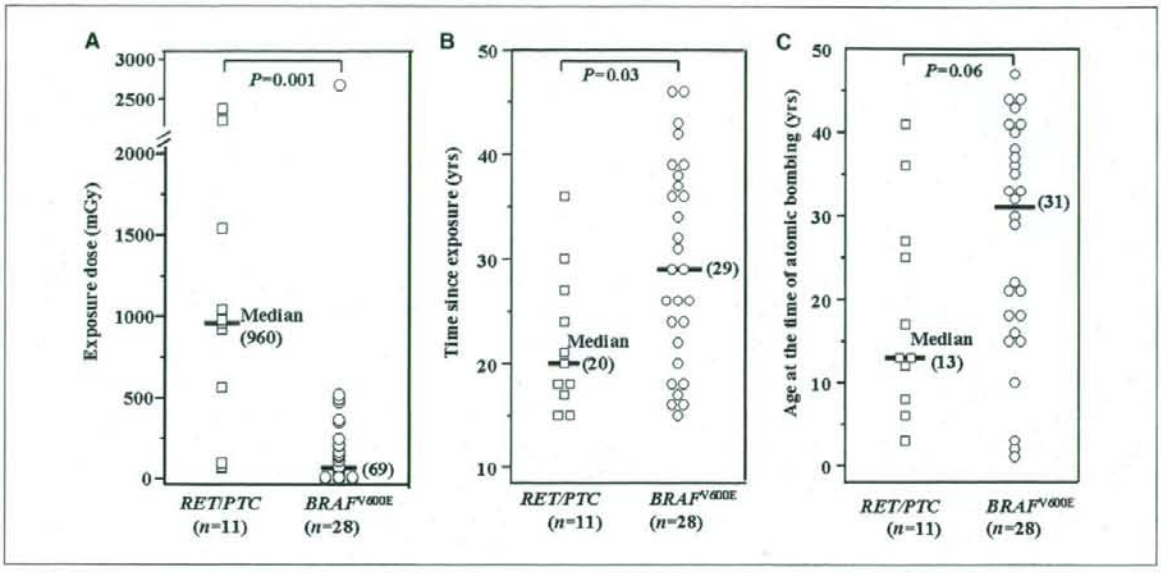

Figure 2. Comparison of *RET/PTC* and $BRAF^{V600E}$ alterations in PTC patients. *A*, radiation dose; *B*, time since exposure; *C*, age at the time of atomic bombing. *RET/PTC* *PTC* rearrangement (□) and $BRAF^{V600E}$ mutation (○), respectively, are shown.

rearrangements, and *NTRK1* rearrangements (9) was singularly found, indicating that one such gene alteration is an important early event in development of PTC. Furthermore, a recently identified *AKAP9-BRAF* rearrangement did not coexist with *BRAF* mutation in radiation-associated PTC (30). These data suggest that a single genetic event in the MAPK-signaling pathway may be sufficient for thyroid cell transformation and tumorigenesis.

In this study, pathologic and epidemiologic characteristics, specifically radiation-related ones, of PTC having *RET/PTC* rearrangements contrasted clearly with those of PTC having $BRAF^{V600E}$ mutation. Noting that 17 (81%) and 1 (5%) of 21 nonexposed PTC patients having $BRAF^{V600E}$ mutation and *RET/PTC* rearrangement in this study, respectively, are in agreement with other data on nonexposed adult-onset Japanese PTC (18, 25, 40–42), we for the first time have shown that the frequency of *RET/PTC* rearrangements significantly increased with increased radiation dose as well as shorter time elapsed since radiation exposure and younger age at the time of bombing (Figs. 1*A* and 2; Table 4). *RET/PTC* rearrangements were detected in 50% (8 of 16) of adult-onset PTC patients who were exposed to radiation dose of >0.5 Gy, although this frequency was somehow lower than that (about 80%) reported for French thyroid cancer patients who had received external radiotherapy (18). This difference in frequency of *RET/PTC* rearrangements may be due to the different radiation conditions (i.e., single or repeated irradiation and dose). On the other hand, $BRAF^{V600E}$ mutation significantly decreased frequency with increased radiation dose (Fig. 1*A*). This finding seems to be consistent with our parallel observations, shorter time elapsed since exposure, and younger age at the time of bombing in PTC patients with *RET/PTC*, compared with those in the patients with $BRAF^{V600E}$ (Fig. 2; Table 4). Taken together, our findings imply that *RET/PTC* rearrangements, not $BRAF^{V600E}$ mutation, are closely associated with radiation-associated adult-onset PTC.

The existence of a molecular mechanism other than *RET/PTC* rearrangement is suggested from Fig. 1*B*: *RET/PTC* rearrangements showed a peak at 20 to 30 years since radiation exposure and relatively low frequency of 20% in <20 years since exposure, in contrast to 53% of unidentified alterations other than *RET/PTC* and $BRAF^{V600E}$. Because *RET/PTC* and $BRAF^{V600E}$ account for 82% of nonexposed PTC and about 60% to 70% of PTC in the Japanese general population (18, 25, 40–42), this increase of unidentified alterations in <20 years is thought to be caused by radiation. This unidentified mechanism may be involved in radiation-associated PTC, which occurred earlier after radiation exposure than did PTC having *RET/PTC*. However, regarding *NTRK1* rearrangements and the *BRAF* fusion gene, the *TRK-T2* gene lacking five nucleotides was

Table 4. Logistic regression analysis of 39 exposed PTC patients with *RET/PTC* rearrangements or $BRAF^{V600E}$ mutation

Variables	β*	P
Radiation dose (mGy)	0.002	0.012
Age at the time of atomic bombing (y)	−0.113	0.031
Year since exposure (y)	−0.192	0.034
Gender, male vs. female	2.674	0.204
Histology, conventional vs. follicular variant	0.157	0.927

NOTE: A dependent variable was defined as follows: rearranged *RET* and wild *BRAF* = 1; wild *RET* and mutated *BRAF* = 0.
*Regression coefficients in the logistic regression model.

detected in only one exposed case. Therefore, the unidentified alterations may be involved in pathways other than the MAPK-signaling pathway.

We need to confirm our findings with an increased number of study patients, given that the present study covered only about 36% of PTC found in the LSS cohort among A-bomb survivors during 1958 to 1993 for whom tissue specimens could be obtained. Toward this end, an efficient system to collect archival specimens from A-bomb survivors, which are dispersed over a number of hospitals in Hiroshima and Nagasaki, will be necessary in cooperation with the institutions concerned (it took 3 years to collect 90 PTC specimens, 71 of which were used in the present study). Because the specimens deteriorate as time goes by, it is urgent that our collection and analyses be conducted soon to increase our knowledge, which in turn might lead to improved treatment and prevention of radiation-associated cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References

1. Imaizumi M, Usa T, Tominaga T, et al. Radiation dose-relationships for thyroid nodules and autoimmune thyroid diseases in Hiroshima and Nagasaki atomic bomb survivors 55–58 years after radiation exposure. JAMA 2006;295:1011–22.
2. Kazakov VS, Demidchik EP, Astakhova LN. Thyroid cancer after Chernobyl. Nature 1992;359:21.
3. Astakhova LN, Anspaugh LR, Beebe GW, et al. Chernobyl-related thyroid cancer in children of Belarus: a case-control study. Radiat Res 1998;150:349–56.
4. Thompson DE, Mabuchi K, Ron E, et al. Cancer incidence in atomic bomb survivors. Part II: Solid tumors, 1958–1987. Radiat Res 1994;137:S17–67.
5. Takeichi N, Ezaki H, Dohi K. Thyroid cancer: a review of forty-five years study of Hiroshima and Nagasaki atomic bomb survivors. Thyroid cancer: reports up to date and a review. J Radiat Res 1991;32 Suppl:180–8.
6. Nikiforov YE, Gnepp DR. Pediatric thyroid cancer after the Chernobyl disaster. Cancer 1994;74:748–66.
7. Kimura ET, Nikiforova MN, Zhu Z, et al. High prevalence of *BRAF* mutations in thyroid cancer: genetic evidence for constitutive activation of the RET/PTC-RAS-BRAF signaling pathway in papillary thyroid carcinoma. Cancer Res 2003;63:1454–7.
8. Soares P, Trovisco V, Rocha AS, et al. BRAF mutations and RET/PTC rearrangements are alternative events in the etiopathologenesis of PTC. Oncogene 2003;22:4578–80.
9. Frattini M, Ferrario C, Bressan P, et al. Alternative mutations of *BRAF, RET* and *NTRK1* are associated with similar but distinct gene expression patterns in papillary thyroid cancer. Oncogene 2004;23:7436–40.
10. Pachnis V, Mankoo B, Costantini F. Expression of the c-ret proto-oncogene during mouse embryogenesis. Development 1993;119:1005–17.
11. Schuchardt A, D'Agati V, Larsson-Blomberg L, et al. Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. Nature 1994;367:380–3.
12. Ciampi R, Giordano TJ, Wikenheiser-Brokamp K, et al. HOOK3-RET: a novel type of *RET/PTC* rearrangement in papillary thyroid carcinoma. Endocr Relat Cancer 2007;14:445–52.
13. Patel KN, Singh B. Genetic considerations in thyroid cancer. Cancer Control 2006;13:111–8.
14. Ciampi R, Nikiforov YE. RET/PTC rearrangements and BRAF mutations in thyroid tumorigenesis. Endocrinology 2007;148:936–41.
15. Nikiforov YE, Rowland JM, Bove KE, et al. Distinct pattern of *ret* oncogene rearrangements in morphological variants of radiation-induced and sporadic thyroid papillary carcinomas in children. Cancer Res 1997;57:1690–4.
16. Rabes HM, Demidchik EP, Sidorow JD, et al. Pattern of radiation-induced *RET* and *NTRK1* rearrangements in 191 post-Chernobyl papillary thyroid carcinomas: biological, phenotypic, and clinical implications. Clin Cancer Res 2000;6:1093–103.
17. Tallini G, Asa SL. RET oncogene activation in papillary thyroid carcinoma. Adv Anat Pathol 2001;8:345–54.
18. Suárez HG. Genetic alterations in human epithelial thyroid tumors. Clin Endocrinol (Oxf) 1998;48:531–46.
19. Chu EL, Wu WM, Tran KT, et al. Prevalence and distribution of ret/ptc 1,2, and 3 in papillary thyroid carcinoma in New Caledonia and Australia. J Clin Endocrinol Metab 2000;85:2733–9.
20. Bounacer A, Wicker R, Caillou B, et al. High prevalence of activating *ret* proto-oncogene rearrangements, in thyroid tumors from patients who had received external radiation. Oncogene 1997;15:1263–73.
21. Elisei R, Romei C, Vorontsova T, et al. RET/PTC rearrangements in thyroid nodules: studies in irradiated and not irradiated, malignant and benign thyroid lesions in children and adults. J Clin Endocrinol Metabol 2001;86:3211–6.
22. Mizuno T, Iwamoto KS, Kyoizumi S, et al. Preferential induction of RET/PTC1 rearrangement by X-ray irradiation. Oncogene 2000;19:438–43.
23. Dibb NJ, Dilworth SM, Mol CD. Switching on kinase: oncogenic activation of BRAF and the PDGFR family. Nat Rev Cancer 2004;4:718–27.
24. Davies H, Bignell GR, Cox C, et al. Mutations of the *BRAF* gene in human cancer. Nature 2002;417:949–54.
25. Xing M. *BRAF* mutation in thyroid cancer. Endocr Relat Cancer 2005;12:245–62.
26. Wan PTC, Garnett MJ, Roe SM, et al. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. Cell 2004;116:855–67.
27. Kumagai A, Namba H, Saenko VA, et al. Low frequency of $BRAF^{T1796A}$ mutations in childhood thyroid carcinomas. J Clin Endocrinol Metab 2004;89:4280–4.
28. Lima J, Trovisco V, Soares P, et al. *BRAF* mutations are not a major event in post-Chernobyl childhood thyroid carcinomas. J Clin Endcrinol Metabol 2004;89:4267–71.
29. Nikiforova MN, Ciampi R, Salvatore G, et al. Low prevalence of BRAF mutations in radiation-induced thyroid tumors in contrast to sporadic papillary carcinomas. Cancer Lett 2004;209:1–6.
30. Ciampi R, Knauf JA, Kerler R, et al. Oncogenic *AKAP9-BRAF* fusion is a novel mechanism of MAPK pathway activation in thyroid cancer. J Clin Invest 2005;115:94–101.
31. Powell N, Jeremiah S, Morishita M, et al. Frequency of *BRAF* T1796A mutation in papillary thyroid carcinoma relates to age of patient at diagnosis and not to radiation exposure. J Pathol 2005;205:558–64.
32. Takahashi K, Eguchi H, Arihiro K, et al. The presence of *BRAF* point mutation in adult papillary thyroid carcinomas from atomic bomb survivors correlates with radiation dose. Mol Carcinog 2007;46:242–8.
33. DeLellis RA, Lloyd RV, Heitz PU, Eng C, editors. World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of Endocrine Organs. Lyon: IARC Press; 2004.
34. Hamatani K, Eguchi H,Takahashi K, et al. Improved RT-PCR amaplification for molecular analyses with long-term preserved formalin-fixed, paraffin-embedded tissue specimens. J Histochem Cytochem 2006;54:773–80.
35. Young RW, Kerr GD, edit. Reassessment of the Atomic Bomb Radiation Dosimetry for Hiroshima and Nagasaki-Dosimetry System 2002-. Radiation Effects Research Foundation, 2006.
36. Kondo T, Ezzat S, Asa SL. Pathogenetic mechanisms in thyroid follicular-cell neoplasia. Nat Rev Cancer 2006;6:292–306.
37. Melillo RM, Castellone MD, Guarino V, et al. The RET/PTC-RAS-BRAF linear signaling cascade mediates the motile and mitogenic phenotype of thyroid cancer cells. J Clin Invest 2005;115:1068–81.
38. Mitsutake N, Miyagishi M, Mitsutake S, et al. BRAF mediates RET/PTC-induced mitogen-activated protein kinase activation in thyroid cells: functional support for requirement of the RET/PTC-RAS-BRAF pathway in papillary thyroid carcinogenesis. Endocrinology 2006;147:1014–9.
39. Ouyang B, Knauf JA, Smith EP, et al. Inhibitors of RAF kinase activity block growth of thyroid cancer cells with *RET/PTC* or *BRAF* mutations *in vitro* and *in vivo*. Clin Cancer Res 2006;12:1785–93.
40. Motomura T, Nikiforov YE, Namba H, et al. Ret rearrangements in Japanese pediatric and adult papillary thyroid cancers. Thyroid 1998;8:485–9.
41. Kitamura Y, Minobe K, Nakata T, et al. Ret/PTC3 is the most frequent form of gene rearrangement in papillary thyroid carcinomas in Japan. J Hum Genet 1999;44:96–102.
42. kumagai A, Namba H, Akanov Z, et al. Clinical implications of pre-operative rapid BRAF analysis for papillary thyroid cancer. Endocr J 2007;54:399–405.

