Global Health & Medicine. 2021; 3(4):226-235.
ORIGINAL ARTICLE
DOI: 10.35772/ghm.2021.01001

A newly established monoclonal antibody against ERCC1 detects major isoforms of ERCC1 in gastric cancer

Takayuki Oishi[1,2,3,4,§], Yuka Sasaki[1,2,4,§], Ying Tong[1], Lichao Chen[1,4], Takae Onodera[1,2,4], Satoru Iwasa[5], Emiko Udo[6,7], Bungo Furusato[6,7], Hiroaki Fujimori[4], Shoji Imamichi[1,2], Takuya Honda[3,8], Tadayoshi Bessho[9], Junya Fukuoka[6], Kazuto Ashizawa[7,8], Kazuyoshi Yanagihara[10], Kazuhiko Nakao[3], Yasuhide Yamada[11,12], Nobuyoshi Hiraoka[13], Mitsuko Masutani[1,2,4,*]

[1] Department of Molecular and Genomic Biomedicine, Center for Bioinformatics and Molecular Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan;
[2] Laboratory of Collaborative Research, Division of Cellular Signaling, National Cancer Center Research Institute, Tokyo, Japan;
[3] Department of Gastroenterology and Hepatology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan;
[4] Department of Frontier Life Sciences, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan;
[5] Gastrointestinal Medical Oncology Division, National Cancer Center Hospital, Tokyo, Japan;
[6] Department of Pathology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan;
[7] Cancer Genomics Unit, Clinical Genomics Center, Nagasaki University Hospital, Nagasaki, Japan;
[8] Department of Clinical Oncology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan;
[9] Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, Nebraska, USA;
[10] Division of Biomarker Discovery, Exploratory Oncology Research and Clinical Trial Center, National Cancer Center, Tokyo, Japan;
[11] Department of Medical Oncology, Hamamatsu University School of Medicine, Hamamatsu, Japan;
[12] Comprehensive Cancer Center, Center Hospital of the National Center for Global Health and Medicine, Tokyo, Japan;
[13] Division of Pathology, National Cancer Center Hospital, Tokyo, Japan.

Abstract: Identifying patients resistant to cisplatin treatment is expected to improve cisplatin-based chemotherapy for various types of cancers. Excision repair cross-complementing group 1 (ERCC1) is involved in several repair processes of cisplatin-induced DNA crosslinks. ERCC1 overexpression is reported as a candidate prognostic factor and considered to cause cisplatin resistance in major solid cancers. However, anti-ERCC1 antibodies capable of evaluating expression levels of ERCC1 in clinical specimens were not fully optimized. A mouse monoclonal antibody against human ERCC1 was generated in this study. The developed antibody 9D11 specifically detected isoforms of 201, 202, 203 but not 204, which lacks the exon 3 coding region. To evaluate the diagnostic usefulness of this antibody, we have focused on gastric cancer because it is one of the major cancers in Japan. When ERCC1 expression was analyzed in seventeen kinds of human gastric cancer cell lines, all the cell lines were found to express either 201, 202, and/or 203 as major isoforms of ERCC1, but not 204 by Western blotting analysis. Immunohistochemical staining showed that ERCC1 protein was exclusively detected in nuclei of the cells and a moderate level of constant positivity was observed in nuclei of vascular endothelial cells. It showed a clear staining pattern in clinical specimens of gastric cancers. Antibody 9D11 may thus be useful for estimating expression levels of ERCC1 in clinical specimens.

***Keywords*:** excision repair cross-complementing group 1, gastric tumor, cisplatin, predictive biomarker

Introduction

Platinum compounds such as cisplatin (CDDP), carboplatin and oxaliplatin have been used as the standard of care for various cancer patients including gastric, non-small cell lung, head and neck, and cervical cancers (*1-5*). These anti-cancer agents induce cell death accompanying inhibition of DNA repair pathways. CDDP forms intra-strand DNA adducts and inter-strand crosslinks (ICL), which inhibit DNA repair and replication to induce cell death (*6*). DNA lesions caused by CDDP are mainly repaired by nucleotide excision repair (NER) and Fanconi anemia pathway. NER is the major pathway for elimination of DNA adducts generated by CDDP (*7*). In NER pathway, excision repair cross-complementing group 1 (ERCC1) forms a tight complex with xeroderma pigmentosum group F (XPF) to function as a DNA endonuclease (*7*). ERCC1-XPF heterodimer recognizes platinum-DNA adducts and is required for introducing incision at the ICL lesions (*8,9*). For this reason, ERCC1 plays an important role in repair of DNA damage introduced by platinum compounds. It

has been suggested that ERCC1 overexpression may lead to chemoresistance to platinum-based therapy in cancer cells.

It has been reported that ERCC1 overexpression was associated with poor prognosis after CDDP treatment of patients such as gastric and non-small cell lung cancers (*1,10-13*). These reports suggested that high ERCC1 expression levels were correlated with resistance to platinum agents such as CDDP. In advanced gastric cancer, the measurement of *ERCC1* mRNA level showed a positive association with resistance to cisplatin and 5-fluorouracil-based therapy (*1*). Thus, it is considered that ERCC1 overexpression level may become a predictive biomarker for CDDP treatment. Low expression levels of ERCC1 were also reported to increase sensitivity of lung cancer cells to PARP inhibitors (*14,15*).

The ERCC1 gene produces four isoforms (201, 202, 203 and 204) by alternative splicing. Friboulet *et al.* reported that only ERCC1 isoform 202 contributed to develop cisplatin resistance in a xenograft model of lung cancer cells A549 (*16,17*). However, each isoform contains particular domains for different functions and it is not elucidated whether other isoforms function or not in certain cancer types for the repair of CDDP and other platinum agents. Considering this situation, the antibody that detects not only 202 but also other major isoforms of ERCC1 may be useful for evaluation of sensitivity to cisplatin and other platinum compounds.

Recently, several anti-ERCC1 antibodies for immunohistochemical staining of clinical specimen were developed (*16,18-21*). Anti-ERCC1 monoclonal antibody 8F1 was available for evaluating the ERCC1 levels in tumor samples of cancer patients. However, 8F1 did not specifically detect ERCC1 and was found to be cross-reactive with an unrelated protein (*16,18*).

In this study, to evaluate the expression level of ERCC1 in tumor samples by immunohistochemical staining, we developed a novel anti-ERCC1 monoclonal antibody 9D11 using a method to obtain a high affinity antibody. We showed that 9D11 antibody can recognize ERCC1s three major isoforms containing exon 3 coding regions and detected ERCC1 protein in all seventeen gastric cancer cell lines. It was further shown to detect ERCC1 in immunohistochemical staining of tumor specimens from patients.

