

Case Report

## Chromosomal Instability and Double Minute Chromosomes in a Breast Cancer Patient

Hrvoje Lalić<sup>a\*</sup>, Črtomir Volavšek<sup>b</sup>, and Biserka Radošević-Stašić<sup>c</sup>

<sup>a</sup>Department of Occupational and Environmental Medicine, Medical Faculty, University of Rijeka, Croatia,

<sup>b</sup>Department of Medical Cytogenetic and Toxicology, Center for Environmental Health, Ljubljana, Slovenia, and

<sup>c</sup>Department of Physiology and Immunology, Medical Faculty, University of Rijeka, Croatia

Cytogenetic analysis was performed in peripheral blood lymphocytes (PBL) of a woman with ductal breast carcinoma, who as a hospital employee was exposed professionally for 15 years to low doses of ionizing radiation. The most important finding after the chemotherapy in combination with radiotherapy was the presence of double minutes (DM) chromosomes, in combination with other chromosomal abnormalities (on 200 scored metaphases were found 2 chromatid breaks, 10 dicentrics, 11 acentric fragments, 2 gaps, and 3 double min chromosomes). In a repeated analysis (after 6 months), DM chromosomes were still present. To rule out the possibility that the patient was overexposed to ionizing radiation at work, her blood test was compared with a group of coworkers as well as with a group of professionally unexposed people. The data rejected this possibility, but the retroactive analysis showed that the patient even at the time of employment had a moderately increased number of chromosomal aberrations (3.5%) consisting of 3 isochromatids and 4 gaps, suggesting that her initial genomic instability enhanced the later development. The finding of a continuous presence of rare DM chromosomes in her PBL (4 and 10 months after radio-chemotherapy) was considered as an indicator of additional risk, which might have some prognostic significance.

**Key words:** breast carcinoma, chromosomal instability, double minutes, ionizing radiation

**B**reast cancer is the most common tumor occurring in women in the western world. When detected in its early stages, it is highly treatable by surgery, radiation therapy, chemotherapy, and hormonal therapies, but the prognosis and selection of therapy are influenced by patient age, stage of the disease, and pathologic characteristics of the primary tumor, including the presence of estrogen and progesterone-receptor levels in the tumor tissue [1–3]. Additional predictive and prognostic fac-

tors are the presence of inherited germ-line mutations in the genes BRCA1 and BRCA2, localized to chromosome 17q21 and chromosome 13q12–13, respectively, which predispose women to breast, ovarian, and other cancers [4–8], as well as to acquired somatic perturbations, which result in further genetic instability that contributes to most, if not all, human cancers [9, 10]. A loss of genome stability results in a mutator phenotype, which enables cancer cells to bypass the host regulatory processes that control cell location, division, expression, and death, provoking the progression of cancer [11]. However, although this hypothesis predicts that some tumors acquire a mutator phenotype necessary for tumorigenesis,

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\*Corresponding author. Phone: +385-51-371-094; Fax: +385-51-371-094  
E-mail: biserr@medri.hr (H. Lalić)

genomic instability affects processes such as DNA repair and recombination, checkpoint control of the cell cycle, and transcription, which are fundamental not only for tumor cells, but to all cells. In this sense, chromosomal aberrations seen commonly in peripheral blood lymphocytes (PBL) of cancer patients both before [12–16] and after radio or chemotherapy [12–18] imply that tumors might grow due to the concomitant dysfunction of the immune system, which is necessary for recognition and elimination of neoplastic cells. Furthermore, this genetic instability might also be responsible for the appearance of a hypersensitivity to genotoxic factors such as ionizing radiation and chemotherapeutic agents [16, 18], thus affecting the disease outcome.

