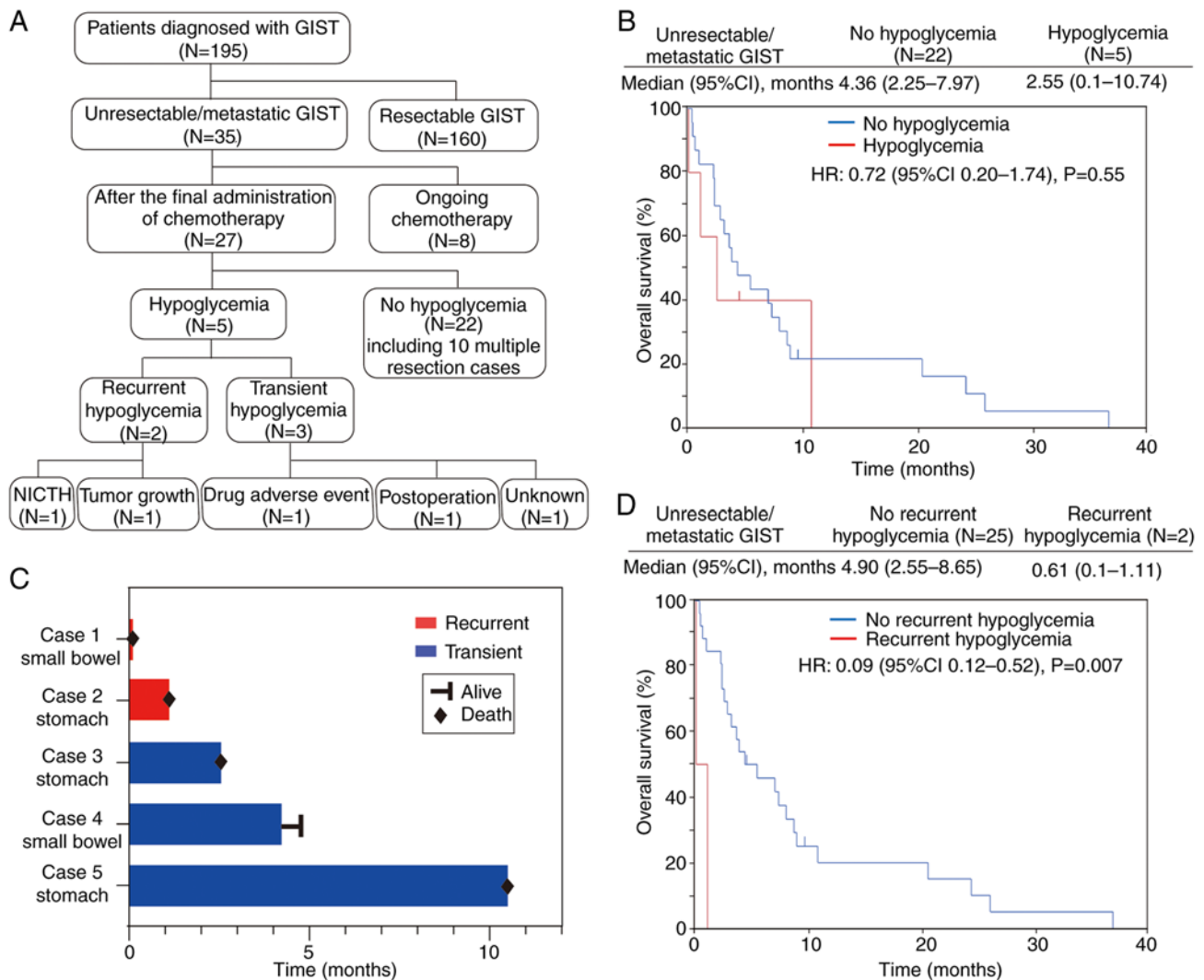


Figure 1. Patients with GISTs with recurrent hypoglycemia have a poor survival. (A) Overview of patients with GISTs and details of patients with unresectable or metastatic GISTs with hypoglycemia who completed chemotherapy. (B) Overall survival curve after the final administration of chemotherapy in patients with unresectable or metastatic GISTs with and without episodes of hypoglycemia. (C) Swimmer's plot after the final administration of chemotherapy for each patient with unresectable or metastatic GISTs with hypoglycemia, separately shown in the recurrent and transient cases. (D) Overall survival curve after the final administration of chemotherapy for patients with unresectable or metastatic GISTs with or without recurrent episodes of hypoglycemia. GIST, gastrointestinal stromal tumor; HR, hazard ratio; NICTH, non-islet cell tumor hypoglycemia.

effect of tumor progression (Fig. S1B) and weight loss (which is a surrogate marker for cachexia). Interestingly, we did not observe significant weight loss which satisfied the criteria of cachexia (weight loss >5% or 'BMI <20 and weight loss >2%' within the past 6 months) (23), while tumor progression was remarkable in both case1 and 2 (Fig. S1C and D). Drug adverse event remained as a possible cause. Indeed, 1 patient out of 3 patients who had an episode of transient hypoglycemia was caused by drug adverse event from the combination of insulin and sunitinib. We identified this cause from the episode that hypoglycemia recovered smoothly after discontinuation of sunitinib. For the remaining 2 cases, there were no possible causality with drug administration and their episode of transient hypoglycemia. Thus, the clinical course and cause of recurrent hypoglycemia in these 2 cases were retrospectively investigated in detail.

