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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Establishment of a New Scirrhous Gastric Cancer Cell Line with FGFR2 Overexpression, OCUM-14

Tomohisa Okuno, MD[1,2,3], Masakazu Yashiro, MD, PhD[1,2,3], Go Masuda, MD, PhD[1], Shingo Togano, MD[1,2,3], Kenji Kuroda, MD[1,2,3], Yuichiro Miki, MD, PhD[1,2,3], Kosei Hirakawa, MD, PhD[1], Masahiko Ohsawa, MD, PhD[4], Hideki Wanibuchi, MD, PhD[5], and Masaichi Ohira, MD, PhD[1]

[1]Department of Surgical Oncology, Osaka City University Graduate School of Medicine, Osaka City, Osaka, Japan; [2]Molecular Oncology and Therapeutics, Osaka City University Graduate School of Medicine, Osaka City, Japan; [3]Cancer Center for Translational Research, Osaka City University Graduate School of Medicine, Osaka City, Japan; [4]Department of Diagnostic Pathology, Osaka City University Graduate School of Medicine, Osaka City, Japan; [5]Molecular Pathology, Osaka City University Graduate School of Medicine, Osaka City, Japan

ABSTRACT

Background. The prognosis of scirrhous gastric carcinoma (SGC), which is characterized by rapid infiltration and proliferation of cancer cells accompanied by extensive stromal fibrosis, is extremely poor. In this study, we report the establishment of a unique SGC cell line from a gastric cancer patient in whom an autopsy was performed.

Methods. A new SGC cell line, OCUM-14, was established from malignant ascites of a male patient with SGC. A postmortem autopsy was performed on the patient. Characterization of OCUM-14 cells was analyzed by microscopic examination, reverse transcription polymerase chain reaction, fluorescence in situ hybridization analysis, immunohistochemical examination, CCK-8 assay, and in vivo assay.

Results. OCUM-14 cells grew singly or in clusters, and were floating and round-shaped. Most OCUM-14 cells had many microvilli on their surfaces. The doubling time was 43.1 h, and the subcutaneous inoculation of 1.0×10^7 OCUM-14 cells into mice resulted in 50% tumor formation. mRNA expressions of fibroblast growth factor receptor 2 (*FGFR2*) and human epidermal growth factor receptor 2 (*HER2*) were observed in OCUM-14 cells. FGFR2, but not HER2, overexpression was found in OCUM-14 cells. The heterogeneous overexpression of FGFR2 was also found in both the primary tumor and metastatic lesions of the peritoneum, lymph node, bone marrow, and lung of the patient. The FGFR2 inhibitors AZD4547 and BGJ398 significantly decreased the growth of OCUM-14 cells, while paclitaxel and 5-fluorouracil significantly decreased the proliferation of OCUM-14 cells, but cisplatin did not.

Conclusion. A new gastric cancer cell line, OCUM-14, was established from SGC and showed FGFR2 overexpression. OCUM-14 might be useful for elucidating the characteristic mechanisms of SGC and clarifying the effect of FGFR2 inhibitors on SGC.

Gastric cancer is the fourth most commonly diagnosed cancer and the second most common cause of cancer-related deaths worldwide.[1] Human scirrhous gastric carcinoma (SGC), also known as linitis plastica-type carcinoma, is characterized by microscopic undifferentiated cancer cell infiltration accompanied by extensive stromal fibrosis.[2] The prognosis of SGC is extremely poor; the 5-year survival rate of these patients is only approximately 15%.[3,4] To improve the outcome of SGC, it is necessary to develop an effective therapy based on the biological behaviors of SGC; however, the mechanisms responsible for the characteristic progression of SGC are not clearly understood. While SGC cell lines

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M. Yashiro, MD, PhD
e-mail: m9312510@med.osaka-cu.ac.jp

might be useful for analyzing the biological behavior of SGC, the number of SGC cell lines remains small; only 18 SGC cell lines have been identified in the literature.[5]

Fibroblast growth factor receptor 2 (FGFR2) has been reported to be present in approximately 10% of gastric cancers and is particularly frequent in SGC (i.e. present in approximately 20–30% of lesions).[6,7] Several clinical studies using FGFR2 inhibitors such as ARQ 087[8] dovitinib,[9,10] AZD4547,[11] BGJ398,[12] JNJ-42756493,[13] and LY2874455[14] to treat solid cancers have been ongoing. Since patients with FGFR2-positive gastric cancer show poor prognosis,[7,15,16] FGFR2 is considered to be one of the molecular targets for gastric cancer, especially for SGC; however, only three existing SGC cell lines have been reported to overexpress FGFR2.[16–18] Establishment of a new SGC cell line with FGFR2 overexpression will be helpful in clarifying the effect of FGFR2 inhibitors on SGC. In this study, we report on a new SGC cell line with FGFR2 overexpression, OCUM-14.

MATERIALS AND METHOD

Immunohistochemistry

Immunohistochemical staining was performed using the following antibodies: anti-FGFR2 antibody (D4L2V; Cell Signaling Technology, MA, USA), anti-cytokeratin (clone AE1/AE3 antibody; Dako, Glostup, Denmark), anti-S100 antibody (Dako), anti-carcinoembryonic antigen (CEA) antibody (clone IB2; IBL, Gunma, Japan), anti-cancer antigen (CA) 19-9 antibody (C241:5:1:4; Nichirei Bioscience, Tokyo, Japan), anti α-smooth muscle actin (αSMA) antibody (clone 1A4; Dako), or anti-human epidermal growth factor receptor 2 (HER2) antibody (Leica, Newcastle, UK).