To emphasize the importance of information obtained from conventional cytogenetic tests, we will present herein a case report showing a high genetic instability in PBL of a female physician with ductal breast carcinoma, who was subjected to radio and chemotherapy. Because the patient had during the last 15 years been exposed professionally to low doses of ionizing radiation, her blood test results were compared with those of a group of coworkers, as well as with professionally unexposed people. The data clearly eliminated the possibility that she was overexposed at work. However, the archived data revealed that even at the time of the employment (15 years ago) the patient had a moderately increased number of chromosomal aberrations (3.5%), suggesting that initial genomic instability probably contributed to the later development.

## Materials and Methods

**Chromosome aberration analysis.** As previously reported, a genotoxic analysis was performed by conventional metaphase analysis of peripheral blood lymphocytes, which were stained by Giemsa staining techniques [19, 20]. Briefly, short-term lymphocyte cultures were prepared using Gibco F10 medium, which was supplemented with 20% fetal calf serum, antibiotics, and phytohemagglutinin (Murex, Biotech Ltd., Dartford, England). Two cultures of each sample were prepared. The cells were harvested at 48 h following stimulation. Colchicine (0.004%) (Sigma, Chemical Co., St. Louis, MO, USA) was added 3 h before harvest. The cultures were centrifuged and subjected to a hypotonic shock (20 min, 0.075 M KCl) at 37 °C. The lymphocytes were then fixed in acetic-methanol (1:3) and air-dried with 5%

aqueous Giemsa solution for 10 min. For each individual, 200 metaphases were analyzed for the presence of structural aberrations such as chromatid and chromosome breaks (CB), acentric (AC) and dicentric (DIC) fragments, as well as for double minutes (DM) and gaps (GAP).

**Patient and disease history.** The female patient, a 47-year-old physician, had been employed for 15 years by the Pulmonary Department of the Clinical Hospital Center-Rijeka. Four years ago, a node had been identified in the upper region of the left breast that was diagnosed as fibroadenoma. A surgeon regularly controlled the tumor, but due to its progressive growth a radical mastectomy was performed in April 2002. The diagnosis was ductal carcinoma T<sub>2-3</sub> N<sub>1</sub>M<sub>0</sub>. Familiar anamnesis was negative, suggesting the appearance of a sporadic tumor, but gene mutations associated with greater risk to breast Ca were not determined. Therapy consisted of four cycles of Endoxan, Adriamycin, and Nolvadex, and 25 cycles of irradiation with 40 Gy, given by a linear accelerator. The acute skin reaction on the breast appearing after the completion of treatment was scored as moderate erythema. Clinically, the patient's illness seems to be cured, but the conventional genotoxic test, made after radio-chemotherapy, revealed a high incidence of chromosomal aberrations (presented in Results). A regularly carried out biodosimetric analysis showed that during her professional exposure, the patient had received a total doses of 133  $\mu$ Sv in the last 5 years, which did not exceed either the permitted total absorption limits (20,000  $\mu$ Sv) or the dose received by other employees working in the same Department. Retroactive analysis made at the time of her employment (15 years ago) showed, however, that she even then had moderately increased levels of chromosomal aberrations in her PBL (presented in Results), thus indicative of an initial condition of genomic instability in this patient.

**Control groups.** Control groups consisted of ten coworkers of similar age who were employed in the same Department and were exposed professionally to the same sources of ionizing irradiation, as well as of 12 age-mated unemployed individuals living in the same town who were not exposed to any known genotoxic factor.

**Statistical analysis.** Differences between the parameters found in the group of collaborators and a group of control unrelated people were analyzed by the Mann-Whitney *U* test and the Person-product moment correlation. The computer program StatSoft was used for

these analyses.

## Results

Chromosomal aberrations were scored by conventional metaphase analysis in peripheral blood lymphocytes of the reported patient 4 and 10 months after combined radio-chemotherapy for breast cancer. These data were compared with an archived chromosome aberration test of the same patient made 15 years ago, at the time when she started to work in the Pulmonary Department, as well as with findings in 10 coworkers currently employed in the same Department. An additional control consisted of 12 unexposed individuals living in the same town (Fig. 1).

