AI at CA-SSR I represents amplification as determined by quantitative real-time PCR

AI assessed by PCR-based microsatellite analysis describes only the relative gene dosages of the two CA-SSR I alleles. To determine whether the AI represents amplification or deletion, we performed a quantitative 5′ nuclease assay. *egfr*-specific primers that flank the CA-SSR I in the first intron as well as a FAM-labelled $(CA)_{15}$ probe were used in this assay. PCR amplification was performed using serial dilutions of leukocyte and tumour DNA normalized with respect to two different housekeeping genes as described in the Materials and methods section.

The sensitivity of the method allows the detection of amplification if the LOH score is less than 0.56 or more than 1.3. As a control, DNA isolated from the MDA-MB-468 cell line was tested and displayed 30- to 60-fold amplification of *egfr* CA-SSR I. Because of the limited sensitivity of the 5′ nuclease assay, amplification could be confirmed in only one-third of cases presenting with AI. However, no tumour with a decreased gene dosage was found by this method. Moreover, the 5′ nuclease assay assessed a 16- to 32-fold increase in the gene dosage of *egfr* in the two tumours with high-level gains of *egfr*, as detected by CGH (Figures 1 and 2).

Distribution of the length of the polymorphic CA dinucleotide stretch at CA-SSR I in German and Japanese breast cancer patients

Comparison of the length of the CA dinucleotide repeat at CA-SSR I yielded a highly significant difference in the germ line allele status between the German and the Japanese patient groups. Tumours from German women harboured, on average, shorter stretches containing 16–18 CA dinucleotides (71.6%) than Japanese women. The Japanese tumours were very homogeneous, with predominantly longer alleles containing 19 or more CA dinucleotides, which covered 67% of all alleles (Figure 3; $p < 0.001$, Wilcoxon test).

Regulation of EGFR expression is associated with allele length and CA-SSR I amplification in German and Japanese tumours

In order to investigate the interplay between *egfr* CA-SSR I length, amplification of the *egfr* CA-SSR, and EGFR expression, we determined the intratumoural EGFR concentration using an enzymatic immunoassay. Significantly lower EGFR expression levels were found in the Japanese tumours than in the German breast cancers (Figure 3; median: 17 versus 59; mean ± SEM: 24 ± 12 versus 65 ± 35 fmol/mg, $p < 0.001$, Wilcoxon test).

In contrast, the Japanese breast tumours that harboured amplifications of the *egfr* CA-SSR I as assessed by the 5′ nuclease assay expressed significantly higher EGF receptor densities compared with the German cases (mean ± SEM: 113.5 ± 31.2 versus 65 ± 35 fmol/mg; $p < 0.05$, t-test; median: 96.1 versus 59 fmol/mg; $p < 0.05$, Wilcoxon test).

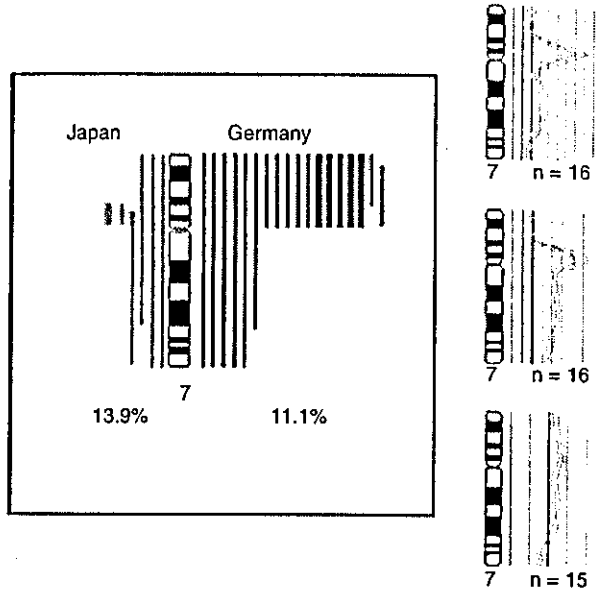

Figure 1. Left: all gains of chromosomal material including 7p in German and Japanese breast tumours are indicated on both sides of the ideogram of chromosome 7 in a cumulative manner. High-level amplifications are indicated using thick bars. The overall frequency of 7p-gains in both subgroups is given below the ideogram. Right: the respective CGH ratio profiles of chromosome 7 for all Japanese patients with high-level amplification of *egfr* are shown. The black central line indicates the fluorescence ratio of balanced DNA sequence copy number state (1.0) between the tumour and reference DNA. The lines to the left represent the 0.75 and 0.5 thresholds for losses, and the lines to the right the 1.25, 1.5, 1.75, etc. thresholds for gains. 95% confidence limits are shown. Chromosome numbers are also indicated. Whereas two Japanese breast tumours demonstrated clear high-level amplifications similar to MDA-MB-468, one case showed a circumscribed low-level gain of genetic material in 7p12–13

Discussion

Members of the *erb*B gene family are attractive targets for new therapeutic strategies in various malignant neoplasms, since their overexpression, especially in breast cancer, is a common finding [14]. The clinical application of such new specific treatment modalities is unfortunately hampered by restricted knowledge regarding reliable diagnostics for identifying those patients who might benefit from such approaches [15].

For c-*erb*B2, the evaluation of immunohistochemical expression and gene dosage measurements by fluorescence *in situ* hybridization (FISH) currently represents the state-of-the-art [16]. For EGFR, the situation is much more complicated. Patients with EGFR overexpression seem to be independent of those with c-*erb*B2 expression [17,18]. Investigation of breast

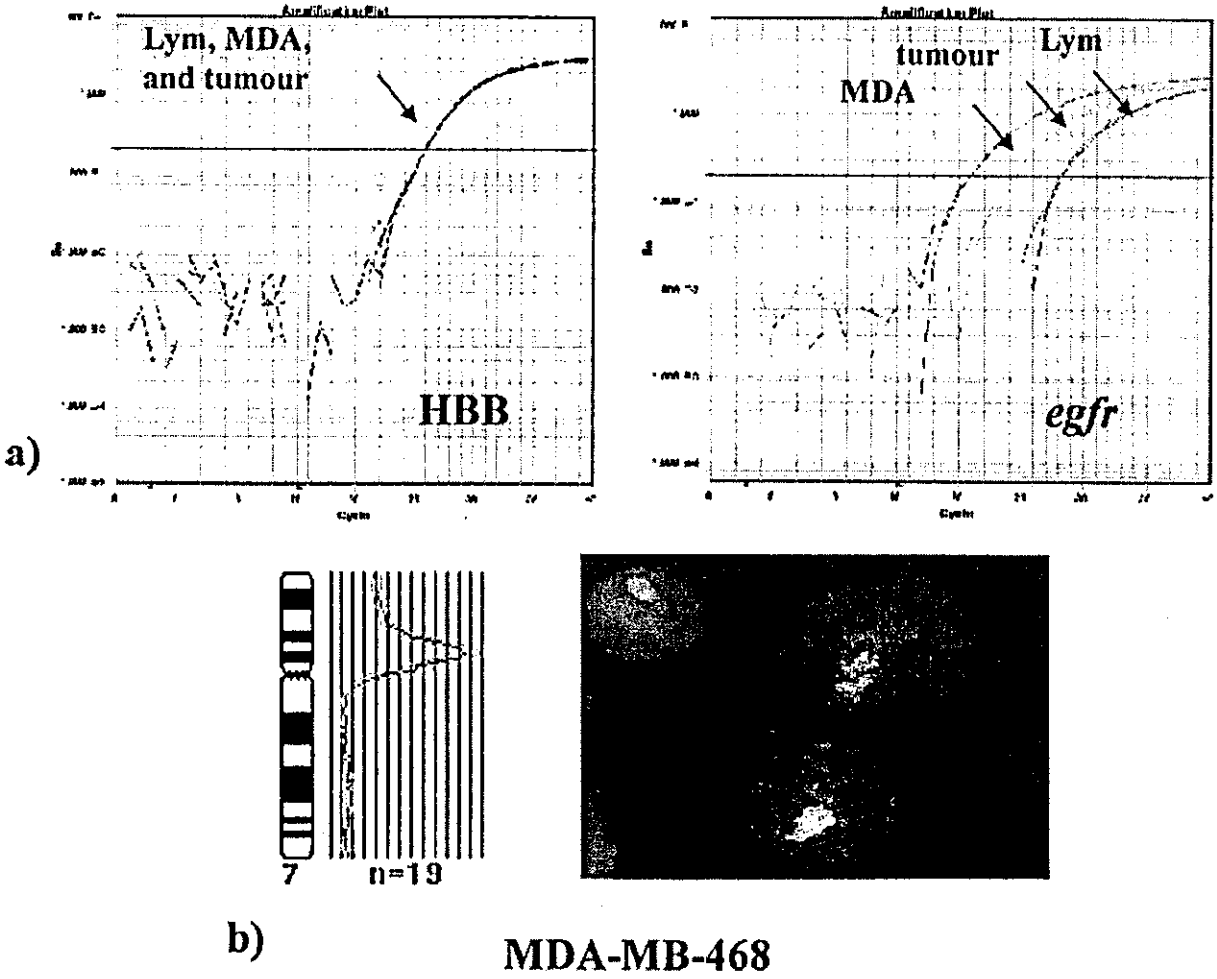

Figure 2. (a) 'Real-time PCR' to confirm *egfr* amplification in one of the Japanese breast tumours that showed amplification of *egfr* by CGH. Real-time PCR was used to quantify *egfr* gene dosages in breast tumour tissue. This example shows 8- to 16-fold amplification of CA-SSR I. Normal lymphocyte, MDA-MB-468, and tumour tissue DNA display identical C_t values using HBB primers and probe, indicating that equal amounts of DNA were analysed. Analysis with *egfr* primers and probe revealed a difference of C_t in both MDA-MB-468 ($\Delta C_t = 7.3$) and this tumour ($\Delta C_t = 3.5$) in comparison with normal lymphocyte DNA. (Rn = fluorescence in a semi-logarithmic plot normalized to a passive reference). (b) The MDA-MB-468 cell line served as an internal control. CGH analysis of this cell line showed a similar CGH ratio profile to that seen in two of the Japanese breast tumours. Additional FISH analysis revealed a cloudy signal indicating high-level amplification of *egfr*

cancer cell lines points towards the importance of a gene dosage effect [19], but in primary breast cancer, amplification of the *egfr* gene using Southern blot techniques or FISH was found at low frequencies [20,21]. Consequently, it has become widely accepted that overexpression of EGFR is due mainly to regulation at the level of transcription [22].