Materials and Methods

Cell culture and reagents

Hybridoma cells were cultured in Hybridoma-SFM (Gibco, NY, USA) and gastric cancer cell lines MKN45, HSC-39, HSC-40A, HSC-43, HSC-44PE, HSC-45, HSC-57, HSC-58, HSC-59, HSC-60, HSC-64, SH101-P4, MKN-1, MKN-28, MKN-74 and KATO-III were cultured in RPMI1640 medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Invitrogen, MA, USA) (*22,23*). Gastric cancer cell line GCIY was cultured in MEM medium (Gibco) supplemented with 15% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Invitrogen). HeLa cells were cultured with MEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C.

E. coli strains and plasmids

Plasmid pET-ERCC1 encodes human ERCC1 isoform 202 downstream of P_{T7} promoter of pET-Duet1. This plasmid contains an N-terminal His6 tag conjugated with ERCC1. For construction of expression plasmids of other ERCC1 isoforms in *E. coli*, the pET-ERCC1 was used as a template. The pET-E201, pET-E203 and pET-E204 all contain the N-terminal His6-tag conjugated human ERCC1 isoforms 201, 203 and 204 cassettes, respectively. These genes were cloned into the *EcoR* I-*Sal* I double digested pET-Duet1 (GenScript, Tokyo, Japan). *E. coli* BL21 (DE3) was utilized to induce expression of ERCC1 isoforms. For expression in human cells, plasmid RC208787 and RC228204 were purchased from ORIGENE to express the ERCC1 isoforms 201 and 203 with C-terminal Myc and FLAG tags, in HeLa cells. For expression of ERCC1 isoform 204 in HeLa cells, plasmid pCDNA-E204 was constructed from pCDNA3-HsERCC1, which contains isoform 202 cDNA. This plasmid encodes human ERCC1 isoform 204 without tags downstream of the cytomegalovirus promoter.

Purification of recombinant human ERCC1 isoform 202 as the antigen

E. coli transformed with plasmid pET-ERCC1 was grown in Luria-Bertani broth containing 100 μg/mL ampicillin at 37°C until OD_{600} reached approximately 0.5. Isopropyl-β-$_D$-1-thiogalactopyranoside (IPTG) at 1 mM was then added to induce expression of ERCC1 and cultured further at 37°C for 5 hr. Cells were centrifuged at 2500 xg for 20 min, washed with PBS (-) harvested by centrifugation and sonicated into buffer A (0.5 M NaCl, 5% (v/v) glycerol, 50 mM Tris-HCl (pH8.0), 1 mM PMSF). After centrifugation at 1700 xg for 25 min, supernatant (soluble fraction) was mixed into 5 ml of TALON Metal Affinity Resin (Clontech, CA, USA) and incubated on ice for 30 min, loaded onto the column and washed with 10 column volumes of buffer A containing 5 mM imidazole. His-tagged proteins were eluted with 5 column volumes of buffer A containing 500 mM imidazole, and pooled using measurement of A_{280}. This fraction was passed through Amicon Ultra (30K MWCO) (Millipore, MA, USA) to exchange the buffer and concentrate recombinant proteins. The purity

of the eluted fraction was confirmed with SDS-PAGE and Western blotting using anti-ERCC1 antibody 8F1 (Abcam, Cambridge, UK).

Immunization and screening of ERCC1 monoclonal antibodies

Recombinant ERCC1 (ProSpec-Tany TechnoGene Ltd., Rehovot, Israel) of isoform 202 harboring N-terminal His6 tag was used to immunize three GANP mice to generate monoclonal antibodies and purified recombinant ERCC1 isoform 202 as described above were used for evaluation of conditioned medium in hybridoma clones by ELISA (TransGenic Inc., Fukuoka, Japan). Briefly, NUNC Maxi Sorp F96 plates (Thermo Fisher Scientific, MA, USA) were coated with 1 μg / ml recombinant human ERCC1 isoform 202 for 1 hr at room temperature and incubated with PBS-T containing 0.5 % gelatin overnight at 4 °C. Then, conditioned medium from hybridoma culture supernatant was added and incubated for 1 hr at room temperature. Substrate solution containing *o*-phenylenediamine (OPD) was added to wells and the reaction was stopped with 1 N H_2SO_4. Plates were analyzed using a plate reader at an absorbance of 490 nm.

Western blot analysis

Western blotting was performed as described previously (*24*). Cell extracts were prepared with Laemmli's buffer and separated by SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes. The following antibodies were used for Western blotting: anti-ERCC1 (8F1, Abcam), anti-ERCC1 (FL297) (Santa Cruz Biotechnology, CA, USA) and anti-β-actin (Sigma-Aldrich, MO, USA). Immune complexes were visualized using a horseradish peroxidase-linked secondary antibody and enhanced chemiluminescence (Millipore, MA, USA). Image quantification was performed with Image J software (NIH).

Quantitative RT-PCR (qRT-PCR)

RNA prepared was reverse transcribed using a High Capacity Reverse Transcription Kit (Thermo Fisher Scientific, MA, USA). The quantitative RT-PCR (qRT-PCR) analysis was performed using SYBR Green with the CFX96 Real-Time System (Bio-Rad, CA, USA) and StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). The mRNA levels were normalized by *GUSB*. The primers for qRT-PCR were synthesized by Eurofins Genomics: For *ERCC1*, forward primer (F2) 5'-GGGAATTTGGCGACGTAATTC-3', and reverse primer (R2) 5'- GCGGAGGCTGAGGAACAG-3' and for *GUSB*, forward primer 5'-GCCTGCGTCCCACCTAGAAT-3' and reverse primer 5'-ACATACGGAGCCCCCTTGTC-3' were used, respectively.

Transfection

MKN45 (2.3×10^5 cells) and HeLa (1.0×10^5 cells) were seeded onto 6-well and 12-well plates, respectively. Transfection with plasmid DNA (pCDNA3 Hs-ERCC1) was performed using Lipofectamine 3000 (Life Technologies, CA, USA).

For siRNA transfection to HeLa cells, Lipofectamine RNAiMAX (Life Technologies) was used. Individual siRNAs were used at a final concentration of 10 nM in Opti-MEM. The siRNAs for ERCC1 (5'-GGCCAAGCC CUUAUUCCGAUCUACA-3', 5'-UGUAGAUCGGAA UAAGGGCUUGGCCAC-3', (hs.Ri.ERCC1.13.1) were purchased from Integrated DNA Technologies. DS NC1 siRNA (51-01-14-04) (Integrated DNA Technologies, IA, USA) was used as a negative control (siN.C.).

Construction of a stable cell line expressing human ERCC1 isoform 202

MKN45 cells (2.3×10^5 cells) were seeded onto 6-well plates and transfected with plasmid pCDNA3 Hs-ERCC1, that was digested with restriction enzyme *Pvu* I and purified with Gene Jet Kit (Thermo Fisher Scientific). In the N- or C-terminal region, tag was not conjugated. Two days after transfection, cells were started in culture with medium containing 800 μg/mL of G418 for 19 days. After treatment of G418, surviving colonies were isolated and cultured further for 4 days. Stable cell lines expressing human ERCC1 were selected by qRT-PCR with SuperPrep Cell lysis and RT kit for qPCR (TOYOBO, Osaka, Japan) and confirmed by Western blotting with anti-ERCC1 (4F9 and FL297).