The data clearly showed that the incidence of chromosomal aberrations in both control groups was very low ( $1.67 \pm 0.5$  and  $1.4 \pm 0.4$  in 200 metaphases, respectively). In contrast, the patient showed moderate genomic instability at the time of employment (15 years ago), consisting of 7 aberrations in 200 metaphases, as well as signs of very high genomic damage after radical mastectomy and radio-chemotherapy. More specifically, 4 and 10 months later, 28 and 17 chromosomal aberrations were found in 200 metaphases, respectively (Fig. 1). Qualitative analysis showed that the patient 15 years previously had 3 isochromatids and 4 GAPs on 200

metaphases (Fig. 2). Four months after radio-chemotherapy she, however, had multiaberrant "rogue" cells consisting of 3 double min chromosomes, 2 chromatid breaks, 10 dicentric fragments, 11 acentric chromosomes, and 2 gaps (Figs. 2 and 3). Six months later the numbers of unstable aberrations had gradually decreased, but the number of DM remained unchanged (Fig. 2).

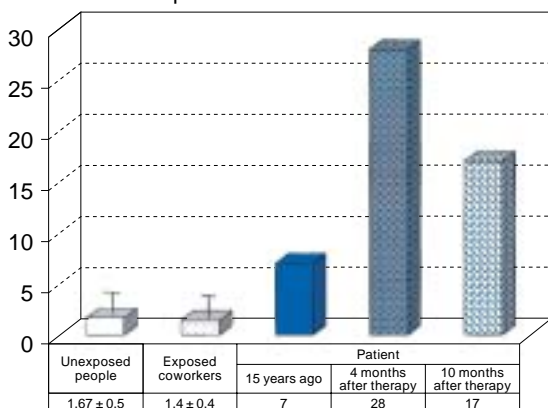
The data also showed that persons from the control group and the co-workers employed in the same department had only occasional CB, AC, and GAPs. Moreover, in co-workers there was also no correlation ( $r = 0.4$ ;  $P > 0.05$ ) between the total number of chromosomal aberrations in their PBL and their years spent working at the hospital (Fig. 4), indicating that occupational protection had been well provided for. Furthermore, the effects of cumulative professional exposure to ionizing irradiation in this patient were excluded by bio-dosimeter analysis, which showed that during the last 5 years she received only  $133 \mu\text{Sv}$  of ionizing irradiation, suggesting that multiaberrant cells might have been a sign of the individual genomic instability of this patient.

## Discussion

Chromosomal instability in PBL of breast cancer patients has been repeatedly demonstrated both before and after therapy [2, 8, 12-18], but the finding of "rogue" cells, consisting of double minutes (DM) chromosomes, in addition of other types of structural aberrations is not as frequent.

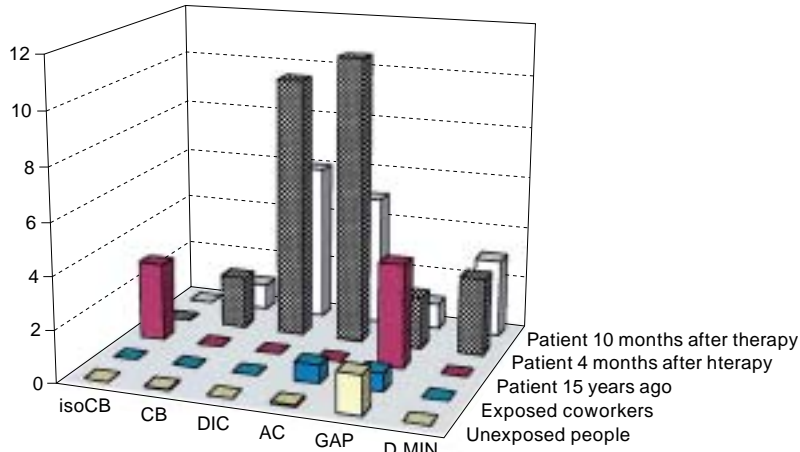
As is widely known, DM chromosomes are small chromatin bodies consisting of genes amplified in an extrachromosomal location [21, 22]. In normal human cells they are very rare, but they may be seen in primary tumors, tumor cell lines, and drug-resistant cells grown *in vitro* [23], as well as in persons occupationally exposed to ionizing radiation, where their appearance might be in positive correlation with the duration of exposure [24]. It is noteworthy that these cells were also found in blood samples from Chernobyl accident-clearance workers [25] and in cultured lymphocytes of persons exposed to the atomic bomb in Hiroshima, where among a total of 1,835 examined persons only 45 were found to exhibit rogue cells [26]. Similar findings have been obtained in astronauts exposed to irradiation with high-LET particles of cosmic origin [27, 28], but generally the incidence of rogue cells has shown a large variability