Previously, our group reported on a polymorphic CA repeat in intron 1 of the *egfr* gene (CA-SSR I), which modulated *egfr* transcription both *in vitro* and *in vivo* [1,5]. We were also able to show that distinct amplifications, almost exclusively limited to this regulatory sequence of *egfr*, are a frequent event in German breast cancer cases and number amongst the first stages of mammary carcinogenesis [6]. Interestingly, we also showed that young breast cancer patients with a family history of breast cancer are also characterized by predominantly longer alleles [8].

Recently, data from preliminary studies support the assumption that the length of the CA-SSR I is related to the response rate of conventional and anti-*egfr*-based therapy in colon and lung cancer [9,10]. Nevertheless, the detection of amplification, either of the regulatory sequence in intron 1 centred around CA-SSR I or of the whole gene as seen by CGH, demonstrates that expression of the EGF receptor in cancer tissue cannot simply be interpreted within the narrow perspective of the length of the CA repeat polymorphism. Moreover, Japanese breast cancers had a higher prevalence of amplification than their German counterparts, an observation found to be statistically significant with CGH. Similar data have been published in other populations, with two of 40 Asian breast cancer cases displaying amplifications at 7p12–13, the chromosomal region containing the *egfr* gene [23]. In contrast, this has been a very infrequent finding

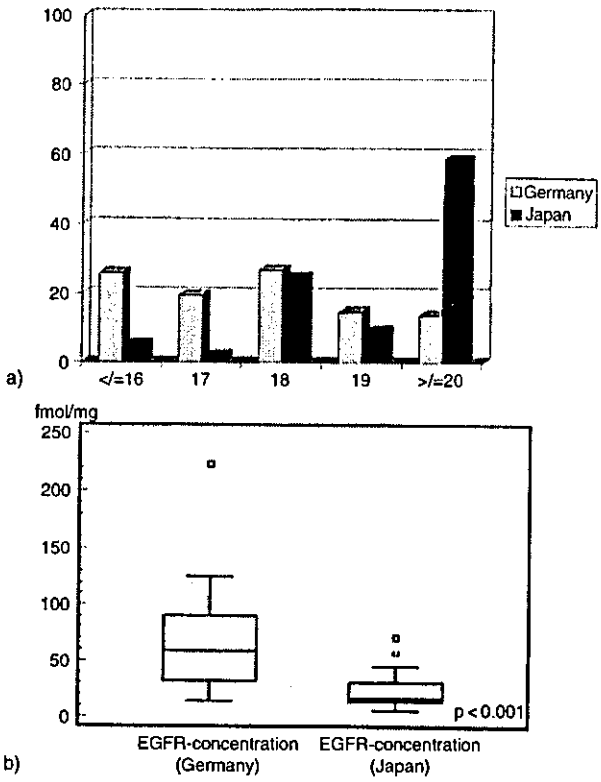

Figure 3. (a) Frequency of CA repeat germ line polymorphisms in intron 1 of *egfr* in Japanese and German breast cancer patients. (b) Comparison of EGFR expression in Japanese and German breast tumours

in CGH studies analysing Caucasian breast tumours [24,25]. Furthermore, it has already been shown that the number of repeats itself affects the mutation rate of nucleotide repeats [26]. One could therefore argue that the acquisition of *egfr* amplification by breast cells might represent a mechanism by which growth advantage is gained during breast carcinogenesis, despite inherited longer alleles, associated with lower baseline *egfr* transcription. Further studies including larger tumour cohorts are required to show whether the frequency of the mutations is associated with the predominance of distinct CA-SSR I lengths. Our results showing that Japanese breast cancer patients mainly harbour alleles with 19 or more repeats might be taken as support for this hypothesis. As a result, the first clinical observations on Japanese patients who responded more frequently to anti-*egfr* therapies [10] might be based on the fact that this ethnic subgroup is more susceptible to *egfr* amplifications, so that EGFR overexpression that is relevant to therapy and prognosis occurs at a higher rate [27]. In conclusion, it seems obvious that EGFR overexpression is the end result of a complicated, delicate network of inherited and acquired genetic factors, as shown in Figure 4. With the present state of knowledge, it therefore seems advisable to include gene dosage measurements for the intron 1 CA repeat and the determination of the CA repeat length in clinical trials in order to predict potential therapeutic outcome.

Acknowledgements

We thank Ulrike Neubert, Petra Meier, and Lydia Grote for technical assistance. This work was supported by grants from the Deutsche Krebshilfe (Nos 10-1681-Bü-I and 10-1660-Br 4).

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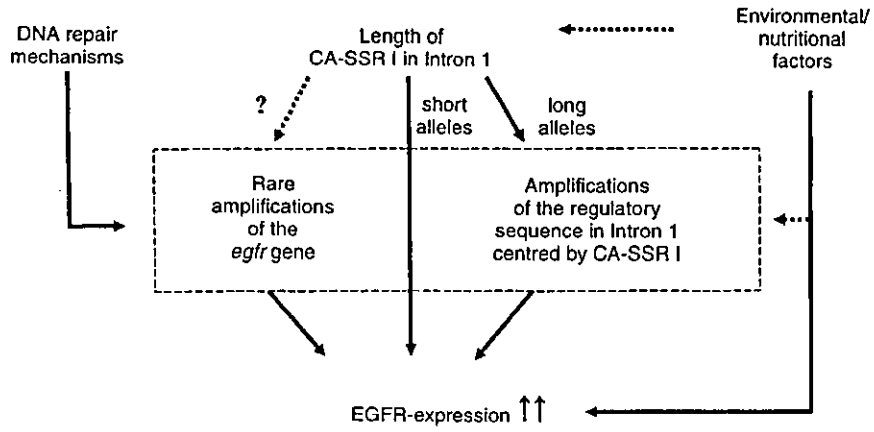

Figure 4. Working hypothesis considering the interaction of inherited and acquired genetic factors in the regulation of EGFR expression in breast cancer. The length of the CA repeat in intron 1 of *egfr* is a modulator of the basic transcriptional activity of *egfr* [1,5]. This holds especially true for patients with short alleles associated with high transcriptional activity. In patients with inherited, longer, alleles, a growth advantage obtained by the acquisition of *egfr* amplification [6] — either of the regulating sequences or of the whole gene — might take place, as shown in the Japanese breast cancer patient, and increases the transcription level. Furthermore, environmental and nutritional factors have the potential to modulate the basic, allele-dependent transcription activity of *egfr* in either way [28]. First epidemiological results yielded additional evidence that these factors in combination with the *egfr* polymorphism status might have a modulating role in the pathogenesis of familial breast cancer [8]

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Radiation-induced apoptosis of stem/progenitor cells in human umbilical cord blood is associated with alterations in reactive oxygen and intracellular pH

Tomonori Hayashi[a,*], Ikue Hayashi[b], Tomoko Shinohara[a], Yukari Morishita[a], Hiroko Nagamura[a], Yoichiro Kusunoki[a], Seishi Kyoizumi[a], Toshio Seyama[c], Kei Nakachi[a]

[a] *Department of Radiobiology/Molecular Epidemiology, Radiation Effects Research Foundation, Hijyama Park, Minami Ward, Hiroshima, Japan*
[b] *Central Research Laboratory, Hiroshima University Faculty of Dentistry, Hiroshima, Japan*
[c] *Yasuda Women's University, Hiroshima, Japan*

Received 4 March 2004; accepted 8 July 2004

Abstract

To investigate the sensitivity of human hematopoietic stem cell populations to radiation and its relevance to intracellular events, specifically alteration in cellular energy production systems, we examined the frequency of apoptotic cells, generation of superoxide anions ($O_2^{\bullet -}$), and changes in cytosol pH in umbilical cord blood (UCB) $CD34^+/CD38^-$, $CD34^+/CD38^+$ and $CD34^-/CD38^+$ cells before and after 5 Gy of X-irradiation. Human UCB mononucleated cells were used in this study. After X-irradiation and staining subgroups of the cells with fluorescence (FITC, PE, or CY)-labeled anti-CD34 and anti-CD38 antibodies, analyses were performed by FACScan using as stains 7-amino-actinomycin D (7-AAD) for the detection of apoptosis, and hydroethidine (HE) for the measurement of $O_2^{\bullet -}$ generation in the cells. For intracellular pH, image analysis was conducted using confocal laser microscopy after irradiation and staining with carboxy-SNAFR-1. The frequency of apoptotic cells, as determined by cell staining with 7-AAD, was highest in the irradiated $CD34^+/CD38^-$ cell population, where the level of $O_2^{\bullet -}$ detected by the oxidation of HE was also most highly elevated. Intracellular pH measured with carboxy-SNARF-1-AM by image cytometer appeared to be lowest in the same irradiated $CD34^+/CD38^-$ cell population, and this intracellular pH decreased as early as 4 h post-irradiation, virtually simultaneous with the significant elevation of $O_2^{\bullet -}$ generation. These results suggest that the $CD34^+/CD38^-$ stem cell population is sensitive to radiation-induced apoptosis as well as production of intracellular $O_2^{\bullet -}$, compare to more differentiated $CD34^+/CD38^+$ and $CD34^-/CD38^+$ cells and that its intracellular pH declines at an early phase in the apoptosis process.