Case 1: 60-year-old man diagnosed with small bowel GIST. The patient's clinical course is shown in Fig. 2A. The patient

underwent small bowel resection in 2011. The patient had a recurrence of GIST with peritoneal metastasis in 2015, and imatinib mesylate (400 mg) treatment was initiated. A rash was considered as an adverse event, and the treatment was switched to sunitinib (37.5 mg). Sunitinib was replaced with imatinib mesylate (300 mg) in 2018 after progressive disease. Without therapeutic response, the treatment plan of the patient changed into best supportive care. During his final phase of this clinical course, he was comatose due to severe persistent hypoglycemia, and the patient died after 2 days of hospital admission. There were no signs of liver failure from tumor involvement, and the patient did not have diabetes. The patient's blood glucose and glycated hemoglobin (HbA1c) levels at the outpatient clinic were within the normal range (Fig. S2A). Owing to the sudden onset, case 1 had remarkably limited blood checkups, and no tissue sample collection which made it difficult to approach the cause of hypoglycemia retrospectively. From the initial screening and the following clinical information, we concluded the cause of hypoglycemia in case1 as tumor growth.

Table I. Clinical characteristics of unresectable or metastatic gastrointestinal stromal tumor associated with hypoglycemia.

Variables	Recurrent hypoglycemia (n=2)	Transient hypoglycemia (n=3)	No hypoglycemia (n=22)
Median age, years (range)	59 (57-61)	61 (56-67)	65 (48-84)
Male sex, n (%)	2 (100)	2 (67)	14 (64)
Primary tumor, n (%)			
Stomach	1 (50)	2 (67)	7 (32)
Duodenum	0 (0)	0 (0)	2 (9)
Small bowel	1 (50)	1 (33)	12 (55)
Others	0 (0)	0 (0)	1 (5)
Metastasis, n (%)			
Liver	2 (100)	3 (100)	13 (59)
Peritoneum	2 (100)	0 (0)	14 (64)
Lung	1 (50)	0 (0)	2 (9)
Subcutaneous	0 (0)	1 (33)	0 (0)
Surgical site			
Stomach, n (%)	1 (50)	2 (67)	5 (23)
Partial gastrectomy	1	2	5
Total gastrectomy	0	0	0
Duodenum, n (%)	0 (0)	0 (0)	2 (9)
Small bowel, n (%)	1 (50)	1 (33)	11 (41)
Median length of resection, cm (range)	22	20	21 (10-28)
Liver, n (%)	1 (50)	0 (0)	10 (45)
Peritoneum, n (%)	0 (0)	0 (0)	6 (27)
Subcutaneous, n (%)	0 (0)	1 (33)	0 (0)
Median number of regimens (range)	2.5 (2-3)	2 (1-3)	2 (1-4)
Regimen, n (%)			
Imatinib	2 (100)	3 (100)	22 (100)
Sunitinib	2 (100)	2 (67)	14 (64)
Regorafenib	1 (50)	0 (0)	7 (32)
KIT mutation, n (%)			
Exon 9	1 (50)	0 (0)	3 (14)
Exon 11	1 (50)	0 (0)	5 (23)
Exon 17	1 (50)	0 (0)	3 (14)
Unmeasured	0 (0)	3 (100)	15 (68)
Cause of hypoglycemia, n (%)			
NICTH	1 (50)	0 (0)	-
Tumor growth	1 (50)	0 (0)	-
Drug adverse events	0 (0)	1 (33)	-
Postoperation	0 (0)	1 (33)	-
Unknown	0 (0)	1 (33)	-

NICTH, non-islet cell tumor hypoglycemia.

Case 2: 68-year-old man diagnosed with gastric GIST. The patient's clinical course and sample collection are shown in Fig. 2B. The patient underwent a partial gastrectomy in August 2010. Relapse was observed in September 2012, and treatment with imatinib mesylate (400 mg) was initiated. During medical treatment, limited progression was observed in the relapsed liver metastasis, and partial hepatectomy was performed in November 2016. The pathological micrometastases remained

in the liver, and the patient continued treatment with imatinib. After 7 years of imatinib treatment, imatinib was switched to sunitinib (50 mg) owing to the appearance of new metastatic lesions in the liver and lung. After 5 months of treatment, sunitinib was switched to regorafenib (160 mg) because of disease progression. After 8 months of treatment, with the appearance of peritoneal metastasis, the treatment regimen was changed with the reintroduction of imatinib. In August

