HIGH RESOLUTION GENOME-WIDE ANALYSIS OF GENETIC MARKERS AND RETROSPECTIVE BIOLOGICAL DOSIMETRY OF ABSORBED RADIATION

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Abstract

In this paper it has been evaluated the potentialities offered by DNA arrays for the detection of submicroscopic structural chromosome aberrations as a retrospective dose estimation of human exposure to ionizing radiation. To this aim we performed studies in established human cell cultures. We studied the effects of ionizing radiation (at doses 2-5 Gy) on immortalized lymphocyte cell lines using accelerated protons, in modulated beams. We determined cell growth after irradiation, morphological cell changes, clonogenic cell survival (by colony-counting), flow-cytometer cell cycle, apoptosis, necrosis, evaluation of genome-wide DNA copy numbers in >1.800.000 genetic markers by high density oligonucleotide SNP arrays.

INTRODUCTION

The major consequence of ionizing radiation (IR) exposure in cells is the generation of single or double-stranded breaks in DNA. Damage to DNA elicits a cellular stress response that includes DNA damage recognition and cell cycle arrest, followed by DNA repair or apoptosis. Although the vast majority of DNA lesions are rapidly repaired a low percentage can escape repair system and, if compatible with cell life, can persist in the cell population. Indeed, quantification of radiation-induced chromosome aberrations has been used as a retrospective dose estimation of human exposure to ionising radiation.

Structural chromosome aberrations are sensitive indicators of a preceding exposure of the hematopoietic system to ionizing radiation and cytogenetic investigations have therefore become routine tools for an assessment of absorbed radiation doses and their biological effects after occupational or therapeutical exposure or radiation accidents. Two important application fields are represented by space flights and radiotherapy of tumors. In long-duration space missions radiation environments are governed mainly by galactic cosmic ray particles, solar flares particles, and protons trapped in the Earth's radiation belts.

However the low resolution and sensitivity of the available methods for quantification of simple and complex chromosome exchanges and the large interindividual susceptibility to radiation-damage hampered the usefulness of routine applications. For many years, fluorescence in situ hybridisation (FISH) and in particular so-called "FISH-painting" has been used for the quantification of radiation-induced chromosome aberrations (for reviews see Refs. [1,2]). However such methods can offer only a partial genome analysis. More recently new techniques for the detection of chromosome aberrations have been established that allow a simultaneous detection of each of the 24 human chromosomes in individual colours. Two widely used methods are available to perform such analysis: the filter-based multi-fluor FISH (mFISH) approach [5] and the interferometer-based 'spectral karyotyping' (SKY) approach [6]. Both techniques are based on the combinatorial labelling of whole chromosome-specific painting probes for all human chromosomes using five different fluorochromes and the subsequent classification in 24 different computer-generated colours. These techniques have been applied very successfully to the analysis of cytogenetic alterations in tumors (e.g. [7,8]) and radiation-induced cytogenetic changes [9,10]. However, karyotyping only detects abnormalities at low resolutions (larger than ~5 Mb). More recently, emphasis has been placed on characterizing variations of the genome that fall in the range between the single nucleotide and visible chromosomal changes — submicroscopic structural variants (those that involve less than 5 Mb).

Recently a new range of molecular techniques based on DNA arrays has sharpened our ability to detect submicroscopic chromosomal aberrations [11]. DNA probes are arrayed on a chip and comparative genomic hybridization (CGH) is used to test for increased or decreased dosage of chromosomal regions of interest. With a single test, array CGH can detect genomic errors

for disorders that are usually identified by cytogenetic analysis and multiple FISH tests. Array CGH has been widely used in the detection of chromosomal imbalances in solid tumours, mental retardation, subtelomeric rearrangements and other constitutional chromosomal abnormalities. The detection limits of copy-number differences by array CGH depend on the probe density and the resolution of the platform used. The DNA probes range from genomic clones, most often BAC clones (80–200 kb), to oligonucleotides (25–85 bp). If genomic clones are used as the hybridization target, the size of each individual target and the distance between targets defines the size limits of what is detectable. For example, if BACs are spaced at 1-Mb intervals, any copy number change that occurs between the BACs and is smaller than 1 Mb will not be detected. Spacing BACs at 0.5-Mb intervals results in a twofold improvement in the ability of the array to detect changes. Oligonucleotide arrays can detect gains or losses of shorter stretches of the genome, and if oligonucleotides for a given region are densely arrayed, the sensitivity for detecting alterations of that region is greatly enhanced. Commercially available platforms that typically utilize oligonucleotides representing from tens of thousands to more than 1 million SNPs distributed across the genome are now frequently being used to assess copy number. These arrays or chips are used for genotyping studies, but groups of adjacent SNPs can be interrogated to determine copy number for a given chromosomal region. In addition, when combined with appropriate analytical tools, SNP-based arrays can detect other types of genomic alteration. For example, regions of copy-number neutral loss of heterozygosity could be encountered in uniparental disomy (UPD) of a genomic interval.