Keywords: Apoptosis; Stem cell; Radiation; Reactive oxygen species; Intracellular pH

* Corresponding author. Tel.: +81 822613169; fax: +81 822613710.
E-mail address: tomo@rerf.or.jp (T. Hayashi).

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doi:10.1016/j.mrfmmm.2004.07.002

1. Introduction

Apoptosis is an important mechanism in the selective elimination of mammalian cells: It is distinct from the process of the cell death by necrosis [1], and is basically characterized by cellular shrinkage, marked condensation and margination of chromatin, and nuclear and cellular fragmentation with well preserved cell organelles. Radiation-induced cell death has been studied extensively in a wide variety of cell types and cell lines. Ionizing radiation causes breaks in chromosomal DNA resulting in upregulation of stress-activated proteins including p53, p21, Gadd45, and manganese superoxide dismutase [2–6]. Apoptosis and classical necrosis are two genetically, biochemically and morphologically different types of cell death, and the differences have been recognized as depending not only on the cell type but also on the radiation dose. In apoptosis by radiation, following localization of pro-apoptotic proteins, such as Bax, to the mitochondria, there is a decrease in mitochondrial membrane permeability and release of cytochrome-C into the cytoplasma, followed by induction of typical apoptotic features including chromatin condensation and nuclear fragmentation accompanied with caspase-3 activation and poly-ADP-ribosyl polymerase cleavage [7–9]. The mechanism by which these subcellular events occur has remained unclear.

The reactive oxygen species (ROS), especially superoxide anion ($O_2^{\bullet -}$), play an important role in signaling mechanisms and are also in chromosomal aberrations, cell proliferation and cell death [10–13]. Radiation-induced $O_2^{\bullet -}$ generation among different cell types may be of interest for understanding sensitivity to radiation-induced chromosomal aberrations in the survived cells. Hydroethidine (HE), a non-fluorescent lipophilic marker, is oxidized by superoxide anion to the fluorescent hydrophilic product ethidium (Eth), and indicates the accumulation of ROS in the cells [14,15]. In several models of apoptosis, increased formation of ROS has been noted as an early event in apoptosis and as a main cause of chromosomal aberrations in survived cells [15,16]. A decline in the pH of the cell interior (pHi) has also been reported as a concomitant of apoptosis in HL-60 cells and CTLL cells [17–20], and is thought to be an early feature of apoptosis in neutrophils [21]. However, whether ROS or pHi is the primary trigger/inducer of the radiation-induced apoptotic pathway has not been determined.

The hemopoietic stem cell seems to be one of the most radiosensitive cells in the body [22]. However, the death-signaling mechanism and susceptibility of hemopoietic stem cells to ionizing radiation have not been well understood. The stem cell marker CD34 is expressed on the surface of stem and early progenitor cells, and hemopoietic progenitor cells in bone marrow or umbilical cord blood (UCB) have proved divisible into various subpopulations in terms of CD34 and CD38 antigen expressions [23]. Thus, for example, $CD34^+CD38^-$ cells were found to be multilineage stem cells, whereas $CD34^+CD38^+$ cells and $CD34^-CD38^+$ cells were differentiated cells, respectively [24]. In this study, we examined the sensitivity of human stem/progenitor/differentiated cells to radiation-induced apoptosis and determined the relationship between $O_2^{\bullet -}$ generation and intracellular pH in the apoptotic process.

2. Materials and methods

2.1. Human UCB cells

Human UCB mononucleated cells were obtained from BioWhittaker Inc. (Walkersville, MD). After thawing cryopreserved cells by stepwise dilution in Earle's Balanced Salt Solution (EBSS) containing 2.5% fetal calf serum (FCS), the cells were washed with EBSS containing 2.5% FCS and cultured with 10% heat-inactivated FCS, and 90% RPMI 1640 (Nikken Biomedical Lab., Kyoto, Japan) until used for flow cytometric analysis or subset purification.

2.2. Irradiation

UCB cells or sorted cells were X-irradiated using a Shin-ai II unit (Shimadzu Corp., Tokyo, Japan) at 200 kVp, 20 mA, with 0.5 mm Al and 0.5 mm Cu filters. Dose rate was 0.61 Gy/min. Immediately after irradiation, cells were suspended in RPMI 1640-10% FCS and cultured at 37 °C humidified air containing 5% CO_2.

2.3. Cytofluorometric analyses of $O_2^{\bullet -}$ generation and apoptosis (cell viability)

Many different methods have been described previously for the quantification of programmed cell death

at an individual cell level by flow cytometry [25,26]. Most of these techniques, however, do not permit the simultaneous evaluation of FITC and phycoerythrin (PE) cell-surface staining due to either a fixation or a sample preparation method that could not preserve cell surface staining and/or to the use of DNA dyes with fluorescence emission spectra that overlap extensively with PE. One technique for measuring apoptosis has been developed with 7-amino-actinomycin D (7-AAD), whose fluorescence emission can be clearly discriminated from PE fluorescence emission [27]. We therefore decided to use a combination of 7-AAD, FITC-CD34 and PE-CD38 to evaluate the apoptotic cell proportion in each hemopoietic cell subset.

For staining of CD34 and CD38 antigens in human UCB cells, 20 μl each of FITC-conjugated antibody against CD34 (FITC-CD34) and CY or PE-conjugated antibody against CD38 (CY-CD38 or PE-CD38, respectively, PharMingen, San Diego, CA) in 100 μl of phosphate buffered saline (PBS without Ca^{2+} and Mg^{2+}, Nikken Biomedical Lab., Kyoto, Japan) containing 1% BSA were added to 1×10^7 UCB cells followed by incubation for 30 min on ice. In the case of double staining, either combination of HE and CY-CD38 or PE-CD38 and 7-AAD were selected. After one wash with 1 ml of PBS, the supernatant was removed, and the cell pellet was resuspended in 1 ml of PBS. To measure $O_2^{\bullet -}$ generation, the cell suspension was stained for 15 min at 37 °C with 2.5 μl of a 63.5 mM HE (Polysciences, Inc., Warrington, PA) solution in N,N-dimethylformamide (DMF), washed once with PBS containing 1% BSA and 0.01% sodium azide (PBS-BSA/AZ), and resuspended in 1 ml of PBS-BSA/AZ. To measure cell viability, the cell suspension was stained with 20 μg/ml 7-AAD (Wako pure chemicals, Tokyo, Japan) in PBS-BSA/AZ for 15 min on ice protected from light; the cells were then analyzed with a FACScan flow cytometer (Beckton Dickinson Immunocytometry Systems, BDIS, San Jose, CA) in their staining solution.

2.4. Subset purification for pHi analysis

CY- or PE-stained cells could not be used for pHi analysis due to the interference of the fluorescent dye. To overcome this technical difficulty, we used the high-gradient magnetic cell sorting (MACS) system (Miltenyi Biotech GmBH, Bergisch Gladback, Germany) [28] and fluorescence-activating cell sorting (FACS) system for the collection of each cell subset before the analysis of pHi. Briefly, human UCB cells were fractionated into $CD34^+$ and $CD34^-$ cell populations by positive and negative selection using MACS system. The purified $CD34^+$ cells and $CD34^-$ cells were stained with FITC-CD38 for 30 min on ice in RPMI 1640 containing 1% FCS. After incubation, the cells were washed twice, resuspended in RPM I1640 containing 1% FCS, and sorted into $CD34^+/CD38^-$, $CD34^+/CD38^+$ and $CD34^-/CD38^+$ cell populations using a FACStar (BDIS).

2.5. Image cytometric analysis of intracellular pH

Cells were cultured for the indicated times in serum-free RPMI 1640 medium, then loaded with 10 μM Carboxy-SNARF-1-AM (Molecular Probes Inc., Eugene, OR) for 30 min in HBSS, centrifuged, and resuspended in HBSS containing 20 mM Hepes (pH 7.4). Image analysis was performed on an Ultima Interactive Laser Cytometer (Meridian Instruments, Inc., Okemos, MI) with excitation at 488 nm and emission ratio analysis at 575 and 620 nm. Ten to twenty cells were analyzed, and emission ratios were converted to pH values by comparison to ratios observed in cells treated with nigericin in high potassium buffer at a defined pH [29].

3. Results

3.1. $CD34^+$ cells from human UCB are sensitive to radiation-induced apoptosis

Separated human UCB cells were irradiated in vitro, harvested after 16 h, and subsequently flow-cytometrically analyzed with 7-AAD after staining with FITC-CD34 and PE-CD38. The cells investigated were gated on 2-color flow cytometry as described in Fig. 1, with the exclusion of monocytes, platelets and cell debris in a light-scattering profile. FITC-CD34/PE-CD38 stained cells are shown for a 16 h culture, demonstrating the plots obtained for unirradiated and 5-Gy irradiated cell samples, respectively.