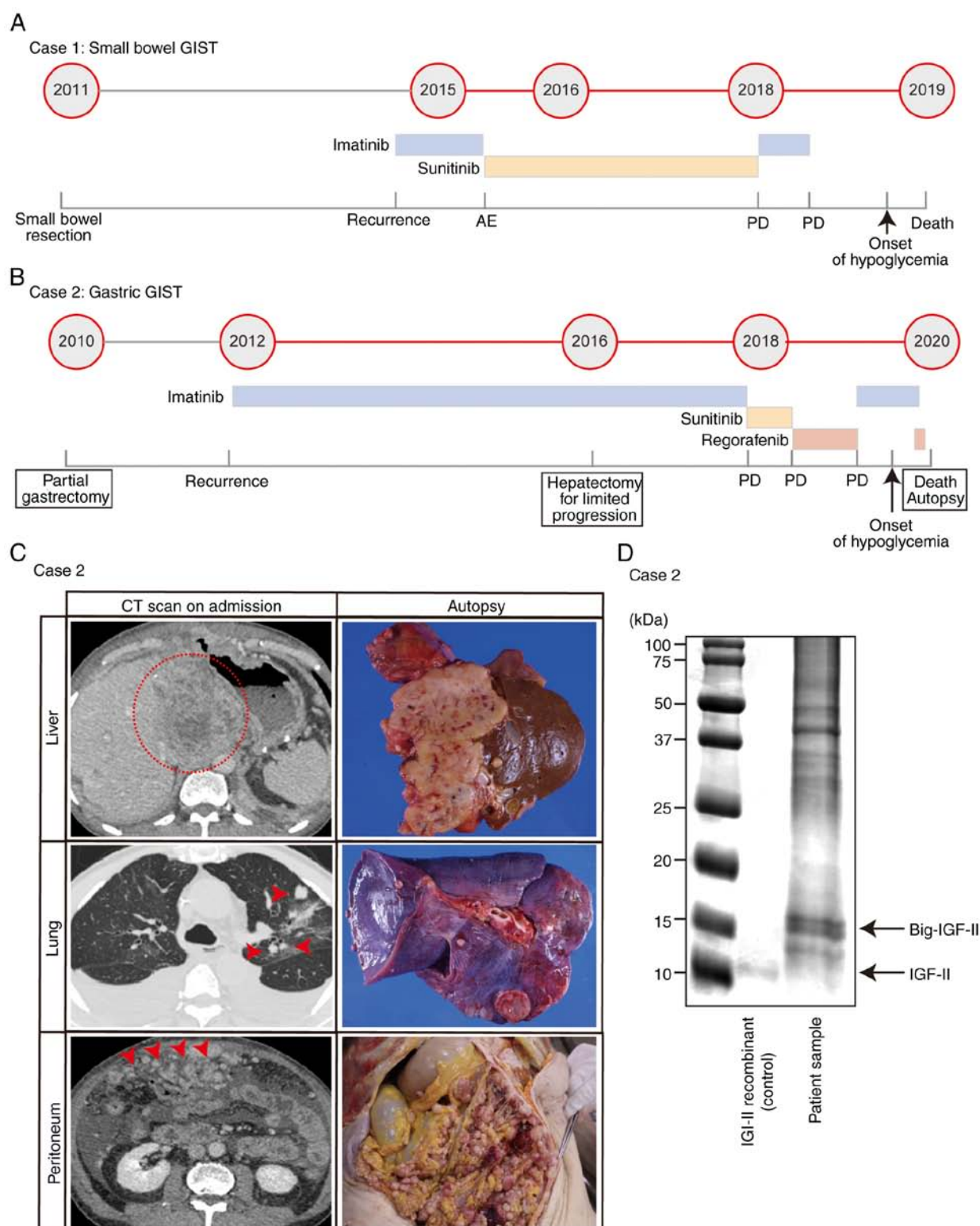


Figure 2. Clinical course and retrospective diagnosis of patients with metastatic GISTs with recurrent hypoglycemia. Clinical courses of unresectable or metastatic GISTs with recurrent episodes of hypoglycemia in (A) case 1 and (B) case 2. Numbers in the circle indicate the year. Timepoints of sample collection are shown at the bottom and indicated with a black square for case 2. (C) Metastatic lesion on admission (CT scan) and autopsy in case 2. On the CT scans, the red circle indicates liver metastasis, and red arrows indicates lung and peritoneal metastases. (D) Detection of IGF-II with protein analysis in case 2. Recombinant IGF-II (control) is shown on the left, and the protein extracted from the patient pathology sample was loaded on the right. The protein bands that include big-IGF-II and IGF-II are indicated with arrows. AE, adverse event; GIST, gastrointestinal stromal tumor; IGF-II, insulin-like growth factor II; PD, progressive disease.

2020, the patient was comatose and was admitted to a nearby hospital. His blood glucose level was 40 mg/dl upon arrival. The patient's blood glucose and HbA1c levels at the outpatient

clinic were within the normal range (Fig. S2B). Liver function and other hormone levels were also within the normal range, except for growth hormone (0.37 ng/ml), insulin ($<0.5 \mu\text{U/ml}$),