Aberrations in 200 metaphases



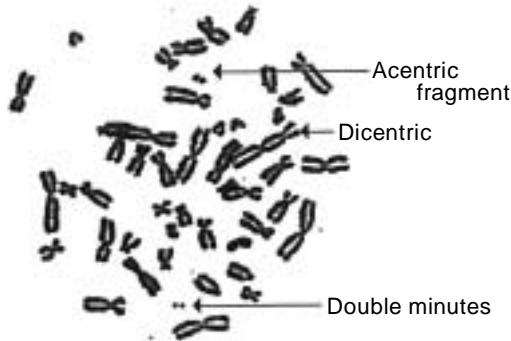
**Fig. 1** Total number of chromosome aberrations found by conventional metaphase analysis in 200 peripheral blood lymphocytes, stained by Giemsa, in unexposed people ( $N = 12$ ), medical staff professionally exposed to the ionizing condition ( $N = 10$ ), and in the patient at the time of employment (15 years ago), as well as 4 and 10 months after radio-chemotherapy for breast cancer. Data in the control groups represent mean  $\pm$  standard error.

Aberrations in 200 metaphases



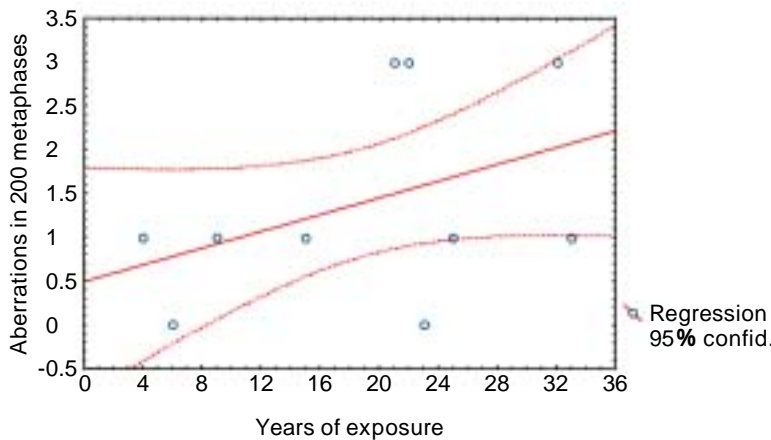
	isoCB	CB	DIC	AC	GAP	D.MIN
Unexposed people	0	0.08 ± 0.07	0	0.08 ± 0.07	1.5 ± 0.4	0
Exposed coworkers	0	0	0	0.7 ± 0.3	0.7 ± 0.2	0
Patient 15 years ago	0	0	0	0	4	0
Patient 4 months after therapy	0	2	10	11	2	3
Patient 10 months after therapy	3	1	6	5	1	3

**Fig. 2** The frequency of structural chromosome aberrations found at the time of the patient's employment and 4 and 10 months after radio-chemotherapy for breast cancer in comparison with chromosomal aberrations found in unexposed people (N = 12) and co-workers (N = 10) isoCB-isochromosomes, CB-chromatid breaks, DIC-dicentric fragments, AC-acentric fragments, D.MIN-double minutes. Data in the control groups represent mean ± standard error.



