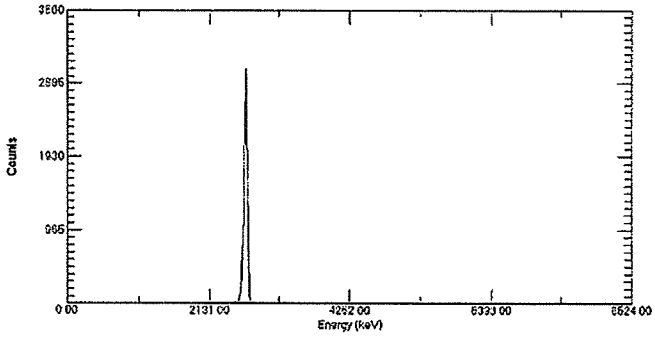

Fig. 19. A monoenergetic peak in the energy spectrum of the single-proton microbeam.

ween the collimator exit and the cell dish. A photomultiplier tube (PMT, Hamamatsu, type R7400U-4) mounted above the cell dish collects the scintillation photons produced on the particle passage through the scintillator (See Fig. 17). When the system detects the present number of particles, the scaler generate a signal to turn off the beam. The turn-off time of the deflection is approximately 250 ns.

Cell image processing and cell irradiation

The most important factor in determining the throughput of a microbeam system for irradiating cells is the ability of the microscopic image analysis system to recognize the targets and move them into position. In the cell image processing, the sample cells are grown on a 3.5-μm Mylar dish with a density of 800–1000 cells per 10 mm × 10 mm area. During the irradiation procedure, the cells are visualized after stained using fluorescent dyes and then can be easily distinguished from the background. To minimize the UV dose delivered to the cells during the imaging process, a fast mechanical light shutter is incorporated to limit illumination on the cells only for the time necessary for the CCD camera. The images are transmitted to a PC where every cell is recognized using automatic image processing routines (programmed by our group). After image processing, each cell is sequentially moved over the beam exit by scanning the sample stage (step length 0.025 μm) according to the established relative coordinates. Then particles are delivered with the preset number. The beam is turned on until the chosen number of particles has arrived. This operation is repeated until all the cells are irradiated.

Accuracy determination

To determine the single-ion detection efficiency and the target location accuracy, we employed the 10 × 10 single-hit grids etched on CR39 nuclear track detector. Figure 20 shows a result by the CAS-LIBB microbeam. The measured results show that a single-particle detection efficiency > 98% and a target positioning deviation ≤± 2 μm are successfully achieved.

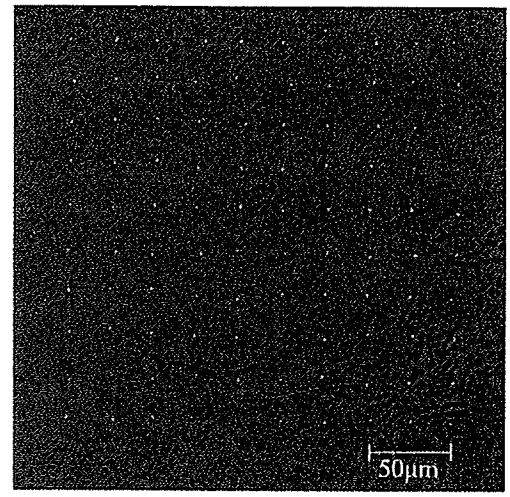

Fig. 20. A 10 × 10 single-hit grid etched on CR39 by the CAS-LIBB microbeam.

Biological studies with CAS-LIBB single-particle microbeam

To determine whether long-distance bystander/abscopal effects exist in whole organisms and to clarify the problem of intercellular communication, a specific cell group, the shoot apical meristem in model plant system *Arabidopsis* embryo, was irradiated with a defined number of protons by CAS-LIBB single-particle microbeam and examined for root development. The results showed that after direct damage to the shoot apical meristem from ion traversals, root hair differentiation, primary root elongation and lateral root initiation were all inhibited significantly in postembryonic development, suggesting that radiation-induced long-distance bystander/abscopal responses might exist in the whole organism. To further scrutinize the mechanism(s) underlying these inhibitory effects, a DR5-*GUS* transgenic *Arabidopsis* was used. The results showed that accumulation of the reporter *GUS* gene transcript in irradiated shoot apical meristem embryos decreased in the postembryonic development. Treatment with either 2,4-dichlorophenoxyacetic acid, a synthetic plant auxin, or DMSO, a effective reactive oxygen species (ROS) scavenger, could rescue the reporter GUS enzyme accumulation and the length of primary root in irradiated shoot apical meristem embryos, indicating that ROS or probably the ROS related auxin and auxin-dependent transcription process may be involved in radiation-induced long-distance bystander/abscopal effects.

REFERENCES

1. Folkard, M., Vojnovic, B., Prise, K. M., Bowey, A. G., Locke, R. J., Schettino, G. and Michael, B. D. (1997) A charged-particle microbeam: Part I: Development of an experimental system for targeting cells individually with counted particles,

Int. J. Radiat. Biol. **72**: 375–385.
2. Randers-Pehrson, G., Geard, C. R., Johnson, G., Elliston, C. D. and Brenner, D. J. (2001) The Columbia University single-ion microbeam. Radiat. Res. **156**: 210–214.
3. Zhou, H., Suzuki, M., Randers-Pehrson, G., Vannais, D., Chen, G., Trosko, J. E., Waldren, C. A. and Hei, T. K. (2001) Radiation risk to low fluences of alpha particles may be greater than we thought. Proc. Natl. Acad. Sci. USA **98**: 14410–14415.
4. Shao, C., Stewart, V., Folkard, M., Michael, B. D. and Prise, K. M. (2003) Nitric oxide-mediated signaling in the bystander response of individually targeted glioma cells. Cancer Res. **63**: 8437–8442.
5. Zhou, H., Ivanov, V. N., Gillespie, J., Geard, C. R., Amundson, S. A., Brenner, D. J., Yu, Z., Lieberman, H. B. and Hei, T. K. (2005) Mechanism of radiation-induced bystander effect: role of cyclooxygenase-2 signaling pathway. Proc. Natl. Acad. Sci. USA **102**: 14641–14646.
6. Shao, C., Folkard, M., Michael, B. D. and Prise, K. M. (2004) Targeted cytoplasmic irradiation induces bystander responses. Proc. Natl. Acad. Sci. USA **101**: 13495–13500.
7. Zhou, H., Ivanov, V. N., Lien, Y. C., Davidson, M. and Hei, T. K. (2008) Mitochondrial function and nuclear factor-kappa B-mediated signaling in radiation-induced bystander effects. Cancer Res. **68**: 2233–2240.
8. Funayama, T., Hamada, N., Sakashita, T. and Kobayashi, Y. (2008) Heavy-ion microbeams – development and applications in biological studies. IEEE Trans. Plasma Sci. **36**: 1432–1440.
9. Hamada, N. (2009) Recent insights into the biological action of heavy-ion radiation. J. Radiat. Res. **50**: 1–9.
10. Sugimoto, T., Dazai, K., Sakashita, T., Funayama, T., Wada, S., Hamada, N., Kakizaki, T., Kobayashi, Y. and Higashitani, A. (2006) Cell cycle arrest and apoptosis in *caenorhabditis elegans* germline cells following heavy-ion microbeam irradiation. Int. J. Radiat. Biol. **82**: 31–38.
11. Tanaka, A., Kobayashi, Y., Hase, Y. and Watanabe, H. (2001) Positional effect of cell inactivation on root gravitropism using heavy-ion microbeams. J. Exp. Bot. **53**: 683–687.
12. Kiguchi, K., Shirai, K., Kanekatsu, R., Kobayashi, Y., Tu, Z. L., Funayama, T. and Watanabe, H. (2003) Radiosurgery using heavy ion microbeams for biological study: Fate mapping of the cellular blastoderm-stage egg of the silkworm, *bombyx mori*. Nucl. Instr. Meth. Phys. Res. B **210**: 312–315.
13. Fukamoto, K., Shimura, S., Shirai, K., Kanekatsu, R., Kiguchi, K., Sakashita, T., Funayama, T. and Kobayashi, Y. (2006) Effects of heavy-ion irradiadion on the differentiation of epidermal cells in the silkworm, *bombyx mori*. J. Insect Biotech. Sericol. **75**: 107–114.
14. Fukamoto, K., Shirai, K., Sakata, T., Sakashita, T., Funayama, T., Hamada, N., Wada, S., Kakizaki, T., Shimura, S., Kobayashi, Y. and Kiguchi, K. (2007) Development of the irradiation method for the first instar silkworm larvae using locally targeted heavy-ion microbeam. J. Radiat. Res. **48**: 247–253.
15. Miyazawa, Y., Sakashita, T., Funayama, T., Hamada, N., Negishi, H., Kobayashi, A., Kaneyasu, T., Ooba, A., Morohashi, K., Kakizaki, T., Wada, S., Kobayashi, Y., Fujii, N. and Takahashi, H. (2008) Effects of locally targeted heavy-ion and laser microbeam on root hydrotropism in Arabidopsis thaliana. J. Radiat. Res. **49**: 373–379.
16. Funayama, T., Wada, S., Kobayashi, Y. and Watanabe, H. (2005) Irradiation of mammalian cultured cells with a collimated heavy-ion microbeam. Radiat. Res. **163**: 241–246.
17. Kobayashi, Y., Funayama, T., Wada, S., Furusawa, Y., Aoki, M., Shao, C., Yokota, Y., Sakashita, T., Matsumoto, Y., Kakizaki, T. and Hamada, N. (2004) Microbeams of heavy charged particles. Biol. Sci. Space **18**: 235–240.
18. Funayama, T., Wada, S., Yokota, Y., Fukamoto, K., Sakashita, T., Taguchi, M., Kakizaki, T., Hamada, N., Suzuki, M., Furusawa, Y., Watanabe, H., Kiguchi, K. and Kobayashi, Y. (2008) Heavy-ion microbeam system in JAEA for microbeam biology. J. Radiat. Res. **49**: 71–82.
19. Shao, C., Furusawa, Y., Kobayashi, Y., Funayama, T. and Wada, S. (2003) Bystander effect induced by counted high-LET particles in confluent human fibroblasts: A mechanistic study. FASEB J. **17**: 1422–1427.
20. Yokota, Y., Funayama, T., Kobayashi, Y., Sakashita, T., Wada, S., Hase, Y., Shikazono, N., Tanaka, A. and Inoue, M. (2003) Development of an ion microbeam system for irradiating single plant cell[s]. Biol. Sci. Space **17**: 298–301.
21. Shao, C., Furusawa, Y., Kobayashi, Y. and Funayama, T. (2006) Involvement of gap junctional intercellular communication in the bystander effect induced by broad-beam or microbeam heavy ions. Nucl. Instr. Meth. Phys. Res. B **251**: 177–181.
22. Oikawa, M., Satoh, T., Sakai, T., Miyawaki, N., Kashiwagi, H., Kurashima, S., Okumura, S., Fukuda, M., Yokota, W. and Kamiya, T. (2007) Focusing high-energy heavy ion microbeam system at the JAEA AVF cyclotron. Nucl. Instr. Meth. Phys. Res. B **260**: 85–90.
23. Kurashima, S., Miyawaki, N., Kamiya, T., Oikawa, M., Yoshida, K., Fukuda, M., Okumura, S., Satoh, T., Nara, T., Agematsu, T., Ishibori, I., Yokota, W. and Nakamura, Y. (2007) Improvement in beam quality of the JAEA AVF cyclotron for focusing heavy-ion beams with energies of hundreds of MeV. Nucl. Instr. Meth. Phys. Res. B **260**: 65–70.
24. Hino, M., Wada, S., Tajika, Y., Morimura, Y., Hamada, N., Funayama, T., Sakashita, T., Kakizaki, T., Kobayashi, Y. and Yorifuji, H. (2007) Heavy ion microbeam irradiation induces ultrastructural changes in isolated single fibers of skeletal muscle. Cell Struct. Funct. **32**: 51–56.
25. Hamada, N., Ni, M., Funayama, T., Sakashita, T. and Kobayashi, Y. (2008) Temporally distinct response of irradiated normal human fibroblasts and their bystander cells to energetic heavy ions. Mutat. Res. **639**: 35–44.
26. Hamada, N., Hara, T., Omura-Minamisawa, M., Ni, M., Funayama, T., Sakashita, T., Sora, S., Nakano, T. and Kobayashi, Y. (2008) Heavy-ion microbeam irradiation induces bystander killing of human cells. Biol. Sci. Space **22**: 46–53.
27. Iwakawa, M., Hamada, N., Imadome, K., Funayama, T., Sakashita, T., Kobayashi, Y. and Imai, T. (2008) Expression profiles are different in carbon ion-irradiated normal human fibroblasts and their bystander cells. Mutat. Res. **642**: 57–67.
28. Imaseki, H. and Yukawa, M. (2000) Introduction of PIXE