Most unirradiated cells were negative for the 7-AAD staining, whereas about 60% of irradiated $CD34^+/CD38^-$ cells and 40–50% of irradiated $CD34^+/CE38^+$ cells were stained strongly with 7-

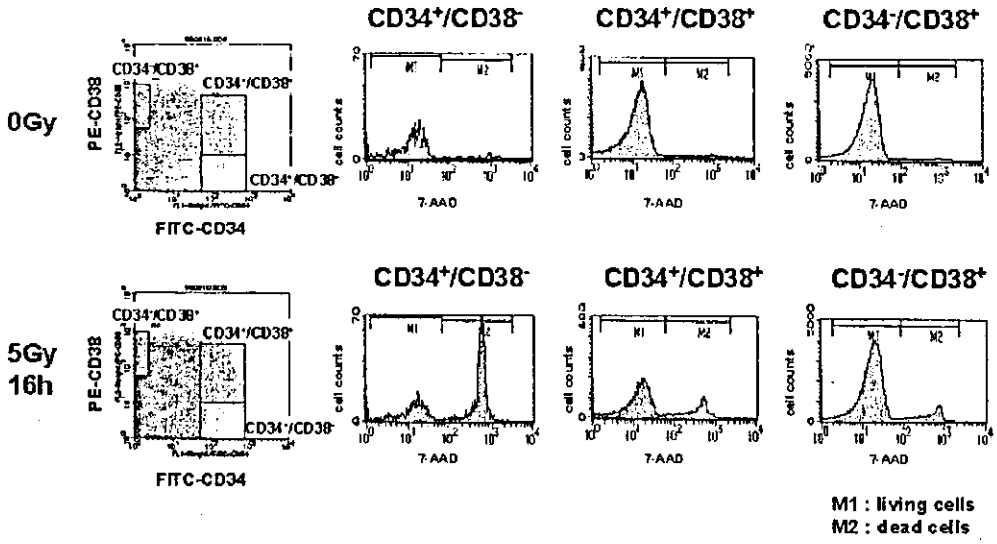

Fig. 1. Triple staining of cells for the simultaneous determination of apoptotic cells among different cell populations. Human UCB cells were stained with FITC-CD34, PE-CD38, and 7-AAD, and subjected to cytometric analyses. Cells were analyzed at 16 h post-irradiation with 5 Gy. Flow cytometer histograms show 7-AAD stained cells. M1: living cells; M2: apoptotic cells.

AAD (Fig. 1). On the other hand, only a small fraction of irradiated $CD34^-/CD38^+$ cells were stained. Fig. 2 shows the percentages of living cells of each cell population at 4 and 16 h post-irradiation with 5 Gy: It is obvious that $CD34^+/CD38^-$ stem cells were more sensitive to radiation than more differentiated $CD34^+/CD38^+$ cells. Also of note is that few cells appeared to reach the stage of apoptosis at 4 h after 5-Gy irradiation.

3.2. A higher level of $O_2^{\bullet -}$ generation in irradiated $CD34^+/CD38^-$ cells

$O_2^{\bullet -}$ generation in irradiated cells (4 or 16 h post-irradiation with 5 Gy) was analyzed by flow cytometry with FITC-CD34, CY-CD38, and HE. The levels of $O_2^{\bullet -}$ generation in unirradiated cells depended on the cell type, i.e., $CD34^+/CD38^+ > CD34^+/CD38^-$

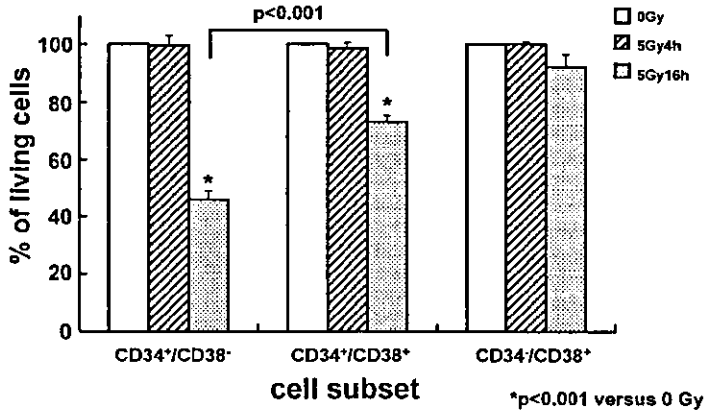

Fig. 2. The percentage of living cells in irradiated human UCB cell subsets. Human UCB cells were stained with FITC-CD34, PE-CD38, and 7-AAD, and subjected to cytometric analyses. Cells were analyzed at 4 and 16 h post-irradiation with 5 Gy. The percentage of living cells was calculated from the 7-AAD no-stained fraction in each UCB subset cells. The values represent mean and S.D. of three experiments.

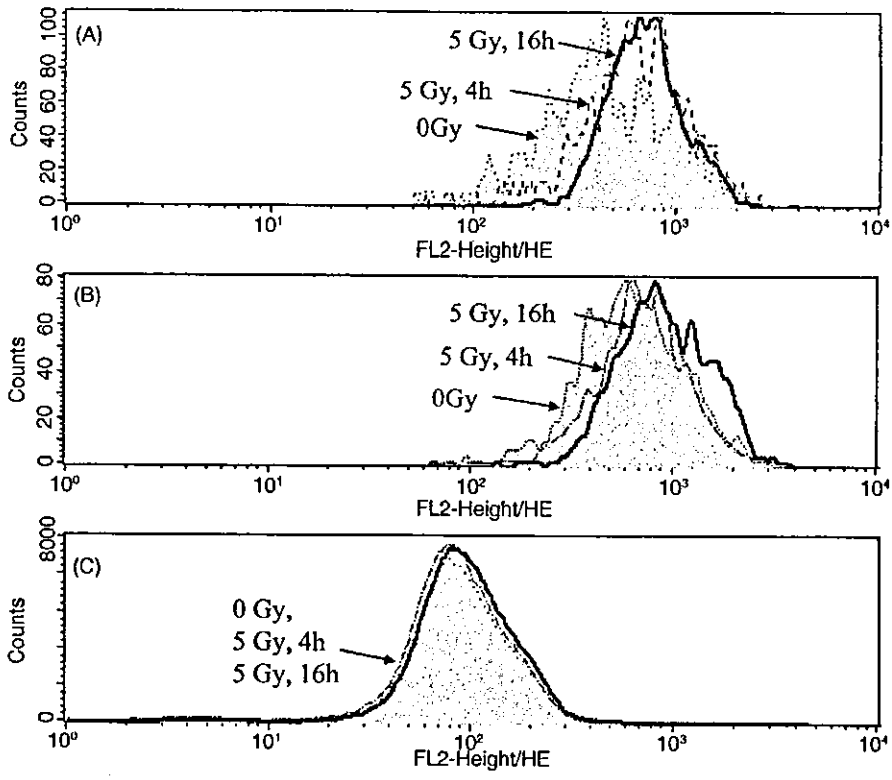

Fig. 3. Changes of $O_2^{\bullet -}$ generation in irradiated human UCB cell subsets. Irradiated human UCB cells were stained with FITC-CD34, CY-CD38, and HE, and subjected to cytometric analyses. (A) $CD34^+/CD38^-$ cells, (B) $CD34^+/CD38^+$ cells, (C) $CD34^-/CD38^+$ cells.

> $CD34^-/CD38^+$ (Figs. 3 and 4). $O_2^{\bullet -}$ generation levels in the $CD34^+/CD38^-$ and $CD34^+/CD38^+$ cell populations appeared to rise as early as 4 h after irradiation and had significantly increased 16 h after irradiation. In contrast, the levels of $O_2^{\bullet -}$ generation in the $CD34^-/CD38^+$ cell populations had not increased at even 16 h after irradiation. Overall changes and fold-induction in $O_2^{\bullet -}$ generation levels 16 h after irradiation were largest for the $CD34^+/CD38^-$ among these three cell populations. These results on cell-type-dependent $O_2^{\bullet -}$ generation after irradiation appear to be consistent with observations of radiation-induced apoptosis in the same cell types.

3.3. Intracellular pH of the $CD34^+/CD38^-$ cell population decreases in an early stage post-irradiation

We took note of intracellular pH as an indicator of the events taking place in live cells. Each of the

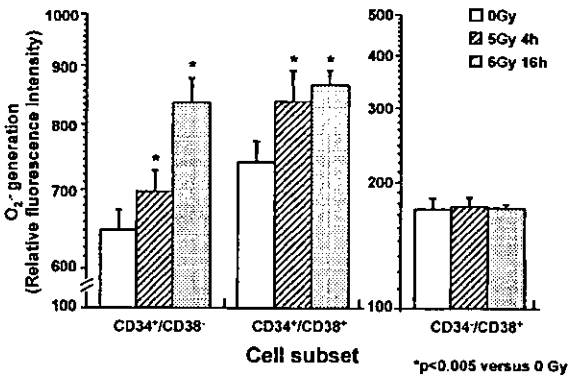

Fig. 4. $O_2^{\bullet -}$ generation in irradiated human UCB cell subsets. Irradiated human UCB cells were stained with FITC-CD34, CY-CD38, and HE, and subjected to cytometric analyses. The level of $O_2^{\bullet -}$ generation was monitored by the geometric mean fluorescence intensity of ethidium derived from HE. Cells were analyzed at 4 and 16 h post-irradiation with 5 Gy. The values represent mean and S.D. of three experiments.