**Fig. 3** Multiaberrant cell containing double minute chromosome, dicentric, and acentric fragments, found by conventional metaphase analysis in peripheral blood lymphocytes found 4 months after radio-chemotherapy in the breast carcinoma patient.

Years of exposure and total number of chromosomal aberrations



**Fig. 4** Correlation between the total number of chromosomal aberrations found in 200 metaphases and years spent at work in a group of hospital-co-workers (N = 10).  $y = 0.49043 + 0.04787 * x$ , correlation;  $r = 0.41681$  ( $P > 0.05$ ).

between studies and individuals.

Besides their significance in radiation-exposed populations, DM chromosomes in PBL are uncommon findings in hematological malignancies such as preleukemia, leukemia, and myelodysplastic syndrome [29–33]. In most of these cases, the DM chromosomes have derived from amplification of the MYC oncogene or, less frequently, the MLL transcription factor [32, 34], as a primary cytogenetic abnormality often associated with trisomy 4 [31, 35], trisomy 6 [36], or X chromosome loss [37].

There is also a conflicting hypothesis that genetic damage in lymphocytes reflects similar damage in cells undergoing carcinogenesis, implying that PBLs might be used as the best surrogate tissue for various target organs in research for cancer and many non neoplastic diseases [38]. Moreover, some studies have shown that the ability of chromosomal aberrations in lymphocytes to predict cancer is independent of exposure to carcinogens [39], pointing to the importance of cytogenetic analysis for future cancer onset [40].

Despite the controversy in this field and the only putative causal association between chromosomal aberrations in lymphocytes and cancer risk [41], the cytogenetic studies today have considerably expanded the group of oncogenes undergoing amplification, implying that the overexpression of oncogene products might play a major role in the development of tumors and, at the same time, reflect the genetic instability of tumor cells [42–44]. Thus, in a high percentage of breast cancer patients cytogenetic analysis has revealed a variety of numerical and structural karyotypic changes in the primary tumor, suggesting that chromosomal instability might be useful in the prediction of the biological aggressiveness of breast cancers [45]. Chromosomal imbalances and the appearance of oncogene amplifications in tumor cells may occur even earlier than aneuploidy [46]. The most commonly amplified oncogenes in breast cancer involve oncogenes HER-2/neu and alterations in the expression of bcl-2, p53, c-erbB-2 proteins or N-myc oncoprotein [47–49], implying that the genetic alterations found in primary breast cancer might be of great interest with regard to disease outcome.

In light of this evidence, we can only speculate about the factors that contributed to the appearance of “rogue” cells in PBL in our case report. The patient’s family history was negative for breast cancer, suggesting the appearance of a sporadic tumor, but unfortunately the estimation of mutations in BRCA genes or other genes

that confer a high risk of breast cancer was not carried out. Her clinical past history was unspecific, providing no explanation of the first finding of moderate genomic instability in her PBL obtained 15 years earlier. Although she had since then been employed in a hospital, where she was occupationally exposed to low doses of ionizing radiation, the dosimeter analysis and chromosome aberration analysis in persons working in the same department (Figs. 1, 2 and 4) do not support the possibility that she was professionally overexposed to ionizing radiation. However, due to the initial genomic instability seen in this patient, we can not exclude the possibility that an individual hypersensitivity had led to cumulative radiation-induced genetic damage, which probably contributed to both tumor appearance and an increased reactivity to curative radio-chemotherapy.