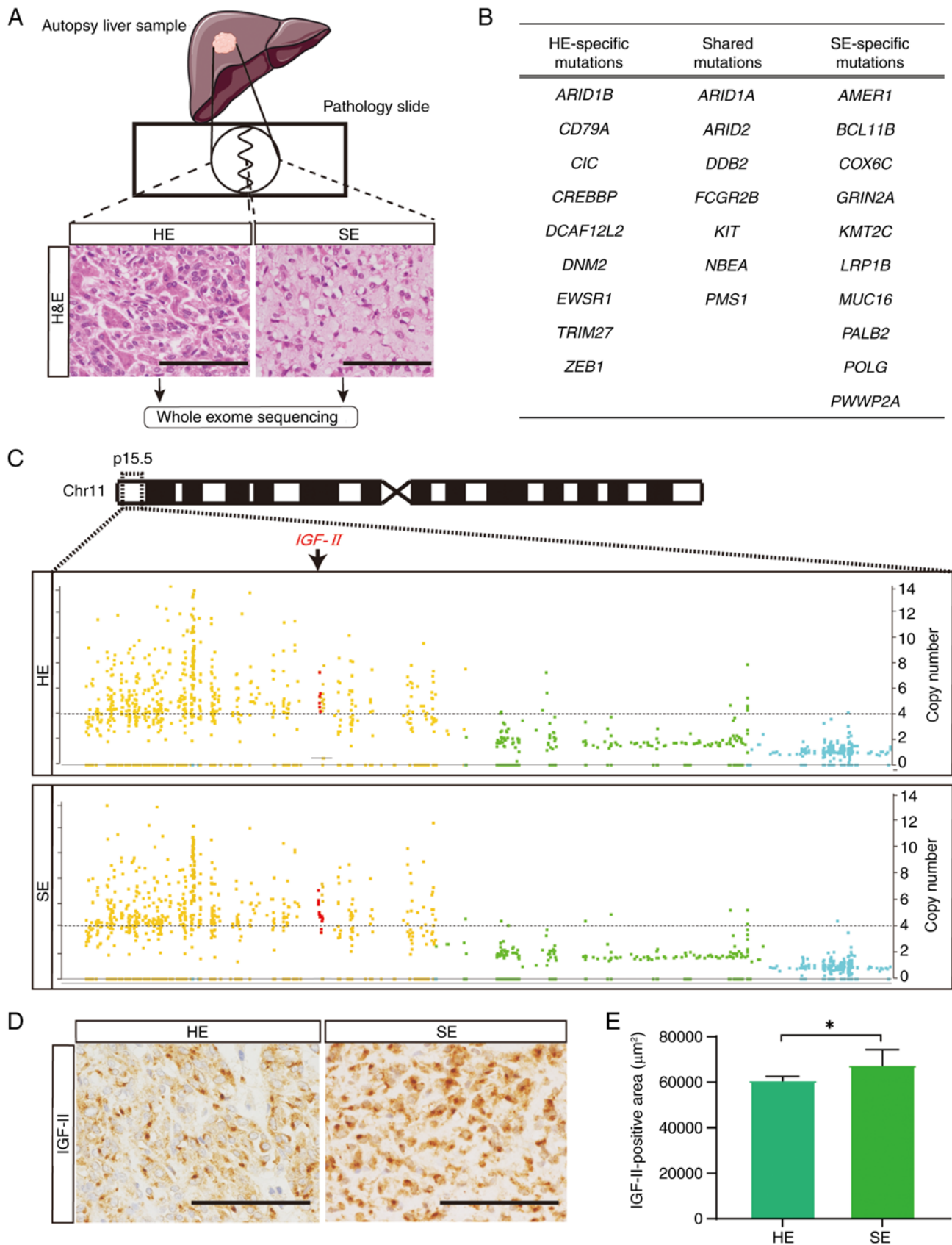


Figure 3. Genomic analysis of two distinct histological components expressing IGF-II in case 2 (patient with non-islet cell tumor hypoglycemia-gastrointestinal stromal tumor). (A) Schematic of the genome extraction from formalin-fixed paraffin-embedded samples. Each histological tumor component was dissected and used for whole exome sequencing. Scale bar, 100 μm . (B) Genomic mutations from each component. HE-specific mutations are on the left, shared mutations between the two components are in the middle and SE-specific mutations are on the right. These genomic mutations are restricted to COSMIC census genes. (C) Copy number variant of 11p15.5 chromosomal area. HE is indicated on the top and SE on the bottom. The y-axis indicates the copy numbers. Blue dots indicate $\text{CN}<2$, green dots indicate $2<\text{CN}<4$ and yellow dots indicate $\text{CN}>4$. IGF-II is indicated as red dots. (D) IGF-II immunohistochemistry staining of HE and SE components in liver metastasis. Scale bar, 100 μm . (E) Quantitative analysis of the positive area of IGF-II. * $P<0.05$, paired Student's t-test. CN, copy number; HE, hypercellular epithelioid; IGF-II, insulin-like growth factor II; SE, sclerosing epithelioid.

