XRCC4 deficiency in human subjects causes a marked neurological phenotype but no overt immunodeficiency

Chaowan Guo, PhD,[a,b,c]* Yuka Nakazawa, PhD,[a,b,c]* Lisa Woodbine, PhD,[d]* Andrea Björkman, PhD,[e] Mayuko Shimada, BS,[b,c] Heather Fawcett, PhD,[d] Nan Jia, PhD,[a,b,c] Kaname Ohyama, PhD,[b,f] Tao-Sheng Li, MD, PhD,[g] Yuji Nagayama, MD, PhD,[c] Norisato Mitsutake, MD, PhD,[b,h] Qiang Pan-Hammarström, MD, PhD,[e] Andrew R. Gennery, MD,[i] Alan R. Lehmann, PhD,[d] Penny A. Jeggo, PhD,[d] and Tomoo Ogi, PhD[a,b,c,j]

Nagoya, Nagasaki, and Mishima, Japan, Brighton and Newcastle upon Tyne, United Kingdom, and Stockholm, Sweden

Background: Nonhomologous end-joining (NHEJ) is the major DNA double-strand break (DSB) repair mechanism in human cells. The final rejoining step requires DNA ligase IV (LIG4) together with the partner proteins X-ray repair cross-complementing protein 4 (XRCC4) and XRCC4-like factor. Patients with mutations in genes encoding LIG4, XRCC4-like factor, or the other NHEJ proteins DNA-dependent protein kinase catalytic subunit and Artemis are DSB repair defective and immunodeficient because of the requirement for NHEJ during V(D)J recombination.

Objective: We found a patient displaying microcephaly and progressive ataxia but a normal immune response. We sought to determine pathogenic mutations and to describe the molecular pathogenesis of the patient.

Methods: We performed next-generation exome sequencing. We evaluated the DSB repair activities and V(D)J recombination capacity of the patient's cells, as well as performing a standard blood immunologic characterization.

Results: We identified causal mutations in the *XRCC4* gene. The patient's cells are radiosensitive and display the most severe DSB repair defect we have encountered using patient-derived cell lines. In marked contrast, a V(D)J recombination plasmid assay revealed that the patient's cells did not display the junction abnormalities that are characteristic of other NHEJ-defective cell lines. The mutant protein can interact efficiently with LIG4 and functions normally in *in vitro* assays and when transiently expressed *in vivo*. However, the mutation makes the protein unstable, and it undergoes proteasome-mediated degradation.

Conclusion: Our findings reveal a novel separation of impact phenotype: there is a pronounced DSB repair defect and marked clinical neurological manifestation but no clinical immunodeficiency. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: *DNA double-strand break repair, nonhomologous end-joining, double-strand break repair deficiency, XRCC4/LIG4, immunodeficiency, microcephaly*

Double-strand breaks (DSBs) arise from oxidative damage and during processes such as meiosis or V(D)J recombination. They are also the major lethal lesions generated in cellular DNA by ionizing radiation. Although DSBs do not arise at high frequency endogenously, they are a critical lesion causing cell death or carcinogenesis if misrepaired. Additionally, the development of the immune response involves introduction of DSBs to generate the requisite genetic diversity.[1] V(D)J recombination represents one such process.[2] Class-switch recombination (CSR), which involves switching of immunoglobulin isotypes, also involves introduction and rejoining of DSBs.[3]

Nonhomologous end-joining (NHEJ) is the major DSB repair process in mammalian cells. The process is initiated by the binding of the Ku heterodimer to double-stranded DNA ends, followed by recruitment of the DNA-dependent protein kinase (DNA-PK) catalytic subunit, generating the DNA-PK holoenzyme and activating DNA-PK activity.[4] DNA-PK has a role in end-tethering and regulates the steps of end-processing. The final step of rejoining involves the recruitment of a ligation complex encompassing DNA ligase IV (LIG4), X-ray repair cross-complementing protein 4 (XRCC4), and XRCC4-like factor (XLF). LIG4 and

From [a]the Department of Genetics, Research Institute of Environmental Medicine (RIeM), Nagoya University, Furo-cho, Chikusa-ku, Nagoya; [b]Nagasaki University Research Centre for Genomic Instability and Carcinogenesis (NRGIC); the Departments of [c]Molecular Medicine, [g]Stem Cell Biology, and [h]Radiation Medical Sciences, Atomic Bomb Disease Institute, and [f]the Course of Pharmaceutical Sciences, Graduate School of Biomedical Sciences, Nagasaki University; [d]the Genome Damage and Stability Centre, University of Sussex, Falmer, Brighton; [e]the Department of Laboratory Medicine, Karolinska Institutet, Karolinska University Hospital, Stockholm; [i]the Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne; and [j]the Microbial Genetics Laboratory, Genetic Strains Research Center, National Institute of Genetics, Mishima.