Preparation of tumor xenografts derived from stable cell lines overexpressing human ERCC1 isoform 202 in mice

MKN45 cells or stable cell lines expressing human ERCC1 isoform 202 (1×10^6 cells) were mixed with Growth Factor Reduced Matrigel (BD Biosciences, NJ, USA) and injected subcutaneously into flanks of 5-week-old Balb/c-*nu*/*nu* nude mice (Japan SLC, Inc., Shizuoka, Japan). Tumor diameters were measured with micrometer calipers, and tumor volumes were calculated using the following formula: (largest diameter) × (smallest diameter)^2/2. Animal studies were approved by the Animal Experimental Committee of the National Cancer Center and performed following the Guidelines for Animal Experiments of the National Cancer Center. When tumor volumes reached approximately 100-220 mm^3, mice were sacrificed and tumors were fixed by formalin. Then, paraffin-embedded sections were prepared.

Immunohistochemical staining

For immunohistochemical staining of paraffin-

embedded tissue and tumor sections, the sections were deparaffinized in xylene and rehydrated. Antigen retrieval was performed with Envision FLEX target retrieval solution (Dako Japan Inc., Kyoto, Japan), Low or High pH (Dako Japan Inc) by autoclave treatment at 121°C for 10 min. After blocking of endogenous peroxidase activity with EnVision FLEX Peroxidase Blocking Reagent (Dako Japan Inc.) for 5-10 min, samples were washed with Wash buffer (Dako Japan Inc.) and incubated with anti-human ERCC1 antibody diluted with Antibody Diluent (Dako Japan Inc.) for 20 min at room temperature. After washing the sections, samples were incubated with Polymer Reagent (Dako Japan Inc.) for 20 min at room temperature. After washing the sections, antibody complexes on the slides were detected with 3,3'-diaminobenzidine substrate (Dako Japan Inc.). Immunohistochemical analyses were also carried out with Ventana Bench Mark XT Automated Stainer (Ventana Medical Systems Inc., Tucson, AZ, USA) using OptiView DAB Universal Kit with the procedure of OptiView DAB IHC v4 following standard protocols.

Study samples

Histologically proven gastric cancer patient specimens used for this study were approved by the Institutional Ethics Committees of Nagasaki University and National Cancer Center, within which the work was undertaken and it conforms to the provisions of the Declaration of Helsinki.

Purification of anti-ERCC1 monoclonal antibody (clone No. 9D11)

Fifty ml of Protein G-Sepharose (GE Healthcare Japan, Tokyo, Japan) was mixed with 2,500 ml of conditioned medium. After washing the column with PBS (-), the antibody was eluted with glycine buffer (pH 3.0) and collected into new tubes (6 ml/fraction). After neutralization, 280 nm absorbance was measured and the fractions containing antibodies were pooled and dialyzed with PBS (-) and concentrated with ultrafilters.

Expression of ERCC1 isoforms in E. coli and preparation of whole cell lysates

Plasmids containing an expression cassette of human ERCC1 isoform 201, 202, 203 and 204, respectively, were transformed into *E. coli* BL21 (DE3) and cultured in LB medium containing 100 μg/ml ampicillin until OD_{600} reached 0.4-0.5. Then, after treatment with 1 mM IPTG approximately for 5 hr, cells were harvested by centrifugation at 5700 *xg* for 10 min. *E. coli* expressing ERCC1 were lysed into Laemmli's buffer containing Complete Protease Inhibitor Cocktail (Roche, Basel, Swiss) and the resulting supernatant was obtained by centrifugation at 13,000 rpm for 10 min (MA-2024 rotor, KUBOTA, Tokyo, Japan) and used as whole cell lysates.

Immunofluorescence staining

HeLa cells were seeded and transfected with siRNA of negative control (siN.C.) or *ERCC1* in chamber slides and cultured at 37°C for 3 days. Cells were fixed with methanol and treated with PBS(-)-1% BSA-10% FBS for 2 hr. The following antibodies were used for immunofluorescence staining: anti-ERCC1 (9D11). Immune complexes were detected using AlexaFluor 594-conjugated mouse secondary antibodies (A-11005) (Molecular Probes, OR, USA). Finally, cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) using VECTASHIELD Mounting Medium (H-1200) (VECTOR Laboratories, CA, USA).

Results

Screening of novel monoclonal antibodies against human ERCC1

To evaluate the expression level of ERCC1 in human tumor tissues, we developed novel monoclonal antibodies targeting human ERCC1 that are useful for immunohistochemical staining. To establish monoclonal antibodies to ERCC1, GANP mice (*25*), which can develop high affinity antibodies, were immunized with the purified recombinant ERCC1 isoform 202 (Figure S1, *https://www.globalhealthmedicine.com/site/supplementaldata.html?ID=23*). Thirty clones of hybridoma cells were obtained and the conditioned media, which can recognize human ERCC1, were screened. Analysis with ELISA showed that all of the conditioned media could bind to the purified ERCC1 (Figure S2, *https://www.globalhealthmedicine.com/site/supplementaldata.html?ID=23*). To evaluate antigen specificities of antibodies in conditioned media, we also performed Western blot analysis using cell lysate of gastric cancer cells MKN45, which transiently overexpressed human ERCC1 isoform 202. Twenty-six clones of conditioned media showed detection of ERCC1 at a molecular weight of 37 kDa (data not shown).

Next, we assessed reactivity of antibodies against ERCC1 in immunohistochemistry. For this purpose, to generate xenograft models of human ERCC1 overexpressing and parental tumors, we established stable cell lines of human stomach cancer MKN45 cells that overexpress ERCC1. Clone 14, 2, and 21 expressed 3.6, 2.5, and 1.2-fold levels of ERCC1, respectively, compared with parental MKN45 cells in Western blots using commercially available antibody 8F1 against ERCC1 (Figure 1A). These MKN45 clones and parental cells were subcutaneously transplanted into nude mice to obtain tumor xenografts. Paraffin-embedded tumor