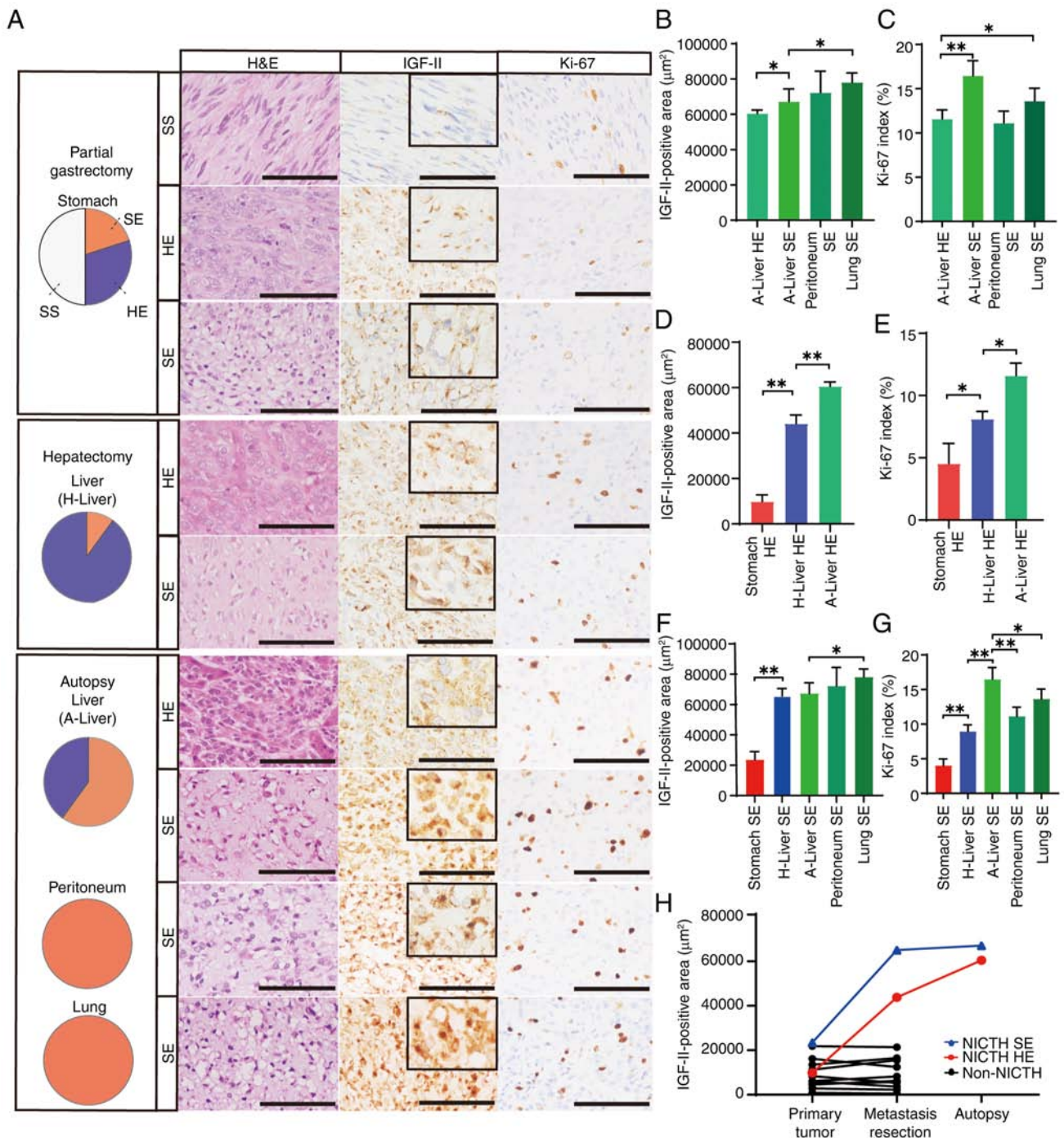


Figure 4. Chronological IGF-II expression in each histological tumor component of case 2 (NICTH-GIST). (A) H&E, IGF-II and Ki-67 staining of liver, peritoneal and lung metastases from the partial gastrectomy (primary), hepatectomy and autopsy samples. The circle indicates the proportion of each pathological component observed in the tumor. Insets show images of IGF-II-stained slides. Original magnification, x20. Scale bar, 100 μm. (B) Quantitative analysis of IGF-II-positive area for each metastatic site at autopsy. (C) Quantitative analysis of Ki-67 (%) for each metastatic site at autopsy. (D) Quantitative analysis of IGF-II-positive area in the HE component at each time point. (E) Quantitative analysis of Ki-67 (%) in the HE component at each time point. (F) Quantitative analysis of IGF-II-positive area in the SE component at each time point. (G) Quantitative analysis of Ki-67 (%) in the SE component at each time point. (H) Comparison of IGF-II expression in paired samples between 1 NICTH GIST case and 10 non-NICTH GIST cases. NICTH-GIST SE and HE components are shown as blue and red lines, respectively. * $P < 0.05$, ** $P < 0.01$. Comparisons of more than two groups were performed with repeated-measures ANOVA followed by Bonferroni's multiple comparison test. A, autopsy; GIST, gastrointestinal stromal tumor; H, hepatectomy; HE, hypercellular epithelioid; IGF-II, insulin-like growth factor II; NICTH, non-islet cell tumor hypoglycemia; SE, sclerosing epithelioid; SS, sclerosing spindle.

and IGF-I (20 ng/ml), which showed low serum levels, and the IGF-II/IGF-I ratio (20.7). The patient was initiated on treatment for hypoglycemia with the continuous use of glucocorticoid and glucose doses (Fig. S2C). Despite these efforts, the patient died in November 2020, only 3 months after the

first episode of hypoglycemia, and underwent an autopsy with the patient's consent.

For Case 2, extensive blood testing and autopsy were conducted. In this case, multiple metastases were observed in the liver, lung, and peritoneum on computed tomography

Table II. Characteristics of 10 patients with non-non-islet cell tumor hypoglycemia-gastrointestinal stromal tumors who underwent resection of primary and metastatic lesions.

Patient no.	Age, years	Sex	Primary lesion	Metastatic lesion	Maximum diameter, cm ^a	Plasma glucose, mg/dl ^b
1	56	F	Stomach	Liver	2.8	99
2	35	M	Stomach	Peritoneum	3.0	119
3	62	M	Duodenum	Liver	3.0	104
4	62	M	Duodenum	Liver	7.0	118
5	79	F	Small bowel	Liver	1.3	136
6	77	F	Small bowel	Peritoneum	2.8	103
7	59	M	Small bowel	Peritoneum	4.5	98
8	55	F	Small bowel	Peritoneum	7.5	135
9	64	M	Small bowel	Peritoneum	7.5	119
10	70	M	Small bowel	Liver	15.0	134

^aMaximum diameter of the resected metastasis sample. ^bPlasma glucose level before surgical metastasectomy. F, female; M, male.