Fluorescence In Situ Hybridization Analysis

Fluorescence in situ hybridization (FISH) of OCUM-14 cells was performed using a PathVysion™ HER-2 DNA Probe Kit (Abbot Japan, Tokyo, Japan) or FGFR2/CEN10p Dual Color FISH Probe (GSP Laboratories, Inc.).

Patient

OCUM-14 was derived from the malignant ascites of a 64-year-old male with peritoneal dissemination of SGC. Upper gastrointestinal endoscopy of the patient revealed a macroscopic Borrmann type 4 gastric tumor (Fig. 1a). The histopathological findings of the biopsy specimen from the primary tumor showed poorly differentiated adenocarcinoma with abundant stromal cells, and the macroscopic and microscopic findings indicated that the gastric tumor was SGC.[19] The patient was at an advanced stage of SGC, and after three courses of cisplatin and S-1, a 5-fluorouracil analog, the patient developed malignant ascites (Fig. 1b). Ten months after the diagnosis, the patient died of the tumor. A postmortem autopsy examination was performed after obtaining permission from the patient's family members.

Autopsy Examination

A postmortem autopsy examination revealed advanced gastric cancer of scirrhous type measuring 130 mm in the gastric body (Fig. 1c). Histologically, the tumor cells appeared as a poorly differentiated adenocarcinoma (Fig. 1d) with immunopositivity for AE1 and AE3 (Fig. 1e), but negativity for S100. Cancer cells were negative on CEA and CA19-9 staining (data not shown). Most stromal cells in the cancer microenvironment were positive for αSMA (Fig. 1f). The tumor had metastasized to the peritoneum surface, lymph nodes, bone marrow, and lung.

Establishment of Cell Line and Cell Culture

Malignant ascites from a patient with carcinomatous peritonitis was collected aseptically during the one course of weekly paclitaxel, and informed consent was obtained from patients when the ascites was obtained. The pellet was suspended in 10 ml of culture medium (Dulbecco's modified Eagle's medium [DMEM; Wako, Osaka, Japan] with fetal bovine serum [FBS; Nichirei] and 0.5 mM sodium pyruvate [Sigma]). Floating cells were collected and re-suspended in medium. The floating cell lines were designated OCUM-14, and the OCUM-14 cell was carried for more than 24 months and passaged for more than 120 generations. This study was approved by the Osaka City University Ethics Committee.

Tumorigenicity

Tumorigenicity was carried out on OCUM-14 cells at the 50th passage. Cell suspensions containing 1×10^7 OCUM-14 cells were inoculated subcutaneously into nude mice (Oriental Kobo, Osaka, Japan), and, after 8 weeks, the tumor incidence was determined. The tumor specimens were washed in phosphate-buffered saline (PBS) and fixed in 10% formalin for paraffin section. Animal experiments were performed in compliance with the guidelines of the Osaka City University Ethics Committee.

Growth Kinetics

The doubling time of OCUM-14 cells was determined between the 90th and 100th passage using a Coulter Counter Industrial D (Coulter Electronics, Luton, UK).