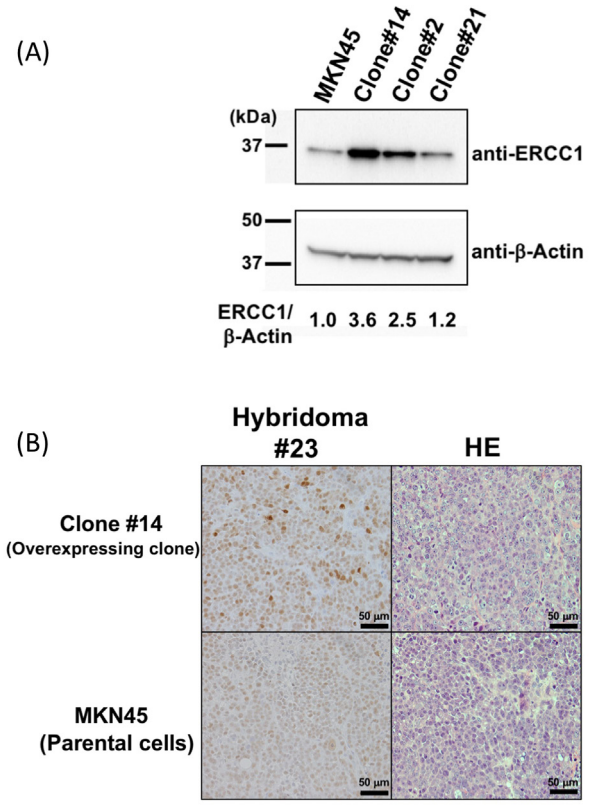

Figure 1. Establishment and evaluation of ERCC1 monoclonal antibody 9D11. (A) Construction of MKN45 stable cell lines expressing human ERCC1 isoform 202. Expression of ERCC1 in stable cell lines was verified by Western blotting with antibody 4F9. The expression level of ERCC1 was 3.6-fold higher in MKN45 clone #14 as compared with the parent cell line MKN45. **(B)** Evaluation of conditioned media of the hybridoma clone #23 that produces antibody 9D11 by immunohistochemical staining. Expression of ERCC1 in the xenograft derived from MKN45 clone #14 (the upper panels) that overexpresses ERCC1 showed a higher positivity compared with the xenograft derived from parental MKN45 cells (the lower panels). Bar, 50 μm.

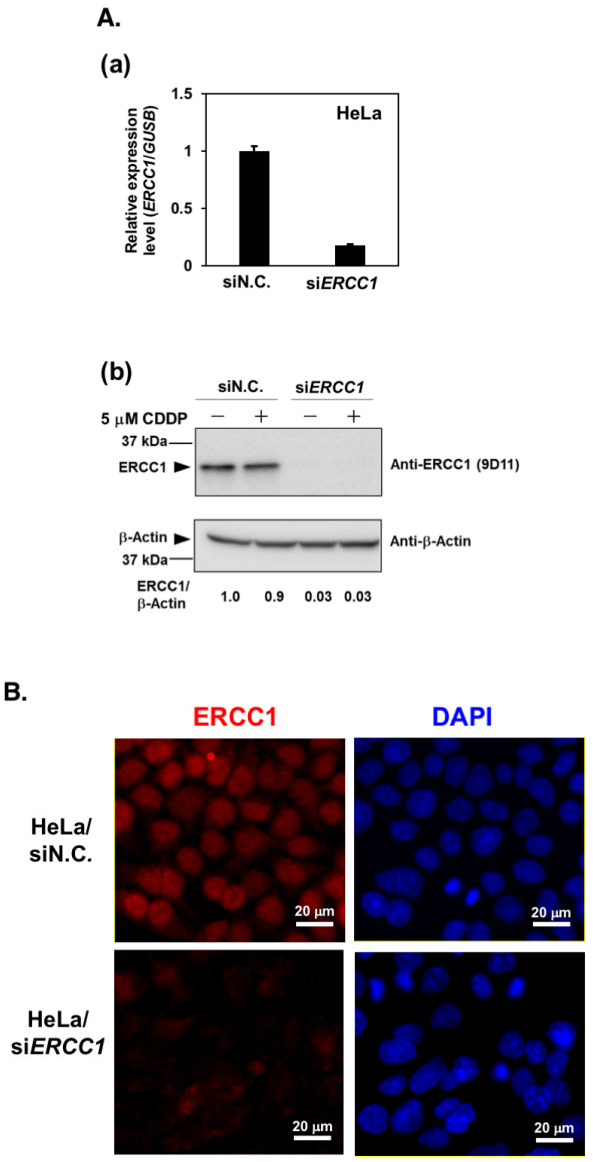

Figure 2. Immunofluorescent staining with antibody 9D11 in ERCC1 knock-down HeLa cells. (A) (a) Real-time PCR analysis of ERCC1 mRNA in knock-down cells three days after transfection of siRNAs, ERCC1 or siN.C. into HeLa cells. The siRNA treated HeLa showed a decreased *ERCC1* mRNA compared to siN.C. condition. (b) Western blot analysis of ERCC1 protein level with antibody 9D11 in knock-down cells and control cells (siN.C.) after treatment with 5 μM CDDP or in the absence of CDDP for 10 hr two days after transfection. **(B)** ERCC1 was detected by immunofluorescent staining with antibody 9D11 (red color) with DAPI staining (blue color) in HeLa cells three days after transfection of si*ERCC1* or control (siN.C.). Bars, 20 μm.

sections were then prepared from ERCC1-overexpressed tumors. We next performed immunohistochemical staining to validate twenty-six clones using hybridoma conditioned media. It revealed that hybridoma conditioned media of six clones 3, 12, 17, 23, 24, and 28 more intensely stained ERCC1 in the nuclei of the tumors derived from MKN45 clone 14 that overexpresses ERCC1 as compared with those derived from parental MKN45. Especially, hybridoma conditioned media of clone 23 (antibody was named 9D11) showed a most clear nuclear staining pattern in ERCC1-overexpressed tumor sections (Figure 1B). Accordingly, we focused on 9D11 as a novel anti-ERCC1 antibody and purified the antibody from conditioned medium. ELISA analysis showed that 9D11 antibody could recognize recombinant ERCC1 isoform 202, in a concentration-dependent manner (data not shown).

Characterization of anti-ERCC1 monoclonal antibody 9D11

To validate whether antibody 9D11 specifically recognizes ERCC1, *ERCC1* was knocked down with siRNA in HeLa cells. Real-time PCR using F2-R2 primers for exon 4-5, which can amplify all isoforms and Western blots supported the reduced level of ERCC1 (Figure 2A a & b). We noted that HeLa cells express isoform 202 and/or 203 but not 201 and 204.