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Caspase-independent cell death without generation of reactive oxygen species in irradiated MOLT-4 human leukemia cells

Kengo Yoshida [a], Yoshiko Kubo [a], Yoichiro Kusunoki [a], Yukari Morishita [a], Hiroko Nagamura [a], Ikue Hayashi [b], Seishi Kyoizumi [a,c], Toshio Seyama [d], Kei Nakachi [a], Tomonori Hayashi [a,*]

[a] Department of Radiobiology/Molecular Epidemiology, Radiation Effects Research Foundation, 5-2 Hijiyama Park, Minami-ku, Hiroshima 732-0815, Japan
[b] Central Research Laboratory, Hiroshima University Faculty of Dentistry, Hiroshima, Japan
[c] Faculty of Human Ecology, Yasuda Women's University, Hiroshima, Japan
[d] Faculty of Pharmaceutical Sciences, Yasuda Women's University, Hiroshima, Japan

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ABSTRACT

To improve our understanding of ionizing radiation effects on immune cells, we investigated steps leading to radiation-induced cell death in MOLT-4, a thymus-derived human leukemia cell. After exposure of MOLT-4 cells to 4 Gy of X-rays, irradiated cells sequentially showed increase in intracellular reactive oxygen species (ROS), decrease in mitochondrial membrane potential, and eventually apoptotic cell death. In the presence of the caspase inhibitor z-VAD-fmk, irradiated cells exhibited necrotic characteristics such as mitochondrial swelling instead of apoptosis. ROS generation was not detected during this necrotic cell death process. These results indicate that radiation-induced apoptosis in MOLT-4 cells requires elevation of intracellular ROS as well as activation of a series of caspases, whereas the cryptic necrosis program—which is independent of intracellular ROS generation and caspase activation—is activated when the apoptosis pathway is blocked.

1. Introduction

A single cell-death-inducing stimulus such as ionizing radiation seems to evoke multiple and discrete mechanisms of cell death, and the physiological condition of the cells may determine the eventual outcome—whether apoptosis or necrosis, or another type of cell death [1]. Apoptosis and necrosis reveal varying postmortem features based on morphology, molecular events, and metabolic variations [2]. Immune responses to these two types of cell death may also differ in the event of radiation exposure including cancer radiotherapy (i.e., immune suppression in apoptosis vs. potent immune responses and inflammation in necrosis). Thus, elucidation of the cell-death mechanisms is essential to an improved understanding of radiation effects on a host. Early intracellular responses to radiation such as the possible involvement of reactive oxygen species (ROS)[1] in the process of apoptosis and/or necrosis, may determine the fate of irradiated cells and will be of particular interest in prevention and treatment of radiation-induced insults.

Apoptotic cell death is an active, programmed process of cell dismantling, which occurs under normal physiological and pathological conditions. Apoptotic features include chromatin condensation and fragmentation, overall cell shrinkage, formation of condensed cell bodies (apoptotic bodies), and minor changes in the cytoplasmic organelles. The molecular pathways of apoptosis, in which a series of cysteine proteases known as caspases play a central role, have been extensively studied over the last decade [3–5], but the upstream initiating events of radiation-induced apoptosis as well as the roles of mitochondria and ROS in these pathways remain to be elucidated [6].

Necrosis has thus far been considered passive and accidental cell death caused by extreme environmental perturbation, and characterized by plasma membrane rupture and swelling of the cytoplasmic organelles, particularly mitochondria. However, our current knowledge of what happens in the molecular pathways of radiation-induced necrosis is very limited. Recent studies using various cell types have shown that blockage of the apoptosis pathways failed to prevent cell death and instead led to necrotic cell death, which implies that necrosis is a physiologically alternative process of cellular dismantling under particular conditions [7–9].

Therefore, we investigated radiation-induced intracellular events, particularly ROS generation, to their conclusion in apoptosis or necrosis, using MOLT-4 cells with or without a caspase inhibitor z-VAD-fmk. Our results show that a 4 Gy X-ray irradiation mainly induced apoptotic cell death in MOLT-4 cells, but resulted in necrotic cell death in the presence of z-VAD-fmk. Moreover, generation of intracellular ROS was observed during the apoptotic

* Corresponding author. Fax: +81 82 261 3170.
E-mail address: tomo@rerf.or.jp (T. Hayashi).

[1] *Abbreviations used:* PI, propidium iodide; ROS, reactive oxygen species; z-VAD-fmk, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

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process of irradiated MOLT-4 cells but not during the necrotic process of these cells pretreated with z-VAD-fmk: It is therefore possible that radiation may induce necrotic cell death through ROS-independent pathways.

2. Materials and methods

2.1. Cell culture

Human leukemia T cell line MOLT-4 [10] was obtained from the Japanese Cancer Resources Bank (Tokyo, Japan) and was grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), L-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml) in a 5% CO_2 humidified atmosphere. All experiments were performed with cells in the exponential growth phase.

2.2. Irradiation

Irradiation of cells was performed using an X-ray generator (Shimadzu HF-320; 220 kVp, 8 mA) with a 0.5 mm aluminum and 0.3 mm copper filter at a dose rate of approximately 0.8 Gy/min. Cells in the exponential growth phase were irradiated in RPMI 1640 with 10% FCS in a plastic dish at room temperature under normal oxygen tension.

2.3. Mitochondrial membrane potential and superoxide anions

To measure mitochondrial membrane potential ($\Delta\psi m$) and generation of the superoxide anions O_2^-, cells (5×10^5) were stained with 0.26 μM Rhodamine123 (Rh123; Molecular Probes, Eugene, OR) and 20 μM hydroethidine (HE; Molecular Probes), respectively, for 20 min at 37 °C [11–15]. Cells were washed once with phosphate buffered saline (PBS) containing 1% FCS, and then analyzed with a FACScan flow-cytometer (BD Biosciences, Franklin Lakes, NJ). To isolate certain cell fractions, cells were sorted using a FACS Vantage SE (BD Biosciences).

2.4. Phosphatidyl serine externalization and cell death

Externalization of the plasma membrane phosphatidyl serine, an early process of apoptosis, and cell death were assessed by measuring annexin V-FITC- and propidium iodide (PI)-stained cells, respectively, using a kit from MBL (Nagoya, Japan). After incubation in the reaction buffer (130 μl of binding buffer, 0.5 μl of annexin V-FITC, and 1.0 μl of PI) for 10 min, cells were analyzed using a FACScan flow-cytometer.

2.5. Catalytic activity of caspases

To suppress the activity of caspases, cells were pretreated with z-VAD-fmk (*N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; Peptide Institute, Osaka, Japan). Cells were suspended in the medium containing 100 μM z-VAD-fmk for 30 min prior to irradiation. Catalytic activity of a series of caspases was determined based on colorimetric assays. Cells (5×10^5) were lysed in lysis buffer (10 mM Tris–HCl, pH 7.4, 25 mM NaCl, 0.25% Triton X-100, 1 mM EDTA). The lysates were centrifuged at 16,000*g* at 4 °C for 30 min, and the supernatants were stored at −80 °C until analysis. Each supernatant (25 μg) was then incubated with 100 μl of caspase buffer (50 mM HEPES, pH 7.2, 100 mM NaCl, 1 mM EDTA, 10% sucrose, 0.1% CHAPS, 5 mM dithiothreitol) containing 100 μM substrates conjugated with 7-amino-4-methylcoumarin (Ac-VDVAD-AMC for caspase-2, Ac-DNLD-AMC for caspase-3, Ac-DEVD-AMC for caspase-3 and caspase-7, Ac-IETD-AMC for caspase-8, and Ac-LEHD-AMC for caspase-9) at 37 °C for 60 min. The release of 7-amino-4-methylcoumarin was measured using a spectrometer.

2.6. Cytochrome c release from mitochondria

Flow-cytometry was used to analyze cytochrome *c* release from mitochondria, as reported by Stahnke et al. [16]. Briefly, irradiated cells were fixed and permeabilized with 4% paraformaldehyde and 0.2% saponin for 20 min at 4 °C, and washed twice with Perm/Wash Buffer including fetal bovine serum, sodium azide, and saponin (BD Biosciences). Cells were incubated with 5 μg/ml mouse IgG to block nonspecific binding. Anti-cytochrome c antibody (1:20, clone 7H8.2C12, BD Biosciences) was then added, and cells were incubated at 4 °C for 20 min. Finally, cells were treated with goat anti-mouse IgG-FITC (1:20, Southern Biotech, Birmingham, AL) at 4 °C for 20 min and subjected to a FACScan flow-cytometer.

2.7. Transmission electron microscopy

Prefixation was performed with 2% glutaraldehyde in 100 mM cacodylate buffer, followed by washing in 100 mM cacodylate buffer overnight and subsequent postfixation with 2% osmium tetroxide in distilled water for 3 h. Cells were then dehydrated through a graded series of ethanol before being embedded in gelatin capsule with epoxy resin. Samples were sectioned into ultrathins (70–80 nm) using a LKB-8800 ultramicrotome (LKB, Bromma, Sweden), stained with 2% uranyl acetate in distilled water for 10 min and with lead staining solution for 5 min, and then examined using a JEM-2000EX (JEOL, Tokyo, Japan).