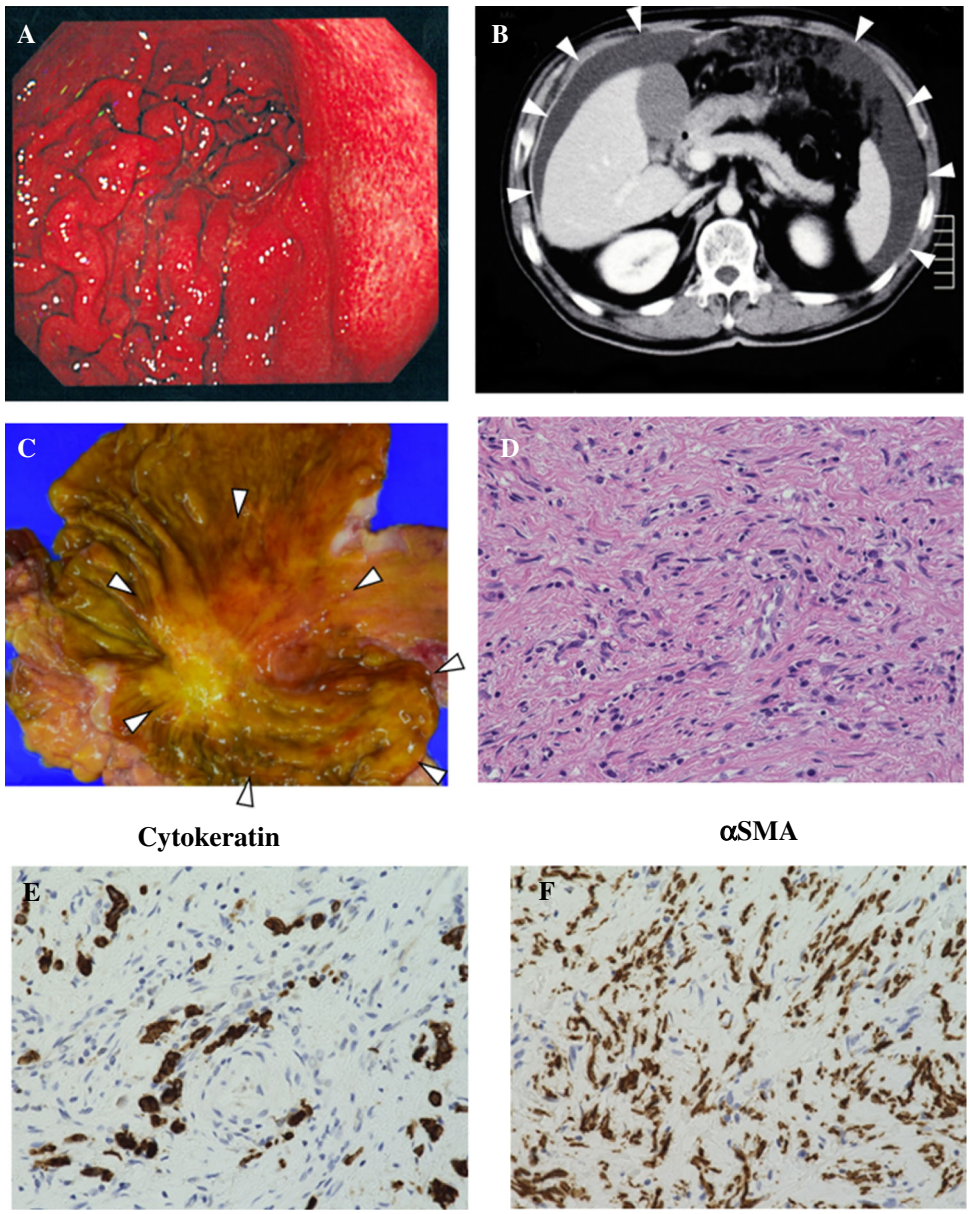


FIG. 1 Clinicopathologic features of patients. **a** Gastric endoscopy. Macroscopic findings of gastric tumor showed Borrmann type 4 with thickened mucosal folds on the greater curvature side of the body in which ulceration was not prominent. **b** Abdominal computed tomography showed ascites (arrowheads). Postmortem autopsy examination of gastric tumor. **c** Macroscopic findings of the primary gastric tumor showed diffusely infiltrating carcinomas in a wide range of stomach. **d**–**f** Histologic findings of gastric tumor: **d** primary tumor showed poorly differentiated adenocarcinoma with abundant stromal cells; **e** cancer cells were positive for cytokeratin; **f** stromal cells in the tumor microenvironment were positive for α-SMA. *SMA* smooth muscle actin

Chromosome Analysis

Cells at the 50th passage were karyotyped using a standard air-dried method[20] using trypsin G banding.

Short Tandem Repeat Analysis

Short tandem repeat (STR) profiling to exclude cross-contamination of cell lines was performed using services provided by the JCRB Cell Bank, as previously reported.[21]

Reverse Transcription Polymerase Chain Reaction

mRNA expression level of receptor tyrosine kinase genes was examined by reverse transcription polymerase chain reaction (RT-PCR), as previously reported.[22]

Growth Inhibition Assay

Two FGFR inhibitors, AZD4547 (LC Laboratories, MA, USA)[23,24] and BGJ398 (AdooQ bioscience, CA, USA),[25] and three chemotherapeutic agents, cisplatin, paclitaxel, and 5-fluorouracil, were used. The effect of FGFR inhibitors and anticancer drugs on OCUM-14 cell viability was determined using a Cell Counting Kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan).

RESULTS

Establishment of a New Gastric Cancer Cell Line, OCUM-14

A new SGC cell line, OCUM-14, was successfully established from the malignant ascites of the patient with SGC. On phase-contrast microscopic examination, OCUM-14 cells grew singly or in clusters and were floating and round-shaped (Fig. 2a). OCUM-14 cells showed various-sized irregular nuclei by hematoxylin and eosin (H&E) staining (Fig. 2b). On electron microscopic observation, most OCUM-14 cells were found to have many microvilli on their surfaces (Fig. 2c), and a few cells in clusters had tight junctions (Fig. 2d). Figure 2e shows the many microvilli and mitochondria, and Fig. 2f shows the mitosis of OCUM-14 cells. The doubling time estimated from the growth curve of OCUM-14 cells was 43.1 h (electronic supplementary Fig. S1), and the percentage of the matched loci of OCUM-14 cells was under 80% in the STR profile databases (electronic supplementary Fig. S2). The levels of tumor-associated antigens CEA, CA19-9, α-fetoprotein (AFP), and SPan-1 were within the normal range. The subcutaneous inoculation of 1.0×10^7 OCUM-14 cells into mice resulted in 50% tumor formation (6/12).

Chromosome Analysis

Ten of 40 metaphase spreads examined were karyotyped. Figure 3a shows the representative karyotype features of OCUM-14 cells. The number of chromosomes of OCUM-14 cells ranged from 130 to 163, with a modal number of 138.

Expression of Growth Factor Receptors and Growth Factors in OCUM-14 Cells

mRNA expressions of *FGFR2*, *FGFR3*, *FGFR4*, *VEGFC*, *EGFR* and *HER2* were observed in OCUM-14 cells, but those of *VEGFRs*, *cKit*, *cMet FGF7*, *PDGFRa*, *PDGFRβ Flt3*, *IGF1R*, *IGF2R*, *HER3*, and *HER4* were not. The expression levels of *FGFR2* and *HER2* in OCUM-14 cells were high compared with those of *FGFR3*, *FGFR4*, *VEGFC*, and *EGFR* (Fig. 3b).

Fibroblast Growth Factor Receptor 2 (FGFR2) Overexpression of Xenografted Tumor by OCUM-14 Cells

The xenografted tumors formed by OCUM-14 cell inoculation appeared as poorly differentiated adenocarcinoma with medullary growth. The tumors developed from OCUM-14 cells showed FGFR2 overexpression (Fig. 3c), and OCUM-14 showed FGFR2 expression by Western blot analysis (Fig. 3d). *FGFR2* amplification was also found in OCUM-14 cells using FISH analysis (Fig. 3e); the mean FGFR2 and CEN10 signals were 26.33 and 2.10, respectively. Since the *FGFR2/CEN10* ratio was 12.54, OCUM-14 cells were recognized to have *FGFR2* amplification.