analysis system in NIRS. Int. J. PIXE. **10**: 77–90.
29. Imaseki, H., Ishii, K., Ishikawa, T., Iso, H., Shinomiya, T., Hamamo, T. and Yukawa, M. (2005) Development of droplet-PIXE system for environmental monitoring samples. Int. J. PIXE, **15**: 293–299.
30. Ishikawa, T., Iso, H., Oikawa, M., Konishi, T., Kitamura, H., Higuchi, Y., Suya, N., Hamano, T. and Imaseki, H. (2009) Development of a real-time beam current monitoring system for microbeam scanning-PIXE analysis using a ceramic channel electron multiplier. Nucl. Instr. and Meth. B. *in press.*
31. Imaseki, H., Ishikawa, T., Iso, H., Konishi, T., Suya, N., Hamano, T., Wang, X., Yasuda, N. and Yukawa, M. (2007) Progress report of the single particle irradiation system to cell (SPICE). Nucl. Instr. and Meth. B. **260**: 81–84.
32. Konishi, T., Ishikawa, T., Iso, H., Yasuda, N., Oikawa, M., Higuchi, Y., Kato, T., Hafer, K., Kodama, K., Hamano, T,. Suya, N. and Imaseki, H. (2009) Biological studies using mammalian cell lines and the current status of the microbeam irradiation system, SPICE., Nucl. Instr. and Meth. B. *in press.*
33. Yasuda, N., Namiki, K., Honma, Y., Umeshima, Y., Marumo, Y., Ishii, H. and Benton, E. R. (2005) Development of a high speed imaging microscope and new software for nuclear track detector analysis. Radiat. Meas. **40**: 311–315.
34. Soh, N. (2006) Recent advances in fluorescent probes for the detection of reactive oxygen species. Anal. Bioanal. Chem. **386**: 532–543.
35. Hafer, K., Konishi, T. and Schiestl, R. (2008) Radiation-induced long-lived extracellular radicals do not contribute to measurement of intracellular reactive oxygen species using the dichlorofluorescein method. Radiat. Res. **169**: 469–473.
36. Kamiya, T., Yokota, W., Kobayashi, Y., Cholewa, M., Krochmal, M. S., Laken, G., Larsen, I. D., Fiddes, L., Parkhill, G. and Dowsey, K. (2001) Development of an automated single cell irradiation system combined with a high-energy heavy ion microbeam system, Nucl. Instr. Meth. B. **181**: 27–31.
37. Kobayashi, K., Usami, N., Maezawa, H., Hayashi, T., Hieda, K. and Takakura, K. (2006) Synchrotron X-ray microbeam irradiation system for radiobiology. J. Biomed. Nanotechnol. **2**: 116–119.
38. Hatori, S., Kurita, T., Hayashi, Y., Yamada, M., Yamada, H., Mori, J., Hamachi, H., Kimura, S., Shimoda, T., Hiroto, M., Hashimoto, T., Shimada, M., Yamamoto, H., Ohtani, N., Yasuda, K., Ishigami, R., Sasase, M., Ito, Y., Hatashita, M., Takagi, K., Kume, K., Fukuda, S., Yokohama, N., Kagiya, G., Fukumoto, S. and Kondo, M. (2005) Developments and applications of accelerator system at the Wakasa Wan Energy Research Center. Nucl. Instr. and Meth. **B241**: 862–869.
39. Yasuda, K., Hatashita, M., Hatori, S., Inomata, T., Ishigami, R., Ito, Y., Kurita, T., Sasase, M. and Takagi, K. (2003) Ion microbeam facility at the Wakasa Wan Energy Research Center. Nucl. Instr. and Meth. **B 210**: 27–32.
40. Folkard, M., Schettino, G., Vojnovic, B., Gilchrist, S., Michette, A. G., Pfauntsch, S. J., Prise, K. M. and Michael, B. D. (2001) A focused ultrasoft X-ray microbeam for targeting cells individually with submicrometer accuracy. Radiat. Res. **156**: 796–804.
41. Hei, T. K., Zhou, H., Ivanov, V. N., Hong, M., Lieberman, H. B., Brenner, D. J., Amundson, S. A. and Geard, C. R. (2008) Mechanism o radiation-induced bystander effects: a unifying model. J. Pharm. Pharmacol. **60**: 945–950.
42. Shao, C., Prise, K. M. and Folkard, M. (2008) Signaling factors for irradiated glioma cells induced bystander response in fibroblasts. Mutat. Res. **638**: 139–145.
43. Koyama, S., Kodama, S., Suzuki, K., Matsumoto, T., Miyazaki, T. and Watanabe, M. (1998) Radiation-induced long-lived radicals which cause mutation and transformation. Mutat. Res. **421**: 45–54.
44. Suzuki, K., Kodama, S. and Watanabe, M. (2001) Extremely low-dose ionizing radiation causes activation of mitogen-activated protein kinase pathway and inhances proliferation of normal human diploid cells. Cancer Res. **61**: 5396–5401.
45. Kumagai, J., Masui, K., Itagaki, Y., Shiotani, M., Kodama, S., Watanabe, M. and Miyazaki, T. (2003) Long-lived mutagenic radicals induced in mammalian cells by ionizing radiation are mainly localized to proteins. Radiat. Res. **60**: 95–102.
46. Hauptner, A., Dietzel, S., Drexler, G. A., Reichart, P., Krucken, R., Cremer, T., Friedl, A. A. and Dlooinger, G. (2004) Microirradiation of the cells with energetic heavy ions. Radiat. Environ. Biophys. **42**: 237–245.
47. Schettino, G., Folkard, M., Prise, K. M., Vojnovic, B., Held, K. D. and Michael, B. D. (2003) Low-dose studies of bystander cell killing with targeted soft X-rays. Radiat. Res. **160**: 505–511.
48. Kobayashi, K., Usami, N., Hieda, K., Takakura, K., Maezawa, H. and Hayashi, T. (2001) Development of microbeam irradiation system for radiobiology. Nucl. Instrum. Methods **A467–468**: 1329–1332.
49. Maeda, M., Usami, N. and Kobayashi, K. (2008) Low-dose hypersensitivity in nucleus-irradiated V79 cells studied with synchrotron X-ray microbeam. J. Radiat. Res. **49**: 171–180.
50. Hu, Z. W., Yu, Z. L. and Wu, L. J. (2003) An optimization control program for the ASIPP microbeam. Nucl. Instr. Meth. A. **507**: 617–621.
51. Folkard, M., Vojnovic, B., Hollis, K. J., Bowey, A. G., Watts, S. J., Schettino, G., Prise, K. M. and Michael, B. D. (1997) A charged-particle microbeam: Part II: A single-particle micro-collimation and detection system, Int. J. Radiat. Biol. **72**: 387–395.
52. Folkard, M., Vojnovic, B., Gilchrist, S., Prise, K. M. and Michael, B. D. (2003) The design and application of ion microbeams for irradiating living cells and tissues, Nucl. Instr. Meth. B. **210**: 302–307.
53. Wu, L. J., Hei, T. K. and Randers-Pehrson, G. (1999) Columbia University microbeam: Development of an experimental system for targeting cells individually with counted particles, Nucl. Sci. & Tech. **10**(3): 143–148.
54. Zirkle, R. E. and Bloom, W. (1953) Irradiation of parts of cells, Science **117**: 487–493.
55. Legge, G. J. F. (1997) A history of ion microbeams, Nucl. Instr. Meth. B **130**: 9–19.
56. Michelet, C., Moretto, Ph., Barberet, Ph., Balana, A., Dutta, R. K. and Aguer, P. (2002) A focused microbeam for targeting cells with counted multiple particles, Radiat. Res. **158**: 370–371.
57. Greif, K. D., Brede, H. J., Frankenberg, D. and Giesen, U.

(2004) The PTB single ion microbeam for irradiation of living cells, Nucl. Instr. Meth. B. **217**: 505–512.
58. Hei, T. K., Wu, L. J., Liu, S. X., Vannais, D., Waldren, C. A. and Randers-Pehrson, G. (1997) Mutagenic effects of a single and an exact number of α particles in mammalian cells, Proc. Natl. Acad. Sci. USA **94**: 3765–3770.
59. Wu, L. J., Randers-Pehrson, G., Xu, A., Waldren, C. A., Geard, C. R., ZengLiang, Y. Z. and Hei, T. K. (1999) Targeted cytoplasmic irradiation with alpha particles induces mutations in mammalian cells, Proc. Natl. Acad. Sci. USA **96**: 4959–4964.
60. Geard, C. R., Randers-Pehrson, G., Marino, S. A., Jenkins-Baker, G., Hei, T. K., Hall, E. J. and Brenner, D. J. (2000) Intra- and intercellular responses after cell site-specific microbeam irradiation, Radiat. Res. **153**: 233.

Received on January 19, 2009
Revision received on January 23, 2009
Accepted on January 26, 2009

Original Article

Genomic Instability in the Epidermis Induced by Atomic Bomb (A-Bomb) Radiation

A Long-Lasting Health Effect in A-Bomb Survivors

Yuki Naruke, MD[1]; Masahiro Nakashima, MD, PhD[2]; Keiji Suzuki, PhD[3]; Hisayoshi Kondo, PhD[4]; Tomayoshi Hayashi, MD, PhD[5]; Midori Soda, MD, PhD[6]; and Ichiro Sekine, MD, PhD[1,2]

BACKGROUND: Radiation etiology is suggested in the occurrence of basal cell carcinoma (BCC) of the skin among atomic bomb (A-bomb) survivors. Any genotoxicity, including ionizing radiation, can induce a DNA damage response (DDR), leading to genomic instability (GIN), which allows the accumulation of mutations during tumorigenesis. In this study, the authors evaluated the presence of GIN in the epidermis of survivors as a late effect of A-bomb radiation. **METHODS:** In total, 146 BCCs, including 23 cases arising from nonexposed skin, were identified in survivors from 1968 to 1999. The incidence rate (IR) of BCC was calculated with stratification by distance in kilometers from the hypocenter ($\leq$1.5 km, 1.6-2.9 km, and $\geq$3 km). Nineteen epidermal samples surrounding BCC at the nonexposed sites were collected and tested for p53 binding protein 1 (53BP1) expression with immunofluorescence. 53BP1 rapidly forms nuclear foci at the sites of DNA double strand breaks (DSBs). Because 1 manifestation of GIN is the induction of endogenous DSBs, the level of 53BP1-focus formation (DDR type) can be considered as a marker for GIN. **RESULTS:** The incidence rate of BCC increased significantly as exposure distance approached the hypocenter. Of the 7 epidermal samples from the proximal group ($\leq$1.5 km), 5 samples predominantly expressed DDR and an abnormal type of 53BP1 expression. In contrast, 4 of 5 samples from the distal group ($\geq$3 km) and all samples from the control group predominantly expressed the stable type of 53BP1 expression in the epidermis. **CONCLUSIONS:** The current results demonstrated the endogenous activation of DDR in the epidermis surrounding BCC in the proximal group, suggesting the presence of a GIN in the survivors as a late effect of A-bomb radiation, which may indicate a predisposition to cancer. **Cancer 2009;115:3782–90. © 2009 American Cancer Society.**

KEY WORDS: atomic bomb, genomic instability, p53 binding protein 1, radiation, basal cell carcinoma.