3. Results

3.1. Radiation-induced apoptotic cell death of MOLT-4 is characterized by decrease in mitochondrial membrane potential along with increase in intracellular ROS

Since a number of previous studies showed the crucial roles of mitochondria and ROS generation during apoptotic cell death by various stimuli [17–19], we first examined levels of mitochondrial membrane potential ($\Delta\psi m$) and intracellular superoxide anions (ROS) in X-ray-irradiated MOLT-4 cells. At 16 h after a 4 Gy irradiation, membrane potentials of irradiated cells had significantly decreased compared with those in unirradiated cells (Fig. 1A), while most irradiated cells showed elevated intracellular ROS (Fig. 1B). The irradiated MOLT-4 cells also exhibited several apoptotic features, including externalization of plasma membrane phosphatidyl serine (data not shown) and retention of normal mitochondrial shapes (Fig. 2B).

We next investigated temporal changes in irradiated MOLT-4 cells, focusing on mitochondrial membrane potential and intracellular ROS. Flow-cytometric analysis of MOLT-4 cells at 16 h after a 4 Gy irradiation identified five cell fractions (Fig. 3A): a fraction identical to unirradiated cells or viable cell fraction (R1), a low-ROS generating fraction (R2), a high-ROS generating fraction (R3), a moderate-to-high-ROS generating and low-$\Delta\psi m$ fraction (R4), and a low-ROS and low-$\Delta\psi m$ fraction (R5). Because almost all cells in the R4 and R5 fractions were stained with PI, they were considered dying or dead cells (data not shown). To elucidate the sequential process of radiation-induced cell death of MOLT-4, at 16 h after the irradiation we separated all fractions using a cell sorter and then cultured them for additional 8 h before analyzing them again by flow-cytometry. The cells that were in the R1 fraction (Fig. 3A) scattered and were found in R2, R3, R4, and R5 (Fig. 3B-R1). In a similar manner, cells in R3 remained

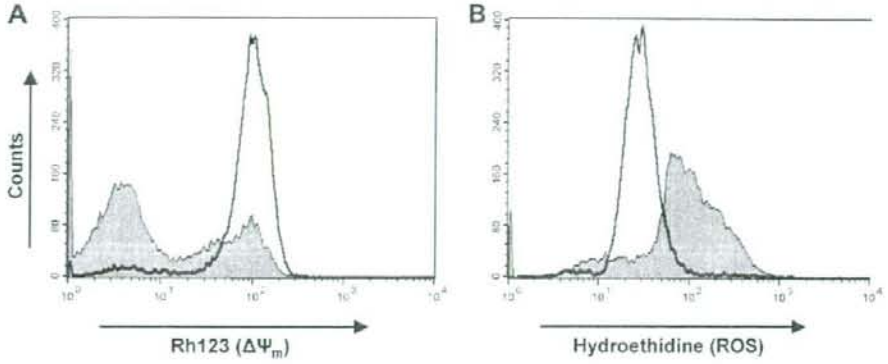

Fig. 1. Mitochondrial membrane potential and intracellular ROS in irradiated MOLT-4 cells. MOLT-4 cells were analyzed by flow-cytometry at 16 h after a 4 Gy X-ray irradiation. (A) Signal intensity of Rhodamine123 (Rh123), reflecting the membrane potential (Δψm), decreased in irradiated MOLT-4 cells (shaded histogram) compared with that in unirradiated cells (open histogram). (B) Hydroethidine signals represent levels of intracellular superoxide anions. The signals of irradiated cells (shaded histogram) were elevated, compared with those of unirradiated cells (open histogram).

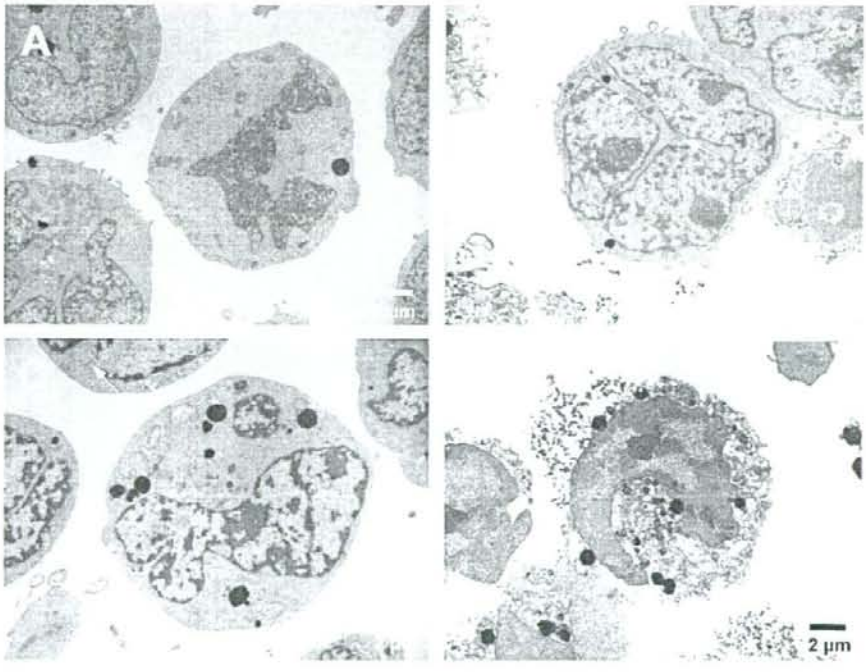

Fig. 2. Cellular morphology of irradiated MOLT-4 cells under the electron microscope. (A) Unirradiated MOLT-4 cells. (B) Irradiated MOLT-4 cells in the low-Δψm R4 fraction. Normal-shaped mitochondria (indicated by white arrows) were observed at 16 h after a 4 Gy irradiation. (C and D) Irradiated MOLT-4 cells pretreated with 100 μM z-VAD-fmk in the low-ROS R2 and low-Δψm R4 fractions, respectively. Swollen mitochondria (indicated by arrows) were observed at 16 h after a 4 Gy irradiation. Scale bar, 2 μm.

in R3 or moved to R4 and R5 (Fig. 3B-R3) and cells in R4 remained in R4 or moved to R5 (Fig. 3B-R4). However, cells in R2 moved only to R4 or remained in R2 (Fig. 3B-R2). It follows that most irradiated MOLT-4 cells may shift from R1 to R3, next to R4, and finally to R5; on the other hand, cells in R2 may shift to R4, and eventually R5. These observations suggest that generation of intracellular ROS is a step before a reduction of mitochondrial membrane potential in the radiation-induced apoptotic process of MOLT-4 cells.

3.2. Radiation-induced necrotic cell death of MOLT-4 without increase in intracellular ROS in the presence of a caspase inhibitor

We next investigated the involvement of caspases in X-ray-induced cell death of MOLT-4 using a broad-range caspase inhibitor, z-VAD-fmk. In the absence of z-VAD-fmk, living cell percentage of irradiated MOLT-4 cells (i.e., the cells not stained with PI) and the cell percentage of the R1 fraction were 20% and 15%, respectively, at 16 h after a 4 Gy irradiation (Fig. 4A-b, A-e, and B). In the pres-

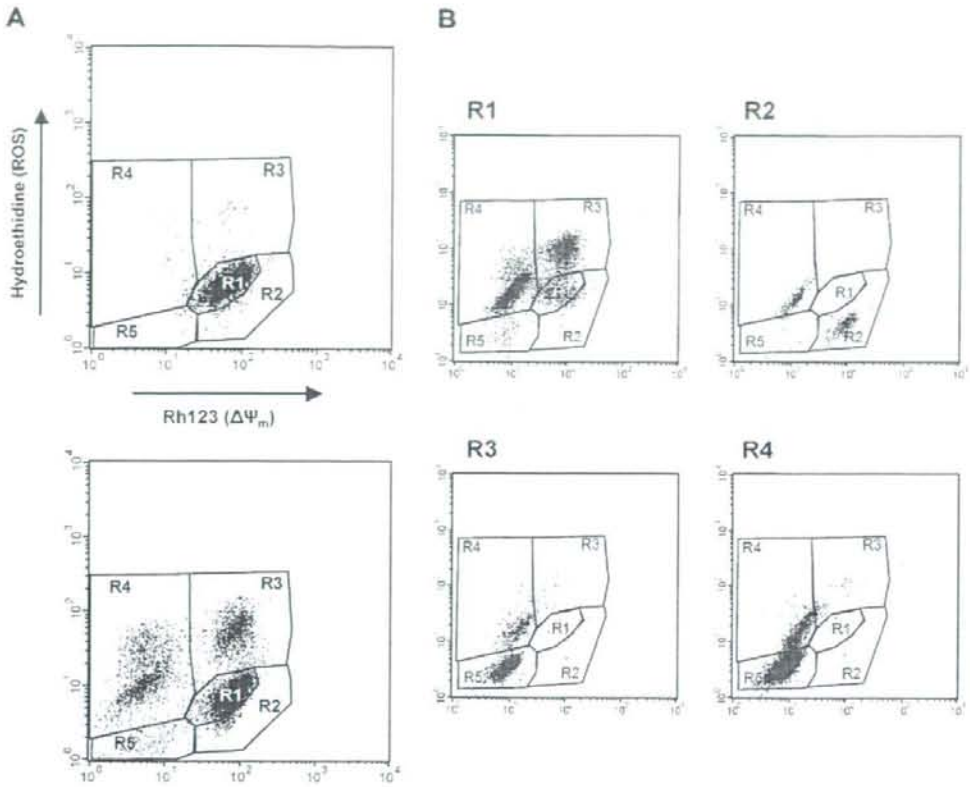

Fig. 3. Temporal changes in irradiated MOLT-4 in terms of mitochondrial membrane potential and intracellular ROS. (A) Upper and lower panels show non-irradiated control and irradiated cells, respectively. In the lower panel, five cell fractions were identified at 16 h after a 4 Gy irradiation: a viable cell fraction (R1), a low-ROS fraction (R2), a high-ROS fraction (R3), a moderate-to-high ROS and low-$\Delta\psi$m fraction (R4), and a low-ROS and low-$\Delta\psi$m fraction (R5). (B) Scattered pattern of cells in each fraction in Fig. 3A after a lapse of another 8 h. Cells in R1, R2, R3, and R4 fractions in the lower panel of Fig. 3A were isolated, cultured for another 8 h, and analyzed again by flow-cytometry.

ence of the caspase inhibitor, the percentages of living cell and the R1 fraction were 75% and 50%, respectively (Fig. 4A-c, A-f, and C). After irradiation with the z-VAD-fmk pretreatment, a number of cells were found in the R4 and R5 fractions (Fig. 4A-c), showing that cell death still occurred after caspase inhibition. Furthermore, in the case of irradiation with z-VAD-fmk, more viable cells were found in the low-ROS R2 fraction than in the high-ROS R3 fraction (Fig. 4A-c), implying that cell death occurs without excess generation of intracellular ROS. In addition, the cells in the low-ROS R2 fraction were considered to be viable, since they were not stained with PI (Fig. 4A-f).

3.3. Time-course analyses of irradiated MOLT-4 cells in the absence or presence of a caspase inhibitor

The time-course analysis clearly shows that irradiated MOLT-4 cells shifted from the R1 fraction to the partially low-ROS R2 or predominantly high-ROS R3 fraction, and eventually to the low-$\Delta\psi$m R4 and R5 fractions (Fig. 4B). On the other hand, cells pretreated with z-VAD-fmk traveled a different course from R1 to low-ROS R2, and eventually to low-$\Delta\psi$m R4 and R5, with no cells going to high-ROS R3 (Fig. 4C). In fact, when the cells in R2 were isolated and their temporal changes were analyzed by flow-cytometry, the R2 cells eventually died via R4 and R5, not R3 (data not shown).

3.4. Suppression of caspase activities by z-VAD-fmk

To confirm the suppressive effects of z-VAD-fmk in irradiated MOLT-4 cells, we next examined catalytic activity of caspases by using colorimetric substrates that mimic the cleavage sites of caspases. In the absence of z-VAD-fmk, activities of caspases—including caspase-3—were clearly enhanced by irradiation and peaked at 8 h after irradiation (Fig. 5). Up-regulation of caspase-3 expression was also confirmed by Western blotting (data not shown). In contrast, the presence of z-VAD-fmk effectively suppressed activities of all caspases examined (Fig. 5). These results were consistent with previous studies [8,20,21] which reported that caspase-3 and other caspases were activated in MOLT-4 cells following ionizing radiation. The results again demonstrated that cell death of irradiated MOLT-4 with z-VAD-fmk was independent of caspase activation.