*These authors contributed equally to this work.

Supported by a foreign researcher grant from the Japan Society for the Promotion of Science (JSPS; P14093) to C.G.; a grant for Research for overcoming intractable diseases (H26-general-046) from the Ministry of Health, Labour and Welfare, Japan, KAKENHI Grants-in-Aid for Scientific Research (B [26291005]) from JSPS, KAKENHI Grants-in-Aid for Scientific Research (A [Overseas Academic Research, 15H02654]) from JSPS, and a science research grant from the Uehara Memorial Foundation to T.O.; KAKENHI Grants-in-Aid for Young Scientists (A [15H05333]) from JSPS, a research grant from the Uehara Memorial Foundation, a medical research grant from Daiichi-Sankyo Foundation of Life Science, and a medical research grant from Takeda Science Foundation and Special Coordination Funds for Promoting Science and Technology from the Japan Science and Technology Agency (JST) to Y. Nakazawa; and the Swedish Research Council and European Research Council (242551-ImmunoSwitch; to Q.P.-H.).

Disclosure of potential conflict of interest: L. Woodbine has been supported by an MRC grant, is employed by the University of Sussex, and has received payment for diagnostic analysis. A. R. Lehmann has received research support from and is a scientific consultant for UK NHS and has received support for travel to conferences. P. A. Jeggo has received research support from and is employed by MRC and has received payment for reviewing papers and for diagnostic analysis. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication February 20, 2015; revised May 27, 2015; accepted for publication June 8, 2015.

Corresponding author: Tomoo Ogi, PhD, Department of Genetics, Research Institute of Environmental Medicine (RIeM), Nagoya University, Furo-cho, Chikusa-ku, Nagoya, 464-8601, Japan. E-mail: togi@riem.nagoya-u.ac.jp. Or: Penny A. Jeggo, PhD, Genome Damage and Stability Centre, University of Sussex, Falmer, Brighton BN1 9RQ, United Kingdom. E-mail: p.a.jeggo@sussex.ac.uk.

0091-6749/$36.00

http://dx.doi.org/10.1016/j.jaci.2015.06.007

Abbreviations used
CID: Combined immunodeficiency
CS: Cockayne syndrome
CSR: Class-switch recombination
DNA-PK: DNA-dependent protein kinase
DSB: DNA double-strand break
GST: Glutathione S-transferase
IR: Ionizing radiation
LIG4: DNA ligase 4
3-MA: 3-Methyladenine
NHEJ: Nonhomologous end-joining
NMD: Nonsense-mediated mRNA decay
RAG: Recombination-activating gene
SCID: Severe combined immunodeficiency
WT: Wild-type
XLF: XRCC4-like factor
XRCC4: X-ray repair cross-complementing protein 4

XRCC4 interact strongly through a tandem BRCA1 carboxyl terminal domain in LIG4 and a coiled-coil region in XRCC4, forming a highly stable complex.[5,6] XRCC4 is required for LIG4 stabilization, but recent findings have shown an excess of XRCC4 relative to LIG4.[7] Mutations that abolish LIG4/XRCC4 interaction prevent all NHEJ activity. XLF is a weaker binding partner. XRCC4 and XLF are structurally similar; both have a globular head domain, but the coiled-coil region of XLF is shorter than that of XRCC4.[8,9] Recent studies have reported that dimers of XRCC4 and XLF interact to form long superhelical filaments in solution, which have been proposed to promote end-tethering or end-bridging downstream of the DNA-PK complex.[8,10-12]

Loss of LIG4 or XRCC4 confers embryonic lethality in mice because of extensive neuronal apoptosis.[13,14] In contrast, XLF-null mice are viable and do not show marked immunodeficiency.[15] Mutations in *LIG4*, *DCLRE1C* (Artemis), *NHEJ1* (XLF), and *PRKDC* (DNA-PK catalytic subunit) have been described.[16-20] LIG4 syndrome, which is caused by hypomorphic mutations in *LIG4*, is characterized by growth delay, microcephaly, and combined immunodeficiency (CID) or severe combined immunodeficiency (SCID). LIG4 syndrome cell lines show radiosensitivity, diminished ability to repair DSBs, and impaired V(D)J recombination. XLF-defective patients, some of whom have null mutations, display features similar to but often more severe than those of patients with LIG4 syndrome.[18] Given the close interaction and requirement of XRCC4 for LIG4 stability, the expectation was that XRCC4 deficiency in patients would resemble LIG4 syndrome.