(Fig. 2C). Based on the result of IGF-II/IGF-I ratio from the blood test, we suspected NICTH as the cause of hypoglycemia. For the diagnosis, we extracted protein from the paraffin block of the autopsied liver metastasis and attempted to detect big-IGF-II following previously reported diagnostic criteria (16). As expected, a band was observed at 11-18 kDa, which is considered the molecular weight of big-IGF-II from the literature, compared with 7.5-10 kDa, which is the standard for IGF-II (24) (Fig. 2D). Based on these results, we concluded that the recurrent hypoglycemia in case 2 was due to NICTH.

Genomic analysis of two distinct histological components expressing IGF-II in NICTH-GIST. To determine the cause of NICTH in case 2, we focused on autopsied liver metastasis, in which big-IGF-II protein was detected. Interestingly, we observed two distinct histological phenotypes of liver metastasis: hypercellular epithelioid (HE) and sclerosing epithelioid (SE) (Fig. 3A) (25). HE tumors were highly cellular with well-defined cell borders, and SE, the most common variant of gastric GISTs, showed variably sclerosing stroma without visible cell borders, as previously described (25). HE and SE constituted approximately 40% and 60% of the autopsied liver metastasis, respectively (Table SII). To verify the two components from a genomic perspective and to investigate the mechanism underlying big-IGF-II production, we performed WES for each component (Fig. 3A). DNA was extracted from the paraffin sections and processed for WES. The quality of the DNA genome is confirmed with DNA integrity number which was above 2.3 (26). The genomic profiles of the two histological components were remarkably different (Fig. 3A). In particular, the TMB differed between the two components, with 90 non-synonymous SNVs in SE and 200 non-synonymous SNVs in HE. Specific mutations that differed between the two components were epigenetic mutations, such as *CREBBP* and *ARID1B* in HE and *KMT2C* in SE (Fig. 3B). Although these two histological phenotypes were genomically distinct, there were several shared mutations including *KIT* (Exon 11,17), suggesting a common ancestor of the two phenotypes (Fig. 3B). Since we detected

big IGF-II on our protein analysis, we next focused on the IGF-II region (chr11p15.5). Unfortunately, we did not detect a mutation in the open reading frame of *IGF-II*, but instead, we detected an amplification of *IGF-II* regions (CNV=4) which may have served as the source of IGF-II expression forming big-IGF-II (Fig. 3C). Furthermore, *IGF-II* amplification was present in both tumor components. To evaluate the expression of big-IGF-II in both histological components, we performed IGF-II IHC, which reflects big-IGF-II expression, following the evaluation method described in previous studies (27). Although IGF-II expression was higher in the SE component, it was also strongly expressed in the HE component, suggesting the presence of IGF-II in both components in line with the genomic results (Fig. 3D and E). Overall, we identified genetic commonalities and differences between the two histologically distinct components of the tumor, indicating that *IGF-II* amplification was the source of big-IGF-II.

Chronological IGF-II expression in each histological tumor component of NICTH-GIST. To identify whether liver metastasis was the only source of IGF-II, we evaluated other metastatic sites (lung and peritoneum) obtained during autopsy. First, we investigated tumor heterogeneity as observed in liver metastases (Fig. 4A). Interestingly, the peritoneal and lung metastases showed only SE histology (Fig. 4A). We also evaluated IGF-II staining and observed that the samples were positive for IGF-II, similar to the liver metastasis sample from the autopsy (Fig. 4A). Furthermore, comparison of each metastatic site within the autopsy sample indicated that the HE component of the liver metastasis had the lowest IGF-II expression at all sites, and peritoneal SE and lung SE showed higher expression than liver SE (Fig. 4B). Regarding the Ki-67 index, the liver SE showed the highest expression (Fig. 4C). This result indicated that not only liver metastasis but also peritoneal and lung metastases were sources of big-IGF-II, suggesting that the IGF-II-expressing clone may have appeared prior to metastasis.

Next, we attempted to determine when the tumors began to produce IGF-II. Since tumor resection with limited

Table III. Literature review on NICTH-GIST.