Corresponding author: Masahiro Nakashima, MD, PhD, Tissue and Histopathology Section, Division of Scientific Data Registry, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan; Fax: (011) 81-95-849-7130; moemoe@nagasaki-u.ac.jp

[1]Department of Tumor and Diagnostic Pathology, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan; [2]Tissue and Histopathology Section, Division of Scientific Data Registry, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan; [3]Department of Molecular Medicine, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan; [4]Biostatics Section, Division of Scientific Data Registry, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan; [5]Department of Pathology, Nagasaki University Hospital, Nagasaki, Japan; [6]Radiation Effects Research Foundation, Nagasaki, Japan

We thank Ms. Noguchi for her secretarial assistance in preparing this article.

Received: August 21, 2008; **Revised:** January 6, 2009; **Accepted:** January 9, 2009

Published online: June 10, 2009 © 2009 American Cancer Society

DOI: 10.1002/cncr.24405, www.interscience.wiley.com

Sixty-three years have elapsed since 2 atomic bombs (A-bombs) were exploded on Hiroshima and Nagasaki, Japan in August 1945. The survivors who were exposed at young ages already have reached the cancer-prone age. The incidence of several types of leukemia peaked during the period from 5 years to 10 years after the A-bomb explosions. Meanwhile, an increased risk of cancer has continued for decades, and the incidence of certain types of cancer has remained higher in survivors than in controlled populations.[1-3] We recently described a higher IR of multiple primary cancers (MPCs) in A-bomb survivors, particularly for those who were exposed at a younger age and at a closer distance to the hypocenter.[4] The occurrence of MPCs is considered a reflection of systematic exposure to carcinogens or a predisposition to cancer that serves as an indicator of genomic instability (GIN). These results provide evidence for the involvement of A-bomb radiation in the occurrence of MPCs among the survivors. Thus, a higher risk of cancer still persists in survivors. Although a long-lasting radiation effect is considered to be a contributing factor in tumorigenesis in A-bomb survivors, to date, the molecular mechanisms involved are not fully understood.

It has been postulated that ionizing radiation induces breast cancers among A-bomb survivors.[3] Our recent study demonstrated the association of human epidermal growth factor receptor 2 or HER-2 and cellular myelocytomatosis or C-MYC oncogene amplification in breast cancers among A-bomb survivors with radiation exposure.[5] It is believed that oncogene amplification is associated with GIN and with the main characteristic of solid tumors.[6] It is conceivable that radiation from the A-bomb 63 years ago may have induced a higher level of GIN in A-bomb survivors as a long-lasting health effect associated with the development of oncogene amplifications and subsequent carcinogenesis.

Ionizing radiation effectively induces DNA double strand breaks (DSBs) in normal cells and activates DNA damage response (DDR) pathways to maintain genomic integrity. Thus, defective DDR can result in GIN, which generally is considered central to any carcinogenic process.[7,8] Alternatively, the presence of activated DDR can be a hallmark of an enhanced carcinogenic process. P53-binding protein 1 (53BP1) belongs to a family of evolutionarily conserved DDR proteins with C-terminal breast cancer 1 (BRCA1) C-terminus domains.[9,10] 53BP1 is a nuclear protein that rapidly localizes at the sites of DSBs and activates p53 along with other kinases.[11-16] Subsequently, activated p53 plays a critical role in cellular responses to genomic injury, such as cell cycle arrest, DNA repair, and apoptosis.[17,18] It has been well documented in vitro with immunofluorescence that 53BP1 exhibits diffuse nuclear staining in untreated primary cells. However, after exposure to radiation, 53BP1 localizes at the sites of DSBs and forms discrete nuclear foci.[11,12,19,20] We recently demonstrated in formalin-fixed, paraffin-embedded mouse intestine that immunofluorescence analysis specifically detected the 53BP1 nuclear foci as a state of DDR induced by radiation.[21] Because 1 manifestation of GIN is the induction of endogenous DDR,[22] the level of 53BP1-focus formation can be considered a cytologic marker for GIN.

Skin cancers are relatively rare in Japan. The age-adjusted IR of approximately 1.0 to 5.5 per 100,000 person-years (PY) for nonmelanoma skin cancer in Japan are much lower than the rates among US white of approximately 250 per 100,000 PY.[23,24] A previous study indicated that the crude IR of basal cell carcinoma (BCC) of the skin among residents in Nagasaki city who were not exposed to A-bomb radiation was 3.1 per 100,000 PY.[25] In addition to breast cancer, the incidence of BCC also reportedly was elevated in A-bomb survivors, suggesting a radiation etiology in skin carcinogenesis as well.[26] The skin is the primary barrier for humans against the external environment. Therefore, epidermal cells are the first cells to be exposed to physical and chemical genotoxic agents, such as ultraviolet (UV) radiation, ionizing radiation, and superoxide. In the current study, by using immunofluorescence staining for 53BP1, we demonstrated the sporadic activation of DDR in the epidermis surrounding BCC resected from survivors who were exposed to A-bomb radiation proximal to the hypocenter. The results suggest that the presence of increased GIN as a late effect of radiation from the A-bomb can predispose survivors to the development of cancer.

MATERIALS AND METHODS

Identification of Basal Cell Carcinoma in Atomic Bomb Survivors

Clinical data were available on 91,890 A-bomb survivors who were registered after 1968 at the Division of Scientific Data Registry, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical

Sciences. The population that was used in this study was confined to residents if Nagasaki city who were exposed directly to the A-bomb. To identify cases of skin BCC in survivors, we used a database compiled by the Nagasaki Tumor Tissue Registries. This database contains 301,673 pathologic reports of patients living in south Nagasaki prefecture, including Nagasaki city, that were collected from 1961 to 1999. The database includes patient age, sex, tumor site, histologic diagnosis, and date of diagnosis.

Association Between Basal Cell Carcinoma and Atom Bomb Radiation

An event of BCC in each survivor was considered to occur with a pathologic diagnosis. PY of observation were cumulated from the date on which an individual survivor's data were registered in our database, beginning in 1968 and continuing until there was either a diagnosis of BCC, time of death, termination of follow-up (emigration from Nagasaki city), or the end of study (December 31, 1999). These BCC cases were divided into 3 different distance groups: individuals who were exposed at a distance $\leq$1.5 km from the hypocenter (proximal group), individuals who were exposed at a distance $>$1.5 km but $<$3 km from the hypocenter (intermediate group), and individuals who were exposed at a distance $\geq$3 km from the hypocenter (distal). Then, the IR of BCC per 100,000 PY among A-bomb survivors was calculated with stratification by distance in kilometers from the hypocenter ($\leq$1.5 km, 1.6-2.9 km, $\geq$3 km).

The exposure distance was used as a measure of the estimated irradiated dose, as documented previously in several unique epidemiological studies on Nagasaki survivors.[4,26-29] The estimated doses in Nagasaki survivors who were not shielded at the time of explosion are were 924.7 centigrays (cGy) at 1 km, 120.7 cGy at 1.5 km, 17.9 cGy at 2 km, and 2.9 cGy at 2.5 km from the hypocenter.[28] The experimental protocol was approved by the Ethics Review Committee of Nagasaki University Graduate School of Biomedical Sciences (Protocol No. 0305150036-2).

Survivors Studied With Immunofluorescence for p53 Binding Protein 1 Expression

For this study, we used samples of the epidermis surrounding BCCs that had been surgically resected from A-bomb survivors. For controls, samples of the epidermis surrounding BCCs from calendar year-matched patients who were not exposed to the A-bomb were analyzed. All samples were formalin-fixed, paraffin-embedded tissues that were archived in the pathologic records of our department. The sun-exposed epidermis is exposed continuously to a low level of physical and chemical genotoxic agents, such as UV radiation, ionizing radiation, and oxidative stress. Thus, the level of GIN in the sun-exposed epidermis may be influenced by a genotoxic injury induced by external environmental factors. Therefore, to evaluate the level of GIN in the epidermis from A-bomb survivors and control populations, samples that were resected only from a nonexposed site, such as chest, vulva, axilla, thigh, buttock, and inguinal regions, were tested for 53BP1 expression with immunofluorescence.

Immunofluorescence for p53 Binding Protein 1 Expression

After antigen retrieval with microwave treatment in citrate buffer, deparaffinized sections were preincubated with 10% normal goat serum. Tissue sections were then reacted with anti-53BP1 rabbit polyclonal antibody (Bethyl Laboratories, Montgomery, Tex) at a 1:200 dilution. The slides subsequently were incubated with Alexa Fluor 488-conjugated goat antirabbit antibody (Invitrogen, Carlsbad, Calif). Specimens were counterstained with 4′6-diamidino-2-phenylindole dihydrochloride (DAPI-I; Vysis Inc., Downers Grove, Ill) and were studied and photographed using a high-standard, all-in-1 fluorescence microscope (Biorevo BZ-9000; KEYENCE, Japan, Osaka, Japan). Signals were analyzed in 10 viewing areas per specimen at $\times$1000 magnification.

Evaluation of Immunofluorescence Results

The pattern of 53BP1 immunoreactivity, as described in our previous report,[21] was classified into 4 types: 1) the stable type (faint and diffuse nuclear staining), 2) the low DDR type (1 or 2 discrete nuclear foci), 3) the high DDR type ($\geq$3 discrete nuclear foci), and 4) the abnormal type (intense, heterogeneous nuclear staining with occasional several small foci). The percentage of epidermal cells that expressed each type of 53BP1 immunoreactivity in each viewing area was calculated in each specimen.

Table 1. Crude Incidence Rates of Basal Cell Carcinoma in Nagasaki Atomic Bomb Survivors by Sex and Exposure Distance Group

	Men			Women			Total		
Distance, km	PY	Cases	IR	PY	Cases	IR	PY	Cases	IR
≤1.5	41,020	16	39.0	55,282	11	19.9	96,302	27	28.0
1.6-2.9	157,616	16	10.2	264,060	16	6.1	421,676	32	7.6
≥3.0	384,561	32	8.3	654,842	55	8.4	1,039,403	87	8.4
Overall	583,197	64	11.0	974,184	82	8.4	1,557,381	146	9.4

PY indicates person-years; IR, incidence rate.

Statistical Analyses

The effect of exposure distance on the IR of BCC in A-bomb survivors was measured as a hazard ratio (HR) with 95% confidence interval (CI) using a multivariate Cox proportional hazards model. The Cochran-Armitage trend test was used to evaluate associations between the type of 53BP1 expression in the epidermis and exposure distance groups. Furthermore, effects of sex and exposure distance on the incidence of BCC in nonexposed and sun-exposed sites among A-bomb survivors were evaluated as odds ratios (OR) with 95% CIs using a multivariate logistic regression model. The PHREG procedure in SAS software (version 8.2; SAS Institute, Cary, NC) was used for calculations. All tests were 1-tailed, and a P value $<.05$ was considered statistically significant.

RESULTS

The Incidence Rate of Basal Cell Carcinoma and Its Association With Atom Bomb Radiation

Overall, 91,890 A-bomb survivors have been followed for 1,557,381 PY, and 146 patients have had a confirmed diagnosis of BCC, including 64 men and 82 women. The crude IR of BCC was 9.4 per 100,000 PY in the overall study population. The crude IRs of BCC in survivors by sex and by exposure distance are summarized in Table 1. The IR of BCC decreased significantly as distance increased from the hypocenter (HR per 1-km increment, 0.77; 95% CI, 0.68-0.88).