3.5. Apoptotic and necrotic features related to cellular morphology and the cytochrome c release

We further characterized cell death in irradiated MOLT-4 with transmission electron microscopy, since its morphologic description remains the best way to discriminate apoptosis and necrosis [22]. After cells in each fraction separated with Rh123- and hydroethidine-staining were sorted, an electron microscopic review was performed. In the absence of z-VAD-fmk, mitochondria of irradi-

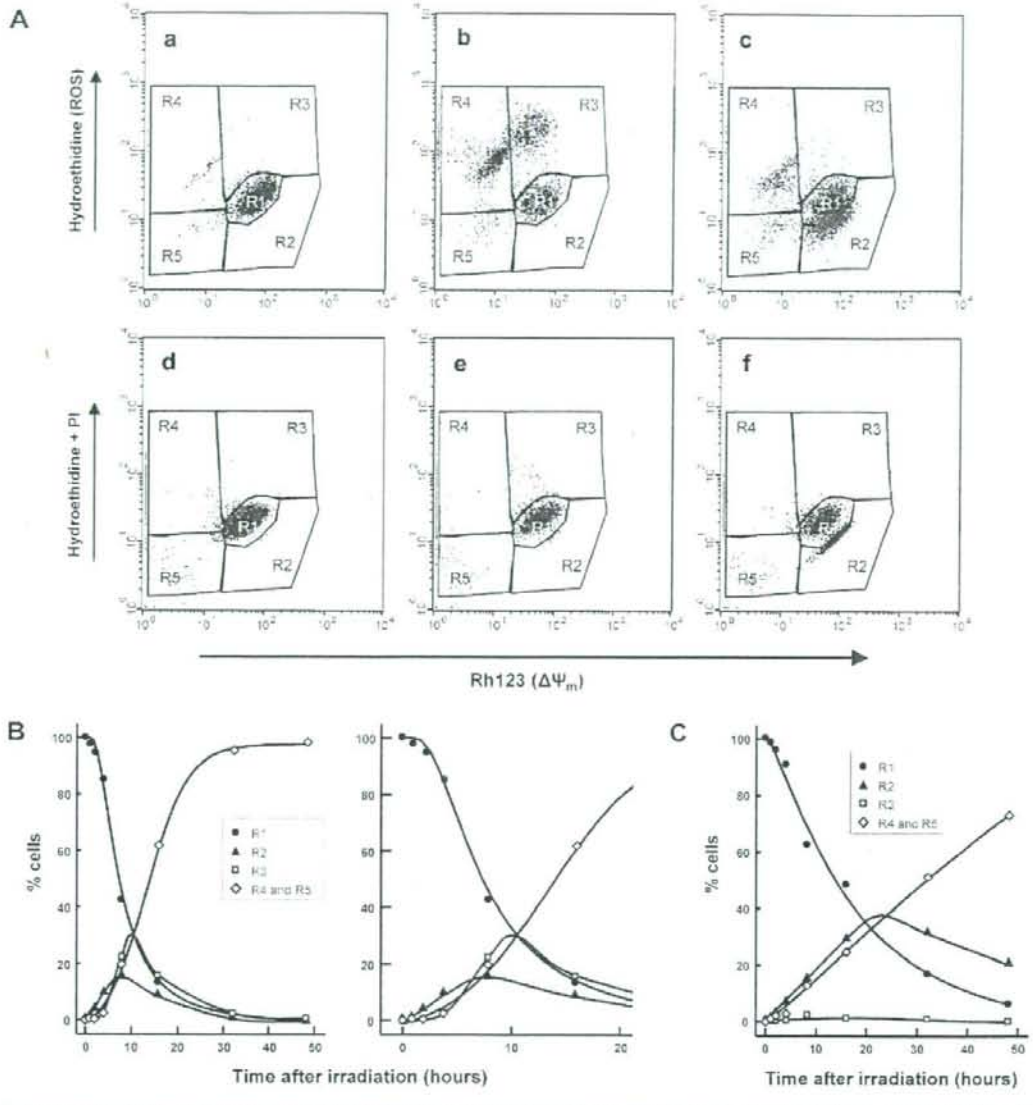

Fig. 4. Effects of caspase inhibition on radiation-induced cell death in MOLT-4. (A) Upper panels show flow-cytometric analyses with Rh123- and hydroethidine-staining on (a) unirradiated MOLT-4 cells, (b) cells at 16 h after a 4 Gy irradiation, and (c) irradiated cells pretreated with 100 μM z-VAD-fmk (note that many cells are located in the low-ROS R2 fraction). Lower panels show PI-staining in addition to Rh123- and hydroethidine-staining on (d) unirradiated cells, (e) irradiated cells, and (f) irradiated cells with z-VAD-fmk, corresponding to panels a, b, and c, respectively. In the lower panels, most PI-stained cells moved out of the displayed region due to their intense signals. (B) Time-course analyses of cells in R1 (viable cells, indicated by circle), R2 (low-ROS, triangle), R3 (high-ROS, square), and R4 and R5 (low-$\Delta\psi$m, rhombus) fractions after a 4 Gy irradiation. An enlarged illustration of the left panel from 0 to 20 h is shown in the right panel. (C) Time-course analyses of cells after a 4 Gy irradiation with a pretreatment with 100 μM z-VAD-fmk. Each cell fraction in Fig. 4B and C corresponds to a fraction in the upper panels of Fig. 4A.

ated MOLT-4 cells—even those in the low-$\Delta\psi$m R4 fraction—retained their normal shapes as already described (Fig. 2B). On the other hand, the organelles were swollen in the low-ROS R2 fraction in the presence of z-VAD-fmk (Fig. 2C). Those observations reinforced the concept that irradiated MOLT-4 cells ended in apoptosis when mediated by caspases, and necrosis proceeded under caspase inhibition. Finally, we examined cytochrome *c* release from mitochondria by flow-cytometry in order to further investigate the molecular mechanisms of radiation-induced necrosis in MOLT-4 cells. Cytochrome *c* release represents a key step in the cell death pathway, and it reportedly plays a role in radiation-induced apoptosis of MOLT-4 cells as well [23]. Cells in viable or early cell death phase, gated according to forward/side scatter properties, were analyzed with anti-cytochrome *c* antibody (clone 7H8.2C12), which binds only to mitochondrial cytochrome *c* [16]. At 16 h after a 4 Gy irradiation, the cytochrome *c* signals were significantly reduced, reflecting the release of cytochrome *c* (Fig. 6B), whereas no obvious changes in cytochrome *c* signals were detected when

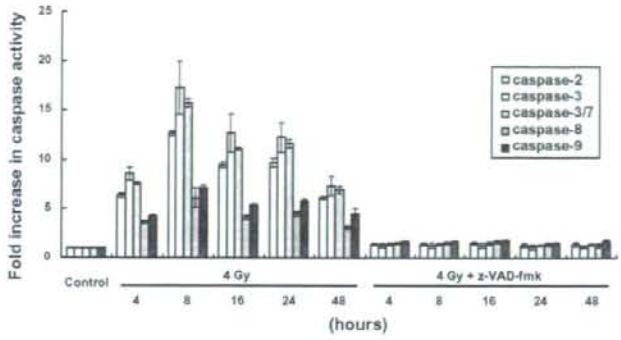

Fig. 5. Caspase activity of irradiated MOLT-4 cells in the absence or presence of a caspase inhibitor. Activities of caspases 2, 3, 3/7, 8, and 9 were examined by colorimetric assay with or without a pretreatment with 100 μM z-VAD-fmk, using fluorogenic substrates for these caspases. Fold increases in caspase activities are shown, compared with unirradiated control cells. An average of two measurements is used.

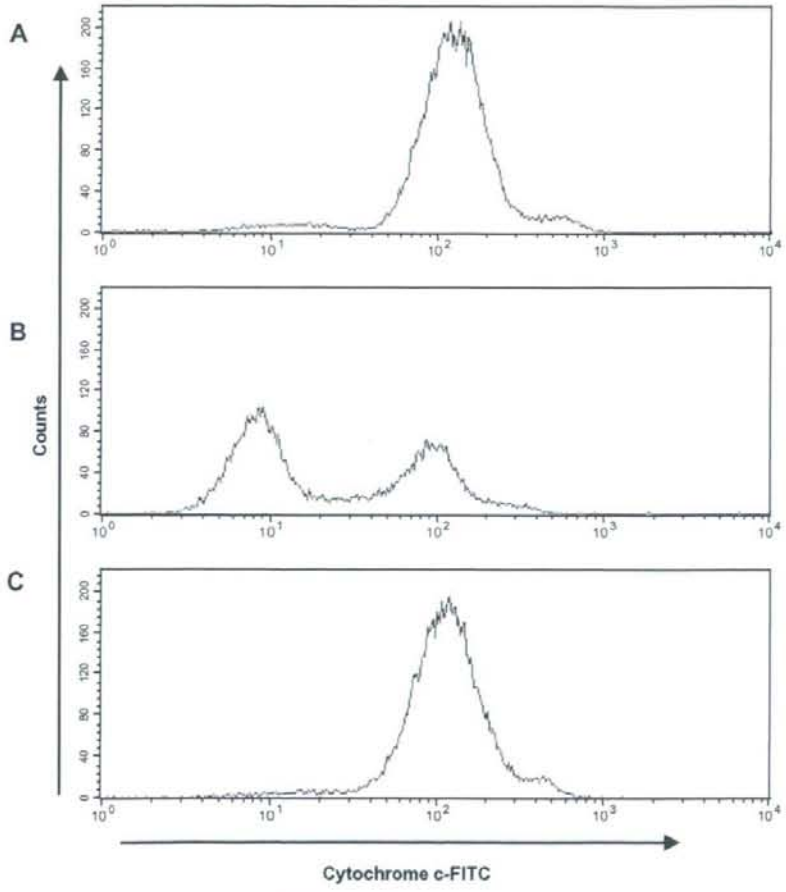

Fig. 6. Release of cytochrome *c* from mitochondria in irradiated MOLT-4 cells. Mitochondrial cytochrome *c* was detected by using anti-cytochrome *c* antibody (clone 7H8.2C12) in fixed and permeabilized MOLT-4 cells. This antibody binds only to mitochondrial cytochrome *c*, not to cytosolic one. Cells were gated according to forward/side scatter properties into viable or early phase of cell death, and then analyzed by flow-cytometry. (A) Unirradiated MOLT-4 cells. (B) MOLT-4 cells at 16 h after a 4 Gy irradiation. (C) MOLT-4 cells at 16 h after a 4 Gy irradiation in the presence of 100 μM z-VAD-fmk.

the cells were pretreated with z-VAD-fmk (Fig. 6C). These results suggest that the release of cytochrome *c* might not invariably precede necrotic cell death in irradiated MOLT-4.

4. Discussion

In the present study, apoptotic cell death was induced by a 4 Gy irradiation in MOLT-4, a thymus-derived T cell line, and the apoptosis was characterized by a decrease in mitochondrial membrane potential and an increase in intracellular ROS. On the one hand, when caspase activities were suppressed by the z-VAD-fmk pretreatment, irradiated MOLT-4 avoided apoptosis, and died exhibiting necrotic features. We demonstrated for the first time that there was no increase in intracellular ROS in the process of this necrotic cell death of MOLT-4. These results indicate that apoptosis, as the main pathway of radiation-induced cell death in MOLT-4, requires both elevation of intracellular ROS and activation of a series of caspases, while the cryptic necrosis program—independent of ROS generation and caspase activation—becomes active when the apoptosis pathway is blocked.

In agreement with our observations, this shift from apoptosis to necrosis has been reported in several cell types when the apoptosis pathways were blocked by z-VAD-fmk (U-937 cell death induced by camptothecin [24], mouse thymocyte death induced by dexamethasone and etoposide [7], and irradiated MOLT-4 cell death [8]). Enhanced expression of Bcl-2 also generated a similar shift from apoptosis to necrosis in HL-60 cells treated with oxidized low density lipoproteins [25]. In addition, interdigital cells from mice genetically lacking the caspase activator Apaf-1 underwent necrosis, not apoptosis, during embryonic development [26]. Therefore, it is plausible to imagine that cell death, including radiation-induced death, can be achieved through multiple molecular pathways—typically apoptotic or necrotic—depending on cellular physiological status and available effecter molecules.