FGFR2 Overexpression of Autopsy Specimens

FGFR2 overexpression was found in primary gastric cancer cells at the muscularis propria, subserosa, or perigastric lymph node of the primary tumor, but not in the cancer cells at the mucosa or submucosa. On the other hand, HER2 expression of the mucosa or submucosa of the primary tumor was equivocal (Fig. 4a), while HER2 overexpression and amplification was not found in either cancer cells at the subserosa and perigastric lymph node of the primary tumor, or the xenografted tumor by OCUM-14 cells. Metastatic cancer cells were found at the peritoneum, lymph nodes, lung, and bone marrow. Histologic findings of these metastatic tumors showed poorly differentiated adenocarcinoma. Figure 4b shows the high magnification of stromal fibroblasts of primary SGC by both H&E and FGFR2 staining. The stromal fibroblasts express FGFR2, but the expression level was weak. FGFR2 overexpression was observed in metastatic tumors (Fig. 4c), while HER2 expression was not (data not shown).

Effect of FGFR2 Inhibitors and Anticancer Drugs on the Proliferation of OCUM-14 Cells

The FGFR2 inhibitors AZD4547 and BGJ398 significantly decreased the growth of OCUM-14 cells. The half maximal inhibitory concentration (IC_{50}) values of OCUM-14 by AZD4547 and BGJ398 were 1.76 and 3.04 nM, respectively (Fig. 5a). Paclitaxel and 5-fluorouracil significantly decreased the proliferation of OCUM-14 cells, but cisplatin did not (Fig. 5b).

DISCUSSION

A large body of biological knowledge regarding SGC has been obtained from experimental studies using SGC cell lines; however, the number of SGC cell lines is still insufficient for comprehensive understanding of the behaviors of SGC. In this study, we established a new SGC cell line, OCUM-14, from malignant ascites of an SGC patient with carcinomatous peritonitis. OCUM-14 cells are floating and round-shaped, features similar to most of the reported SGC cell lines. Subcutaneous injection of OCUM-14 cells into mice resulted in tumors, while histologic findings of these xenografted tumors showed medullary

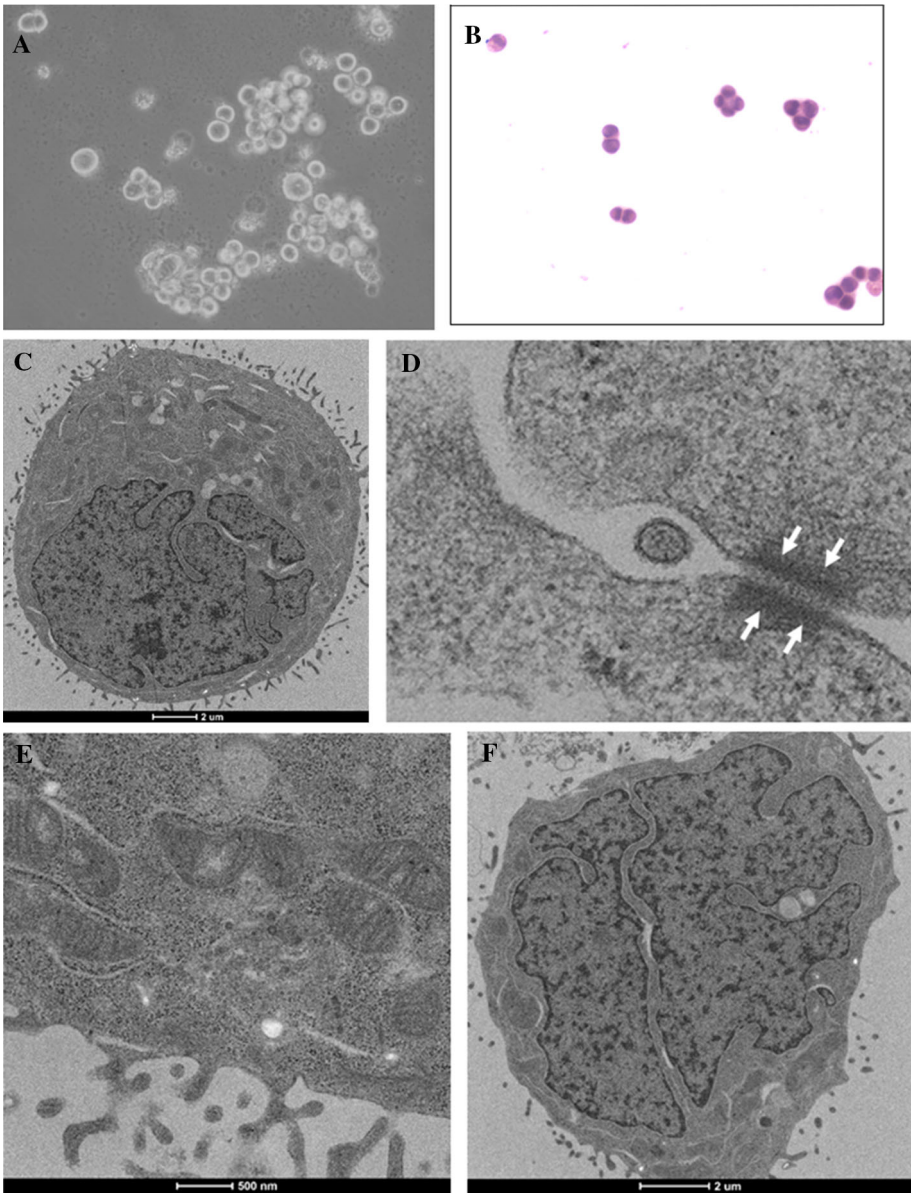
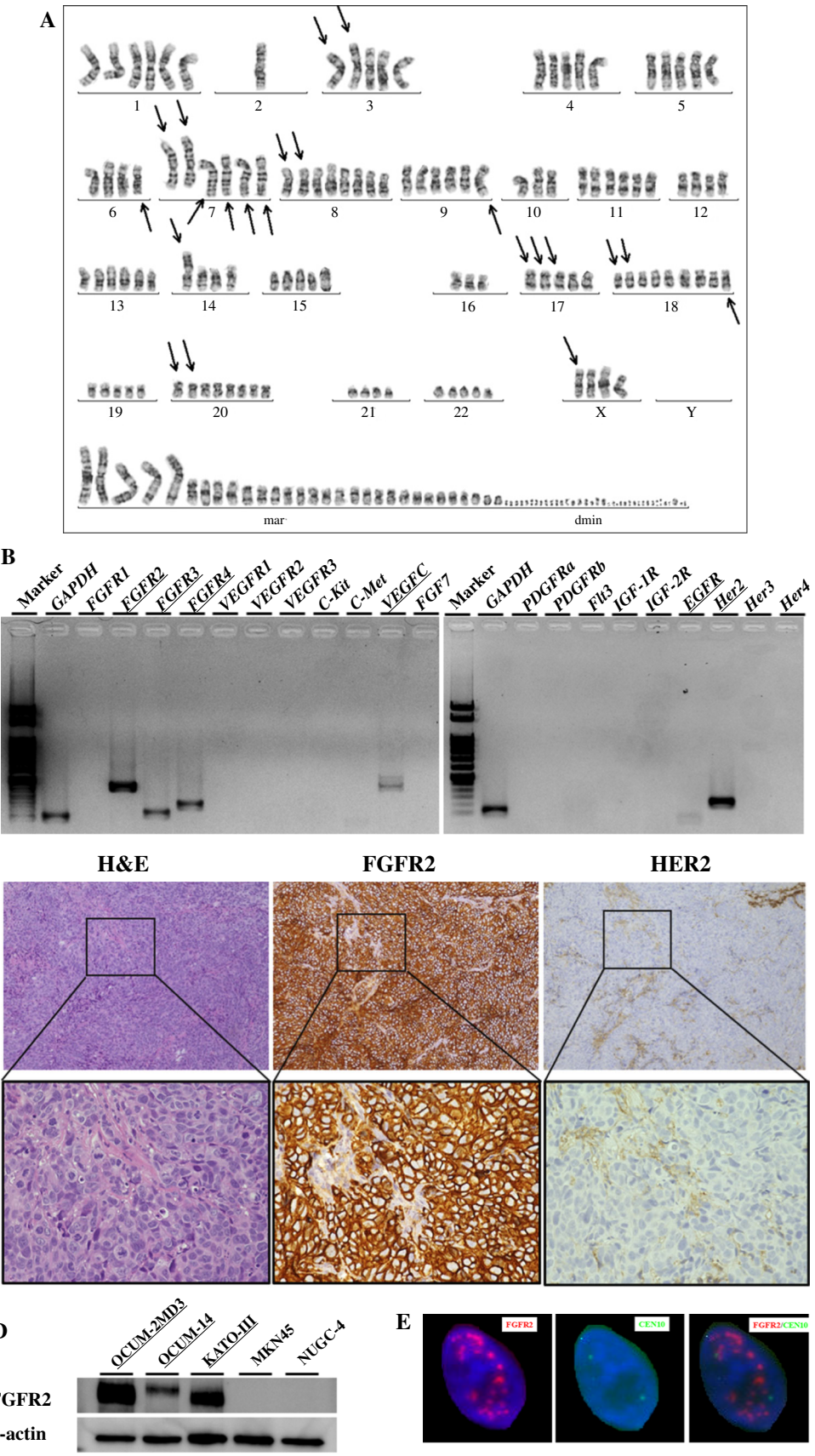