In particular the Affymetrix® Genome-Wide Human SNP Array 6.0 interrogates 906,600 SNPs by combining the Nsp I and Sty I PCR fractions prior to the DNA purification step and through a reduction in the absolute number of features associated with each individual SNP on the array. This array also contains 945,826 copy number probes designed to interrogate copy number variations (CNVs) in the genome; 115,000 of these probes interrogate previously identified CNVs while the remaining 831,000 are distributed across the genome for improved CNV detection.

The combination of Affymetrix SNP 6.0 arrays, the Cytogenetics Copy Number Assay, and Genotyping Console 2.1 software allows to perform high-resolution genome-wide DNA copy number analysis. The Affymetrix solution for cytogenetics also provides genotyping information, enabling detection of loss of heterozygosity (LOH), which can be used to detect uniparental disomies (UPDs). The combined high resolution DNA copy number data and the ability to detect gains, losses, and UPDs on a single array makes the Affymetrix Cytogenetics Solution a great tool for next generation cytogenetics studies.

In the present paper we propose to evaluate the utility of SNP Array 6.0 in the detection of radiation induced chromosomal aberrations. Since microgram amounts of DNA are necessary, a cell cloning step will be performed after irradiation of cell cultures.

We studied the effects of ionizing radiation on human immortalized lymphocyte cell line, at doses up to 5 Gy, using accelerated protons, in modulated beams. After irradiation, sensitivity to radiation has been evaluated by standard clonogenic survival assays. Clonal cell cultures have been prepared from different surviving clones and genomic DNA was extracted for evaluation of stable submicroscopic chromosomal aberrations by DNA arrays.

MATERIALS AND METHODS

Cell cultures. Immortalized lymphocyte cell lines are grown in "RPMI 1640" Gibco-BRL containing 10% (vol/vol) heat-inactivated FCS, 50 units/ml penicillin, and 50 mg/ml streptomycin at 37°C in a humidified incubator with 5% CO_2. Exponentially growing cells are adjusted to a density of 1-2 x10^6 cells/ml in flasks (Growth Area 75 cm^2, Working Volume 15-40 mL) the day before the irradiation experiment. Cells are plated at a density of 1-2 x 10^6 cells/flask (Growth Area 75 cm^2, Working Volume 15-40 mL) and exposed to two doses of radiation: 2 and 10 Gy.

Irradiation conditions. Cell cultures (7 ml) were irradiated in 50 ml- centrifuge tubes placed vertically. Clinical proton beams accelerated by the superconducting cyclotron at LNS-INFN, Catania (Italy) were used for proton-irradiation at a dose rate of 11.76 Gy/min. The proton beams were modulated to give uniform distribution of the absorbed dose in the entire cell suspension. The proton energy was 54.12 MeV at the entrance, and 15.43 MeV at the exit from the cell sample. A plane-parallel advanced PTW 34045 Markus ionization chamber was adopted as a reference dosimeter. The dose measurements were performed in a water phantom, according to International Atomic Energy Agency (IAEA) TRS 398. The absorbed dose to water per monitor unit (cGy/M.U.) is measured at isocenter, at the depth corresponding to the middle of the modulated beam, with the reference circular collimator (diameter = 25 mm).

Cell viability and cell growth assay. Cell suspensions are distributed in cell culture flasks at a density of 0.2 × 10^6 cells/ml. At indicated time points cells in aliquots of 50 μl are counted in a Burker-Tuerk hemocytometer. Cell viability is tested by the Trypan Blue exclusion method.