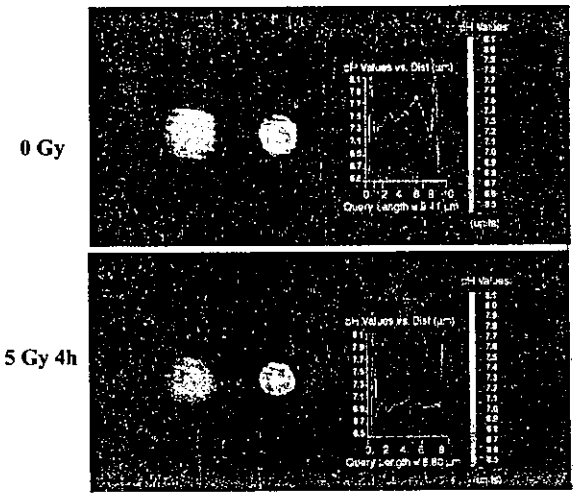

Fig. 5. Visualized imaging analysis of intracellular pH of $CD34^+/CD38^-$ cells using Carboxy-SNARF-1-AM. Purification of $CD34^+$ and $CD34^-$ cells was performed by positive selection using MACS system. Purified $CD34^+$ cells and $CD34^-$ cells were stained with FITC-CD38 and separated into $CD38^+$ and $CD38^-$ cells by a FACStar. Emission ratios were converted to pH values by comparison with ratios observed in cells treated with nigericin in high potassium buffer at a defined pH [29].

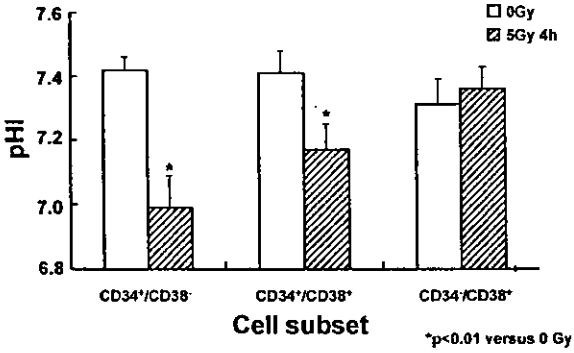

Fig. 6. Intracellular pH of irradiated UCB cell subsets. Ten to twenty cells were analyzed as depicted in Fig. 5. Emission ratios were converted to pH values by comparison with ratios observed in cells treated with nigericin. The values represent mean and S.D. of three experiments.

$CD34^+/CD38^-$, $CD34^+/CD38^+$, and $CD34^-/CD38^+$ cell fractions was sorted using magnet beads and FACStar, and intracellular pH in each cell population was evaluated using image analysis before, and 4 h after, 5-Gy irradiation (Fig. 5). The pH in irradiated cells was much lower than that in non-irradiated cells, and this radiation effect was more obvious in the $CD34^+/CD38^-$ cell population than in the $CD34^+/CD38^+$ cell population. As for the $CD34^-/CD38^+$ cell population, there was no significant radiation effect on the intracellular pH. These results indicate that a remarkable decline of intracellular pH in the $CD34^+/CD38^-$ cell population occurs along with the $O_2^{\bullet -}$ generation that appears in the same cell population (Fig. 6).

4. Discussion

In the present study, we found that the $CD34^+/CD38^-$ stem cell population was more sensitive to radiation-induced apoptosis than were more differentiated cell populations, and that a decline in intracellular pH and an accumulation of intracellular ROS in the stem cell population were early features prior to apoptosis.

Susceptibility to cell-death signal in early hemopoietic stem-progenitor cells has been partially clarified: It has been reported that hemopoietic stem-progenitor cells express membrane-bound FasL (mFasL) as well as Fas, which suggests autocrine and/or paracrine regulation of the Fas/FasL system in hemopoiesis [30–32]. It has also been reported that growth factor deprivation induces apoptosis of hemopoietic stem-progenitor cells through the Fas/FasL system, and that radiation-induced apoptosis can be prevented by the addition of a combination of KIT ligand, FLT-3 ligand, thrombopoietin, and interleukin-3 [33,34]. The radiation-induced apoptotic pathway, however, is different from the Fas-mediated apoptosis pathway: the apoptotic stimuli resulting from ionizing radiation induce a rapid decline of cytosol pH, followed by cytochrome c (cytC) release, caspase activation and mitochondrial swelling and depolarization, events that are not involved in the mitochondria-independent apoptosis found in Fas-mediated apoptosis [35]. Although there is general agreement that active mitochondria are needed for the variety of energy-required processes occurring during apoptosis, it seems apparent that energy charge is a key control point of cell death by either apoptosis or necrosis, and that cells with impaired mitochondrial energy metabolism reach a bioenergetic threshold that triggers apoptosis [36–41]. The background level of ROS in $CD34^+/CD38^+$ cells appeared to be higher than that

in $CD34^+/CD38^-$ cells. This may be due to that ROS generation during the continuous energy production for cell proliferation is pronounced in the former type of cells than in the latter. Such a difference in the background ROS generation may not be involved in different radiosensitivity between these cell populations. Our observation that declining intracellular pH accompanied ROS accumulation in irradiated $CD34^+/CD38^-$ cells may account for the significance of cellular energy charge that is decisive of radiation-induced apoptosis.

The accumulation of ROS is known to be associated with reduced levels of the cellular antioxidants GSH and NADPH, and the $O_2^{\bullet -}$ generated by the xanthine/xanthin oxidase system in mitochondria, where generation of a large amount of $O_2^{\bullet -}$ causes rapid cytC release to the cytosol. Madesh and Hajnoczky [42] examined the molecular machinery used by ROS to trigger apoptosis in the mitochondria phase. They found that cytC release induced by $O_2^{\bullet -}$ may have widespread significance in apoptosis, since in a number of pathological conditions – such as ischemia/reperfusion injury, drug insults, and inflammatory responses – large amounts of $O_2^{\bullet -}$ are produced by the xanthine oxidase-mediated catabolism of purine nucleotides, by increased electron transport chain activity or by activation of NADPH oxidase, particularly in neutrophils [43]. On the other hand, it has been reported that the efficiency of caspase activation by cytC is dependent on intracellular pH, with a pH optimum of 6.3–6.8 in vitro [35]. Furthermore, when changes in cytosolic pH induced by Bax were prevented by a use of F_0F_1-ATPase inhibitors or protonophores, caspase activation was impaired and fewer cells underwent apoptosis. Thus, mitochondria-induced cytosol acidification may promote cytC-mediated activation of caspases, a notion which our present results support.

When exploring the reasons for differences in cellular radiosensitivity, factors such as bcl-2 and p53 will need to be taken into account. In this study, decreased intracellular pH was closely associated with increased levels of $O_2^{\bullet -}$ generation as well as increased radiosentitivity among different types of blood cell populations. Thus, the ability of cells to control cellular energy charge may partly determine their susceptibility to radiation-induced apoptosis, and the molecules involved in this cellular control mechanism may play important roles in cell death. In addition, the significant elevation of intracellular ROS observed in the $CD34^+/CD38^-$ stem cells, in response to radiation, suggested that the survived stem cells may proliferate bearing increased chromosomal aberrations. Further investigations of the molecular mechanisms by which DNA-damaged cells control their energy charges are therefore warranted.

Acknowledgements

The Radiation Effects Research Foundation (RERF), Hiroshima and Nagasaki, Japan is a private, non-profit foundation funded by the Japanese Ministry of Health, Labour and Welfare (MHLW) and the U.S. Department of Energy (DOE), the latter through the National Academy of Sciences. This publication was supported by RERF Research Protocol(s) RP No. 1-93 and in part by Grants-in-Aid for Scientific Research (No. 15510065) from the Japanese Ministry of Education, Science, Sports and Culture and the MHLW.

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Hum Genet (2004) 115: 372–376
DOI 10.1007/s00439-004-1155-1

ORIGINAL INVESTIGATION

Hiroyuki Shimada · Kimiko Shimizu · Sachiyo Mimaki · Tokuki Sakiyama · Tetsuya Mori · Noriko Shimasaki · Jun Yokota · Kei Nakachi · Tsutomu Ohta · Misao Ohki

First case of aplastic anemia in a Japanese child with a homozygous missense mutation in the *NBS1* gene (I171V) associated with genomic instability

Received: 9 March 2004 / Accepted: 20 May 2004 / Published online: 24 August 2004

Abstract The *NBS1* gene is strongly linked to several factors involved in genome integrity. Functional disruption of NBS1 could therefore induce genomic instability and carcinogenesis. Four children with acute lymphoblastic leukemia have been reported to be heterozygous for a germline and/or somatic missense mutation in *NBS1*, leading to the I171V substitution. We screened healthy controls and pediatric patients with hematological malignancies and aplastic anemia (AA) for the presence of I171V. Of the 62 patients, one individual with AA was confirmed to harbor a homozygous I171V mutation. Genetic analysis of *NBS1* in this patient and her healthy parents indicated that she inherited the germline I171V mutation from her father and the wild-type allele from her mother, and that the second I171V hit occurred on the wild-type allele early in embryonic development. Furthermore, cytogenetic analysis of lymphoblastic cell lines from the patient indicated a remarkable increase in numerical and structural chromosomal aberrations in the absence of clastogens, suggesting that she potentially carried genomic instability. This is the first report of AA with a homozygous I171V mutation. We hypothesize that *NBS1* may play an important role in the pathogenesis of AA.