First author/s, year	Case	Age, years	Sex	Primary lesion	Metastatic lesion	Histological subtype	Max ^a , cm	Diagnosis of NICTH	KIT status	Treatment of NICTH	Outcome	Autopsy/ WES (Refs.)
Pink <i>et al</i> , 2005	1	35	M	Small bowel	Liver/peritoneum	N.S.	N.S.	Plasma IGF-I/II	N.S.	Surgery/Imatinib	Cured	-/- (36)
Escobar <i>et al</i> , 2007	2	39	M	Small bowel	Liver	N.S.	40	Plasma pro-IGF-IIE, IHC	Exon 9	Surgery	Cured	-/- (37)
Tan <i>et al</i> , 2011	3	63	F	Small bowel	Peritoneum/liver	N.S.	15.9	Plasma IGF-I	N.S.	Surgery	Cured	-/- (38)
Dimitriadis <i>et al</i> , 2015	4	70	F	Small bowel	Abdomen/liver/ pelvis	Hypocellular/ spindle	14	Plasma IGF-I/II	N.S.	Glucocorticoids/ Imatinib	Cured	-/- (39)
Hirai <i>et al</i> , 2016	5	61	F	Small bowel	Liver	Spindle/ epithelioid	10	Protein analysis	N.S.	Glucocorticoids/ Imatinib	Cured	-/- (10)
Wilson <i>et al</i> , 2017	6	60	M	Small bowel	Lung/rib	Spindle	24	Plasma IGF-I	Exon 11	Imatinib	N.S.	-/- (40)
Pink <i>et al</i> , 2005	7	59	M	Duodenum	Liver	N.S.	10	Plasma IGF-I/II	N.S.	Imatinib	Cured	-/- (36)
Haeri <i>et al</i> , 2021	8	59	F	Duodenum	Liver	N.S.	N.S.	Plasma IGF-I/II	N.S.	Glucocorticoids	Died	-/- (41)
Takebayashi <i>et al</i> , 2020	9	65	M	Small bowel	Pelvis/ peritoneum	Spindle	N.S.	IHC	Exon 11	Glucose infusion/ glucocorticoids	Died	-/- (42)
Davda and Seddon, 2007	10	44	F	Small bowel	Peritoneum	N.S.	N.S.	Plasma IGF-I/II	N.S.	Glucocorticoids/ Imatinib	Died	-/- (43)
Hamberg P, 2006	11	57	M	N.S.	Liver	N.S.	N.S.	Plasma pro-IGF-IIE	N.S.	Glucose infusion	Cured	-/- (44)
Hamberg <i>et al</i> , 2006	12	52	F	N.S.	Intra-abdominal	N.S.	N.S.	Plasma pro-IGF-IIE	N.S.	Imatinib	Died	-/- (44)
Beckers <i>et al</i> , 2003	13	69	M	Pelvic mass	None	Spindle	15	Plasma pro-IGF-IIE, IHC	N.S.	Surgery	Cured	-/- (45)
Dean <i>et al</i> , 2012	14	54	F	Pelvic mass	None	N.S.	13	N.S.	N.S.	Surgery	Cured	-/- (46)
Singhal <i>et al</i> , 2017	15	65	F	Pelvic mass	None	N.S.	18	Plasma insulin	N.S.	Glucocorticoids/ Imatinib	Cured	-/- (47)
Saeed <i>et al</i> , 2016	16	64	F	Ovary	Peritoneum	Spindle	7	Plasma IGF-I/II	N.S.	Glucocorticoids/ Imatinib	Cured	-/- (48)
Rikhsaf <i>et al</i> , 2005	17	50	M	Retroperi- toneum	Liver	N.S.	N.S.	Plasma pro-IGF-IIE	N.S.	Glucose infusion	Died	-/- (49)
Guiteau <i>et al</i> , 2006	18	65	F	Stomach	Peritoneum	Spindle	44	N.S.	N.S.	Surgery	Cured	-/- (50)
Yamasaki <i>et al</i> , 2020	19	84	M	Stomach	None	Spindle	11.6	Protein analysis, IHC	N.S.	Surgery	Cured	-/- (27)
Dhali <i>et al</i> , 2021	20	46	F	Stomach	None	Epithelioid	5	Protein analysis, IHC	N.S.	Surgery	Cured	-/- (51)
Present case	Present case	68	M	Stomach	Peritoneum/liver	Hypocellular/ sclerosing	16	Protein analysis, IHC	Exon 11, 17	Glucocorticoids/ Imatinib	Died	+/-

^aMaximum diameter of the tumor. F, female; GIST, gastrointestinal stromal tumor; IGF, insulin-like growth factor; IHC, immunohistochemistry; M, male; NICTH, non-islet cell tumor hypoglycemia; N.S., not specified; WES, whole exome sequencing.

progression (referred to as debulking surgery) improves the prognosis of patients with GISTs (28,29), this patient had two resected samples that were available for analysis (Fig. 2B). We also evaluated the histological heterogeneity of the previously resected samples. The archived samples showed that the initial primary gastric tumor had a sclerosing spindle (SS) component (50%), an HE component (30%), and an SE component (20%) (Fig. 4A). The SS component was absent from the resected liver sample, which consisted only of the HE (90%) and SE (10%) components (Fig. 4A). As expected, IGF-II was expressed in all the components. We evaluated the proclivity of IGF-II expression in tumor samples. Interestingly, IGF-II was highly expressed in the autopsy samples compared with the rest of the samples, regardless of the components, and the Ki-67 index showed the same proclivity as IGF-II (Fig. 4D-G). These results suggest that both HE and SE contribute to IGF-II expression causing NICTH.

Based on these observations, we investigated a clinical method to predict the development of NICTH-GIST. Since IGF-II expression is also observed in non-NICTH-GIST from the literature (44%) (30), we hypothesized the chronological increase of IGF-II expression as a unique observation in NICTH-GIST. To demonstrate this hypothesis, we selected 10 non-NICTH-GIST cases with a history of multiple resections to confirm the IGF-II expression changes in patients without hypoglycemia (Fig. 1A; Table II). We stained 10 non-NICTH-GIST-paired samples and compared them with the NICTH sample. As expected, while NICTH case showed remarkable increase of IGF-II expression, no chronological elevation in IGF-II expression were observed in the 10 non-NICTH-GIST cases (Fig. 4H). Furthermore, we noticed a difference in distribution of IGF-II staining, while some of the non-NICTH-GIST samples showed slight expression of IGF-II limited to the Golgi apparatus, the expression of IGF-II in NICTH-GIST was broadly distributed in the Golgi apparatus and cytoplasm (Fig. S3).