Immunofluorescence staining showed that 9D11 antibody exhibited positive staining of HeLa cell nuclei,

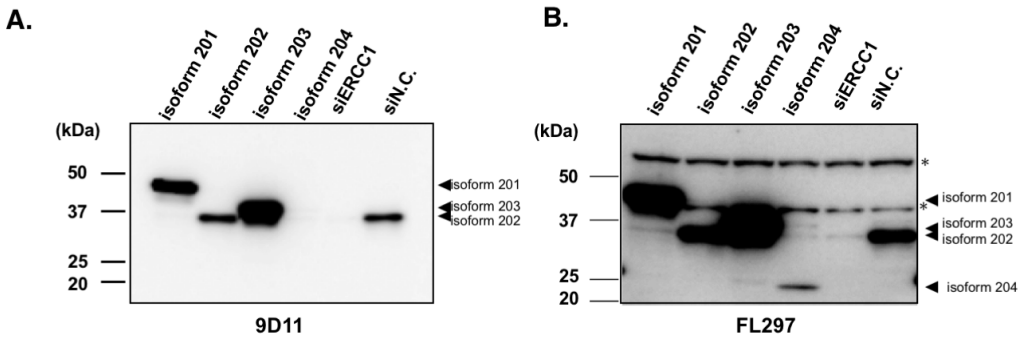

Figure 3. 9D11 antibody recognizes ERCC1 isoforms 201, 202, and 203 but not 204. Isoforms 201, 202, 203 and 204 were transiently expressed in *ERCC1* knock-down HeLa cells and whole cell extracts were subjected to Western blot analysis using anti-ERCC1 antibodies, 9D11 **(A)**, and FL297 **(B)**. *, non-specific band.

whereas almost no staining was observed in the knocked down cells with *ERCC1* siRNA (Figure 2B). The results suggested that 9D11 antibody specifically recognizes ERCC1 protein.

9D11 antibody recognizes isoforms 201, 202, and 203 but not 204

To clarify which isoforms are recognized by 9D11 antibody, we transiently transfected isoforms 201, 202, 203, and 204 expression vectors into ERCC1 knocked-down HeLa cells, respectively. As shown in Figure 3A, antibody 9D11 could detect isoforms 201, 202, and 203 but not isoform 204 and no other unrelated proteins, whereas polyclonal antibody FL297 detected all isoforms but non-specific bands were also detected (asterisks in Figure 3B).

9D11 antibody detects ERCC1 in seventeen gastric cell lines

To examine whether 9D11 detects major isoforms of ERCC1 in gastric cancer, we next examined whether 9D11 antibody can detect ERCC1 protein in seventeen gastric cancer cell lines of various types. When we measured *ERCC1* mRNA expression levels by real-time PCR using F2-R2 primer set that detects all four isoforms of ERCC1, all cell lines showed expression of ERCC1 at various levels (Figure 4A). We next carried out Western blot analysis of ERCC1 using 9D11. As shown in Figure 4B, all seventeen cell lines expressed ERCC1 protein at 37 kDa that matches the size of isoforms 202 and 203. In these gastric cancer cell lines, the presence of isoform 204 at 20-25 kDa was not detected with a commercially available polyclonal antibody FL297 that can recognize all four isoforms (Figure 4C). The bands (marked with asterisks) at the upper position of the 202/203 isoform band detected by FL297 were considered to be cross-reactive non-specific bands, because in HeLa cells, knockdown with siRNA did not affect the expression of bands at similar sizes (Figure 3B). These Western blot results with 9D11 and FL297 antibodies suggested that 202 and/or 203 could be the major isoforms in gastric cancer cells. We also noted that the mRNA and protein levels of ERCC1 did not show a clear correlation. The specific detection of ERCC1 major forms by 9D11 antibody suggests that 9D11 could be useful to detect ERCC1 levels in gastric cancers.

Evaluation of staining with clinical specimens

To further evaluate the reactivity of 9D11, we performed immunohistochemical staining using the paraffin-embedded sections of gastric cancers (Figure 5A). As a result, conditioned medium of hybridoma 9D11 showed a clear pattern of nuclear staining of ERCC1 in the specimen of poorly differentiated adenocarcinoma. Ganglion cells exhibited a high staining level of ERCC1, which was consistent with a previous report using ERCC1 antibody 4F9 (*26*), while little cytoplasmic staining was observed (Figure 5A). We also observed that vascular endothelial cells show a moderate level of constant staining.

9D11 antibody was also prepared from a large scale hybridoma culture and evaluated by immunohistochemical staining with paraffin-embedded tumor sections from several gastric cancer patients. As in the case with the small scale preparation, 9D11 antibody intensely stained nuclei (Figure 5B). Specimens from different patients showed diverse levels of staining. Taken together, analysis by Western blotting, ELISA and immunohistochemical staining demonstrated that antibody 9D11 can specifically recognize human ERCC1.

Discussion

We developed a novel ERCC1 monoclonal antibody 9D11 useful for detection of three isoforms 201, 202, and 203 containing exon 3 using GANP mouse, from which high affinity antibodies can be generated because of higher rates of mutation in the variable region of antibodies (*25*). Our results using knockdown experiments with siRNA of *ERCC1* showed that