Breast carcinoma, as well as other carcinomas, arises because of multiple changes in the genome of normal epithelial cells, with these changes often occurring in a stepwise manner. The number of natural, spontaneous DNA lesions due to thermodynamic processes and the action of free radicals (such as OH, peroxides, and reactive oxygen species) is usually about 70 million per cell per year; nevertheless, formation of the cancer is considered to be a billion-iterative system function, since after a total malignant transformation the cell has to divide some billions of times before a cancer forms [50]. The malfunction of mismatch repair genes, which produces nuclear and mitochondrial genome instability [51], as well as the exposure to ionizing radiation and chemotherapeutic drugs, which activate a range of gene products, including the DNA repair pathways and cell-cycle checkpoints, might, however, play an important role in the origin of some hereditary and sporadic human cancers [52, 53]. Controversy still remains, however, since at least 3 changes (continuous growth stimulation, immortalization, and changes on the cell surface) must first take place in order to induce a complete tumor transformation, leading to only one cancer death per 50 billion people exposed to a dose of 1 mSv of irradiation [54]. These data suggest that in our patient the prolonged professional exposure to low ionizing irradiation in the hospital was irrelevant to the later appearance of her breast carcinoma, although the contribution of radiation-induced genomic instability might be more prominent in individuals already possessing gene mutations associated with instability phenomenon. Furthermore, current knowledge emphasizes that ionizing radiation might lead to 2 unexpected

phenomena: a “bystander effect,” which can be demonstrated at low doses as a transferable factor(s) causing radiobiological effects in unexposed cells, and low-dose hyper-radiosensitivity and increased radioresistance, which can be demonstrated collectively as a change in the dose-effect relationship occurring around 0.5–1 Gy of low-LET radiation [55]. The damage signals leading to these changes seem to be transmitted from irradiated to bystander cells by gap junction-mediated intercellular communication, resulting in the activation of multiple signal-transduction pathways in bystander cells, including a significant modulation of the expression levels of p53, p21, and MDM2, and an induction of genomic instability [56]. Because extracellular signaling is in turn modulated by cytokines and growth factors from the microenvironment, it has been hypothesized that radiation-induced bystander effects and genomic instability might be, respectively, positive and negative manifestations of homeostatic processes responsible for the elimination of abnormal cells and an inhibition of neoplastic behavior [57], but numerous questions regarding the effects of radiation on membrane domains, protein-lipid interactions, membrane-bound growth factors, and proapoptotic molecules remain unanswered [58]. It should also be emphasized that radiation-induced genetic damage can take the form of early chromosomal changes that are unstable and lethal, as well as of delayed, latent damage, which is transmissible down cell generations and results in a persistently enhanced frequency of *de novo* mutations and chromosomal abnormalities [16]. In tumors, it has been proposed that induced genomic instability might contribute to the radiosensitivity of primary tumors as well as to a lower incidence, longer latency, and slower growth rate of recurrences and metastases. However, in normal tissues such as lymphocytes and keratocytes, it seems that the induced delayed chromosomal instability might contribute to a more severe or prolonged reaction to radiotherapy as a consequence of the increased cell loss, greater residual injury, and longer time required for recovery [16].

Radiation-induced bystander effects seem to be supported by the finding that stable chromosomal aberrations in circulating lymphocytes of patients treated for breast carcinoma correlate with irradiation of the internal mammary chain and the supraclavicular lymph node area and the volume of irradiated blood vessels [17], as well as with finding of intensive genotoxic effects of ionizing radiation and chemotherapy in patients with Hodgkin's

disease and other types of malignancy [59, 60]. It has been speculated that in most of these conditions, the presence of DM chromosomes in PBL point to the development of a resistance to chemotherapy [29, 61], leading occasionally to the growth of additional tumors [62, 63] and a poor disease outcome [64], but the diagnostic value of DM chromosomes remains questionable [29, 33, 36, 37]. Our data show that the PBL of a breast cancer patient, subjected to radio-chemotherapy inducing high genetic instability and the long-lasting presence of DM chromosomes, contribute to many unclear events, pointing to a possible interaction of multiple factors.

However, regardless of the unclear mechanisms involved in the patient's disease, the results emphasize the need for further continuous monitoring of her disease, indicating that chromosomal aberration analysis in PBL might be a useful technique for a better preoperative and postoperative definition of the biologic characteristics of breast carcinoma.

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