Taken together, these results suggest that the IGF-II-expressing cells may have emerged before debulking surgery of the liver metastasis (i.e., 4 years prior to the episode of hypoglycemia), and the increase of IGF-II expression may potentially predict the onset of NICTH-GIST.

Discussion

We catalogued the characteristics of GISTs with hypoglycemia through a single-institution retrospective analysis. The prevalence of unresectable/metastatic GIST was 17.9% (35/195) of our whole cohort, which is lower than that of previous reports (31,32). Hypoglycemia was observed in 4.1% of patients with GISTs (all stages) and 14.2% of patients with unresectable/metastatic GISTs. Moreover, 2 out of 35 patients with unresectable/metastatic GISTs had recurrent hypoglycemic episodes, and they had a worse prognosis than patients who had no or a single episode of hypoglycemia. From our initial analysis for the cause of 2 recurrent hypoglycemia cases, we could not find a reasonable explanation of hypoglycemia such as metastatic sites, history of surgical resection, and cachexia.

For one of these cases, we succeeded in retrospective diagnosis of NICTH. NICTH is a serious paraneoplastic

syndrome associated with several types of cancers, including GISTs (9,11,33). As far as we know, there are 20 GIST cases which has been described in the past literature (Table III), and we present the first case of the autopsy and genetic sequencing of NICTH-GIST. Through the autopsy, we obtained the opportunity to investigate the histological differences between tumors and this led us to the identification of two histologically and genetically distinct components. Although two cases from the small intestine and a single case of colon cancer with NICTH also reported on a mixed histological subtype, we succeeded in demonstrating that these histological subtypes were most likely derived from a common ancestor through genomic analysis (34). The genomic analysis also provided us with a novel mechanism of NICTH. NICTH is known to be induced by protein-folding abnormalities, prohormone convertase abnormalities, and imprinting of IGF-II (13-15). In our case, we identified an additional potential mechanism of IGF-II abnormality, namely, *IGF-II* amplification. Specific mechanism how *IGF-II* amplification contributes to the generation of big-IGF-II is unclear. However, considering that there are no other genomic abnormalities in *IGF-II*, we would consider that the amplification of *IGF-II* takes part in the development of NICTH in this case.

The autopsy results of the NICTH case also provided us with an opportunity to evaluate where the IGF-II is produced. Based on the finding that the lung and peritoneal metastases also expressed IGF-II in our case, we identified several sources of IGF-II, which contributes to the tumor growth (FigS1B) (35). This observation is in line with the previous analysis of primary and metastatic NICTH colon cancer (34). This led us to the next question of when the tumor began to express IGF-II. Our result from the chronological analysis using archived samples showed continuous elevation of IGF-II expression specific to NICTH case. This result suggested that the emergence of IGF-II-expressing clones may have occurred in the early phase of tumor development. The genetic analysis data showing *IGF-II* amplification in both histological components also supported this hypothesis by suggesting that these two components derived from a common ancestor with *IGF-II* amplification.

Above all, we consider that our results provide novel data for comprehensive histological and genomic analyses of the natural course of NICTH-GIST. As for the clinical application, we would like to suggest the potential of our findings as a method for early detection of NICTH. We believe that using IGF-II staining in surgically resected samples might help predict the development of NICTH.

As for the limitations, i) this is a single-institutional study which may involve a potential of patients' sampling bias, ii) the discussion of recurrent hypoglycemic cases stems from 2 cases, and the discussion on NICTH stems from one single analysis which was treated with molecular targeted therapy, iii) Although it is unlikely, we cannot clarify the causality of the onset of NICTH and the treatment effect, iv) Comparison of IGF-II expression level of non-NICTH cases and NICTH cases are performed in IHC due to the lack of blood samples. Despite these limitations, we believe that our catalogue still provides a valuable clinical resource in the field of this rare disease condition.

In conclusion, we reported a single-institution retrospective analysis of GISTs with hypoglycemia. We showed that GISTs

with recurrent hypoglycemic episodes had a worse prognosis, and one of these cases was diagnosed with NICTH. Through comprehensive genetic and histological investigations of this single NICTH-GIST case, we identified a novel cause of NICTH with *IGF-II* amplification. Furthermore, while this case presented with sudden onset of hypoglycemic symptoms, our finding suggests that the emergence of clonal changes that leading to hypoglycemia may occur in the early phase of tumor development.

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Availability of data and materials

The whole-exome sequencing data generated in the present study are not publicly available as the study participant did not consent to the public sharing of the genomic data but may be requested from the corresponding author. The other data generated in the present study may be requested from the corresponding author.

Authors' contributions

AC, KK, TaK and YH were involved in the conception and design of the study. AC, JK, HH, ToK, SM, ES, SS, SK, SH, YS, KS, KTs, KTo, KH, HN and YK were involved in acquisition of the data. AC, KK, JK, HH, ToK, SM, ES, SS, SK, SH, YS, KS, KTs, KTo, KH, HN, YK, TaK and YH were involved in the analysis and interpretation of data. KK, TaK and YH acquired funding. AC, KK, HN, YK and TaK provided resources. AC and KK wrote the original draft. KK reviewed and edited the manuscript. AC and KK confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Keio University Hospital (approval no. 20200347; Tokyo, Japan). All gastrointestinal stromal tumor samples for staining and genetic testing were collected after obtaining written informed consent.

Patient consent for publication

Written informed consent for publication of the article was obtained from the patients in whom immunostaining or genomic analysis was performed.

Competing interests

The authors declare that they have no competing interests.

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