The proximal distance group (≤1.5 km; of 27 of 146 patients; 18.5%) included 16 BCCs from sun-exposed sites, 9 BCCs from nonexposed sites, and 2 BCCs from unknown sites. The intermediate distance group (1.6-2.9 km; 32 of 146 patients; 21.9%) included 26 BCCs from sun-exposed site s, 4 BCCs from nonexposed sites, and 2 BCCs from unknown sites. The distal distance group (≥3 km; 87 of 146 patients; 59.6%) included 74 BCCs from sun-exposed sites, 10 BCCs from nonexposed sites, and 3 BCCs from unknown sites. The clinical profiles of the patients with BCC by exposure distance group and site are summarized in Table 2.

p53 Binding Protein 1 Expression in Normal Epidermis Surrounding Basal Cell Carcinoma

For immunofluorescence analysis, samples from 7 of 9 patients (mean age, 67 years; men/women, 3/4) with nonexposed epidermis in the proximal distance group and samples from 5 of 10 patients (mean age, 60.6 years; men/women, 2/3) with nonexposed epidermis in the distal distance group were available. For control samples, samples from 7 individuals (mean age, 68.3 years; men/women, 5/2) with nonexposed epidermis also were analyzed. No family histories of skin cancer were evident in our patients. The clinical profiles of all patients with BCC in this study are summarized in Table 3. The results from immunofluorescence staining patterns for 53BP1 in the epidermis of each sample also are presented in Table 3. The mean numbers (±standard deviation) of epidermal cells that were analyzed for 53BP1 expression were 161.7 ± 39.1 cells (range, 111-224 cells), 174.6 ± 38.5 cells (range, 145-239 cells), and 191.6 ± 33.1 cells (range, 145-240 cells) per sample in the proximal distance, distal distance, and control groups, respectively. Statistical analysis with the Welch t test revealed no significant differences in the number of epidermal cells that were examined between the 3 groups. Of the 7 samples of nonexposed

Table 2. Summary of Each Clinical Factor by Exposure Distance Group and Site of Basal Cell Carcinoma in Atomic Bomb Survivors

	Distance Group								
	Proximal, ≤1.5 km (n=27)			Intermediate, 1.6-2.9 km (n=32)			Distal, ≥3 km (n=87)		
Variable	Nonex	Sun-ex	Uk	Nonex	Sun-ex	Uk	Nonex	Sun-ex	Uk
No.	9	16	2	4	26	2	10	74	3
No. of men/women	4/5	10/6	2/0	1/3	12/14	1/1	4/6	25/49	3/0
Distance, km	1.1	1.1	1.3	2.4	2.3	2.2	3.7	4.6	3.9
ATB, y	20.5	23	35.5	35.5	33.3	24.5	16.9	29.1	39.7
ATD, y	65.6	68.6	77.1	82.9	76.3	53.1	64.2	72.9	79.9

Nonex indicates nonexposed; Sun-ex, sun exposed; Uk, unknown; ATB, age at time of bombing; ATD, age at time of diagnosis.

Table 3. Clinical Profiles of Each Basal Cell Carcinoma Sample Used in Immunofluorescence Analysis and Results of Typing for p53 Binding Protein 1 Expression in Normal Epidermis

						53BP1 Expression, %			
Group	Age, y	Sex	Distance, km	Disease Site	PH	Stable	Low DDR	High DDR	Abnormal
Proximal distant group									
1	60	Woman	0.5	Chest	Thyroid, breast	0	11.2	2.3	86.5
2	70	Woman	0.7	Vulva		67.5	27.8	4.6	0
3	69	Man	1.0	Inguinal		26.1	18	54.1	1.8
4	66	Man	1.1	Buttock	Prostate	77.9	17.5	4.6	0
5	82	Man	1.2	Buttock		5.8	0.9	0	93.3
6	69	Woman	1.3	Back		47.1	36	16.9	0
7	53	Woman	1.4	Heel		49.6	21.5	28.9	0
Mean	67		1.03			39.1	19	15.9	25.9
Distal distant group									
1	68	Woman	3	Buttock	Skin*	74.5	16.8	8.7	0
2	53	Man	3.6	Back		86.6	10	2.9	0.4
3	65	Man	3.7	Back		67.8	26.1	5.6	0.6
4	66	Woman	3.8	Abdomen		91.3	8.8	0	0
5	51	Woman	5	Buttock		91.7	8.3	0	0
Mean	60.6		3.82			82.4	14	3.4	0.2
Control group									
1	79	Woman	—	Back		91	8.4	0.6	0
2	64	Man	—	Back		84.8	14	1.1	0
3	65	Man	—	Back		82.8	15.9	1.4	0
4	58	Man	—	Axilla	Thyroid	89.1	8.4	1.5	1
5	66	Woman	—	Back		82.5	10.4	3.3	3.8
6	65	Man	—	Back		77.9	16.3	5.8	0
7	81	Man	—	Pubic		77.4	17.3	3.1	2.2
Mean	68.3		—			83.6	13	2.4	1

53BP1 indicates p53 binding protein 1; PH, past history of other malignancy; DDR, DNA damage response.
*Squamous cell carcinoma.

epidermis in the proximal distance group, 5 samples (71%) predominantly expressed DDR and abnormal type cells in keratinocytes around the basal layer (Fig. 1A,D). The other 2 samples (29%) predominantly had stable cell types but also included a small number (up to 5%) of high DDR types in the basal layer. In contrast, 4 of 5 samples in the distal distance group (Fig. 1B,E) and all samples in the control groups (Fig. 1C,F) predominantly expressed stable types in >70% of epidermal cells but also had up to 20% of low DDR type cells in the basal layer of the epidermis. The Cochran-Armitage trend test revealed that the mean percentage of cells expressing DDR and an

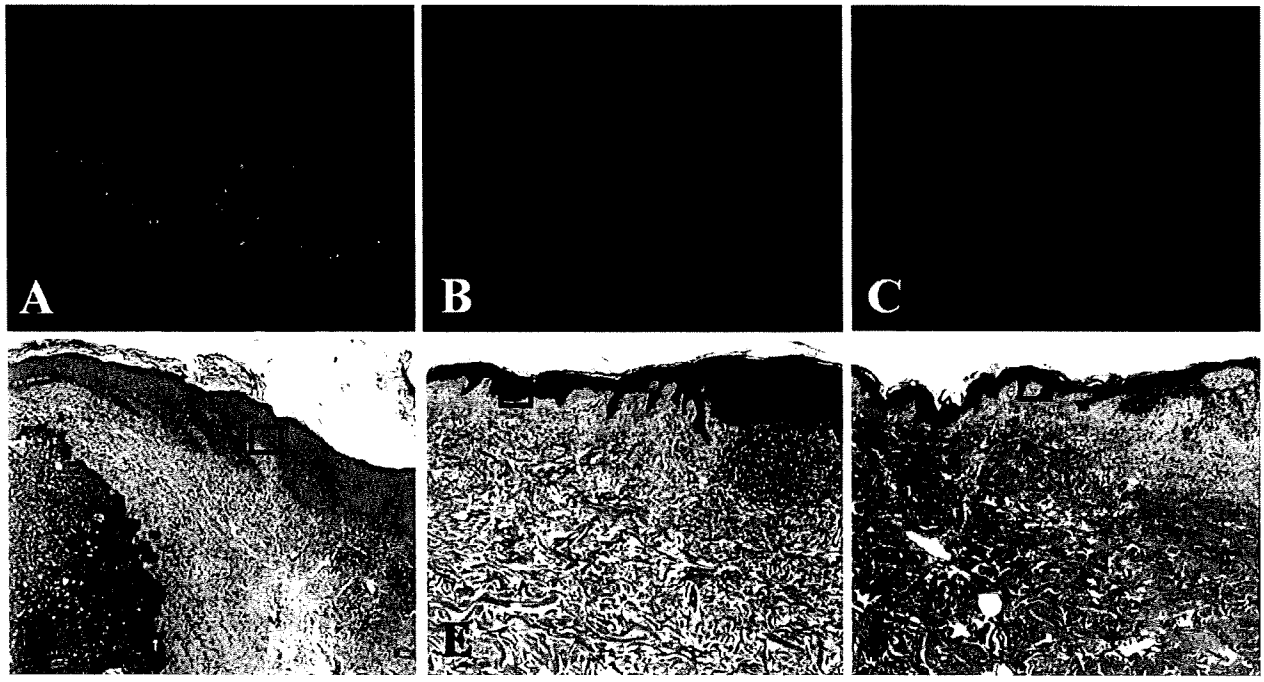

FIGURE 1. Immunofluorescence of p53 binding protein 1 (53BP1) expression in the epidermis surrounding basal cell carcinoma at the nonexposed site. (A) This sample from the proximal distance group of atomic bomb survivors reveals many discrete nuclear 53BP1 foci in the epidermis around the basal layer. In contrast to A, samples from (B) the distal distance group of atomic bomb survivors and (C) the control group reveal only a few nuclear 53BP1 foci in the epidermal cells. Squares in D, E, and F with hematoxylin and eosin staining reveal corresponding areas of A, B, and C, respectively.

abnormal type of 53BP1 in the epidermis of the proximal distance group had a significant tendency to be higher than the percentages in both the distal distance group and the control group ($P < .001$). However, there was no significant difference in the type of 53BP1 expression in the epidermis between the distal distance group and the control group.

Comparisons for Effects of Sex and Atom Bomb Radiation in the Incidence of Basal Cell Carcinoma Among Atom Bomb Survivors by Site

The effects of sex and exposure distance on the incidence of BCC at nonexposed and sun-exposed sites among A-bomb survivors were evaluated as ORs with 95% CIs using a multivariate logistic regression model. These results are presented in Table 4. When analyses were stratified by sex, the IR of BCC was significantly higher in men than in women for both sun-exposed sites (OR [vs women], 1.63; 95% CI, 1.09-2.43) and total sites (OR [vs women], 1.51; 95% CI, 1.04-2.19). However, there was no significant difference in the IR of BCC between men and women in for nonexposed sites (OR [vs women], 1.05; 95% CI, 0.40-2.75). Furthermore, when the analysis was stratified by distance group, the IR of BCC was significantly higher in the proximal distance group than in the distal distance group both for nonexposed sites (OR [vs the distal distance group], 5.86; 95% CI, 2.19-15.6) and for total sites (OR [vs the distal distance group], 2.17; 95% CI, 1.32-3.57). However, there was no significant difference in the IR of BCC between the proximal and distal distance groups at sun-exposed sites (OR [vs the distal distance group], 1.62, 95% CI, 0.89-2.94) or between the intermediate and distal distance groups for any sites.

DISCUSSION

The epidemiologic and molecular analyses of carcinogenesis in A-bomb survivors require clinical data from individuals and biologic materials with pathologic data on tumors. In this study, 2 databases were used to identify BCC resected from survivors: a clinical database providing exposure distance on Nagasaki survivors registered at

Table 4. Comparisons of the Effect of Sex and Distance in the Incidence of Basal Cell Carcinoma Among Atomic Bomb Survivors

	Nonexposed Site (n=23)		Sun-exposed Site (n=116)		Total (n=139)	
Factor	OR	95% CI	OR	95% CI	OR	95% CI
Sex						
Men	1.05	0.40-2.75	1.63	1.09-2.43	1.51	1.04-2.19
Women	1		1		1	
Distance, km						
0-1.5	5.86	2.19-15.6	1.62	0.89-2.94	2.17	1.32-3.57
1.6-2.9	0.26	0.03-2.00	0.89	0.56-1.42	0.81	0.51-1.27
≥3	1		1		1	

OR indicates odds ratio; CI, confidence interval.

our institute, which was established in 1972, and a pathologic database from the Nagasaki Tumor Tissue Registries, which were established in 1974. Our retrospective search of these independently established databases identified a total of 146 patients with BCC who were tracked from 1968 to 1999 and were exposed directly to Nagasaki A-bomb radiation. Among these survivors, an increased risk has been demonstrated for the development of BCC in individuals who were exposed at a closer distance. This concurs with previous reports[26,30] and provides further evidence that A-bomb radiation is associated significantly with skin carcinogenesis.