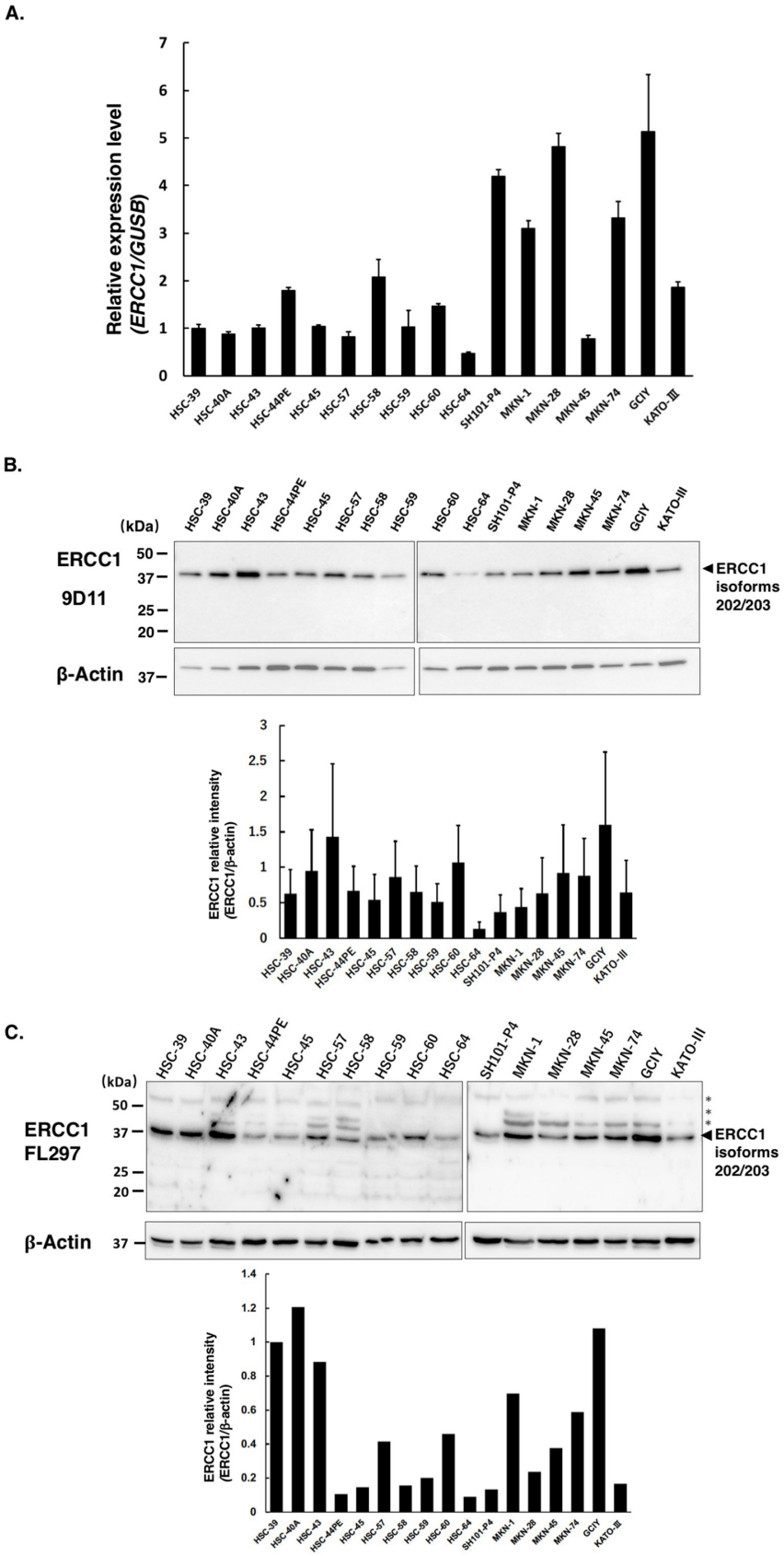

Figure 4. Analysis of *ERCC1* mRNA expression and ERCC1 protein isoforms in seventeen gastric cancer cell lines. (A) The mRNA levels of seventeen gastric cancer cell lines were analyzed by real-time PCR using F2-R2 primer set (mean + SE). **(B)** The Western blot analysis of ERCC1 using 9D11 antibody (upper panel) and the normalized ERCC1 levels by β-actin levels are shown in the lower panel (n = 2-3). **(C)** The Western blot analysis of ERCC1 detected with FL297 polyclonal antibody (upper panel) and the normalized ERCC1 levels by β-actin level are shown in the lower panel. ERCC1 isoforms 202 and/or 203 are detected at 37 kDa. The upper bands (asterisks) are cross reactive non-specific bands.

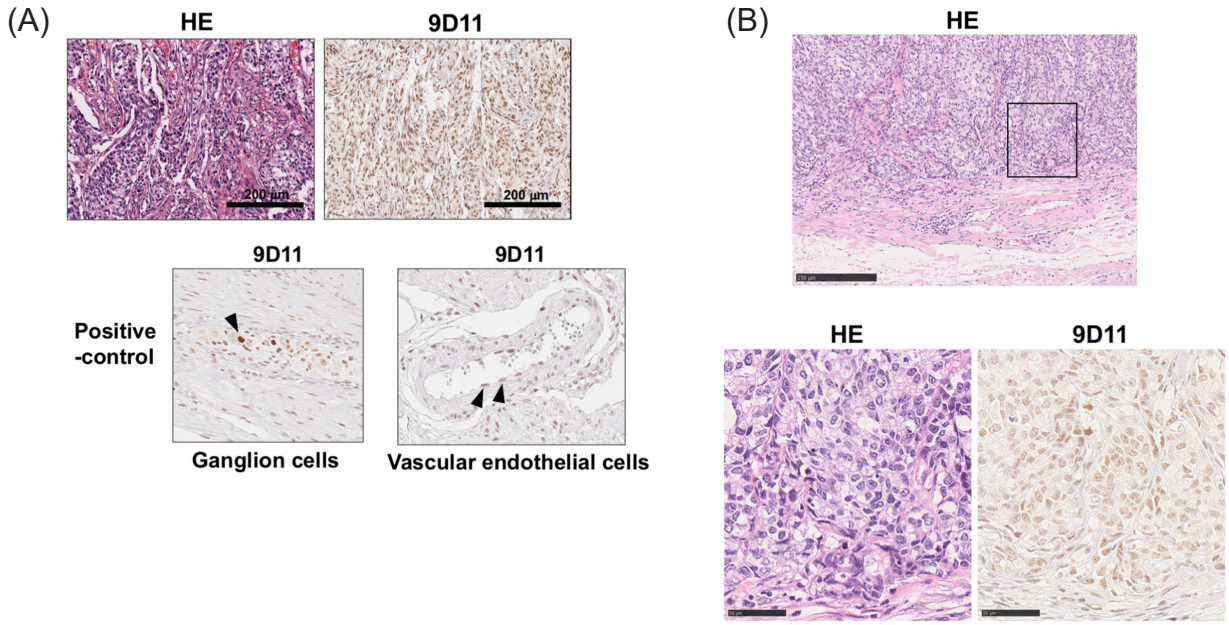

Figure 5. Immunohistochemical staining with 9D11 antibody in paraffin-embedded tumor tissue samples of gastric cancer patients. (A) A gastric cancer specimen of poorly differentiated adenocarcinoma (the top left panel, hematoxylin-eosin staining) was stained with 9D11 antibody intensely in nuclei (the top right panel) as well as ganglion cells (the bottom left panel, arrow head) and vascular endothelial cells (the bottom right panel, arrow heads). Bars, 200 μm. **(B)** 9D11 antibody was also prepared from the large scale hybridoma culture and evaluated by immunohistochemical staining with paraffin-embedded sections of poorly differentiated adenocarcinoma from gastric cancer patients. In the top panel, hematoxylin-eosin staining is shown at a lower magnification. The immunostaining of the surrounded square area is shown in the lower right panel with hematoxylin-eosin staining (the lower left panel). The bar in the top panel, 250 μm. Bars in lower panels, 50 μm.

antibody 9D11 is a specific antibody against ERCC1. We further evaluated whether this antibody can accurately detect the expression level of ERCC1 and is useful for immunohistochemical staining of tumor tissues using paraffin-embedded sections. Immunohistochemical staining using sections from gastric cancers showed an intense staining of nuclei (Figure 5A and 5B). Taken together, this study suggested that 9D11 antibody will be valuable as a novel monoclonal antibody to detect major isoforms of ERCC1 specifically in gastric cancers.

In this study, we showed that 9D11 antibody detected three isoforms 201, 202 and 203 but not isoform 204. We noted that polyclonal FL-297 antibody, which is able to recognize all four isoforms, detected only isoform 202 and/or 203 in HeLa cells and seventeen kinds of gastric cancer cell lines. Therefore, ERCC1 isoform 202 and/or 203 could be major isoforms at least in gastric cancers. This also implies that 9D11 might be useful to evaluate ERCC1 protein levels in gastric cancer specimen.