In contrast to this expectation, an XRCC4-deficient patient, CSL16NG, whose cellular defects we describe in this article, displayed unique clinical features similar to those of patients with Cockayne syndrome (CS), namely progressive neuronal degeneration, including onset of ataxia, but no clinical immunodeficiency. Our cellular analysis revealed that the mutational changes result in substantially reduced levels of XRCC4 protein, a marked defect in DSB repair, but seemingly efficient V(D)J recombination. Based on comparative studies with LIG4- and XLF-deficient cell lines, our results reveal a separation of impact phenotype in which marked deficiency in radiation-induced DSB repair capacity can be uncoupled from defective V(D)J recombination. Our results provide novel insight into the roles of XRCC4 during DSB repair.

METHODS

Human studies

Patient and control samples were obtained with local ethical approvals (the University of Sussex Research Ethics Committee; the Nagasaki University Ethical, Legal and Social Implications (ELSI) Committee; and the Ethics Committee for Human Genome Studies in Research Institute of Environmental Medicine, Nagoya University). Written informed consent was obtained from the patient.

Exome sequencing and bioinformatics

Details are described in the Methods section in this article's Online Repository at www.jacionline.org. Briefly, genomic DNA prepared from CSL16NG fibroblasts was enriched by using the SureSelect All Exon Kit v5 (Agilent Technologies, Santa Clara, Calif), followed by sequencing on the Illumina HiSeq 2500 Sequencer (Illumina, San Diego, Calif). The sequence data were analyzed by using a standard exome pipeline. According to a recessive inheritance model, we selected genes that carried at least 1 novel deleterious homozygous or more than 2 heterozygous changes in 1 gene locus (see Table E1 in this article's Online Repository at www.jacionline.org).

Cell cultures

Human primary or human telomerase reverse transcriptase–immortalized fibroblasts were derived from the patient with XRCC4 deficiency (CSL16NG), patients with LIG4 syndrome (F07/614, 180BR, 411BR, 2303, and 495GOS), patients with XLF deficiency (2BN and F07/402), a patient with xeroderma pigmentosum (XP15BR), or healthy donors (1BR.3 and 48BR). All primary fibroblasts were cultured in Dulbecco modified Eagle medium or modified Eagle medium supplemented with 10% to 15% FBS and 1× penicillin-streptomycin. HEK293FT cells (Invitrogen, Carlsbad, Calif) were grown in Dulbecco modified Eagle medium supplemented with 10% FBS, 1× penicillin-streptomycin, 5 mmol/L L-glutamine, 0.1 mmol/L nonessential amino acids, and 1 mmol/L sodium pyruvate. Human colon cancer HCT116 (RCB2979) and HCT116-XRCC4$^{-/-}$ (RCB2981) cells were provided by the RIKEN BRC through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Reagents and antibodies

Antibodies used were as follows: XRCC4 (C4 and C20; Santa Cruz Biotechnology, Dallas, Tex), LIG4 (D8; Santa Cruz Biotechnology; Ab80514, Abcam, Cambridge, United Kingdom; 12695-1-AP, Proteintech Group, Chicago, Ill), p89 (S-19, Santa Cruz Biotechnology), XPG (8H7, Santa Cruz Biotechnology), CSB (Bio Matrix Research, Chiba, Japan), V5-tag (MBL, Woburn, Mass), Flag-tag (MBL), actin (Ab8227, Abcam), α-tubulin (Invitrogen), XLF (ab33499, Abcam; FL-299, Santa Cruz Biotechnology), 53BP1 (Bethyl Laboratories, Montgomery, Tex), and γH2A.X (ser139, Millipore, Temecula, Calif). MG132, E64D, 3-methyladenine (3-MA), epoxomicin, and cycloheximide (Sigma-Aldrich, St Louis, Mo).

Quantitative RT-PCR

Details are as described previously.[21] Total RNA was extracted with the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). The high-capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, Calif) was used for first-strand synthesis. Quantitative PCR was performed by using the Thermal Cycler Dice Real-Time system (TaKaRa Bio, Clontech, Mountain View, Calif) with a QuantiTect SYBR Green PCR Kit (Qiagen). For each sample, relative mRNA expression levels were normalized by using the hypoxanthine phosphoribosyltransferase 1 *(HPRT1)* gene. Error bars represent the SDs of means of triplicate experiments.

Lentivirus experiments

Details are as described previously.[22] Recombinant lentivirus particles expressing *XRCC4* and its mutants were produced. Human *XRCC4* cDNA was