Several epidemiologic reports have suggested that an increased risk of cancer has continued for decades, and a higher risk of certain types of cancers still persists in survivors.[1-4] Thus, a long-lasting health effect is considered to be a contributing factor in tumorigenesis in A-bomb survivors. In the current study, we demonstrated that there were several nuclear 53BP1 foci in the epidermis surrounding BCC of nonexposed skin in survivors who were exposed at a distance proximal to the hypocenter, suggesting a constitutive activation of DDR in the epidermis of A-bomb survivors. Human cancers develop through a multistep process that involves the accumulation of genetic mutations.[31] It is well established that any DNA damage can induce DDR leading to GIN in injured cells. GIN results in the accumulation of genetic mutations that are implicated in both the initiation and the progression of cancers. Thus, we submitted that, compared with our control group of individuals who were not affected with A-bomb radiation, A-bomb radiation may induce a higher state of GIN in the epidermis of survivors that can be a long-lasting health effect contributing to tumorigenesis. Ionizing radiation effectively induces GIN, which is manifested in several DSBs in a dose-dependent manner. DSBs are repaired through error-prone nonhomologous end joining, single-strand annealing, and/or error-free homologous recombination.[32] Although most DNA damage is repaired correctly or is eliminated through tumor suppressor function of p53, it is well known that the repair process disrupts the genomic structure, which may result in the induction of a mutation, gross rearrangement of chromatin, and consequent promotion of tumorigenesis.[33-36] A-bomb radiation may induce minor disruption of the genomic structures, which may result in GIN for an extended time in the epidermis and, subsequently, may affect skin carcinogenesis in the survivors.

In our recent reports, we have proposed that immunofluorescence analysis of 53BP1 expression can be a useful tool for estimating the level of GIN in tissue sections and may serve as a valuable molecular marker of malignant potential even in formalin-fixed materials.[21,37] GIN seems to be induced at the precancerous stage during skin carcinogenesis, because actinic keratosis revealed a high DDR type of 53BP1 immunoreactivity.[37] Furthermore, we noticed that, in the control group, a low DDR type of 53BP1 immunoreactivity frequently was observed in sun-exposed epidermis, whereas few 53BP1 nuclear foci were observed in nonexposed epidermis.[37] Thus, the sun-exposed epidermis seems to have suffered frequently from a minor genotoxic injury induced by external environmental factors, whereas the endogenous DDR was

observed only in the nonexposed epidermis of the control group. It is noteworthy that, in the current study, we demonstrated that a high level of the high DDR and abnormal type of 53BP1 expression in the epidermis surrounding BCC at the nonexposed site was observed only in the proximal distance group and not in the distal distance or control groups. UV exposure from sunlight is a major causative factor for skin cancer at sun-exposed sites. Although squamous cell carcinoma of the skin usually develops from UV-induced precancerous lesions, the pathogenesis of sporadic BCC is more controversial.[38] Indeed, as noted in our patients, BCC did not always arise on sun-exposed areas. Thus, etiologies other than UV should be considered in BCC at nonexposed areas. It also is worth noting that the percentage of BCC observed on nonexposed areas was higher in the proximal distance group (9 of 27 patients; 33.3%) than in the intermediate distance group (4 of 32 patients; 12.5%) or the distal distance groups (10 of 87 patients; 11.5%). Furthermore, our multivariate analyses revealed that exposure to A-bomb radiation at a closer distance to the hypocenter was a strong risk factor for the incidence of BCC at nonexposed sites, but not at sun-exposed sites, in survivors. It appears that an elevated risk of BCC among the survivors is caused mainly by the higher IR of BCC on nonexposed skin. Furthermore, although the incidence of BCC at sun-exposed sites was significantly higher in men than in women, no sex effect was observed in the incidence of BCC at nonexposed sites among the survivors. The predominance risk among men for BCC at sun-exposed skin sites is plausible, because Japanese women generally dislike sun tanning from the cosmetic point of view and tend to shield their skin from UV exposure. In contrast, because we observed no effect for sex, contributing factors other than UV should be considered in the risk of BCC at nonexposed sites. Thus, it appears that radiation etiology is associated more strongly with tumorigenesis of BCC in the nonexposed skin of survivors.

The BCCs in survivors are unusual, in that they potentially are attributable to 2 types of radiation: UV and ionizing. This study demonstrated several nuclear 53BP1 foci in the epidermis surrounding BCC at nonexposed sites resected from A-bomb survivors who were exposed at a distance proximal to the hypocenter, similar to the foci reported in irradiated cells, suggesting a constitutive activation of DDR in the epidermis of survivors. It is suggested that ionizing radiation from the A-bomb may affect the development of BCC by inducing a higher level of GIN mainly in the basal layer of the epidermis among survivors. The survivors, particularly those who were exposed at a younger age, even now still have a high risk for cancers in various organs, including MPCs. The crucial mechanisms that can account for the continuously higher incidence of cancers in A-bomb survivors for decades remain to be determined. Further research on the molecular mechanisms to maintain a long-lasting GIN in the epidermis from survivors can contribute to an understanding of both radiation-associated carcinogenesis and the predisposition to cancers.

Conflict of Interest Disclosures

Supported in part through Nagasaki University Global Center of Excellence Program, by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Science, Sports, and Culture (20590367), and by a Grant for Research Project of Atomic Bomb Diseases from the Japanese Ministry of Health, Labor, and Welfare.

References

1. Preston DL, Shimizu Y, Pierce DA, Suyama A, Mabuchi K. Studies of mortality of atomic bomb survivors. Report 13: solid cancer and noncancer disease mortality: 1950-1997. *Radiat Res.* 2003;160:381-407.
2. Ron E, Lubin JH, Shore RE, et al. Thyroid cancer after exposure to external radiation: a pooled analysis of 7 studies. *Radiat Res.* 1995;141:259-277.
3. Carmichael A, Sami AS, Dixon JM. Breast cancer risk among the survivors of atomic bomb and patients exposed to therapeutic ionising radiation. *Eur J Surg Oncol.* 2003;29: 475-479.
4. Nakashima M, Kondo H, Miura S, et al. Incidence of multiple primary cancers in Nagasaki atomic bomb survivors: association with radiation exposure. *Cancer Sci.* 2008;99: 87-92.
5. Miura S, Nakashima M, Ito M, et al. Significance of HER2 and C-MYC oncogene amplifications in breast cancer in atomic bomb survivors: associations with radiation exposure and histologic grade. *Cancer.* 2008;112:2143-2151.
6. Zimonjic DB, Zhang H, Shan Z, et al. DNA amplification associated with double minutes originating from chromosome 19 in mouse hepatocellular carcinoma. *Cytogenet Cell Genet.* 2001;93:114-116.
7. Lengauer C, Kinzler KW, Vogelstein B. Genetic instabilities in human cancers. *Nature.* 1998;396:643-649.
8. Coleman WB, Tsongalis GJ. The role of genomic instability in human carcinogenesis. *Anticancer Res.* 1999;19:4645-4664.

9. Bork P, Hofmann K, Bucher P, Neuwald AF, Altschul SF, Koonin EV. A superfamily of conserved domains in DNA damage-responsive cell cycle checkpoint proteins. *FASEB J.* 1997;11:68-76.
10. Joo WS, Jeffrey PD, Cantor SB, Finnin MS, Livingston DM, Pavletich NP. Structure of the 53BP1 BRCT region bound to p53 and its comparison to the Brca1 BRCT structure. *Genes Dev.* 2002;16:583-593.
11. Ward IM, Minn K, Jorda KG, Chen J. Accumulation of checkpoint protein 53BP1 at DNA breaks involves its binding to phosphorylated histone H2AX. *J Biol Chem.* 2003; 278:19579-19582.
12. Schultz LB, Chehab NH, Malikzay A, Halazonetis TD. p53 binding protein 1 (53BP1) is an early participant in the cellular response to DNA double-strand breaks. *J Cell Biol.* 2000;151:1381-1390.
13. Rappold I, Iwabuchi K, Date T, Chen J. Tumor suppressor p53 binding protein 1 (53BP1) is involved in DNA damage-signaling pathways. *J Cell Biol.* 2001;153:613-620.
14. Anderson L, Henderson C, Adachi Y. Phosphorylation and rapid relocalization of 53BP1 to nuclear foci upon DNA damage. *Mol Cell Biol.* 2001;21:1719-1729.
15. Xia Z, Morales JC, Dunphy WG, Carpenter PB. Negative cell cycle regulation and DNA damage-inducible phosphorylation of the BRCT protein 53BP1. *J Biol Chem.* 2001; 276:2708-2718.
16. Shiloh Y, Kastan MB. ATM: genome stability, neuronal development, and cancer cross paths. *Adv Cancer Res.* 2001; 83:209-254.
17. Xu Y, Baltimore D. Dual roles of ATM in the cellular response to radiation and in cell growth control. *Genes Dev.* 1996;10:2401-2410.
18. Xu Y, Yang EM, Brugarolas J, Jacks T, Baltimore D. Involvement of p53 and p21 in cellular defects and tumorigenesis in Atm−/− mice. *Mol Cell Biol.* 1998;18:4385-4390.
19. Mochan TA, Venere M, DiTullio RA Jr, Halazonetis TD. 53BP1 and NFBD1/MDC1-Nbs1 function in parallel interacting pathways activating ataxia-telangiectasia mutated (ATM) in response to DNA damage. *Cancer Res.* 2003;63: 8586-8591.
20. Rakhorst HA, Tra WM, Posthumus-Van Sluijs ST, et al. Quantitative analysis of radiation-induced DNA break repair in a cultured oral mucosal model. *Tissue Eng.* 2006; 12:3395-3403.
21. Nakashima M, Suzuki K, Meirmanov S, et al. Foci formation of P53-binding protein 1 in thyroid tumors: activation of genomic instability during thyroid carcinogenesis. *Int J Cancer.* 2008;122:1082-1088.
22. Suzuki K, Yokoyama S, Waseda S, Kodama S, Watanabe M. Delayed reactivation of p53 in the progeny of cells surviving ionizing radiation. *Cancer Res.* 2003;63:936-941.
23. Parkin DM, Whelan SL, Ferlay J, Teppo L, Thomas DB. Cancer incidence in five continents. Vol. VIII. *IARC Sci Publ.* 2002;155:1-781.
24. Scotto J, Fears TR, Kraemer KH, Fraumeni JFJ. Nonmelanoma skin cancer. In: Schottenfeld D, Fraumeni JFJ, eds. Cancer Epidemiology and Prevention. New York, NY: Oxford University Press; 1996:1313-1330.
25. Kishikawa M, Koyama K, Iseki M, et al. Histologic characteristics of skin cancer in Hiroshima and Nagasaki: background incidence and radiation effects. *Int J Cancer.* 2005; 117:363-369.
26. Sadamori N, Mine M, Hori M. Skin cancer among atomic bomb survivors [letter]. *Lancet.* 1989;1:1267.
27. Shibata S, Sadamori N, Mine M, Sekine I. Intracranial meningiomas among Nagasaki atomic bomb survivors [letter]. *Lancet.* 1994;344:1770.
28. Sadamori N, Shibata S, Mine M, et al. Incidence of intracranial meningiomas in Nagasaki atomic bomb survivors. *Int J Cancer.* 1996;67:318-322.
29. Honda S, Shibata Y, Mine M, et al. Mental health conditions among atomic bomb survivors in Nagasaki. *Psychiatry Clin Neurosci.* 2002;56:575-583.
30. Ron E, Preston DL, Kishikawa M, et al. Skin tumor risk among atomic-bomb survivors in Japan. *Cancer Causes Control.* 1998;9:393-401.
31. Hahn WC, Weinberg RA. Modelling the molecular circuitry of cancer. *Nat Rev Cancer.* 2002;2:331-341.
32. van Gent DC, Hoeijmakers JH, Kanaar R. Chromosomal stability and the DNA double-stranded break connection. *Nat Rev Genet.* 2001;2:196-206.
33. Liang F, Han M, Romanienko PJ, Jasin M. Homology-directed repair is a major double-strand break repair pathway in mammalian cells. *Proc Natl Acad Sci U S A.* 1998; 95:5172-5177.
34. Moynahan ME, Jasin M. Loss of heterozygosity induced by a chromosomal double-strand break. *Proc Natl Acad Sci U S A.* 1997;94:8988-8993.
35. Richardson C, Jasin M. Frequent chromosomal translocations induced by DNA double-strand breaks. *Nature.* 2000; 405:697-700.
36. Pipiras E, Coquelle A, Bieth A, Debatisse M. Interstitial deletions and intrachromosomal amplification initiated from a double-strand break targeted to a mammalian chromosome. *EMBO J.* 1998;17:325-333.
37. Naruke Y, Nakashima M, Suzuki K, et al. Alteration of p53-binding protein 1 expression during skin carcinogenesis: association with genomic instability. *Cancer Sci.* 2008; 99:946-951.
38. Holikova Z, Massi D, Lotti T, Hercogova J. Insight into the pathogenesis of sporadic basal cell carcinoma. *Int J Dermatol.* 2004;43:865-869.