FIG. 2 Morphologic findings of OCUM-14 cells. **a** Phase contrast photomicrography of living OCUM-14 cells. Most cells were floating and round-shaped. **b** H&E staining of OCUM-14 cells. A high nuclear cytoplasmic ratio was observed. **c**, **d** Electron micrograph of OCUM-14 cells. Most OCUM-14 cells have many microvilli. A few tight junctions (arrows) were observed. **e** Many mitochondria were observed in the cytoplasm. **f** Mitosis of OCUM-14 cells was observed. *H&E* hematoxylin and eosin

growth with a poorly differentiated adenocarcinoma, similar to that of the original human tumor. Since cross-contamination remains a common event during establishment of a cell line,[26] STR profiling of OCUM-14 cells was performed to examine the possibility of cross-contamination. The percentage of the matched loci of OCUM-14 cells was under 80% in the STR profile databases of cell lines from ATCC, DSMZ, JCRB and RIKEN, indicating that OCUM-14 is a unique cancer cell line of SGC.

FGFR2 signaling is frequently linked to the proliferative activity of SGC cells, with amplification of the K-*sam-II* oncogene, which was isolated from an SGC cell line, KATO-III,[27,28] and which has a product identical to FGFR2. We previously reported that gastric cancer with FGFR2-positive cancer cells was significantly correlated with a high invasion depth and infiltrative growth pattern.[29] These findings might suggest that FGFR2 expression in cancer cells contributed to high infiltration activity of cancer cells. On the other hand, Fig. 4b showed that fibroblasts weakly expressed FGFR2 (*arrowheads*), while SGC cells highly expressed FGFR2 (*asterisks*), as previously reported.[30] In contrast, we previously reported that transforming growth factor (TGF)-β from cancer cells stimulated the proliferation of fibroblasts.[2,31] These findings suggest that the growth of fibroblasts might be affected by FGFR2 signaling in part, but mainly by TGFβ signaling.