H. Shimada and K. Shimizu contributed equally to this work

H. Shimada · T. Mori · N. Shimasaki
Department of Pediatrics, Keio University School of Medicine, Tokyo, Japan

K. Shimizu (✉) · M. Ohki
Molecular Oncology Division, National Cancer Center Research Institute,
5-1-1 Tsukiji, Chuo-ku,
Tokyo, 104-0045, Japan
e-mail: kkikuchi@gan2.res.ncc.go.jp
Tel.: +81-3-35422511
Fax: +81-3-35420688

S. Mimaki · T. Sakiyama · T. Ohta
Center for Medical Genomics, National Cancer Center Research Institute,
Tokyo, Japan

J. Yokota
Biology Division, National Cancer Center Research Institute,
Tokyo, Japan

K. Nakachi
Department of Epidemiology, Radiation Effects Research Foundation,
Hiroshima, Japan

Introduction

Nijmegen breakage syndrome (NBS) is an autosomal recessive chromosomal instability disorder that is characterized by microcephaly, growth retardation, immunodeficiency, and predisposition to malignancy. Approximately 40% of NBS patients to date have developed a malignancy before the age of 21, the majority of which were lymphomas (The International Nijmegen Breakage Syndrome Study Group 2000). Other malignancies have also been described, including acute lymphoblastic leukemia (ALL), acute myeloblastic leukemia (AML), rhabdomyosarcoma, medulloblastoma, and glioma. In addition, development of aplastic anemia (AA) has recently been reported (Resnick et al. 2002). The gene responsible for NBS, *NBS1*, encodes a 95-kDa protein (Matsuura et al. 1998; Varon et al. 1998; Carney et al. 1998) that interacts with MRE11 and RAD50 in a DNA repair complex (Carney et al. 1998), and is localized at the telomere ends in association with telomere-repeat binding factor (TRF) proteins (Zhu et al. 2000; Wu et al. 2000). The majority of NBS patients are homozygous for the founder mutation within *NBS1*, 657del5, that leads to a prematurely truncated protein (Matsuura et al. 1998; Varon et al. 1998; Carney et al. 1998). NBS carriers also have a high incidence of malignancy (Seemanova 1990). Therefore, the potential pathogenetic role of *NBS1* mutations in sporadic malignant disease, especially lymphoid malignancies, has recently been investigated. Several studies on patients with non-Hodgkin's lymphoma (NHL) and breast cancer have shown that *NBS1* mutations are not a major

cause of carcinogenesis (Hama et al. 2000; Stanulla et al. 2000; Stumm et al. 2001; Cerosaletti et al. 2002; Carlomagno et al. 1999), while Varon et al.(2001), in their study on 47 children with a first relapse of ALL, identified seven cases with four novel missense mutations in *NBS1*. Of the novel mutations, an I171V mutation of germline and/or somatic origin was found in four cases and was not observed in normal controls, suggesting that I171V may be a susceptibility factor in the pathogenesis of childhood ALL. In this study, we analyzed 53 patients with hematological malignancies, nine patients with AA and 413 healthy controls for the presence of the I171V mutation and found one patient with AA who carried a homozygous I171V mutation associated with genomic instability.

Materials and methods

Patient samples and case report

The study comprised 46 patients with leukemia, seven patients with lymphoma, and nine patients with AA, all of Japanese origin, who had been diagnosed before16 years of age (Table 1). The patients had neither the features nor a family history of NBS. Most patients were examined after achieving complete remission.

Patient NCC56 is an 11-year-old Japanese girl who was diagnosed with idiopathic severe AA when she was nine-years-old. After successful immunosuppressive therapy, her current blood analysis is normal except for mild thrombocytopenia. Morphologic and cytogenetic analyses of her bone marrow have always yielded normal results. GPI-anchored protein-deficient clones have never been observed in her peripheral blood (PB), and her lymphocytes have exhibited no increase in chromosome breakage when treated with mitomycin C. Informed consent for this study was obtained according to protocols approved by the institutional review boards of the National Cancer Center and Keio University School of Medicine.

Table 1 Distribution of diseases screened for I171V in *NBS1* (*ALL* acute lymphoblastic leukemia, *AML* acute myeloblastic leukemia, *CML* chronic myelogenous leukemia, *CMMoL* chronic myelomonocytic leukemia, *JMML* juvenile myelomonocytic leukemia, *NHL* non-Hodgkin's lymphoma)

Disease	*n*
Leukemias	46
ALL	29
AML	13
CML	1
CMMoL	1
JMML	2
Lymphomas	7
NHL	6
Hodgkin's lymphoma	1
Aplastic anemia	9
Idiopathic	8
Hepatitis-associated	1
Total patients	62

PCR, template preparation, and pyrosequencing

Genomic DNA was extracted from PB mononuclear cells obtained from the patients using a Blood Maxi Kit (Qiagen, Tokyo, Japan). A panel of 413 DNAs isolated from PB mononuclear cells of unrelated healthy volunteers, all of Japanese origin, was screened as controls. Exon 5 of *NBS1* was amplified by PCR using a forward biotinylated primer (5′-TGGATGTAAA-CAGCCTCTTTGT-3′) and a reverse unlabeled primer (5′-GGCTGCTTCTTGGACTGAAC-3′). After immobilization of the PCR products on streptavidin-coated beads (Dynabeads M-280-streptavidin, Dynal Biotech, Oslo, Norway) and elution of single-stranded DNA, sequencing primers were annealed to the template. Pyrosequencing was performed automatically on a PSQ96 system using enzymes and reagents from a PSQ96 SNP Reagent Kit (Pyrosequencing, Uppsala, Sweden). The sequencing primer (5′-AAAATATTCTGGCTTTA-3′) was designed so that the terminal residue hybridized to the base immediately adjacent to the mutation (A→G) at nucleotide 511 in *NBS1*.

Cytogenetic and SKY analyses

Lymphoblastoid cell lines (LCLs) were derived from the lymphocytes of patient NCC56, her father, and five healthy volunteers who were used as controls. Cytogenetic analysis was performed on metaphase spreads obtained from these LCLs using standard protocols (Harnden and Klinger 1985), without prior incubation with clastogens. At least 40 metaphase figures from each sample were examined for spontaneous chromosomal aberrations. SKY and DAPI staining were performed according to the manufacturer's instructions (Applied Spectral Imaging, Migdal Ha-Emck, Israel). Images of chromosome spreads were acquired using a charge-coupled device camera.

Results

We screened for the I171V mutation in *NBS1* by pyrosequencing of genomic DNA extracted from the blood cells of a total of 62 children with hematological malignancies and AA and 413 healthy controls. While none of the 53 patients with hematological malignancies showed I171V, one of the nine patients with AA, patient NCC56, had an A→G transition in exon 5 at nucleotide 511 of *NBS1*, leading to the I171V substitution. The allelic pattern on the pyrogram indicated that patient NCC56 is homozygous for the A→G transition (Fig. 1A). Subsequently, we analyzed blood cells from her healthy parents, which demonstrated that her mother is homozygous for the wild-type A, whereas her father is heterozygous A/G and therefore a carrier of the mutation (Fig. 1A). To investigate whether the homozygous change in patient NCC56 occurred only in her blood cells, we performed pyrosequencing of her hair and nails. The obtained pyrograms

demonstrated the same allelic pattern as the pyrogram from her blood cells (Fig. 1B), indicating that the homozygous I171V mutation is present in her hair and nail cells as well as in her blood cells. In contrast to the pyrosequencing results, conventional cycle sequencing of her blood cells indicated that she is heterozygous for I171V, just like her father (data not shown). Since pyrosequencing is very quantitative, unlike cycle sequencing, we concluded that the majority of her blood cells are homozygous for I171V and the minority are heterozygous, rather than her mother being a germline mosaic with respect to I171V. Furthermore, the analysis of the adjacent polymorphic site at nucleotide 553 by pyrosequencing and cycle sequencing showed that the patient and her mother are both heterozygous G/C and that her father is homozygous for C (data not shown). We therefore postulated that patient NCC56 inherited the germline I171V mutation from her father and the wild-type allele from her mother, and that the second I171V hit occurred on the wild-type allele early in embryonic development. The patient's wild type allelic frequency was estimated to be less than 5% by the proportion of C- and T-peak heights on the pyrograms (Fig. 1A).

To investigate whether I171V is involved in genomic instability, we performed a cytogenetic analysis of LCLs, in the absence of clastogens, from patient NCC56, her father, and five unrelated healthy controls. The chromosomes from patient NCC56 and her father exhibited a more than six-fold increase and a more than four-fold increase, respectively, in the frequency of polyploidy ($n \geq 69$), mainly near-tetraploid, compared with the controls (Fig. 2A). Furthermore, patient NCC56 had a substantially higher frequency of chromosomal structural aberrations than her father and the controls, while her father had a slightly higher frequency of structural aberrations than the controls (Fig. 2B, C). Chromosomal breakages, quadriradial forms, end-to-end fusions, and abnormal configurations of homologous chromosomes are characteristic of the structural aberrations found in patient NCC56; a representative metaphase spread is displayed in Fig. 2D. In addition, SKY analysis of LCLs from patient NCC56 noticeably demonstrated chromosomal breaks and end-to-end fusions (Fig. 2E). These results suggest that a homozygous I171V mutation may affect genomic integrity in the patient's hematopoietic cells.