Previous studies on radiation-induced apoptosis in MOLT-4 have suggested involvement of individual intracellular events (mediation by p53, activation of SAPK/JNK pathway, critical roles for caspase-3, modulation by Bcl-2, and occurrence of ceramide formation and PARP cleavage [8,21,23,27–29]). Even so, the sequential process of apoptosis still remains to be clarified. That is why we analyzed the temporal process of cell death in terms of mitochondrial membrane potential and intracellular ROS in this study. Our time-course analyses indicate that excess generation of ROS precedes the reduction of the membrane potential during radiation-induced apoptosis in MOLT-4 cells, whereas ROS generation is bypassed during radiation-induced necrosis. Given the potential implications of our findings, the use of antioxidants is indeed a promising strategy for prevention of radiation-induced, and ROS-dependent, cell death upon the development of radioprotective agents for cancer radiotherapy [30]. However, our findings also imply that antioxidants as radioprotective agents may be less effective for ROS-independent necrosis. Thus, development of radioprotective strategies that take into account the molecular mechanisms of ROS-independent necrosis is also warranted.

The precise mechanisms of necrosis in MOLT-4 cells remain unclear. Some previous studies have suggested the involvement of intracellular ROS generation during caspase-independent necrotic cell death, e.g., in neutrophil cells [22,31], while our and other studies have observed unaltered levels of ROS [32,33]. This discrepancy regarding ROS generation in necrotic cell death could be due to different types of cells or different experimental procedures used to induce cell death. This would imply that there are multiple pathways even in caspase-independent necrosis, both ROS-dependent and -independent. In fact, a necrotic-signaling pathway involving ROS was thought to be death receptor-mediated [22,34]. That pathway is probably distinct from the necrosis pathway of irradiated MOLT-4 found in this study, because necrosis in MOLT-4 was observed with total suppression of caspase-8, an initiation molecule for the death receptor-mediated pathway.

In addition, several non-caspase proteases, including calpain, cathepsin, and serine protease Omi/HtrA2, have been reported to be major factors in the propagation and execution phases of necrotic cell death, with or without ROS-generation [32,35–37]. Therefore, it seems certain that further studies will be needed to investigate the involvement of these non-caspase proteases in ROS-independent necrotic cell death.

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References

[1] S.L. Fink, B.T. Cookson, Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells, Infect. Immun. 73 (2005) 1907–1916.
[2] G. Rainaldi, R. Romano, P. Indovina, A. Ferrante, A. Motta, P.L. Indovina, M.T. Santini, Metabolomics using 1H-NMR of apoptosis and Necrosis in HL60 leukemia cells: differences between the two types of cell death and independence from the stimulus of apoptosis used, Radiat. Res. 169 (2008) 170–180.
[3] G.M. Cohen, Caspases: the executioners of apoptosis, Biochem. J. 326 (Pt 1) (1997) 1–16.
[4] M. Enari, H. Sakahira, H. Yokoyama, K. Okawa, A. Iwamatsu, S. Nagata, A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD, Nature 391 (1998) 43–50.
[5] T. Lindsten, A.J. Ross, A. King, W.X. Zong, J.C. Rathmell, H.A. Shiels, E. Ulrich, K.G. Waymire, P. Mahar, K. Frauwirth, Y. Chen, M. Wei, V.M. Eng, D.M. Adelman, M.C. Simon, A. Ma, J.A. Golden, G. Evan, S.J. Korsmeyer, G.R. MacGregor, C.B. Thompson, The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues, Mol. Cell 6 (2000) 1389–1399.
[6] A. Konishi, S. Shimizu, J. Hirota, T. Takao, Y. Fan, Y. Matsuoka, L. Zhang, Y. Yoneda, Y. Fujii, A.I. Skoultchi, Y. Tsujimoto, Involvement of histone H1.2 in apoptosis induced by DNA double-strand breaks, Cell 114 (2003) 673–688.
[7] T. Hirsch, P. Marchetti, S.A. Susin, B. Dallaporta, N. Zamzami, I. Marzo, M. Geuskens, G. Kroemer, The apoptosis-necrosis paradox. Apoptogenic proteases activated after mitochondrial permeability transition determine the mode of cell death, Oncogene 15 (1997) 1573–1581.
[8] D. Coelho, V. Holl, D. Weltin, T. Lacornerie, P. Magnenet, P. Dufour, P. Bischoff, Caspase-3-like activity determines the type of cell death following ionizing radiation in MOLT-4 human leukaemia cells, Br. J. Cancer 83 (2000) 642–649.
[9] P. Golstein, G. Kroemer, Cell death by necrosis: towards a molecular definition, Trends Biochem. Sci. 32 (2007) 37–43.
[10] J. Minowada, T. Onuma, G.E. Moore, Rosette-forming human lymphoid cell lines. I. Establishment and evidence for origin of thymus-derived lymphocytes, J. Natl. Cancer Inst. 49 (1972) 891–895.
[11] L.B. Chen, Mitochondrial membrane potential in living cells, Annu. Rev. Cell Biol. 4 (1988) 155–181.
[12] J.J. Ubl, J.Y. Chatton, S. Chen, J.W. Stucki, A critical evaluation of in situ measurement of mitochondrial electrical potentials in single hepatocytes, Biochim. Biophys. Acta 1276 (1996) 124–132.
[13] C. Ferlini, G. Scambia, Assay for apoptosis using the mitochondrial probes, Rhodamine123 and 10-N-nonyl acridine orange, Nat. Protoc. 2 (2007) 3111–3114.
[14] G. Rothe, G. Valet, Flow cytometric analysis of respiratory burst activity in phagocytes with hydroethidine and 2',7'-dichlorofluorescin, J. Leukoc. Biol. 47 (1990) 440–448.
[15] S. Walrand, S. Valeix, C. Rodriguez, P. Ligot, J. Chassagne, M.P. Vasson, Flow cytometry study of polymorphonuclear neutrophil oxidative burst: a comparison of three fluorescent probes, Clin. Chim. Acta 331 (2003) 103–110.
[16] K. Stahnke, A. Mohr, J. Liu, L.H. Meyer, L. Karawajew, K.M. Debatin, Identification of deficient mitochondrial signaling in apoptosis resistant leukemia cells by flow cytometric analysis of intracellular cytochrome c, caspase-3 and apoptosis, Apoptosis 9 (2004) 457–465.

[17] X. Wang, The expanding role of mitochondria in apoptosis, Genes Dev. 15 (2001) 2922–2933.
[18] J.F. Curtin, M. Donovan, T.G. Cotter, Regulation and measurement of oxidative stress in apoptosis, J. Immunol. Methods 265 (2002) 49–72.
[19] P. Tripathi, D. Hilderman, Sensitization of T cells to apoptosis—a role for ROS?, Apoptosis 9 (2004) 515–523
[20] H. Nakano, K. Shinohara, Time sequence analysis of caspase-3-independent programmed cell death and apoptosis in X-irradiated human leukemic MOLT-4 cells, Cell Tissue Res. 310 (2002) 305–311.
[21] O. Inanami, K. Takahashi, M. Kuwabara, Attenuation of caspase-3-dependent apoptosis by Trolox post-treatment of X-irradiated MOLT-4 cells, Int. J. Radiat. Biol. 75 (1999) 155–163.
[22] W.X. Zong, C.B. Thompson, Necrotic death as a cell fate, Genes Dev. 20 (2006) 1–15.
[23] K. Takahashi, O. Inanami, M. Hayashi, M. Kuwabara, Protein synthesis-dependent apoptotic signalling pathway in X-irradiated MOLT-4 human leukaemia cell line, Int. J. Radiat. Biol. 78 (2002) 115–124.
[24] A.T. Sane, R. Bertrand, Caspase inhibition in camptothecin-treated U-937 cells is coupled with a shift from apoptosis to transient G1 arrest followed by necrotic cell death, Cancer Res. 59 (1999) 3565–3569.
[25] O. Meilhac, I. Escargueil-Blanc, J.C. Thiers, R. Salvayre, A. Negre-Salvayre, Bcl-2 alters the balance between apoptosis and necrosis, but does not prevent cell death induced by oxidized low density lipoproteins, Faseb J. 13 (1999) 485–494.
[26] M. Chautan, G. Chazal, F. Cecconi, P. Gruss, P. Golstein, Interdigital cell death can occur through a necrotic and caspase-independent pathway, Curr. Biol. 9 (1999) 967–970.
[27] H. Nakano, K. Shinohara, Correlation between unirradiated cell TP53 protein levels and radiosensitivity in MOLT-4 cells, Radiat. Res. 151 (1999) 686–693.
[28] A. Enomoto, N. Suzuki, K. Hirano, Y. Matsumoto, A. Morita, K. Sakai, H. Koyama, Involvement of SAPK/JNK pathway in X-ray-induced rapid cell death of human T-cell leukemia cell line MOLT-4, Cancer Lett. 155 (2000) 137–144.
[29] E. Takahashi, O. Inanami, T. Asanuma, M. Kuwabara, Effects of ceramide inhibition on radiation-induced apoptosis in human leukemia MOLT-4 cells, J. Radiat. Res. (Tokyo) 47 (2006) 19–25.
[30] J.F. Weiss, M.R. Landauer, Protection against ionizing radiation by antioxidant nutrients and phytochemicals, Toxicology 189 (2003) 1–20.
[31] N.A. Maianski, D. Roos, T.W. Kuijpers, Tumor necrosis factor alpha induces a caspase-independent death pathway in human neutrophils, Blood 101 (2003) 1987–1995.
[32] M. Okada, S. Adachi, T. Imai, K. Watanabe, S.Y. Toyokuni, M. Ueno, A.S. Zervos, G. Kroemer, T. Nakahata, A novel mechanism for imatinib mesylate-induced cell death of BCR-ABL-positive human leukemic cells: caspase-independent, necrosis-like programmed cell death mediated by serine protease activity, Blood 103 (2004) 2299–2307.
[33] A.S. Cowburn, J.F. White, J. Deighton, S.R. Walmsley, E.R. Chilvers, z-VAD-fmk augmentation of TNF alpha-stimulated neutrophil apoptosis is compound specific and does not involve the generation of reactive oxygen species, Blood 105 (2005) 2970–2972.
[34] A. Kawahara, Y. Ohsawa, H. Matsumura, Y. Uchiyama, S. Nagata, Caspase-independent cell killing by Fas-associated protein with death domain, J. Cell Biol. 143 (1998) 1353–1360.
[35] M. Artal-Sanz, N. Tavernarakis, Proteolytic mechanisms in necrotic cell death and neurodegeneration, FEBS Lett. 579 (2005) 3287–3296.
[36] M.J. Spencer, D.E. Croall, J.G. Tidball, Calpains are activated in necrotic fibers from mdx dystrophic mice, J. Biol. Chem. 270 (1995) 10909–10914.
[37] T. Yamashima, Ca^{2+}-dependent proteases in ischemic neuronal death: a conserved 'calpain–cathepsin cascade' from nematodes to primates, Cell Calcium 36 (2004) 285–293.