High Serum Cartilage Oligomeric Matrix Protein Determines the Subset of Patients with Early-Stage Rheumatoid Arthritis with High Serum C-Reactive Protein, Matrix Metalloproteinase-3, and MRI-Proven Bone Erosion

KEITA FUJIKAWA, ATSUSHI KAWAKAMI, MAMI TAMAI, MASATAKA UETANI, SHOICHIRO TAKAO, KAZUHIKO ARIMA, NAOKI IWAMOTO, TOSHIYUKI ARAMAKI, SHINYA KAWASHIRI, KUNIHIRO ICHINOSE, MAKOTO KAMACHI, HIDEKI NAKAMURA, TOMOKI ORIGUCHI, HIROAKI IDA, KIYOSHI AOYAGI, and KATSUMI EGUCHI

ABSTRACT. ***Objective.*** To identify the significance of serum cartilage oligomeric matrix protein (COMP), a marker of cartilage turnover, in patients with early-stage rheumatoid arthritis (RA) in relation to other serologic variables and magnetic resonance imaging (MRI) features.

Methods. Ninety-eight patients with early-stage RA, whose disease duration from onset was less than 2 years, were enrolled. The objective measures at baseline were Disease Activity Score (DAS28), serum C-reactive protein (CRP), serum matrix metalloproteinase-3 (MMP-3), serum antibodies against cyclic citrullinated peptide (anti-CCP), and MRI features of both wrist and finger joints. The MRI features included the number of sites scored positive for synovitis, bone edema, and bone erosion.

Results. Serum COMP concentration was not different among groups identified with low, moderate, and high DAS28-CRP values. However, COMP values were statistically high in subjects positive for bone erosions on MRI compared with the subjects who were negative for bone erosions. A positive correlation of COMP with CRP and with MMP-3 values was also identified.

Conclusion. Elevation of COMP may reflect joint damage that is dependent on the synovial inflammatory process in early-stage RA. (First Release May 15 2009; J Rheumatol 2009;36:1126–9; doi:10.3899/ jrheum.080926)

Key Indexing Terms:
EARLY-STAGE RHEUMATOID ARTHRITIS CARTILAGE OLIGOMERIC MATRIX PROTEIN
C-REACTIVE PROTEIN MATRIX METALLOPROTEINASE-3
BONE EROSION MAGNETIC RESONANCE IMAGING
ANTI-CYCLIC CITRULLINATED PEPTIDE ANTIBODIES

Synovitis in the context of rheumatoid arthritis (RA) leads to pathologic changes in adjacent structures, such as the articular cartilage, the cortical bone surfaces, and the underlying bone marrow, changes that have recently been verified by comparison of magnetic resonance images (MRI) on the day before surgery and the tissue specimens at joint replacement surgery (metacarpophalangeal or proximal interphalangeal joints) in patients with established RA[1]. These findings were obtained in patients with late-stage RA. A similar process should be occurring in early-stage RA, but it is very difficult to prove, since joint replacement surgery is performed infrequently in cases of early-stage RA. In contrast, qualifying pathologic features in the rheumatoid inflammatory process by serologic variables and MRI is recommend-

From the Unit of Translational Medicine, Department of Immunology and Rheumatology; Department of Radiology and Radiation Research; and Department of Public Health, Graduate School of Biomedical Sciences, Nagasaki University School of Health Sciences, Nagasaki, Japan.

Supported by a grant from The Ministry of Health, Labour and Welfare, Japan.

K. Fujikawa MD, PhD; A. Kawakami, MD, PhD; M. Tamai, MD, PhD, Unit of Translational Medicine, Department of Immunology and Rheumatology; M. Uetani, MD, PhD; S. Takao, MD, PhD, Department of Radiology and Radiation Research; K. Arima, MD, PhD; N. Iwamoto, MD; T. Aramaki, MD, PhD; S. Kawashiri, MD; K. Ichinose, MD, PhD; M. Kamachi, MD, PhD; H. Nakamura, MD, PhD, Unit of Translational Medicine, Department of Immunology and Rheumatology; T. Origuchi, MD, PhD, Department of Public Health, Graduate School of Biomedical Sciences; H. Ida, MD, PhD, Unit of Translational Medicine, Department of Immunology and Rheumatology; K. Aoyagi, MD, PhD, Department of Public Health, Graduate School of Biomedical Sciences; K. Eguchi, MD, PhD, Unit of Translational Medicine, Department of Immunology and Rheumatology, Nagasaki University School of Health Sciences.

Address reprint requests to Prof. K. Eguchi, Unit of Translational Medicine, Department of Immunology and Rheumatology, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. E-mail: eguchi@net.nagasaki-u.ac.jp

Accepted for publication December 3, 2008.

ed in early-stage patients rather than late-stage patients, since there may be fewer secondary disease process-independent phenomena in early-stage patients. We recently reported that MRI-proven synovitis, bone edema, and bone erosion in the wrist and finger joints in early-stage RA reflect systemic inflammatory indices of serum C-reactive protein (CRP) and serum matrix metalloproteinase-3 (MMP-3)[2].

An interesting serum biomarker is cartilage oligomeric matrix protein (COMP), which is thought to be increased in the serum early in the course of RA as a sign of cartilage involvement[3]. However, it is difficult to identify early cartilage involvement by radiography, even in MRI. Thus, the estimation of early cartilage involvement in early-stage RA using established variables, including COMP, would be valuable for prognostication. Our study is the first report to qualify serum COMP values in patients with early-stage RA, in conjunction with other inflammatory indices, as well as MRI detection of early joint damage.

MATERIALS AND METHODS

Ninety-eight patients with early stage RA were enrolled from the Early Arthritis Clinic at the Unit of Translational Medicine, Department of Immunology and Rheumatology (First Department of Internal Medicine), Graduate School of Biomedical Sciences, Nagasaki University. They gave their informed consent to the protocol, which was approved by the Institutional Review Board of Nagasaki University.

The mean disease duration from onset of symptoms to entry was 4.9 months. Disease duration for each of the 98 patients was < 2 years, similar to other recent reports[3,4]. Baseline characteristics of the 98 patients are described in Table 1. All patients fulfilled the 1987 criteria of the American College of Rheumatology for RA[5]. Since serum COMP is reported to be high in patients with osteoarthritis (OA) of large joints[6,7], we excluded the cases complicated with OA of hip or knee joint, classified according to the established criteria[8,9].

Table 1. Baseline characteristics of the 98 patients with early-stage RA.

Age, yrs (range)	53 (16–80)
No. female/male	77/21
Duration of disease, mo (range)	3 (1.5–24)
Tender joint count, n (range)	6 (0–28)
Swollen joint count, n (range)	3 (0–24)
Global health, 100 mm VAS (range)	50.5 (0–100)
DAS28-CRP (range)	4.3 (2.1–8.2)
MRI	
Synovitis positivity, %	90
n (range)	11.5 (0–30)
Bone edema positivity, %	47
n (range)	0 (0–15) mean 2.1
Bone erosion positivity, %	33
n (range)	0 (0–11) mean 1.3
Serologic markers	
COMP, U/l (range)	10.3 (0.6–24.9)
CRP positivity, %	66
Titer, mg/dl (range)	0.52 (0.02–12.7)
Anti-CCP antibody positivity, %	66
Titer U/ml (range)	23 (0.2–2115.3)
MMP-3 positivity, %	42
Titer, ng/ml (range)	66.6 (12.5–1160)

VAS: visual analog scale, COMP: cartilage oligomeric matrix protein, MRI: magnetic resonance imaging, CRP: C-reactive protein, CCP: citric citrullinated peptide, MMP: matrix metalloproteinase.

The following variables were examined at entry. Serologic tests included COMP (COMP ELISA®, AnaMar Medical AB, Göteborg, Sweden), CRP (Eiken Chemical Co. Ltd., Tokyo, Japan), MMP-3 (Daiichi Pure Chemicals, Fukuoka, Japan), and anti-CCP antibodies (Diastat Anti-CCP, Axis-Shield, Dundee, UK; cutoff value 4.5 U/ml). Clinical disease activity was qualified by the Disease Activity Score (DAS28-CRP; high disease activity > 4.1, low disease activity < 2.7, remission < 2.3)[10]. MR images of both wrists and finger joints (1.5 Tesla Sigma device; GE Medical Systems, Milwaukee, WI, USA) were evaluated for bone edema, bone erosion, and synovitis in 15 sites in each finger and wrist, i.e., the distal radioulnar joint, radiocarpal joint, mid-carpal joint, first carpometacarpal joint, second–fifth carpometacarpal joint (together), and first–fifth metacarpophalangeal joints (proximal interphalangeal joints) separately (a total of 30 sites in both hands), as we reported[2,11,12]. The MR images were interpreted independently by 2 board certified radiologists experienced in musculoskeletal imaging (MU and ST), who were blinded to the clinical status of the patients. Both radiologists read each image according to the definition, as described[13,14], and disagreements were resolved by consensus. The degree of MRI features was evaluated as we recently published: synovitis; the number of sites scored as positive for MRI synovitis, bone edema; number of bones scored positive for bone edema, bone erosion; and number of bones scored positive for MRI bone erosion[2].

Serum COMP concentration, in general, is reported to be elevated with age[3], as we found in the 98 patients with early-stage RA we examined ($r = 0.39$, $p < 0.001$). Thus, a partial correlation coefficient adjusting for age was calculated. Since MMP-3 was not normally distributed, we conducted logarithmic transformation. Differences between groups were examined using the age-adjusted mean. A test for trend was performed using the general linear modeling method. A p value < 0.05 denoted the presence of a statistically significant difference.

RESULTS

Table 1 shows the baseline characteristics of the 98 patients. Briefly, the median DAS28-CRP at entry was 4.3. Forty-seven percent of subjects were positive for MRI-proven bone edema, and 33% were positive for bone erosions. The median serum COMP at entry was 10.3 U/ml, median titer of anti-CCP antibodies was 20.3 U/ml, and seropositivity to anti-CCP antibodies was found in 66% of subjects. For MMP-3, the median serum concentration was 66.6 ng/ml, and seropositivity for MMP-3, denoted as in the higher than normal range, was 42%. As described[3], a positive correlation was found between serum COMP value and age in the 98 patients ($r = 0.39$, $p < 0.001$, Spearman's rank correlation test), and thus a partial correlation coefficient adjusting for age was calculated in the following data, as described above.