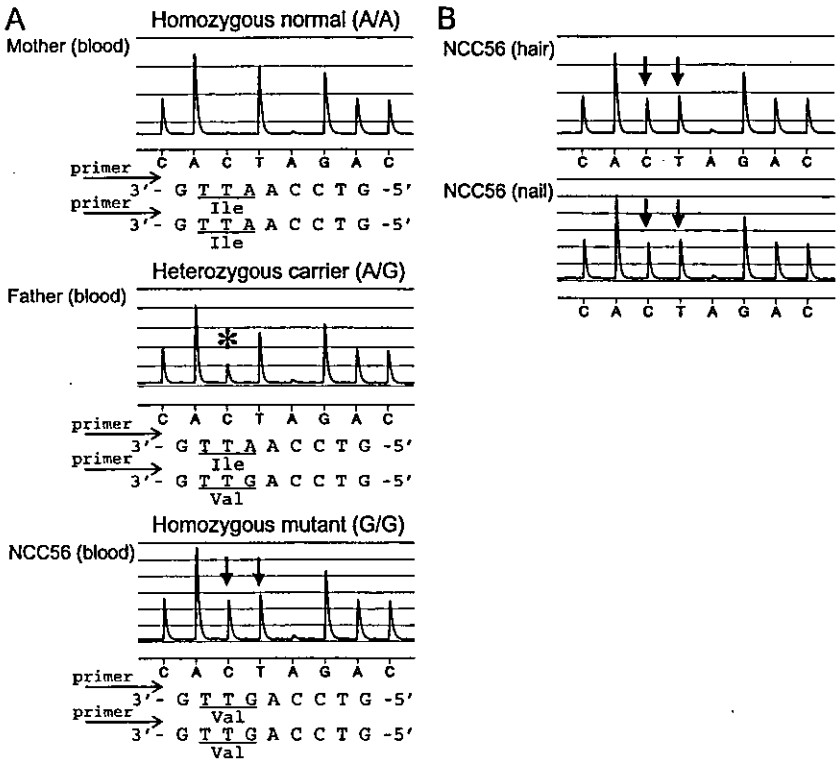

Fig. 1A, B Pyrosequencing analysis of the reverse strand of nucleotides 506–514 of *NBS1*. **A** Mutation analysis of *NBS1* in PB mononuclear cell DNA from patient NCC56 and her parents (sequences read in reverse). Her healthy mother is homozygous for A at nucleotide 511. Her healthy father is heterozygous A/G at nucleotide 511. Note the appearance of the C indicated by an *asterik* and the relative reduction in the T-peak. Patient NCC56 is homozygous for G at nucleotide 511. Note that the C- and T-peaks indicated by the *vertical arrows* are equivalent in height. The *underlined nucleotides* indicate the SNP site (A/G) corresponding to amino acid Ile (ATT) or Val (GTT). The *arrows* show primer position. A-peaks are approximately 20% higher than C-, G-, or T-peaks due to the use of α-thio-dATP in the pyrosequencing reaction system. **B** Mutation analysis of *NBS1* in hair and nail DNA from patient NCC56. Both pyrograms indicate the same allele distribution as the pyrogram obtained from PB mononuclear cell DNA from this patient

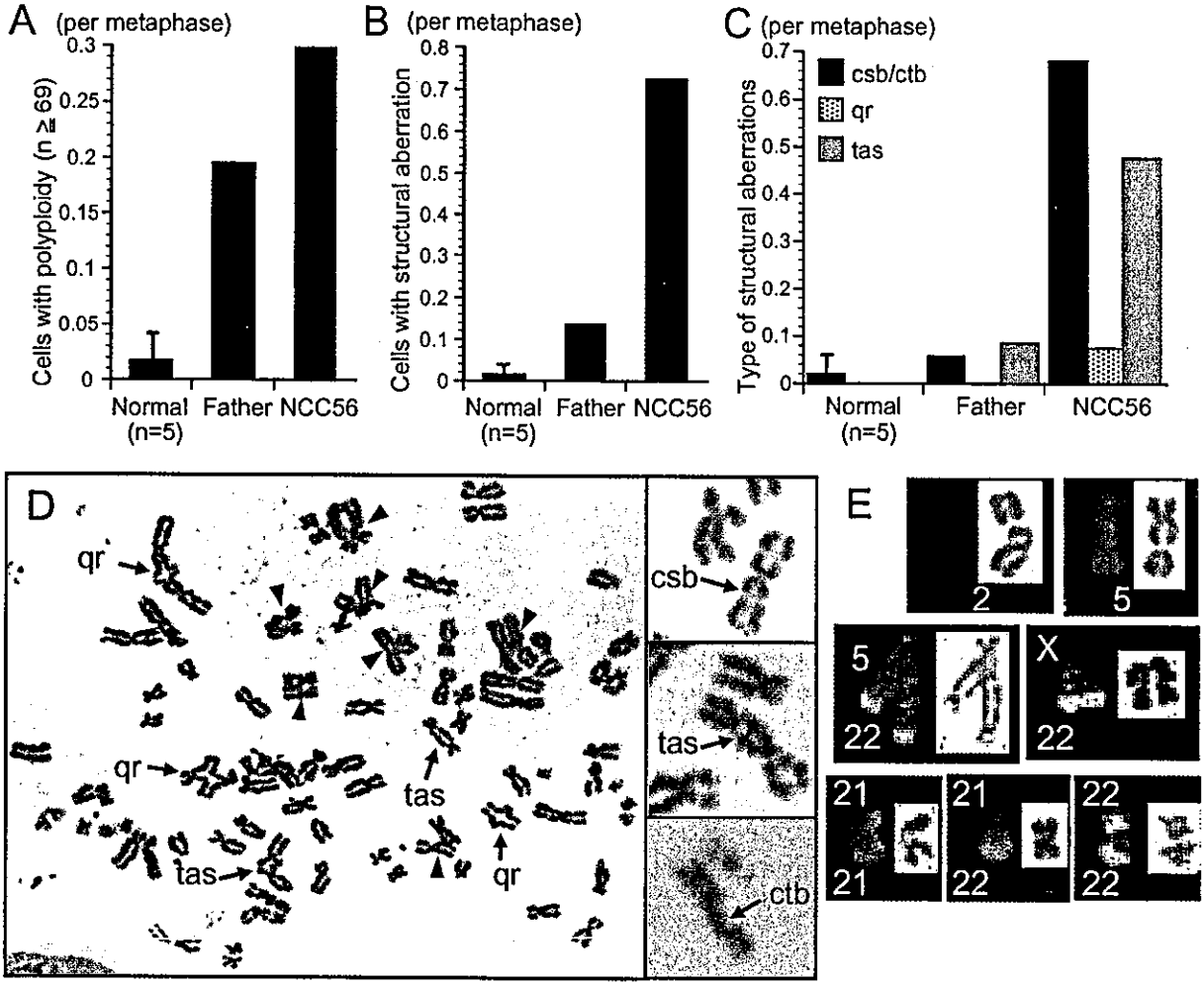

Fig. 2A–E An increase in spontaneous chromosomal aberrations in LCLs from patient NCC56 in the absence of clastogens. Comparison of the frequency of cells with polyploidy (**A**) and structural aberrations (**B**) and the frequency of each type of structural aberration per metaphase spread (**C**) in patient NCC56, her healthy father, and the controls. At least 40 metaphase figures were scored for each sample. The *bars* represent the mean±SD of pooled data from five healthy controls. **D** Representative metaphase spread of LCLs from patient NCC56. Remarkable aberrations are enlarged *on the right*. *Arrows* indicate the characteristic chromosomal breakages, interchromosomal attachments that form "radial" structures, and "telomere–telomere" fusions. *Arrowheads* indicate abnormal configurations of homologous chromosomes. **E** Partial SKY of LCLs from patient NCC56. Chromosomes are presented in SKY display colors (*left*) and the inverted DAPI staining image of the same chromosome (*right*). Chromosomal breaks and end-to-end fusions that were undetectable with G-banding were identified (*csb* chromosome break, *ctb* chromatid break, *qr* quadri-radial formation, *tas* telomeric end-to-end association)

Discussion

We have for the first time identified an *NBS1* mutation in a non-NBS patient with AA. NBS with AA has been previously reported in two Russian patients, who were homozygous for the founder mutation 657del5 (Resnick et al. 2002). The coincidence of AA and *NBS1* mutations suggests that bone marrow failure is etiologically related to defects in NBS1 function. Unlike 657del5, the homozygous I171V mutation did not generate the clinical phenotypic characteristics of NBS, but may have contributed to genomic instability leading to the onset of AA. Although why the patient's condition has presented as typical acquired AA is open to debate, genomic instability may have produced an aberrant protein in her hematopoietic cells thereby provoking an immune response at the onset of AA (Young and Maciejewski 1997). We suspect that patient NCC56 may relapse or develop hematologic clonal disease and even solid malignancies in the future.