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Epidemiology

DNA methylation status is inversely correlated with green tea intake and physical activity in gastric cancer patients

Yasuhito Yuasa [1,†], Hiromi Nagasaki [1], Yoshimitsu Akiyama [1], Yutaka Hashimoto [1], Touichirou Takizawa [2], Kazuyuki Kojima [3], Tatsuyuki Kawano [3], Kenichi Sugihara [3], Kazue Imai [4], Kei Nakachi [4]

[1]Department of Molecular Oncology, Tokyo Medical and Dental University, Tokyo, Japan
[2]Department of Pathology, Tokyo Medical and Dental University, Tokyo, Japan
[3]Department of Surgery, Tokyo Medical and Dental University, Tokyo, Japan
[4]Department of Radiobiology/Molecular Epidemiology, Radiation Effects Research Foundation, Hiroshima, Japan

email: Yasuhito Yuasa (yuasa.monc@tmd.ac.jp)

*Correspondence to Yasuhito Yuasa, Department of Molecular Oncology, Graduate School of Medicine and Dentistry, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan

† Fax: +81-3-5803-0125

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KEYWORDS

DNA methylation ·green tea ·gastric cancer ·*CDX2* ·*BMP-2*

ABSTRACT

Epigenetic silencing of genes by aberrant DNA methylation is recognized as a crucial component of the mechanism underlying tumorigenesis. However, the relationship between DNA methylation and the past lifestyle in cancer patients remains largely unknown. We examined the methylation statuses of 6 tumor-related genes, *CDX2* (homeobox transcription factor), *BMP-2* (bone morphogenetic protein 2), *p16 (INK4A)*, *CACNA2D3* (calcium channel-related), *GATA-5* (transcription factor) and *ER* (estrogen receptor), in 106 primary gastric carcinomas by methylation-specific PCR and compared them with the past lifestyles of the patients. The methylation frequencies of the genes were 23.6, 21.7, 9.4, 32.4, 40.8 and 59.1%, respectively. Significant association was found between a decreased intake of green tea and methylation of *CDX2* and *BMP-2*. More physical activity was correlated with a lower methylation frequency of *CACNA2D3*. Of these 6 genes, the methylation statuses of *CDX2*, *BMP-2* and *p16* revealed a significant interrelationship and those of *CACNA2D3*, *GATA-5* and *ER* did likewise. Thus, some epidemiological factors, such as green tea intake, could be important as to determination of the methylation statuses of selected genes and may influence the development of cancer, including that of the stomach. © 2008 Wiley-Liss, Inc.

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ARTICLE TEXT

Epigenetic changes, particularly methylation of cytosine in CpG dinucleotides in gene promoters, are found in almost every type of human neoplasm and are associated with transcriptional gene silencing.[1][2] Such promoter hypermethylation is as common as the disruption of tumor-suppressor genes in human cancer by mutation. Unlike irreversible genetic changes, epigenetic changes are thought to possibly be reversible by the environment, diet or pharmacological intervention. For example, monozygotic twins are considered genetically identical and are thus ideal for studying the effects of environmental and dietary factors on human health and diseases. In a study of a large cohort of identical twins, the patterns of DNA methylation across the genome were found to be very similar in young monozygotic twins in several cell types, but in older twins the patterns diverged.[3] This strongly suggests that 1 or more environmental factors affect individuals throughout life, modifying gene expression through epigenetic mechanisms that have important implications for health.

Dietary factors are important determinants of cancer risk.[4] Aberrant DNA methylation is associated with dietary factors and other lifestyle factors and may underlie carcinogenesis. The prevalence of promoter hypermethylation of 6 genes, such as *APC*, *p14*^{ARF}, *p16/INK4a* (hereafter *p16*) and *hMLH1*, was higher in colorectal cancers derived from patients with a low folate/high alcohol intake than in ones with a high folate/low alcohol intake, but the differences were not statistically significant.[5] The incidences of hypermethylation of *D17S5* and *p16* in lung cancer are significantly higher in cigarette smokers than in those who have never smoked.[6-8] However, the relationship between DNA methylation and the past lifestyle in cancer patients remains largely unknown.

In 2000, gastric cancer was the second most frequent cause of cancer death worldwide.[9] Infection with *Helicobacter pylori* is a strong risk factor for gastric cancer but is not a sufficient cause for its development.[10] Epidemiological studies have strongly suggested that the risk may be increased with a high intake of salt and salt-preserved foods and decreased with a high intake of fruit and vegetables.[11] The aberrant methylation of many genes has been reported in gastric cancer.[12-14] We previously reported that *CDX2* methylation in men was correlated with a decreased intake of green tea, suggesting that diet could be an important factor determining the methylation status of genes such as *CDX2* and the resultant aberrant expression of genes involved in carcinogenesis.[15] However, these effects may not be universal but gene-specific, and female patients have not been examined. Thus, we analyzed the methylation states of 6 genes in more gastric cancer patients. Five of the 6 genes, that is, *CDX2*,[15] *BMP2*,[16] *p16*,[17] *CACNA2D3*[18] and *GATA5*,[19] were often methylated in gastric cancers but rarely in noncancerous epithelia. We, then, compared the relationship between DNA methylation and the past lifestyle in cancer patients including female ones.

Material and methods

Study population

Cancer tissue specimens were collected from 106 consecutive patients with primary gastric carcinoma in a hospital affiliated to Tokyo Medical and Dental University during 2000-2005. Informed consent was obtained from all patients, and the study was approved by the institutional review committee of Tokyo Medical and Dental University. A self-administered questionnaire was used in this study to assess the lifestyle before cancer onset, covering the disease history, familial history of cancer, medication, cigarette smoking, alcohol consumption, physical activity, intake frequencies of selected food groups and food items, daily consumption of tea (green tea, oolong tea and black tea), regularity of sleep and meals, eating quantity, bowel motion, height and body weight. The food groups were beef, pork, chicken, ham/sausage/bacon, grilled meat, all meat, grilled fish, salted/dried/other processed fish products, pickled vegetables, green leaf vegetables, yellow colored vegetables, cruciferous vegetables, all vegetables, fruits and probiotics-fermented milk. The intake frequencies of these food groups were categorized into not eaten, 1-2 times/month, 1-2 times/week, 3-4 times/week, almost every day and almost every meal. Most lifestyle factors in this questionnaire were selected from those which had previously been reported to be risk or preventive factors for gastric and colon cancers on epidemiological observation.

Tumors were reviewed by a pathologist and microdissected prior to DNA extraction. Histological classification was performed according to the general rules established by the Japanese Gastric Cancer Association[20] and Laurén's classification.[21]

Methylation analyses by the methylation-specific PCR procedure

We extracted genomic DNA from paraffin-embedded tissues by the phenol-chloroform method, and then carried out bisulfite modification and the methylation-specific PCR (MSP) procedure as previously described.[22] The primer sequences of the *CDX2, BMP-2, p16, CACNA2D3, GATA5, and estrogen receptor (ER)* genes for the MSP analyses are shown in Table I. The PCR reaction was performed for 35 cycles in a 25 μl mixture comprising bisulfite-modified DNA (~50 ng), 2.5 μl of 10 × PCR buffer, 1.25 μl of 25 mM dNTP, 10 pmole of each primer and 1 U of JumpStart Red Taq polymerase (Sigma, St. Louis, MO). Each PCR cycle consisted of 95−C for 30 sec, 58−C for 30 sec and 72−C for 30 sec, followed by final extension at 72−C for 5 min. The PCR products were electrophoresed in 2.5% agarose gels. All the MSP procedures were repeated more than twice. The methylation statuses of *CDX2* and *CACNA2D3* in several gastric cancer samples were also analyzed by LightCycler real-time PCR using bisulfite-modified DNA and methylation-specific primers, and the results were concordant with the MSP results.

Table I. PCR Primer Sequences Used for MSP

	Sense	Antisense
CDX2		
U	GAAGTTGTTGGTTTGGGGTTTTGTAT	CCCACAATACTCCACTAACTCCTCACA
M	CGTCGGTTTGGGGTTTCGTAC	GATACTCCGCTAACTCCTCGCG
BMP2		
U	GGATGGTTGTTTTGAGTTATGGGTTGT	CCTTAAAAACCAACACCCAAAAAACACA

	M GGTTGTTTCGAGTTATGGGTCGC	AAAACCAACGCCCGAAAAACGCG
p16		
	U TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA
	M TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA
CACNA2D3		
	U GGATATTGGAGTTTTTGAGTTTTTGTTTGT	ACAACAACCACCCAACCCCACCTCA
	M ATATTGGAGTTTTCGAGTTTTCGTTCGC	ATATTGGAGTTTTCGAGTTTTCGTTCGC
GATA5		
	U TGGAGTTTGTTTTTAGGTTAGTTTTTGGT	AACTTCATAAACCCCAAAAAATCAAACA
	M AGTTCGTTTTTAGGTTAGTTTCGGC	TTCGTAAACCCCGAAAAATCGAACG
ER		
	U GGTGTATTTGGATAGTAGTAAGTTTGT	CCATAAAAAAAACCAATCTAACCA
	M GTGTATTTGGATAGTAGTAAGTTCGTC	CGTAAAAAAAACCGATCTAACCG

Statistical analysis

The promoter methylation status of specific genes, clinico-pathological parameters and lifestyle variables in the patients were computed. Differences in frequency by methylation status were tested using the χ^2 test, and differences in mean values were tested using the *t* test. The association between the methylation status and dietary variables was also analyzed using a nonparametric test (Mann-Whitney U test). We further studied the association using the backward elimination (Wald test) method of logistic regression analysis. In this analysis, the intake frequencies of food groups were dichotomous as follows: ≤6 cups/day *vs.* ≥7 cups/day for green tea, ≤twice/week *vs.* ≥3 times/week for pickled vegetables and ≤twice/week *vs.* ≥3 times/week for drinking. Physical activity was defined as "recreational and voluntary physical exercise for health promotion" and primarily divided into 4 categories in questionnaire as follows: Never, 1-2 hr/week, 3-4 hr/week and ≥5 hr/week. However, in the logistic regression analysis, we combined these categories into 2 groups, ≥1 hr/week *vs.* never. Pearson's contingency coefficients for methylation status of an every pair of the 6 genes were calculated in 106 gastric carcinomas. *p* for trend was calculated by the Cochran-Armitage test. The statistical software used was SPSS software (version 14.0).

Results

Methylation statuses of CDX2, BMP-2, p16, CACNA2D3, GATA5 *and* ER *in primary gastric carcinomas*

The methylation statuses of *CDX2, BMP-2, p16, CACNA2D3, GATA5* and *ER* were determined in 106 primary gastric carcinomas by MSP analyses using the specific primers shown in Table I. The methylation frequencies of these genes were 23.6, 21.7, 9.4, 32.4, 40.8 and 59.1% respectively. When we examined the *CDX2* methylation statuses of noncancerous gastric tissues in 12 patients with methylation-positive gastric cancer and in 13 patients with methylation-negative one, and the *CACNA2D3* methylation statuses of noncancerous tissues in 7 patients with methylation-positive gastric cancer and 19 patients with methylation-negative one, we found no methylation in any samples by MSP, indicating cancer-related methylation of these genes (data not shown).

The relationship between methylation frequencies of the 6 genes and clinicopathological parameters

The clinico-pathological characteristics of the studied patients by the methylation statuses of *CDX2, BMP-2, p16, CACNA2D3, GATA5* and *ER* are shown in Table II. The methylation of *p16* was significantly more frequent in diffuse type (8/49, 16.3%) than in intestinal (2/57, 3.5%) type gastric carcinomas ($p = 0.04$). *CACNA2D3* methylation was more frequently found in lymph node metastasis-positive cases (20/45, 44.4%) than in negative ones (14/60, 23.3%) ($p = 0.03$). In contrast, there was no statistically significant correlation between methylation of 4 of the genes, *CDX2, BMP-2, GATA5* and *ER*, and clinico-pathological parameters (Table II).