FGFR2 overexpression has been reported in only two of the 18 SGC cell lines, KATO-III and OCUM-2M. In this study, we established another FGFR2-overexpressing cell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◀**FIG. 3** Chromosome analysis and expression of growth factor receptors of OCUM-14 cells. **a** G-banding karyotype. G-banding revealed complex dyskaryotypes. The representative karyotype of OCUM-14 was *XXX*, + *X*, − *Y*, − *Y*, − *Y*, + 1, − 2, − 2, − 2, − 2, − 3. *i*(3)(*q*10) × 3, − 4, − 5, − 6, − 6, del(6)(*q*?), add(7)(*p*22), add(7)(*p*22), add(7)(*q*22) × 2, add(7)(*q*32) × 2, + 8, + 8, ?der(9)*t*(8;9)(*q*11.2; *q*13), − 10, − 10, − 11, − 12, − 12, − 14, − 14, − 14, add(14)(*p*11.2), − 16, − 16, − 17, add(17)(*q*11.2) × 3, + 18, + 18, + ?add(18)(*q*21), − 19, + 20, + 20, + 20, − 21, − 21, − 22, + 30–40 mar, 10–70 dmin [cp5]. The *arrows* indicate rearranged chromosomes. **b** Reverse transcription PCR. mRNA expression of *FGFR2*, *FGFR3*, *FGFR4*, VEGFC, *EGFR* and *HER2* was found in OCUM-14 cells. **c**, **e** FGFR2 overexpression and amplification of OCUM-14 cells. Subcutaneous tumor by OCUM-14 cells showed FGFR2 overexpression. FISH analysis indicated that OCUM-14 cells have *FGFR2* amplification. **d** Western blot of OCUM-14, OCUM-2MD3, KATO-III, MKN45 and NUGC4. OCUM-2MD3 is a subtype cell line of OCUM-2M. FGFR2 expression was found in OCUM-14. *EGFR* epidermal growth factor receptor, *FGFR2* fibroblast growth factor receptor 2, *FISH* fluorescence in situ hybridization, *Flt3* Fms-related tyrosine kinase 3, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase, *HER2* human epidermal growth factor receptor 2, *H&E* hematoxylin and eosin, *IGF* insulin-like growth factor, *mRNA* messenger RNA, *PCR* polymerase chain reaction, *PDGFR* platelet-derived growth factor receptor, *VEGFC* vascular endothelial growth factor C

line, OCUM-14, the original tumor of which also overexpressed FGFR2. *FGFR2* mRNA amplification was also found in OCUM-14 cells by FISH analysis. OCUM-14 cells were aneuploid by chromosome analysis, with three chromosomes at chromosome 10. Since the *FGFR2* gene locates at the chromosomal locus 10q26[32] the polyploidy of chromosome 10 might be partly associated with the amplification of *FGFR2*.

In this study, the FGFR2 tyrosine kinase inhibitors AZD4547 and BJC398 significantly inhibited the proliferation of OCUM-14 cells. AZD4547 and BGJ398 inhibit FGFR1, FGFR2, and FGFR3 signaling, but not FGFR4 signaling.[25,33] Furthermore, OCUM-14 cells expressed *FGFR2*, *FGFR3*, and *FGFR4* mRNA, but not *FGFR1*, by RT-PCR analysis. Taken together, these results show that FGFR2 overexpression of OCUM-14 cells was detected using an anti-FGFR2 antibody (D4L2V) specific to FGFR2. These findings suggest that AZD4547 and BJC398 inhibited the FGFR2 phosphorylation of OCUM-14 cells, and that *FGFR2* is a driver gene of OCUM-14 cells. Despite recent advances in diagnostic techniques and therapies for GC, the outcome of SGC remains unsatisfactory.[34] One of the reasons for the poor prognosis of SGC might be the lack of a molecular target therapy. There is still no clinical research showing evidence of a novel key molecule of SGC. In this regard, the FGFR2 inhibitor appears therapeutically promising in cases of SGC with FGFR2 overexpression.

Although HER2 has been one of the molecular targets in the clinical treatment of advanced gastric cancer, SGCs rarely express HER2.[35] Subcutaneous tumors by OCUM-14 showed equivocal expression of HER2, while OCUM-14 cells showed *HER2* mRNA expression by RT-PCR. In contrast, *HER2* amplification was negative on FISH analysis, which might be a reason why subcutaneous tumor by OCUM-14 cells did not overexpress HER2. In addition, we examined the effect of a HER2 inhibitor, trastuzumab, on the proliferation of OCUM-14 cells, and found trastuzumab did not affect the proliferation of OCUM-14 cells. These findings suggest that HER2 signaling might not be a driver of OCUM-14 cells.

Metastatic tumors of the peritoneum, lymph nodes, bone marrow, and lung showed FGFR2 overexpression in the patient from whom OCUM-14 was derived. Because the metastatic tumors overexpressed FGFR2, FGFR2 can be considered a promising target for the patient. The difference in driver–gene expression between the mucosal biopsy specimen and the metastatic specimens in gastric cancer might be a critical issue for precision medicine, and the mucosal biopsy specimen might not be sufficient to determine the applicability of an FGFR2 inhibitor in gastric cancer. It will be necessary to develop a useful tool, such as liquid biopsy, to overcome the heterogeneity of gastric tumors in detecting driver molecules for tumor metastasis.

Tumor stromal cells in the primary gastric cancer were αSMA-positive. Fibroblasts constitute a major stromal compartment of SGC. It has been reported that myofibroblasts, which are distinct from normal fibroblasts in their expression of αSMA, are associated with the characteristic biologic behaviors of SGC.[31,36,37] We previously reported that FGF7 from myofibroblasts might contribute to the remarkable cell proliferation seen in SGCs with FGFR2 overexpression,[17,27,38] and TGFβ might be associated with the differentiation of myofibroblasts.[39] OCUM-14 cells might have a close tumor–stroma interaction, and elucidation of tumor–stroma molecular interactions may provide the basis for new targeted cancer therapies.