Monoclonal antibody 4F9 has been recently reported to specifically recognize isoforms 201, 202 and 203 of ERCC1 and was used to measure ERCC1 level in archival formalin-fixed paraffin-embedded colorectal cancer specimen (*26*). However, the correlation of *ERCC1* mRNA level and ERCC1 protein detection by 4F9 has not been investigated comprehensively. Using a proximity ligation assay method, Kuo *et al.* showed specific detection of functional ERCC1 isoform 202 with newly established monoclonal antibodies 2C11, 7C3 and 10D10 with 4F9 (*27*). However, each isoform contains particular sets of functional domains. Only the C-terminal domain structure of ERCC1 isoform 202 was analyzed by crystal structure analysis (*28,29*) and the structures and functions of the N-terminal domain and other isoforms have not been clarified yet. Several types of platinum agents, including carboplatin and oxaliplatin have been clinically used. Oxaliplatin is reported to kill cells by ribosome biogenesis stress (*30*), suggesting that the different processing of the lesions could be induced by other platinum agents from that induced by CDDP. There may be thus possibilities that other isoforms, such as 201 and 203 may function in DNA repair pathways after induction of lesions by other types of platinum agents. Therefore, the antibody that detects major isoforms of ERCC1, 201, 202, and 203, may be useful for evaluation of sensitivity to cisplatin and other platinum compounds.

In this study, we also showed that the established 9D11 antibody can be used to detect ERCC1 in archival formalin-fixed paraffin-embedded cancer and normal tissues. 9D11 antibody is therefore expected to be useful for measurement of ERCC1 levels in tumor specimens to predict sensitivity to chemotherapeutic agents, including CDDP and other platinum agents. Since other types of cancer and cancer cells were not evaluated in this study, our observation is limited to gastric cancers. It should therefore be examined whether this antibody can detect major isoforms of ERCC1 in other types of cancers. It

is further necessary to study the correlation between the therapeutic effect/prognosis after cisplatin-based chemotherapy and ERCC1 overexpression level in actual clinical practice for gastric and other cancers.

In the TCGA database (*31,32*), amplification of the *ERCC1* gene is found in cancers of various types not only for gastric cancers but also for endometrial carcinoma, pancreatic adenocarcinoma, cervical squamous cell carcinoma, non-small cell lung carcinoma, hepatocellular carcinoma, invasive breast carcinoma and pleural mesothelioma. Deletion of the *ERCC1* gene is found in diffuse glioma, and mature B-cell neoplasm (Figure S3, *https://www.globalhealthmedicine.com/site/supplementaldata.html?ID=23*). It is therefore suggested that both overexpression and decreased levels of ERCC1 protein level could be present in cancers and these ERCC1 states may affect responses to chemotherapies including cisplatin treatment.

As previously reported, cell death is also effectively induced in ERCC1 deficient lung cancer cells with olaparib and niraparib (*14*), poly(ADP-ribose) polymerase (PARP) inhibitors. A low expression level of ERCC1 in cancer cells is reported to be associated with a high sensitivity to ionizing radiation and UV irradiation (*33*). Additionally, it was reported that cells derived from XFE progeroid syndrome patients exhibit low expression levels of ERCC1 and a high sensitivity to mitomycin C (*34*). It should be therefore further evaluated whether 9D11 antibody is useful for prediction of therapeutic efficacy including chemotherapy with CDDP, other platinum agents, mitomycin C, PARP inhibitors and radiotherapy.

Conclusions

In this study, we developed a new ERCC1 monoclonal antibody that detects all ERCC1 major isoforms, 201, 202, 203, in gastric cancer cells. We showed that this antibody can specifically detect the expression level of ERCC1 in immunohistochemical staining of paraffin-embedded tumor sections, suggesting that 9D11 monoclonal antibody will be valuable to detect major isoforms of ERCC1 in gastric cancers and possibly in various types of cancers.

Acknowledgements

We are thankful for kind support from Dr. Toshio Imai of Central Animal Division, National Cancer Center and technical assistance by Noriko Shibata in Nagasaki University. We thank the contributors of TCGA database.

Funding: This research is partially supported by The Practical Research for Innovative Cancer Control from Japan Agency for Medical Research and Development, AMED (16ck0106050h0003, 19ck0106306h0003) and Nagasaki University Priority Research Subject Project Based on Medium-term Goals and Plans, and by the NCGM Intramural Research Fund (20A1014).

Conflict of Interest: Takuya Honda was endowed by Eli Lilly Japan K.K.. Other authors have no conflicts of interest to disclose.

References

1. Yamada Y, Boku N, Nishina T, *et al.* Impact of excision repair cross-complementing gene 1 (ERCC1) on the outcomes of patients with advanced gastric cancer: correlative study in Japan clinical oncology group trial JCOG9912. Ann Oncol. 2013; 24:2560-2565.
2. Liu YP, Ling Y, Qi QF, Zhang YP, Zhang CS, Zhu CT, Wang MH, Pan YD. The effects of ERCC1 expression levels on the chemosensitivity of gastric cancer cells to platinum agents and survival in gastric cancer patients treated with oxaliplatin-based adjuvant chemotherapy. Oncol Lett. 2013; 5:935-942.
3. Dabholkar M, Vionnet J, Bostick-Bruton F, Yu JJ, Reed E. Messenger RNA levels of XPAC and ERCC1 in ovarian cancer tissue correlate with response to platinum-based chemotherapy. J Clin Invest. 1994; 94:703-708.
4. Olaussen KA, Dunant A, Fouret P, *et al.* DNA repair by ERCC1 in non-small-cell lung cancer and cisplatin-based adjuvant chemotherapy. N Engl J Med. 2006; 355:983-991.
5. Bauman JE, Austin MC, Schmidt R, Kurland BF, Vaezi A, Hayes DN, Mendez E, Parvathaneni U, Chai X, Sampath S, Martins RG. ERCC1 is a prognostic biomarker in locally advanced head and neck cancer: results from a randomised, phase II trial. Br J Cancer. 2013; 109:2096-2105.
6. Ghosh S. Cisplatin: The first metal based anticancer drug. Bioorg Chem. 2019; 88:102925.
7. Sijbers AM, de Laat WL, Ariza RR, *et al.* Xeroderma pigmentosum group F caused by a defect in a structure-specific DNA repair endonuclease. Cell. 1996; 86:811-822.
8. McHugh PJ. XPF-ERCC1: Linchpin of DNA crosslink repair. PLoS Genet. 2020; 16:e1008616.
9. Deans AJ, West SC. DNA interstrand crosslink repair and cancer. Nat Rev Cancer. 2011; 11:467-480.
10. Metzger R, Leichman CG, Danenberg KD, *et al.* ERCC1 mRNA levels complement thymidylate synthase mRNA levels in predicting response and survival for gastric cancer patients receiving combination cisplatin and fluorouracil chemotherapy. J Clin Oncol. 1998; 16:309-316.
11. Lord RV, Brabender J, Gandara D, Alberola V, Camps C, Domine M, Cardenal F, Sánchez JM, Gumerlock PH, Tarón M, Sánchez JJ, Danenberg KD, Danenberg PV, Rosell R. Low ERCC1 expression correlates with prolonged survival after cisplatin plus gemcitabine chemotherapy in non-small cell lung cancer. Clin Cancer Res. 2002; 8:2286-2291.
12. Wang X, Zhao J, Yang L, Mao L, An T, Bai H, Wang S, Liu X, Feng G, Wang J. Positive expression of ERCC1 predicts a poorer platinum-based treatment outcome in chinese patients with advanced non-small-cell lung cancer. Med Oncol. 2010; 27:484-490.
13. Huang ZL, Cao X, Luo RZ, Chen YF, Zhu LC, Wen