Table II. Clinicopathological Characteristics of Studied Patients According to the Methylation Statuses of Six Genes

	CDX2 (*n* = 106)			*BMP-2* (*n* = 106)			*p16* (*n* = 106)		
	Methylated (*n* = 25)	Unmethylated (*n* = 81)	*p* value	Methylated (*n* = 23)	Unmethylated (*n* = 83)	*p* value	Methylated (*n* = 10)	Unmethylated (*n* = 96)	*p* value
Age (mean 7 SD)	66.0 7 10.6	63.8 7 10.3	0.35	63.6 7 8.8	64.5 7 10.8	0.73	59.4 7 12.1	64.8 7 10.0	0.12
Sex									
Male	22	57	0.11	17	62	1.00	9	70	0.45
Female	3	24		6	21		1	26	
Size (cm, mean 7	5.7 7 4.3	5.6 7 3.5	0.91	5.6 7 4.6	5.7 7 3.4	0.92	6.1 7 4.0	5.6 7 3.7	0.69

SD)									
Histology									
Intestinal	9	48	0.07	11	46	0.52	2	55	0.04
Diffuse	16	33		12	37		8	41	
Depth of tumor invasion									
m, ms	15	39	0.36	13	41	0.64	3	51	0.20
mp - si	10	42		10	42		7	45	
Lymph node metastasis									
-	16	44	0.49	17	43	0.10	3	57	0.10
+	9	37		6	40		7	39	

	CACNA2D3 (*n* = 105)			*GATA5* (*n* = 98)			*ER* (*n* = 93)		
	Methylated (*n* = 34)	Unmethylated (*n* = 71)	*p* value	Methylated (*n* = 40)	Unmethylated (*n* = 58)	*p* value	Methylated (*n* = 55)	Unmethylated (*n* = 38)	*p* value
Age (mean ± SD)	65.8 ± 11.2	63.8 ± 9.9	0.35	64.9 ± 9.8	64.1 ± 10.9	0.71	65.7 ± 9.9	62.3 ± 10.3	0.12
Sex									
Male	25	54	0.81	27	46	0.24	42	25	0.35
Female	9	17		13	12		13	13	
Size (cm, mean ± SD)	5.9 ± 3.6	5.5 ± 3.7	0.61	6.4 ± 4.2	5.4 ± 3.4	0.23	5.5 ± 3.4	5.8 ± 3.8	0.72
Histology									
Intestinal	17	40	0.68	20	31	0.84	32	17	0.21
Diffuse	17	31		20	27		23	21	
Depth of tumor invasion									
m, ms	16	37	0.68	21	25	0.41	28	19	1.00
mp - si	18	34		19	33		27	19	
Lymph node metastasis									
-	14	46	0.03	22	32	1.00	28	25	0.20
+	20	25		18	26		27	13	

Clinicopathological characteristics between patients with and without methylation were compared using the χ^2 test for categorical data and the *t* test for comparison of mean.

The relationship between methylation frequencies of the 6 genes and epidemiological parameters in gastric carcinoma patients

As shown in Table III, the methylation frequencies of *CDX2* and *BMP-2* were lower in patients consuming 7 cups or more per day of green tea than those consuming 6 cups or less per day (2/25 (8%) *vs.* 22/80 (27.5%), $p = 0.06$ and 1/25 (4%) *vs.* 22/80 (27.5%), $p = 0.02$, respectively). Patients consuming more pickled vegetables exhibited a higher methylation frequency of *GATA5* than ones consuming less ($p = 0.04$). *CACNA2D3* methylation was more frequently found in patients with no physical activity (20/44, 45.5%) than in those with more physical activity (14/59, 23.7%) ($p = 0.03$). In contrast, there was no statistically significant correlation between methylation of 2 of the genes, *p16* and *ER*, and clinico-pathological parameters (Table III).

Table III. Relationships Between the Methylation Status and Lifestyle Factors

	CDX2 (*n* = 106)			*BMP-2* (*n* = 106)			*p16* (*n* = 106)		
	Menthylated (*n* = 25)	Unmethylated (*n* = 81)	Univariate *p* value	Menthylated (*n* = 23)	Unmethylated (*n* = 83)	Univariate *p* value	Menthylated (*n* = 10)	Unmethylated (*n* = 96)	Univariate *p* value
Green tea									
≥7 cups/day	2	23	0.06	1	24	0.02	1	24	0.45
≤6 cups/day	22	58		22	58		9	71	

Pickled vegetables									
≥3 times/week	14	40	0.5	13	41	0.62	5	49	1.00
≤twice/week	10	40		10	40		5	45	
Physical activity									
≥1 hr/week	12	47	0.49	13	46	0.80	5	54	0.74
Never	12	33		9	36		5	40	

	CACNA2D3 (*n* = 105)			*GATA5* (*n* = 98)			*ER* (*n* =93)		
	Menthylated (*n* = 34)	Unmethylated (*n* = 71)	Univariate *p* value	Menthylated (*n* = 40)	Unmethylated (*n* = 58)	Univariate *p* value	Menthylated (*n* = 55)	Unmethylated (*n* = 38)	Univariate *p* value
Green tea									
≥7 cups/day	7	18	0.63	10	15	1.00	15	8	0.63
≤6 cups/day	27	52		30	42		39	30	
Pickled vegetables									
≥3 times/week	19	34	0.54	26	24	0.04	29	19	0.68
≤twice/week	15	35		14	32		24	19	
Physical activity									
≥1 hr/week	14	45	0.03	20	35	0.40	29	23	0.67
Never	20	24		19	22		24	15	

***p* values for χ^2 test.**

When the intake of green tea was stratified, the prevalence of aberrant methylation of *CDX2* and *BMP-2* decreased significantly with a higher intake of green tea (Mann-Whitney U-test, both $p = 0.04$; Cochran-Armitage test, $p_{trend} = 0.03$ and 0.02, respectively) (Figs. 1*a* and 1*b*). A distinct distribution of patients with methylated and unmethylated *CACNA2D3* was also demonstrated for physical activity (Mann-Whitney U-test, $p = 0.03$; Cochran-Armitage test, $p_{trend} = 0.03$) (Fig. 1*c*). On the other hand, an increased intake of pickled vegetables was not associated with an increased methylation frequency of *GATA5* ($p = 0.11$).

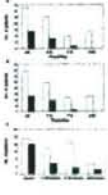

Figure 1. Frequencies of the presence (closed bars) or absence (open bars) of *CDX2* (*a*) and *BMP-2* (*b*) methylation in gastric cancers stratified as to intake of green tea and those of *CACNA2D3* methylation and physical activity (*c*).
[Normal View 11K | Magnified View 25K]

Since dietary factors are closely interrelated, we further performed the backward elimination (Wald) method of logistic regression analysis of the methylation status of each gene in gastric cancer patients including female ones (Table IV). A significant association was found between the intake of green tea and methylation of 2 of the genes, *CDX2* and *BMP-2*. Increased daily consumption of green tea (7 cups or more per day) showed a significant association with decreased methylation frequencies of *CDX2* and *BMP-2* after adjustment ($p = 0.04$ and $p = 0.049$, respectively). On the other hand, an increased methylation frequency of *CACNA2D3* was associated with less physical activity (negative *versus* positive), adjusting for confounding variables ($p = 0.06$) (Table IV). As for factors other than dietary ones, the logistic regression analysis also showed significant associations between *CDX2* methylation and gender or histology, *p16* methylation and histology, and *CACNA2D3* methylation and lymph node metastasis (Table IV).

Table IV. Standardized Partial Regression Coefficients of Variables Related with Methylation Status of Each Gene

	β	SE	*p*

CDX2			
Gender (men vs women)	-1.87	0.78	0.02
Histology (intestinal vs diffuse)	2.61	0.78	0.001
Green tea (≤6 cups/day vs. ≥7 cups/day)	-1.87	0.92	0.04
BMP-2			
Green tea (≤6 cups/day vs. ≥7 cups/day)	-2.08	1.06	0.049
p16			
Histology (intestinal vs. diffuse)	2.06	6.03	0.01
CACNA2D3			
Lymph node metastasis (negative vs. positive)	0.93	0.45	0.04
Physical activity (never vs. ≥1 hr/week)	-0. 86	0.45	0.06

These variables were selected using the backward elimination (Wald) method of logistic regression analysis for the methylation status of each gene.

Interrelationship of the 6 genes relative to their methylation statuses in gastric carcinomas

The methylation statuses of the 6 genes in 106 gastric carcinomas are shown in Figure 2. The methylation patterns of 3 genes, *CACNA2D3*, *GATA-5* and *ER*, were distinct from other 3 genes, *CDX2*, *BMP-2* and *p16*. To determine the relationship of the methylation statuses among the 6 genes, Pearson's contingency coefficients for methylation status of an every pair of the 6 genes were calculated (Table V). On the basis of the contingency coefficients, we found that the 6 genes were divided into 2 groups, Group I (*CDX2*, *BMP-2* and *p16*) and Group II (*CACNA2D3*, *GATA-5* and *ER*), where a statistically significant interrelationship within each group but no intergroup association was noted. The methylation frequencies in gastric carcinomas were lower for Group I genes (*CDX2*, 23.6%; *BMP-2*, 21.7% and *p16*, 9.4%) than Group II ones (*CACNA2D3*, 32.4%; *GATA-5*, 40.8% and *ER*, 59.1%).

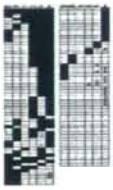

Figure 2. Summary of the methylation statuses of the 6 genes in 106 gastric carcinomas. Each column represents a different gene indicated on the top. Each row represents a primary gastric carcinoma. Black squares, methylated alleles in the carcinoma. White squares, unmethylated alleles in the carcinoma. CACN, *CACNA2D3*; GA5, *GATA5*. NA, not amplified.
[Normal View 16K | Magnified View 32K]

Table V. Contingency Coefficients for Methylation Status Between Two Genes

	CDX2	*BMP2*	*p 16*	*CACNA2D3*	*GATA5*	*ER*
CDX2						
BMP-2	0.24[1]					
p16	0.33[2]	0.22[1]				
CACNA2D3	*0.09*	*0.08*	*0.31*[2]			
GATA5	0.10	0.12	0.09	0.39[3]		
ER	0.01	0.02	0.2	0.36[4]	0.36[4]	

p value for χ^2 test.
[1] $p < 0.05$.
[2] $p < 0.01$.
[3] $p < 0.0001$.
[4] $p < 0.001$.

As described earlier, aberrant methylation of *CDX2* and *BMP-2* was inversely correlated with green tea intake. The prevalence of *p16* methylation was also higher in patients with a lower green tea intake than those with a higher intake, although the difference was not significant. When we analyzed the relationship between green tea intake and the methylation of combinations of Group I genes by the multinominal logistic regression model, the odds ratios of methylation for any 1 gene and ≥2 genes *vs.* no methylation were 4.9 (confidence interval (CI) 1.0-24.3) and 14.8 (CI 1.1-206.7), respectively, in patients consuming 6 cups or less per day of green tea compared with those

consuming 7 cups or more per day. On the other hand, no lifestyle factors were associated with the methylation of combinations of Group II genes.

Discussion

The methylation frequencies of the 6 genes in 106 gastric carcinomas varied from 9.4 to 59.1%. The prevalence of promoter hypermethylation of *CDX2* and *BMP-2* was significantly higher in gastric carcinomas derived from patients with a low green tea intake than those with a high intake. When we analyzed the association between the methylation status and variables using a nonparametric test, increased intake of green tea was found to be significantly associated with decreased methylation frequencies of *CDX2* and *BMP-2*. In a previous study,[15] methylation of 1 of 3 genes, *CDX2*, was correlated with a decreased intake of green tea in 58 male gastric carcinoma patients. Since an inverse relationship with green tea intake was also found for *BMP-2* promoter methylation in 106 gastric carcinoma patients including female ones in this study, the effect of green tea on the decrease of gene promoter methylation might be more common for many genes.

The evidence derived from epidemiologic studies on the relationship between drinking of green tea and cancer-preventive effects is inconclusive; some indicated preventive effects[23][24] and some did not.[25][26] In a detailed study, consumption of green tea was found to be associated with a decreased risk of gastric carcinoma in Japanese women after adjustment for potential confounding factors, whereas no association was observed among Japanese men.[27] The difference between women and men might be explained by the much higher cigarette smoking rate in men than women in Japan, which may play a role as an effect modifier.[28]

Green tea contains several polyphenolic compounds, such as (-)-epigallocatechin-3-gallate (EGCG). A significant inhibitory effect of EGCG on chemical carcinogenesis in the rat stomach has been reported.[29] As for its action on methylation, it was reported that EGCG dose-dependently inhibited DNA methyltransferase activity in several cancer cells, resulting in reactivation of methylation-silenced genes, such as *retinoic acid receptor* β, *p16* and *hMLH1*.[30][31] Polyphenols are rapidly metabolized to forms with quite different bioactivities. But the epithelial surfaces of the gut, particularly those of the esophagus and the stomach, are exposed on the luminal side to high concentrations of tea polyphenols before they undergo metabolism. These characteristics may make gastrointestinal epithelial tissues particularly susceptible to what are probably the beneficial effects of DNA methyltransferase inhibitors.