A partial correlation coefficient adjusting for age showed positive correlation of COMP with CRP ($r = 0.21$, $p = 0.035$) as well as MMP-3 values ($r = 0.20$, $p = 0.046$). Although the difference was not statistically significant, a weak association was determined between the number of MRI-proven bone erosions and the serum COMP ($r = 0.19$, $p = 0.06$). Therefore, we divided the 98 patients into 2 groups according to the MRI-proven bone erosions, and examined the distribution of serum COMP. Figure 1 shows that the age-

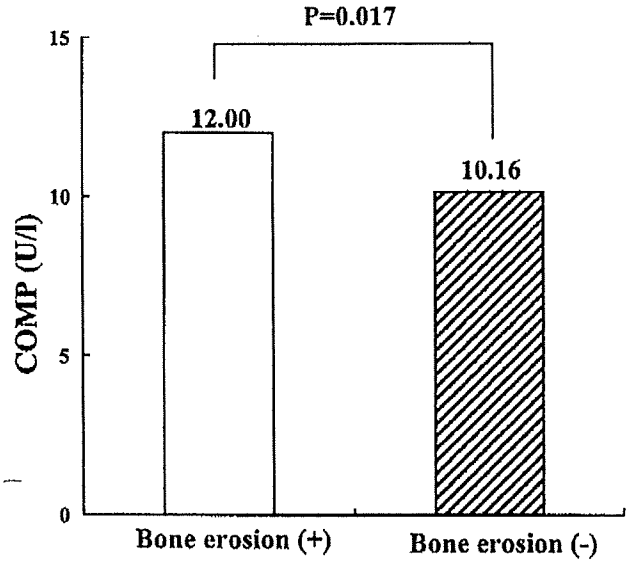

Figure 1. The age-adjusted mean serum COMP concentration was high in subjects with MRI-proven bone erosions (n = 32) compared to those without bone erosions (n = 66).

adjusted mean concentration of serum COMP was statistically significantly high in the subjects who were positive for bone erosions on MRI, compared with subjects who were negative for erosions. As suspected, the mean values of CRP and MMP-3 were also high in the subjects who were positive for bone erosions on MRI (data not shown). We also examined the distribution of serum COMP by DAS28-CRP, and observed no differences among subjects who had low, moderate, and high DAS28-CRP scores (Figure 2). A test for trend showed no association between serum COMP and

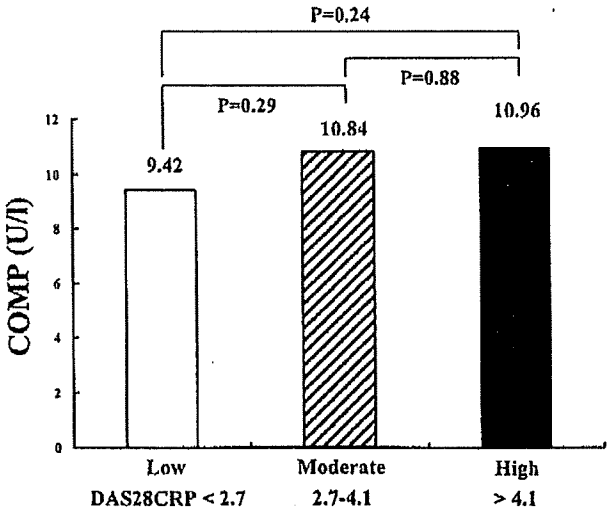

Figure 2. Serum COMP values of 98 patients with early-stage RA were not different among those with low, moderate, and high scores for DAS28-CRP. P value between the groups was calculated by age-adjusted mean. P value was nonsignificant.

DAS28-CRP (p = 0.31). In addition, the age-adjusted mean concentration of serum COMP was not statistically different between subjects with different anti-CCP antibody status (mean COMP concentration was 10.54 U/l in subjects who were anti-CCP antibody-positive; and 11.26 U/l in subjects who were anti-CCP antibody-negative; p = 0.36).

DISCUSSION

COMP is a marker of articular cartilage damage, originally described as the determinant of radiographic progression in OA of the large joints, such as hip OA and knee OA[6,7,15]. Recently, serum COMP was evaluated in patients with RA, and was found to be preferentially elevated not in late-stage RA but in early-stage RA[3]. In addition, high serum COMP at baseline in early-stage RA indicates future radiographic progression[3]. The serum COMP concentration at baseline may also reflect the therapeutic efficacy of radiography in patients with active RA: low COMP at baseline predicted a better radiographic outcome in clinical trials of adalimumab treatment in RA[16]. We have investigated the role of serologic variables as well as MRI features of the wrist and fingers in early-stage RA[2,11,12]; in the present study we focused on the serum COMP value. MR images of the small joints in the wrist and fingers were closely examined, and thus patients with early-stage RA with clinically definite large-joint OA (hip and knee OA) were excluded, since a large amount of COMP could be produced from the affected articular cartilage, which might weaken the significance of the RA-related COMP value.

We initially expected COMP values to correlate with bone damage-prone markers, such as MMP-3, and MRI-proven bone edema and erosion. Raw data also showed an association of COMP with the number of MRI-proven bone erosions (r = 0.33, p = 0.02); however, age-adjusted data identified statistically significant association of COMP with only CRP and MMP-3. Since rheumatoid bone and cartilage damage are suggested to be driven by the neighboring synovial tissues[1], it was recognized that the inflammatory index provided by CRP and MMP-3 correlates with the cartilage turnover marker COMP. In addition, the findings that COMP concentrations were higher in the subjects with MRI-proven bone erosions than in those without erosions may support the speculation that rheumatoid synovial inflammation promotes articular cartilage turnover *in situ* in early-stage RA. Our findings that CRP and MMP-3 levels were high in the subjects with MRI-proven bone erosions also support this. Our recent study showed that MRI-proven bone edema, in early-stage RA, was significantly correlated with inflammatory indexes and DAS[2], but in the present study we did not see a relationship between the serum COMP value and MRI-proven bone edema. These observations may indicate that COMP reflects another aspect of the disease process of RA, which should be confirmed by our continuing prospective study.

This is the first report to investigate the significance of the relation of serum COMP value with MRI detection of early joint damage in early-stage RA. Serum COMP values at entry correlated with CRP and MMP-3 values, and MRI-proven bone erosion also predicted high serum COMP concentrations in study subjects.

REFERENCES

1. Jimenez-Boj E, Nöbauer-Huhmann I, Hanslik-Schnabel B, et al. Bone erosions and bone marrow edema as defined by magnetic resonance imaging reflect true bone marrow inflammation in rheumatoid arthritis. Arthritis Rheum 2007;56:1118-24.
2. Tamai M, Kawakami A, Uetani M, et al. Bone edema determined by magnetic resonance imaging reflects severe disease status in patients with early-stage rheumatoid arthritis. J Rheumatol 2007;34:2154-7.
3. Lindqvist E, Eberhardt K, Bendtzen K, et al. Prognostic laboratory markers of joint damage in rheumatoid arthritis. Ann Rheum Dis 2005;64:196-201.
4. van Gaalen FA, van Aken J, Huizinga TW, et al. Association between HLA class II genes and autoantibodies to cyclic citrullinated peptides (CCPs) influences the severity of rheumatoid arthritis. Arthritis Rheum 2004;50:2113-21.
5. Arnett FC, Edworthy SM, Bloch DA, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315-24.
6. Sharif M, Kirwan JR, Elson CJ, et al. Suggestion of nonlinear or phasic progression of knee osteoarthritis based on measurements of serum cartilage oligomeric matrix protein levels over five years. Arthritis Rheum 2004;50:2479-88.
7. Kelman A, Lui L, Yao W, et al. Association of higher levels of serum cartilage oligomeric matrix protein and N-telopeptide crosslinks with the development of radiographic hip osteoarthritis in elderly women. Arthritis Rheum 2006;54:236-43.
8. Altman R, Alarcon G, Appelrouth D, et al. The American College of Rheumatology criteria for the classification and reporting of osteoarthritis of the hip. Arthritis Rheum 1991;34:505-14.
9. Altman R, Asch E, Bloch DA, et al. Development of criteria for the classification and reporting of osteoarthritis. Classification of osteoarthritis of the knee. Diagnostic and Therapeutic Criteria Committee of the American Rheumatism Association. Arthritis Rheum 1986;29:1039-49.
10. Inoue E, Yamanaka H, Hara M, et al. Comparison of Disease Activity Score (DAS)28-erythrocyte sedimentation rate and DAS28-C-reactive protein threshold values. Ann Rheum Dis 2007;66:407-9.
11. Tamai M, Kawakami A, Uetani M, et al. The presence of anti-cyclic citrullinated peptide antibody is associated with magnetic resonance imaging detection of bone marrow oedema in early stage rheumatoid arthritis. Ann Rheum Dis 2006;65:133-4.
12. Tamai M, Kawakami A, Uetani M, et al. Early prediction of rheumatoid arthritis by serological variables and magnetic resonance imaging of the wrists and finger joints: results from prospective clinical examination. Ann Rheum Dis 2006;65:134-5.
13. McQueen FM, Benton N, Perry D, et al. Bone edema scored on magnetic resonance imaging scans of the dominant carpus at presentation predicts radiographic joint damage of the hands and feet six years later in patients with rheumatoid arthritis. Arthritis Rheum 2003;48:1814-2.
14. Conaghan PG, O'Connor P, McGonagle D, et al. Elucidation of the relationship between synovitis and bone damage: a randomized magnetic resonance imaging study of individual joints in patients with early rheumatoid arthritis. Arthritis Rheum 2003;48:64-71.
15. Clark AG, Jordan JM, Vilim V, et al. Serum cartilage oligomeric matrix protein reflects osteoarthritis presence and severity: the Johnston County Osteoarthritis Project. Arthritis Rheum 1999;42:2356-64.
16. den Broeder AA, Joosten LA, Saxne T, et al. Long term anti-tumour necrosis factor alpha monotherapy in rheumatoid arthritis: effect on radiological course and prognostic value of markers of cartilage turnover and endothelial activation. Ann Rheum Dis 2002;61:311-8.

Arthritis & Rheumatism (Arthritis Care & Research)
Vol. 61, No. 6, June 15, 2009, pp 772–778
DOI 10.1002/art.24711

ORIGINAL ARTICLE

A Prediction Rule for Disease Outcome in Patients With Undifferentiated Arthritis Using Magnetic Resonance Imaging of the Wrists and Finger Joints and Serologic Autoantibodies

MAMI TAMAI, ATSUSHI KAWAKAMI, MASATAKA UETANI, SHOICHIRO TAKAO, KAZUHIKO ARIMA, NAOKI IWAMOTO, KEITA FUJIKAWA, TOSHIYUKI ARAMAKI, SHIN-YA KAWASHIRI, KUNIHIRO ICHINOSE, MAKOTO KAMACHI, HIDEKI NAKAMURA, TOMOKI ORIGUCHI, HIROAKI IDA, KIYOSHI AOYAGI, AND KATSUMI EGUCHI

Objective. To evaluate whether magnetic resonance imaging (MRI) of the wrists and finger joints and an analysis of serologic autoantibodies are clinically meaningful for the subsequent development of rheumatoid arthritis (RA) in patients with undifferentiated arthritis (UA).

Methods. A total of 129 patients with UA, a disease status formally confirmed by a rheumatologist over a period of at least 1 year, were included. Gadolinium-diethylenetriamine–enhanced MRI of both wrists and finger joints and serologic variables were examined upon admission to our Early Arthritis Clinic at Nagasaki University. After a prospective followup of 1 year, a predictive value for the development of RA was determined for each patient.

Results. The subjects were evaluated for their positive or negative status with respect to 3 objective measures at study entry: anti–cyclic citrullinated peptide (anti-CCP) antibodies and/or IgM-rheumatoid factor, MRI-proven symmetric synovitis, and MRI-proven bone edema and/or bone erosion. The patients who were positive for at least 2 of these measures progressed to RA at 1 year with a 79.7% positive predictive value (PPV), 63.0% negative predictive value, 75.9% specificity, 68.0% sensitivity, and 71.3% accuracy. Furthermore, in 22 UA patients positive for both anti-CCP antibodies and MRI-proven bone edema who were considered to have progressed to RA at 1 year, the PPV was increased to 100%. A close correlation was found between the present rule and that established in the Leiden Early Arthritis Cohort.

Conclusion. MRI-proven early joint damage in conjunction with serologic autoantibodies is efficient in predicting progression from UA to RA. This method can be used to identify patients who would benefit from early treatment with disease-modifying antirheumatic drugs.

INTRODUCTION

Early undifferentiated arthritis (UA) is defined as early arthritis that does not fulfill the classification criteria for a more definitive diagnosis, according to the 1987 American College of Rheumatology (ACR; formerly the American Rheumatism Association) criteria for rheumatoid arthritis (RA) (1–3). The natural disease course of UA is variable; therefore, to minimize under- and overtreatment of patients with UA, a model was recently constructed by the Leiden Early Arthritis Cohort to estimate the likelihood of progression to RA in individual patients (2,3). Their prediction rule consists of 9 clinical variables: sex, age, localization of symptoms, morning stiffness, tender joint count,

ISRCTN: 021008-1.
Supported in part by a grant from The Ministry of Health, Labor and Welfare, Japan.
Mami Tamai, MD, PhD, Atsushi Kawakami, MD, PhD, Masataka Uetani, MD, PhD, Shoichiro Takao, MD, Kazuhiko Arima, MD, PhD, Naoki Iwamoto, MD, Keita Fujikawa, MD, PhD, Toshiyuki Aramaki, MD, PhD, Shin-ya Kawashiri, MD, Kunihiro Ichinose, MD, PhD, Makoto Kamachi, MD, PhD, Hideki Nakamura, MD, PhD, Tomoki Origuchi, MD, PhD, Hiroaki Ida, MD, PhD, Kiyoshi Aoyagi, MD, PhD, Katsumi Eguchi, MD, PhD: Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan.