Z. Analysis of ERCC1, BRCA1, RRM1 and TUBB3 as predictors of prognosis in patients with non-small cell lung cancer who received cisplatin-based adjuvant chemotherapy: A prospective study. Oncol Lett. 2016; 11:299-305.
14. Postel-Vinay S, Bajrami I, Friboulet L, Elliott R, Fontebasso Y, Dorvault N, Olaussen KA, André F, Soria JC, Lord CJ, Ashworth A. A high-throughput screen identifies PARP1/2 inhibitors as a potential therapy for ERCC1-deficient non-small cell lung cancer. Oncogene. 2013; 32:5377-5387.
15. Cheng H, Zhang Z, Borczuk A, Powell CA, Balajee AS, Lieberman HB, Halmos B. PARP inhibition selectively increases sensitivity to cisplatin in ERCC1-low non-small cell lung cancer cells. Carcinogenesis. 2013; 34:739-749.
16. Friboulet L, Olaussen KA, Pignon JP, *et al.* ERCC1 isoform expression and DNA repair in non-small-cell lung cancer. N Engl J Med. 2013; 368:1101-1110.
17. Friboulet L, Postel-Vinay S, Sourisseau T, *et al.* ERCC1 function in nuclear excision and interstrand crosslink repair pathways is mediated exclusively by the ERCC1-202 isoform. Cell Cycle. 2013;12:3298-306.
18. Ma D, Baruch D, Shu Y, Yuan K, Sun Z, Ma K, Hoang T, Fu W, Min L, Lan ZS, Wang F, Mull L, He WW. Using protein microarray technology to screen anti-ERCC1 monoclonal antibodies for specificity and applications in pathology. BMC biotechnol. 2012; 12:88.
19. Arbogast S, Behnke S, Opitz I, Stahel RA, Seifert B, Weder W, Moch H, Soltermann A. Automated ERCC1 immunohistochemistry in non-small cell lung cancer: comparison of anti-ERCC1 antibodies 8F1, D-10, and FL-297. Appl Immunohistochem Mol Morphol. 2011; 19:99-105.
20. Bhagwat NR, Roginskaya VY, Acquafondata MB, Dhir R, Wood RD, Niedernhofer LJ. Immunodetection of DNA repair endonuclease ERCC1-XPF in human tissue. Cancer Res. 2009; 69:6831-6838.
21. Bahamon BN, Gao F, Danaee H. Development and validation of an ERCC1 immunohistochemistry assay for solid tumors. Arc Pathol Lab Med. 2016; 140:1397-1403.
22. Yanagihara K, Ito A, Toge T, Numoto M. Antiproliferative effects of isoflavones on human cancer cell lines established from the gastrointestinal tract. Cancer Res. 1993; 53:5815-5821.
23. Y Yanagihara K, Tanaka H, Takigahira M, Ino Y, Yamaguchi Y, Toge T, Sugano K, Hirohashi S. Establishment of two cell lines from human gastric scirrhous carcinoma that possess the potential to metastasize spontaneously in nude mice. Cancer Sci. 2004; 95:575-582.
24. Sasaki Y, Hozumi M, Fujimori H, Murakami Y, Koizumi F, Inoue K, Masutani M. PARG Inhibitors and functional PARG inhibition models. Curr Protein Pept Sci. 2016; 17:641-653.
25. Singh SK, Maeda K, Eid MM, Almofty SA, Ono M, Pham P, Goodman MF, Sakaguchi N. GANP regulates recruitment of AID to immunoglobulin variable regions by modulating transcription and nucleosome occupancy. Nat Commun. 2013; 4:1830.
26. Smith DH, Fiehn AM, Fogh L, Christensen IJ, Hansen TP, Stenvang J, Nielsen HJ, Nielsen KV, Hasselby JP, Brünner N, Jensen SS. Measuring ERCC1 protein expression in cancer specimens: validation of a novel antibody. Scientific reports. 2014; 4:4313.
27. Kuo MS, Adam J, Dorvault N, Robin A, Friboulet L, Soria JC, Olaussen KA. A novel antibody-based approach to detect the functional ERCC1-202 isoform. DNA Repair (Amst). 2018; 64:34-44.
28. Tsodikov OV, Enzlin JH, Scharer OD, Ellenberger T. Crystal structure and DNA binding functions of ERCC1, a subunit of the DNA structure-specific endonuclease XPF-ERCC1. Proc Natl Acad Sci U S A. 2005; 102:11236-11241.
29. Tsodikov OV, Ivanov D, Orelli B, Staresincic L, Shoshani I, Oberman R, Schärer OD, Wagner G, Ellenberger T. Structural basis for the recruitment of ERCC1-XPF to nucleotide excision repair complexes by XPA. EMBO J. 2007; 26:4768-4776.
30. Bruno PM, Liu Y, Park GY, Murai J, Koch CE, Eisen TJ, Pritchard JR, Pommier Y, Lippard SJ, Hemann MT. A subset of platinum-containing chemotherapeutic agents kills cells by inducing ribosome biogenesis stress. Nat Med. 2017; 23:461-471.
31. Cerami E, Gao J, Dogrusoz U, *et al.* The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov. 2012; 2:401-404.
32. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun Y, Jacobsen A, Sinha R, Larsson E, Cerami E, Sander C, Schultz N. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal. 2013; 6: pl1.
33. Orelli B, McClendon TB, Tsodikov OV, Ellenberger T, Niedernhofer LJ, Scharer OD. The XPA-binding domain of ERCC1 is required for nucleotide excision repair but not other DNA repair pathways. J Biol Chem. 2010; 285:3705-3712.
34. Niedernhofer LJ, Garinis GA, Raams A, *et al.* A new progeroid syndrome reveals that genotoxic stress suppresses the somatotroph axis. Nature. 2006; 444:1038-1043.

Received January 10, 2021; Revised April 25, 2021; Accepted May 10, 2021.

Released online in J-STAGE as advance publication May 27, 2021.

§*These authors contributed equally to this work.*
**Address correspondence to*:
Mitsuko Masutani, Department of Molecular and Genomic Biomedicine, Center for Bioinformatics and Molecular Medicine, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan.
E-mail: mmasutan@nagasaki-u.ac.jp