I171V occurs in the breast cancer carboxyl-terminal (BRCT) domain, which is widely conserved in proteins related to DNA repair and cell-cycle checkpoints (Bork et al. 1997). The BRCT domain of NBS1 has been shown to be essential for the interaction between NBS1 and histone γ-H2AX after irradiation (Kobayashi et al. 2002), suggesting that the BRCT domain of NBS1 plays an important role in DNA damage recognition. I171V alters an amino acid that is conserved in *Drosophila*, chicken, rat, and mouse. Furthermore, because I171V has been confirmed in our patient with AA, as well as in four previously known cases with relapsed ALL (Varon et al. 2001), it is possible that I171V plays a pathogenetic role

through an alteration in the function of BRCT. On the other hand, we also found heterozygous I171V mutations in five individuals among the 413 normal controls screened, which corresponds to 1.2% of the Japanese population. Thus, I171V may not be directly linked to pathogenesis; at least, the heterozygous state is unlikely to inactivate NBS1 function through a dominant negative effect. The elevated spontaneous chromosomal instability in LCLs from the AA patient's father suggests an effect of haploinsufficiency of *NBS1* on genome integrity. Given the high incidence of the heterozygous I171V mutation in relapsed ALL (Varon et al. 2001), genomic instability in the heterozygous state may contribute to the advancement of disease that is established through a different mechanism.

The involvement of the *NBS1* mutation with AA suggests the possibility that NBS1 is functionally linked to Fanconi anemia (FA) proteins, because FA is the commonest type of congenital AA and shares several clinical features with NBS (Alter and Young 1998). In support of this possibility, recent studies have reported a direct or a functional interaction of FA proteins with the RAD50/MRE11/NBS1 complex (Nakanishi et al. 2002; Pichierri et al. 2002; Pichierri and Rosselli 2004). We expect that, in a subset of patients, AA may also arise due to dysfunction in other proteins cooperating with NBS1 and FA proteins in DNA-repair processes and cell cycle checkpoint control.

This study suggests that I171V may play a minor role in constitutional susceptibility to childhood hematological malignancies as well as AA, while other *NBS1* variants could contribute to the sporadic occurrence of these diseases. Naturally, further mutation analyses of more patients will be required to verify this assumption. This case is important as it is the first suggestion of a relationship between an *NBS1* mutation and acquired AA.

Acknowledgements We thank Drs. M. Sasaki, K. Sugita, and T. Suzuki for providing patient samples. We also thank M. Mori and C. Hatanaka for technical assistance. This work was supported by the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Drug ADR Relief, R and D Promotion, and Product Review of Japan.

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Cancer Causes and Control 15: 551–558, 2004.

Excess concordance of cancer incidence and lifestyles in married couples (Japan): survival analysis of paired rate data

Shizue Izumi[1,*], Kazue Imai[2] & Kei Nakachi[2]
[1]*Department of Statistics, Radiation Effects Research Foundation (RERF), Hiroshima, Japan;* [2]*Department of Radiobiology/Molecular Epidemiology, Radiation Effects Research Foundation (RERF), Hiroshima, Japan*

Received 9 January 2004; accepted in revised form 2 March 2004

Key words: cohort study, spouses, survival analysis, tobacco smoking, alcohol drinking.

Abbreviations: CI – confidence interval; CR – cross-ratio; ICD – International Classification of Diseases; RR – rate ratio

Abstract

Objective: A population-based cohort study was conducted to examine concordance of cancer incidence and lifestyle in married couples.
Methods: Cancer incidence from 1 September 1986 to 30 June 2000 was followed for a baseline cohort of 2601 community-living married couples aged 40–84 years who were cancer-free and had completed an epidemiologic questionnaire in 1986 in Saitama, Japan. We computed age-adjusted cross-ratios: spouse-with/spouse-without rate ratios as a measure of association for paired rate data, using a bivariate survival analysis method.
Results: Concordance of lifestyles for couples was high for dietary habits, and low for smoking and alcohol habits. During the 14 year follow-up, 464 cancer cases occurred (husbands only: 279, wives only: 119, both: 33 couples). Wives whose husbands developed cancer were at an increased risk of developing cancer themselves, compared with those whose husbands had not had cancer (CR: 1.70, 95% CI: 1.12–2.58). Mean age at diagnosis was 68.2 years for husbands and 66.0 years for wives. And correlation of cancer incidence was further seen among couples who shared habits of smoking and/or drinking.
Conclusions: Our data suggest that shared lifestyles including smoking and drinking habits are associated with an excess concordance of cancer incidence among married couples.

Introduction

Environment, rather than inherited genetic factors, plays the principal role in causing sporadic cancers. The latest study of twins indicates that inherited genetic factors made only a minor contribution to susceptibility to cancer at most sites [1]. In contrast to twins, most married couples are genetically unrelated, and they share home environments and lifestyles such as dietary habits and other exogenous exposures for decades after marriage. Thus, there might be a mutual effect of these factors on cancer occurrence among married couples.

Studies of spousal aggregation of cancer occurrence are now being utilized for the purpose of assessing shared environmental effects using data from various sources: death certificates [2], epidemiologic survey [3], hospital records [4–6], commercially-based medical care program [7], and family cancer registry [8–10]. Furthermore, recent studies have found concordant increases of tobacco-, alcohol-, or infection-related cancers among spouses [7–10]. It is recognized that the risks of developing these cancers increase with selected lifestyle factors, as well as genetic and acquired host factors [11, 12]. However, no cohort studies yet conducted have examined the spousal concordance of cancer risks in

* Address correspondence to: Dr Shizue Izumi, Department of Statistics, RERF, 5-2 Hijiyama Park Minami-ku, Hiroshima 732-0815, Japan. Ph.: +81-82-261-3131; Fax: +81-82-262-9768; E-mail: izumi@rerf.or.jp

relation to lifestyle factors using individual data from both husbands and wives.

In this paper, we examine two working hypotheses using cohort data: whether cancer risks of wives are correlated with those of their husbands (*i.e.*, spousal aggregation of cancer occurrence) and what shared environmental factors affect the strength of the correlation (*i.e.*, causes of spousal aggregation).

Materials and methods

Study population

The Saitama cohort study was founded in 1986 to investigate risk factors of cancer and non-cancer diseases in a sample of 8552 residents (men 4108, women 4444) aged 40 years or older in a town in Saitama Prefecture, Japan. Numerous family clusters were included in the cohort. Details of this study methodology have been published elsewhere [13, 14]. The cohort members completed an epidemiologic questionnaire, based on informed consent, and the Ethical Committee of Saitama Cancer Center approved the research protocol for the epidemiologic studies.

The baseline cohort consisted of 2601 Japanese married couples who shared household environment (the same last name, home address, and phone number), had an age difference no more than nine years, and were cancer-free at baseline in 1986. To reduce recall bias, respondents who were over 85 years of age at the time of survey were excluded. More than half of the couples lived with their offspring and/or their parents. Household information from the subsequent epidemiologic survey in 2001 supported the marriage relationship we used for the present study. Since information regarding age at wedding and duration of marriage was not collected in the surveys, we might have excluded some married couples who used different last names but shared a household.

Assessment of genetic predisposition and lifestyle

The self-administrated epidemiologic questionnaire included questions on parental history of cancer (yes, no), body mass index (<18.5, 18.5–25.0, >25.0 kg/m^2), cigarette smoking (never, past, current), alcohol drinking (never, past, occasional, regular), and dietary habits (daily or almost daily, 2–4 times per week, once per week or less), which assessed the average frequency of intake at the time of survey. The food-frequency questionnaire included nine items: fish (fish, shell fish), meat (chicken, pork, beef, ham, sausages), fruits, soy bean products (tofu, fermented soybeans, boiled soybeans), eggs, dairy products (milk, cheese, yogurt), all vegetables except pickles, green vegetables, and yellow vegetables. No questions were asked about parental age at diagnosis of cancer.

Follow-up/identification of cancer cases

We assessed cancer incidence from 1 September 1986 to 30 June 2000. Cancer incidence was ascertained primarily through death certificate, information from local health centers, and national health insurance receipts. We screened all possible cancer cases upon initial diagnosis of cancer and every year thereafter, and registered only cases that were confirmed as to primary site, histology, and date of diagnosis through inquiry at the hospitals. Cases of cancer were classified based on the *International Classification of Diseases; ninth Revision* (ICD-9) [15]; the end point was cancer at any site (ICD-9 140–208). Among potential etiologic groups, tobacco-related sites (oral cavity, pharynx, esophagus, pancreas, larynx, lung, bladder) and alcohol-related sites (oral cavity, esophagus, larynx, liver) were also considered to examine the effects of common lifestyle and shared environmental factors on excess spousal concordance of cancer occurrence [6]. Latent cancers (*e.g.*, thyroid, carcinoma in situ, non-invasive carcinomas) were not included as cases. We defined age at which the individual ceased to contribute person-years of follow-up (age at diagnosis of first primary cancer, death, or the end of follow-up, whichever occurred first) and final case status (cancer or cancer-free) at that time for analyses.

Statistical methods

First, we examined the concordance of various lifestyle factors for the 2601 couples, computing observed and expected probability of concordance with p values from the chi-square test. Next, we examined any association of these factors with individual cancer rates, computing rate ratios (RRs) with 95% confidence intervals (CIs), adjusting for age, sex, birth year, parental family history of cancer, and age at the time of survey, using Cox regression models. Based on the results, we selected lifestyle risk factors to examine the possible relation to concordance of cancer incidence in the subsequent case-control data analysis.

Finally, paired cancer incidence rates among husbands and wives were analyzed using a bivariate survival analysis method [16, 17]. Cross-ratios (CRs): spouse-with/spouse-without rate ratios were computed to measure an association of paired cancer rates between