Drs. Tamai and Kawakami contributed equally to this work.
Address correspondence to Katsumi Eguchi, MD, PhD, Unit of Translational Medicine, Department of Immunology and Rheumatology, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. E-mail: eguchi@net.nagasaki-u.ac.jp.
Submitted for publication June 24, 2008; accepted in revised form March 2, 2009.

swollen joint count, C-reactive protein (CRP) level, IgM rheumatoid factor (IgM-RF) positivity, and the presence of anti–cyclic citrullinated peptide (anti-CCP) antibodies (2,3). Prediction scores vary from 0 to 14 and correspond to the percent chance of developing RA at 1 year (3). Van der Helm-van Mil et al examined 570 patients with UA and found that, at cutoff levels of ≤6 and ≥8, the negative predictive values (NPVs) and positive predictive values (PPVs) were 91% and 84%, respectively (3), which indicates a score of ≥8 for initiating treatment and a score of ≤6 for withholding treatment.

The above prediction rule indicates that clinical manifestation is still the gold standard in detecting synovitis, which is also mentioned by the European Standing Committee for International Clinical Studies Including Therapeutics (ESCISIT) (4); however, the expert committee of the European League Against Rheumatism defines the importance of imaging methods such as magnetic resonance imaging (MRI) and ultrasonography as being more sensitive than clinical examination or plain radiography for the detection of early joint damage in early arthritis (4). For instance, plain radiography does not detect synovitis, early bone erosion, or bone edema, whereas MRI is able to do so (5). Given the utility of the detection of early joint damage by MRI, our investigation has focused on how to identify patients with early arthritis likely to progress to RA by not using MRI of the wrists and finger joints and serologic variables (6–8). Our previous reports have shown that MRI-proven symmetric synovitis, MRI-proven bone changes (bone edema or bone erosion), and the presence of serologic autoantibodies (anti-CCP antibodies or IgM-RF) upon admission are predictive factors for early-stage RA (6). However, some of the patients examined in the previous studies already fulfilled international disease criteria for RA or osteoarthritis upon admission (6,7), which was a weak point of our previous prediction rule.

The present study is a reevaluation of our prediction rule in patients with UA. Additionally, we examined correlations with a predictive role such as that reported by the Leiden Early Arthritis Cohort.

PATIENTS AND METHODS

Patients. The Early Arthritis Clinic opened in 2001 as a part of the Unit of Translational Medicine, the Department of Immunology and Rheumatology, and the Graduate School of Biomedical Sciences at Nagasaki University. Patients were referred from an area in the western part of Japan, Nagasaki Prefecture, which has ~450,000 inhabitants. From this clinic, 129 patients with UA were included in the present study; their disease status was formally confirmed by a rheumatologist for at least 1 year. We have examined MRI results of both wrists and finger joints for all of the subjects; therefore, all of the 129 patients with UA expressed rheumatic manifestations of the wrists and finger joints at study entry. The characterization of UA upon admission was determined as previously reported (3), i.e., as arthritis that could not be classified according to ACR criteria within 2 weeks after being included in the study, when laboratory and radiographic results were available. At a prospective followup of 1 year, 75 patients were found to have progressed to RA based on the 1987 ACR criteria for RA (1).

Baseline clinical manifestations and variables included sex, age, localization of arthritis, morning stiffness score measured on a 100-mm visual analog scale, the number of tender joints, the number of swollen joints, the CRP level (measured by latex turbidimetric immunosorbent assay; Daiichi Pure Chemicals, Fukuoka, Japan), IgM-RF positivity (measured by latex-enhanced immunonephelometric assay with a cutoff value of 14 IU/ml; Dade Behring, Marburg, Germany), positive status for anti-CCP antibodies (measured by enzyme-linked immunosorbent assay [ELISA] with a cutoff value of 4.5 units/ml; DIASTAT Anti-CCP; Axis-Shield, Dundee, UK), matrix metalloproteinase 3 (measured by ELISA with cutoff values of 59.7 ng/ml for women and 121.0 ng/ml for men; Daiichi Pure Chemicals) (9), and MRI of both wrists and finger joints, as previously described (6–8). All variables were examined on the same day, as previously reported (6–8). Each patient provided a signed consent form to participate in the study, which was approved by the Institutional Review Board of Nagasaki University.

MRI of the wrists and finger joints. MRI of both wrists and finger joints were acquired using a 1.5T system (Sigma; General Electric Medical Systems, Milwaukee, WI) with an extremity coil. Coronal T1-weighted spin-echo (repetition time [TR] 450, echo time [TE] 13) and STIR (TR 3,000, TE 12, T1 160) images were also acquired. The images were evaluated for bone edema, bone erosion, and synovitis in 15 sites in each finger and wrist, including the distal radioulnar joint, the radiocarpal joint, the midcarpal joint, the first carpometacarpal joint, the second through fifth carpometacarpal joints (together), the first through fifth metacarpophalangeal joints, and the first through fifth proximal interphalangeal joints separately (a total of 30 sites in both hands), as we recently reported (6–8). The presence of synovitis, bone edema, and bone erosion was evaluated by 2 experienced radiologists (MU, ST) as described by Lassere et al (10) and Conaghan et al (11), and decisions were reached by consensus. The evaluation of MRI features has been established by several groups (10–13); however, it is a complex task. Therefore, as we previously reported (6–8), we simply determined the presence or absence of synovitis, bone edema, and bone erosion on MRI after the intravenous injection of 0.1 mmoles/kg of gadolinium-diethylenetriamine (Magnevist, Schering, Germany). Our method is qualitative rather than quantitative; however, it is sensitive enough to identify early joint damage in patients with early-stage RA (6–8).

Assessment of disease status at 1 year and statistical analysis. Our previous reports have shown the preferential expression of MRI-proven symmetric synovitis, MRI-proven bone edema, MRI-proven bone erosion, IgM-RF, and anti-CCP antibodies in patients with early-stage RA (6–8). Logistic regression analysis of the previous study identified subjects with positive values for 2 or 3 of the 3 objective measures (anti-CCP antibodies and/or IgM-RF, MRI-proven symmetric synovitis, and MRI-proven bone

Table 1. Baseline characteristics of 129 patients with undifferentiated arthritis*

	RA progression (n = 75)	No RA progression (n = 54)	P†
Age, median (range) years	53 (25–80)	52 (16–79)	NS
Sex, male:female (% female)	17:58 (77.3)	12:42 (77.8)	NS
Duration of symptoms at baseline, median (range) months	3 (0.5–15)	3 (0.5–24)	NS
Morning stiffness, median (range) minutes	60 (0–960)	15 (0–960)	< 0.0005
Tender joint count, median (range)	7 (0–39)	4.5 (0–27)	< 0.05
Swollen joint count, median (range)	3 (0–26)	0 (0–24)	< 0.0001
DAS28 tender joint count, median (range)	6 (0–28)	4 (0–24)	< 0.005
DAS28 swollen joint count, median (range)	3 (0–23)	0 (0–22)	< 0.0001
HAQ score, mean ± SD	7.3 ± 4.3	4.8 ± 3.8	< 0.005
Patients' pain on a 100-mm VAS, mean ± SD	52.6 ± 27.1	52.4 ± 32.0	NS
Patients' global on a 100-mm VAS, mean ± SD	52.5 ± 25.4	56.4 ± 29.5	NS
1987 ACR criteria for RA‡			
Morning stiffness for 1 hour	41 (54.7)	15 (27.8)	< 0.005
Arthritis in ≥3 joints	42 (56.0)	10 (18.5)	< 0.00001
Arthritis of the wrists and finger joints	56 (74.7)	22 (40.7)	0.0001
Symmetric arthritis	41 (54.7)	11 (20.4)	< 0.0001
IgM-RF positivity	39 (52.0)	16 (29.6)	< 0.05
HLA–DRB1*0405 allele carriership	27 (36.0)	13 (24.1)	NS
DAS28-CRP, mean ± SD	4.31 ± 1.22	3.46 ± 1.33	< 0.0001
Serologic variables			
Anti-CCP antibody positivity	43 (57.3)	4 (7.4)	< 0.0001
IgM-RF and/or anti-CCP antibody positivity	50 (66.7)	18 (33.3)	< 0.0005
MMP-3 positivity	27 (36.0)	8 (14.8)	< 0.01
MMP-3 level, median (range) ng/ml	50.2 (0–1,250)	34.8 (10.66–419.6)	< 0.005
CRP positivity	51 (68.0)	16 (29.6)	< 0.0001
CRP level, median (range) mg/dl	0.50 (0.01–18.4)	0.10 (0–8.36)	< 0.0001
MRI features			
Synovitis positivity	68 (90.7)	30 (55.6)	< 0.0001
Symmetric synovitis positivity	56 (74.7)	22 (40.7)	< 0.005
Bone edema positivity	31 (41.3)	5 (9.3)	< 0.0001
Bone erosion positivity	22 (29.3)	5 (9.3)	< 0.0001
Bone edema and/or erosion positivity	36 (48.0)	9 (16.7)	< 0.0001

* Values are the number (percentage) unless otherwise indicated. RA = rheumatoid arthritis; NS = no significant difference; DAS28 = Disease Activity Score in 28 joints; HAQ = Health Assessment Questionnaire; VAS = visual analog scale; ACR = American College of Rheumatology; IgM-RF = IgM rheumatoid factor; CRP = C-reactive protein; anti-CCP = anti–cyclic citrullinated peptide; MMP-3 = matrix metalloproteinase 3; MRI = magnetic resonance imaging.
† Indicates the difference between RA progression and no RA progression.
‡ We did not refer to the duration of the components in the 1987 ACR criteria for RA: morning stiffness for 1 hour, arthritis in ≥3 joints, arthritis of the wrists and finger joints, and symmetric synovitis.

edema and/or bone erosion); the patients were classified as having early-stage RA with 82.5% sensitivity and 84.8% specificity (6). However, our previous study is somewhat inaccurate because some of the subjects were already classified as having early-stage RA or rheumatic diseases other than RA upon admission (6). Therefore, in the present study, all of the selected subjects were classified as having UA upon admission, having been evaluated by the same objective measures in comparison with the prediction rule by the Leiden Early Arthritis Cohort (3). For all tests (chi-square test, Mann-Whitney U test, and Spearman's rank correlation), P values less than 0.05 were considered significant.

RESULTS

Evaluation of the Leiden Early Arthritis Cohort prediction rule in 129 UA patients. We collected the demographic clinical manifestations, serologic data, and MRI features of 129 patients with UA upon admission (Table 1). As expected, these arthritis conditions were condensed in UA that progressed to RA, as compared with UA that did not progress to RA. Although the choice of therapies for the patients was based on the decision of each physician, the difference between clinical manifestations at baseline may reflect on the therapies within the first year. Therefore, the percentage of patients receiving disease-modifying antirheumatic drugs (DMARDs) and glucocorticoids was much higher in 75 patients with UA that progressed to RA than in 54 patients with UA that did not progress to RA. In regard to DMARDs, 63 (84.0%) of 75 patients with UA that progressed to RA were treated with DMARDs, including 39 patients with sulfasalazine, 13 patients with methotrexate, 2 patients with infliximab, and 1 patient with adalimumab, whereas only 3 patients (5.6%) received DMARDs among 54 patients with UA that did not progress to RA ($P < 0.0001$). In regard to glucocorticoids, 45 (60.8%) of 75 patients with UA that progressed to RA were