OCUM-14 cells were shown to be resistant to cisplatin. Indeed, OCUM-14 cells were established from the ascites of a patient who did not respond to cisplatin treatment. OCUM-14 cells might be useful for analyzing the mechanisms behind resistance to cisplatin.

It has been reported that most gastric cancer cells produce some tumor-associated antigens; however, the levels of CEA, CA19-9, AFP, SPan-1, and SLX in OCUM-14 cells were within the normal range. Immunohistochemical analysis also indicated that primary gastric tumor cells did not express CEA or CA19-9. OCUM-14 cells might produce a tumor antigen that is not frequently expressed in gastric cancer.

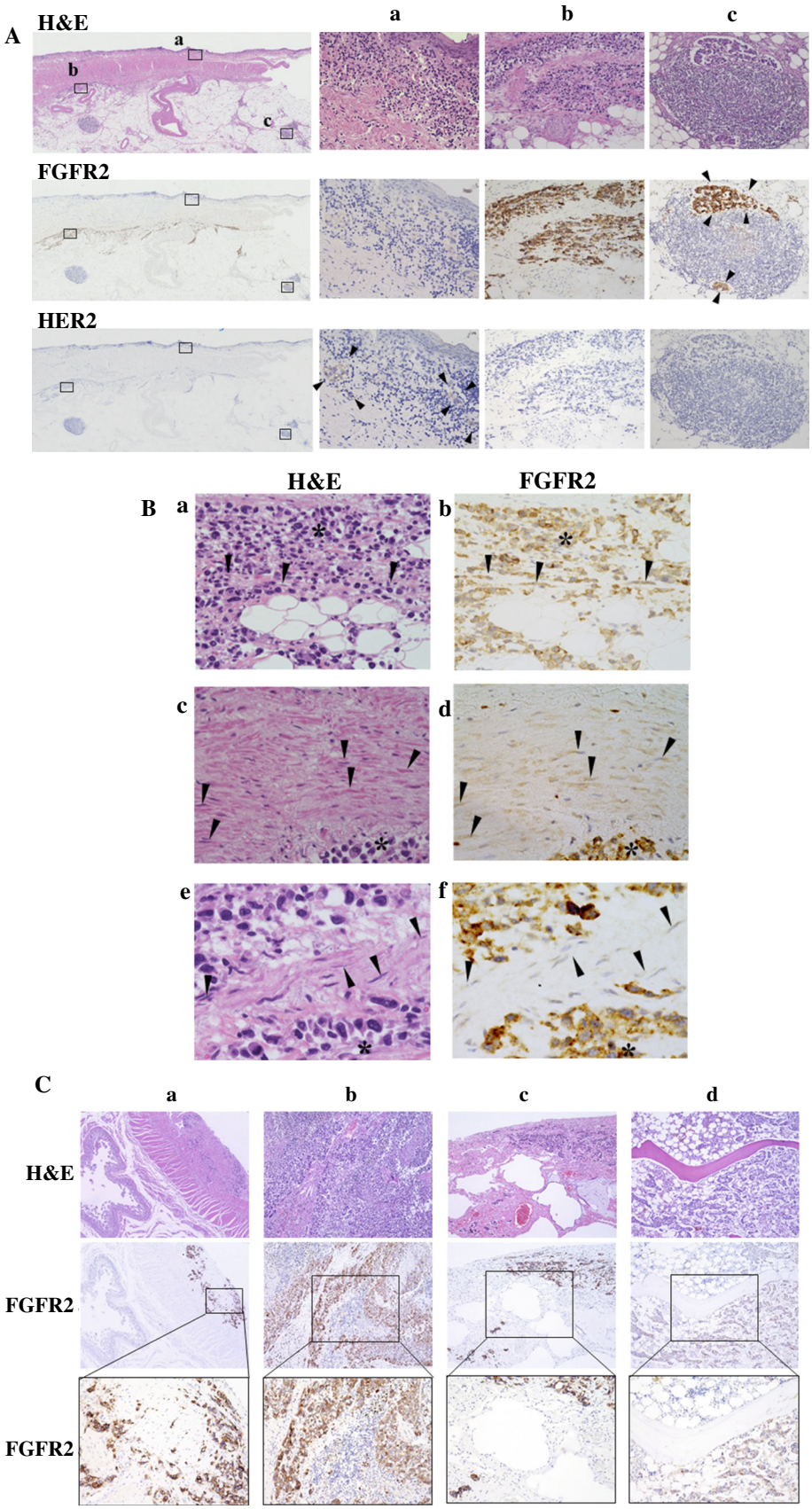

FIG. 4 FGFR2 overexpression of the primary tumor and metastatic tumors. **A** Histologic findings of the primary tumor, which showed poorly differentiated adenocarcinoma by H&E staining. Equivocal expression of HER2 was recognized at the superficial lesion of the primary tumor, but FGFR2 was negative (**a**). In contrast, FGFR2 overexpression was found at the muscularis propria, the subserosa (**b**), or the perigastric lymph node (**c**), while HER2 overexpression and amplification was not found. **B** High magnification of stromal fibroblasts of primary SGC. **a**, **c**, **e** H&E staining, **b**, **d**, **f** FGFR2 staining. Cancer cells (asterisk) and cancer stroma were found. **b**, **d**, **f** Expression of FGFR2 in cancer stroma. The stromal fibroblasts (arrowheads) express FGFR2, but the expression level was weak. **C** Histologic findings of metastatic tumor. Metastatic cancer cells were found at the peritoneum (**a**), lymph nodes (**b**), lung (**c**), and bone marrow (**d**). These tumors showed poorly differentiated adenocarcinoma. FGFR2 overexpression was found on cancer cells of all metastatic tumors. *FGFR2* fibroblast growth factor receptor 2, *HER2* human epidermal growth factor receptor 2, *H&E* hematoxylin and eosin, *SGC* scirrhous gastric carcinoma