In other studies, however, EGCG did not inhibit DNA methyltransferase activity or reactivate genes, whereas nucleoside analogue methylation inhibitors, such as 5-aza-2′-deoxycytidine, were far more effective.[32][33] We also analyzed the effect of EGCG on transcriptional levels of *CDX2* and *BMP-2* in 3 human gastric cancer cell lines. Upregulation of both genes was not found in any cell lines (Hashimoto et al., personal communication). Thus, further studies are necessary to determine how tea polyphenols act on DNA methylation.

CACNA2D3 methylation was more frequently found in gastric carcinoma patients with no physical activity than in those with physical activity. It is known from epidemiological studies that physical activity protects against cancers of the colon, breast (postmenopause) and endometrium.[4] As for gastric carcinoma, a population based case-control study in Canada and a prospective cohort study in Norway indicated that recreational physical activity might have a protective effect against gastric cancer.[34][35] To determine the association of physical activity with promoter hypermethylation of *APC* and *RASSF1A* in breast tissue, a cross-sectional study on 45 women without breast cancer was performed, which revealed that physical activity was inversely associated with promoter hypermethylation of *APC* but not *RASSF1A*.[36] It is, therefore, possible that physical activity may affect the methylation of genes, such as *CACNA2D3*, and gastric carcinogenesis.

There are 2 types of genes according to contingency coefficients for methylation status in gastric carcinomas. The methylation frequencies were lower for Group I genes (9.4-23.6%) than Group II ones (32.4-59.1%). The odds ratios of methylation for any 1 gene and ≥2 genes *vs.* no methylation among the Group I genes were much higher in patients consuming 6 cups or less per day of green tea than in those consuming 7 cups or more per day. These data suggest that green tea intake may be inversely related to the methylation of Group I genes, which may be involved in carcinogenesis. The logistic regression analysis also showed significant associations between histology and the methylation of *CDX2* and *p16*. But there was no association between *BMP-2* methylation and histology. Further investigations are required to clarify the significance of the 2 different types of genes as to methylation in gastric carcinomas.

In conclusion, there were inverse associations between the intake of green tea and the methylation of *CDX2* and *BMP-2*, and between physical activity and *CACNA2D3* methylation. We, therefore, hypothesized that some of the lifestyle factors, which have been reported to be preventive as to gastric cancer on epidemiological observation, may influence the development of gastric cancer through the demethylation or retaining of unmethylated status of selected genes. Because an epigenetic drift may contribute to the development of cancer, strategies involving changes in lifestyle including diet might be highly beneficial in preventing/reversing epigenetic alterations and counteracting cancer.

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REFERENCES

1 Esteller M,Corn PG,Baylin SB,Herman JG. A gene hypermethylation profile of human cancer. *Cancer Res* 2001; **61**: 3225-9. Links

2 Jones PA,Baylin SB. The fundamental role of epigenetic events in cancer. *Nat Rev Genet* 2002; **3**: 415-28. Links

3 Fraga MF,Ballestar E,Paz MF,Ropero S,Setien F,Ballestar ML,Heine-Suñer D,Cigudosa JC,Urioste M,Benitez J,Boix-Chornet M,Sanchez-Aguilera A, et al. Epigenetic differences arise during the lifetime of monozygotic twins. *Proc Natl Acad Sci USA* 2005; **102**: 10604-9. Links

4 Marmot M ,Atinmo T ,Byers T ,Chen J ,Hirohata T ,Jackson A ,James WPT ,Kolonel LN ,Kumanyika S ,Leitzmann C ,Mann J ,Powers HJ , et al., eds. *Food, nutrition, physical activity, and the prevention of cancer: a global perspective*. Washington, DC: World Cancer Research Fund/American Institute for Cancer Research, 2007. 517p.

5 van Engeland M,Weijenberg MP,Roemen GM,Brink M,de Bruïne AP,Goldbohm RA,van den Brandt PA,Baylin SB,de Goeij AF,Herman JG. Effects of dietary folate and alcohol intake on promoter methylation in sporadic colorectal cancer: the Netherlands cohort study on diet and cancer. *Cancer Res* 2003; **63**: 3133-7. Links

6 Eguchi K,Kanai Y,Kobayashi K,Hirohashi S. DNA hypermethylation at the D17S5 locus in non-small cell lung cancers: its association with smoking history. *Cancer Res* 1997; **57**: 4913-5. Links

7 Kim DH,Nelson HH,Wiencke JK,Zheng S,Christiani DC,Wain JC,Mark EJ,Kelsey KT. $p16^{INK4a}$ and histology-specific methylation of CpG islands by exposure to tobacco smoke in non-small cell lung cancer. *Cancer Res* 2001; **61**: 3419-24. Links

8 Belinsky SA. Gene-promoter hypermethylation as a biomarker in lung cancer. *Nat Rev Cancer* 2004; **4**: 707-17. Links

9 Shibuya K,Mathers CD,Boschi-Pinto C,Lopez AD,Murray CJL. Global and regional estimates of cancer mortality and incidence by site. II. Results for the global burden of disease 2000. *BMC Cancer* 2002; **2**: 37. Links

10 Yuasa Y. Control of gut differentiation and intestinal-type gastric carcinogenesis. *Nat Rev Cancer* 2003; **3**: 592-600. Links

11 Tsugane S,Sasazuki S. Diet and the risk of gastric cancer: review of epidemiological evidence. *Gastric Cancer* 2007; **10**: 75-83. Links

12 Toyota M,Ahuja N,Suzuki H,Itoh F,Ohe-Toyota M,Imai K,Baylin SB,Issa JP. Aberrant methylation in gastric cancer associated with the CpG island methylator phenotype. *Cancer Res* 1999; **59**: 5438-42. Links

13 Ushijima T,Nakajima T,Maekita T. DNA methylation as a marker for the past and future. *J Gastroenterol* 2006; **41**: 401-7. Links

14 Kang GH,Lee S,Cho NY,Gandamihardja T,Long TI,Weisenberger DJ,Campan M,Laird PW. DNA methylation profiles of gastric carcinoma characterized by quantitative DNA methylation analysis. *Lab Invest* 2008; **88**: 161-70. Links

15 Yuasa Y,Nagasaki H,Akiyama Y,Sakai H,Nakajima T,Ohkura Y,Takizawa T,Koike M,Tani M,Iwai T,Sugihara K,Imai K, et al. Relationship between *CDX2* gene methylation and dietary factors in gastric cancer patients. *Carcinogenesis* 2005; **26**: 193-200. Links

16 Wen XZ,Akiyama Y,Baylin SB,Yuasa Y. Frequent epigenetic silencing of the bone morphogenetic protein 2 gene through methylation in gastric carcinomas. *Oncogene* 2006; **25**: 2666-73. Links

17 Leung WK,Yu J,Ng EK,To KF,Ma PK,Lee TL,Go MY,Chung SC,Sung JJ. Concurrent hypermethylation of multiple tumor-related genes in gastric carcinoma and adjacent normal tissues. *Cancer* 2001; **91**: 2294-301. Links

18 Wanajo A,Sasaki A,Nagasaki H,Shimada S,Otsubo T,Owaki S,Shimizu Y,Eishi Y,Kojima K,Nakajima Y,Kawano T,Yuasa Y, et al. Methylation of the calcium channel-related gene, *CACNA2D3*, is frequent and a poor prognostic factor in gastric cancer. *Gastroenterology* 2008; **135**: 580-90. Links

19 Akiyama Y,Watkins N,Suzuki H,Jair KW,van Engeland M,Esteller M,Sakai H,Ren CY,Yuasa Y,Herman JG,Baylin SB. *GATA*-4 and *GATA-5* transcription factor genes and potential downstream antitumor target genes are epigenetically silenced in colorectal and gastric cancer. *Mol Cell Biol* 2003; **23**: 8429-39. Links

20 Japanese Gastric Cancer Association. Japanese Classification of Gastric Carcinoma - 2nd English Edition. *Gastric Cancer* 1998; **1**: 10-24. Links

21 Laurén P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. *Acta Pathol Microbiol Scand* 1965; **64**: 31-49. Links

22 Herman JG,Graff JR,Myohanen S,Nelkin BD,Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 1996; **93**: 9821-6. Links

23 Tajima K,Tominaga S. Dietary habits and gastro-intestinal cancers: a comparative case-control study of stomach and large intestinal cancers in Nagoya, Japan. *Jpn J Cancer Res* 1985; **76**: 705-16. Links

24 Mu L-N,Lu Q-Y,Yu S-Z,Jiang Q-W,Cao W,You N-C,Setiawan VW,Zhou X-F,Ding B-G,Wang R-H,Zhao J,Cai L, et al. Green tea drinking and multigenetic index on the risk of stomach cancer in a Chinese population. *Int J Cancer* 2005; **116**: 972-83. Links

25 Tsubono Y,Nishino Y,Komatsu S,Hsieh CC,Kanemura S,Tsuji I,Nakatsuka H,Fukao A,Satoh H,Hisamichi S. Green tea and the risk of gastric cancer in Japan. *N Engl J Med* 2001; **344**: 632-6. Links

26 Hoshiyama Y,Kawaguchi T,Miura Y,Mizoue T,Tokui N,Yatsuya H,Sakata K,Kondo T,Kikuchi S,Toyoshima H,Hayakawa N,Tamakoshi A, et al. A prospective study of stomach cancer death in relation to green tea consumption in Japan. *Br J Cancer* 2002; **87**: 309-13. Links

27 Sasazuki S,Inoue M,Hanaoka T,Yamamoto S,Sobue T,Tsugane S. Green tea consumption and subsequent risk of gastric cancer by subsite: the JPHC Study. *Cancer Causes Control* 2004; **15**: 483-91. Links

28 Sasazuki S,Inoue M,Miura T,Iwasaki M,Tsugane S. Plasma tea polyphenols and gastric cancer risk: a case-control study nested in a large population-based prospective study in Japan. *Cancer Epidemiol Biomarkers Prev* 2008; **17**: 343-51. Links

29 Yamane T,Takahashi T,Kuwata K,Oya K,Inagake M,Kitao Y,Suganuma M,Fujiki H. Inhibition of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced carcinogenesis by (-)-epigallocatechin gallate in the rat glandular stomach. *Cancer Res* 1995; **55**: 2081-4. Links

30 Fang MZ,Wang Y,Ai N,Hou Z,Sun Y,Lu H,Welsh W,Yang CS. Tea polyphenol (-)-epigallocatechin-3-gallate inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines. *Cancer Res* 2003; **63**: 7563-70. Links

31 Lee WJ,Shim JY,Zhu BT. Mechanisms for the inhibition of DNA methyltransferases by tea catechins and bioflavonoids. *Mol Pharmacol* 2005; **68**: 1018-30. Links

32 Chuang JC,Yoo CB,Kwan JM,Li TW,Liang G,Yang AS,Jones PA. Comparison of biological effects of non-nucleoside DNA methylation inhibitors versus 5-aza-2'-deoxycytidine. *Mol Cancer Ther* 2005; **4**: 1515-20. Links

33 Stresemann C,Brueckner B,Musch T,Stopper H,Lyko F. Functional diversity of DNA methyltransferase inhibitors in human cancer cell lines. *Cancer Res* 2006; **66**: 2794-800. Links

34 Campbell PT,Sloan M,Kreiger N. Physical activity and stomach cancer risk: the influence of intensity and timing during the lifetime. *Eur J Cancer* 2007; **43**: 593-600. Links

35 Sjödahl K,Jia C,Vatten L,Nilsen T,Hveem K,Lagergren J. Body mass and physical activity and risk of gastric cancer in a population-based cohort study in Norway. *Cancer Epidemiol Biomarkers Prev* 2008; **17**: 135-40. Links

36 Coyle YM,Xie X-J,Lewis CM,Bu D,Milchgrub S,Euhus DM. Role of physical activity in modulating breast cancer risk as defined by *APC* and *RASSF1A* promoter hypermethylation in nonmalignant breast tissue. *Cancer Epidemiol Biomarkers Prev* 2007; **16**: 192-6. Links

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Sixty years of follow-up of Hiroshima and Nagasaki survivors: Current progress in molecular epidemiology studies

by
Kei Nakachi, Tomonori Hayashi, Kiyohiro Hamatani, Hidetaka Eguchi, Yoichiro Kusunoki

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Elsevier
Radarweg 29
1043 NX Amsterdam
The Netherlands
Tel: +31 (0)20 485 3440
Fax: +31 (0)20 485 3280