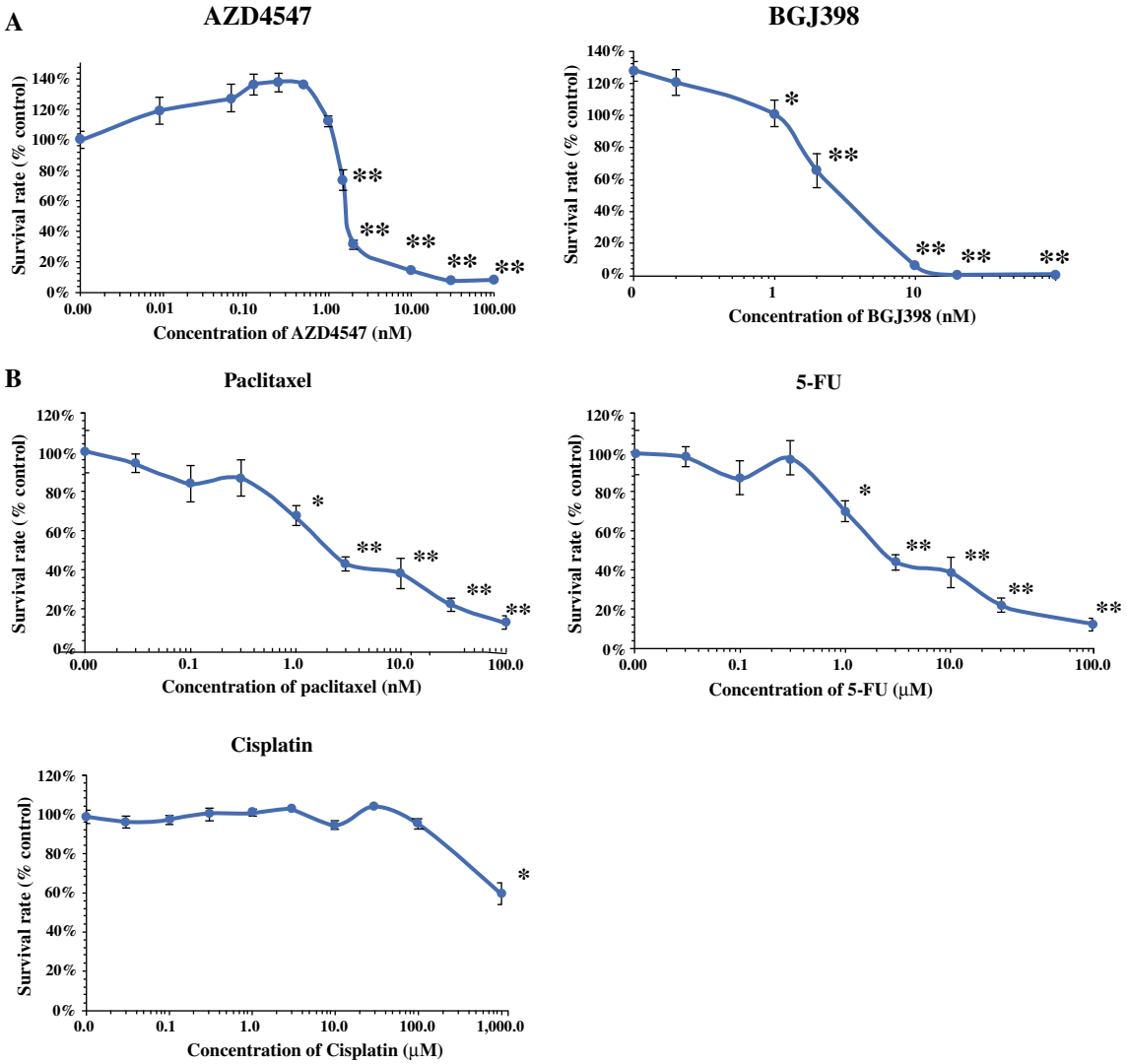

FIG. 5 **a** Effect of the FGFR2 inhibitors AZD4547 and BGJ398 on the proliferation of OCUM-14 cells. Proliferation of OCUM-14 cells was significantly decreased by AZD4547 and BGJ398. IC_{50} of AZD4547 and BGJ398 was 1.76 and 3.04 nM, respectively. **b** Effect of cisplatin, paclitaxel, and 5-fluorouracil on the proliferation of OCUM-14 cells. IC_{50} of paclitaxel, 5-fluorouracil, and cisplatin was 2.19 nM, 2.25 μM, and 1.87 mM, respectively. A significant difference in concentration was observed compared with controls ($*p < 0.05$; $**p < 0.01$). *FGFR* fibroblast growth factor receptor, IC_{50} half maximal inhibitory concentration

CONCLUSION

We have established a new SGC cell line, OCUM-14, from a metastatic lesion of a patient with SGC. *FGFR2* was shown to be a driver gene of OCUM-14 cells. OCUM-14 might be useful for investigating the mechanisms behind the biological behaviors of SGC with FGFR2 overexpression.

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AUTHOR CONTRIBUTIONS Study concept and design: MY; material collection: GM, MO, and MY; data acquisition: YM, TO, KK, GM; analysis and interpretation of data: YM, and MY; drafting of the manuscript: YM and MY; and study supervision: MY, MO, KH, and MO.

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