Clonogenic survival assay. After irradiation cells were harvested and plated in Nunclon TM Microwell TM 96 well plates (Nunc, Roskilde, Denmark) where each well containing 2-1000 cells/100 μl of complete medium containing IL-2 (F.C. 2 ng/ml, Invitrogen - Life Technologies Corporation Carlsbad, California cat. PH0026) + PHA (dil 1:6400, - Life Technologies Corporation Carlsbad, California cat. 10576-015). Separated cell colonies (50-100 cells/colony) were collected from polyclonal irradiated cells 21 days later and grown in flasks (25 cm^2) to reach a confluence of 1 x 10^6 cells/flask.

Flow cytometry. At indicated time points, 10^6 cells were washed with PBS, fixed for at least 2 h in 70% ethanol, and kept at -20°C. Propidium iodide (PI) staining was performed using a PI/RNAse staining buffer (PHARMINGEN 550825) according to the manufacturer's protocol. Briefly, the cell samples fixed in ethanol were washed with PBS and incubated with the staining buffer for 30 minutes at 37ºC in the dark. Cells were analyzed for DNA content by a Becton Dickinson FACS Calibur flow cytometer, CellQuestas dedicated data acquisition software. The freeware WinMDI 2.8 was used for raw data analysis, and the histogram deconvolution software Cylchred for cell cycle distribution quantization. Percentages of cell populations in various cell cycle phases were calculated as averages of values given by the WinMDI software with marker defined histogram regions, and of values given by the deconvolution software Cylchred, from measurements done on at least 2000 single cells. The fraction of apoptotic cells is determined by the sub-G1 population.

Genomic DNA extraction. Genomic DNA (gDNA) was extracted from monoclonal cell colonies expanded in 25 cm^2 flasks using the QIAamp DNA Blood Mini Kit according to the manufacturer's instructions. The concentration and the quality of the DNA was determined using a ND-1000 spectrophotometer (NanoDrop, Thermo Scientific, USA).

High-resolution genome-wide DNA copy number and SNP genotyping analysis. High-resolution genome-wide DNA copy number and SNP genotyping analysis was performed according to the protocol supplied by the manufacturer (Affymetrix, Inc., Santa Clara, CA, USA) for Affymetrix SNP 6.0 arrays that interrogates 906,600 SNPs and 945,826 copy number probes (SNP/CNV array). Briefly, 500 ng of gDNA (50 ng/ul) was digested with NspI and StyI restriction enzymes, ligated to respectively NspI and StyI adaptors, amplified by polymerase chain reaction (PCR) using a single primer with the TITANIUM™ DNA Amplification Kit (Clontech Laboratories, Inc. Montain View, CA). PCR products were purified with Agencourt AMPure® Magnetic Beads (Agencourt Bioscience Corporation, Beverly, MA) and the amplicons were quantified using a ND-1000 spectrophotometer. The 40-70 µg of purified amplicons were fragmented, end-labelled and hybridized to a Genechip Affymetrix SNP 6.0 arrays at 50°C for 16-18 hours in a GeneChip® Hybridization Oven 640 (Affymetrix, Inc.). After washing and staining in a GeneChip® Fluidics Station 450 (Affymetrix, Inc.), the arrays were scanned with a GeneChip® Scanner 3000 7G (Affymetrix, Inc.).

Array scanning and data analysis were performed using Affymetrix® "GeneChip Operating Software" (GCOS) and "Genotyping Console™" (GTC) version 3.0.1". The following algorithms were used: 1) SNP 6.0 Birdseed v2 algorithm for genotyping, 2) BRLMM-P-Plus algorithm and Hidden Markov Model with regional GC correction for copy number analysis, 3) the LOH algorithm. As a quality control of the genotyping and copy number results "Contrast QC value" and "Median Absolute Pairwise Difference" (MAPD) were calculated as implemented in the GTC 3.0.1 software. Median Contrast QC were 2.26 $\pm$0.44, 2.48 $\pm$0.37 for no irradiated and irradiated samples respectively; corresponding MAPD values were 0.30 $\pm$0.02, 0.29 $\pm$0.03.

The log2 ratio between signal for each marker in each sample and the corresponding median value in a reference group (270 HapMap individuals) provides an estimate of copy number. The $\log_2$ ratio has been smoothed using a Gaussian kernel to lower noise to improve per marker Signal to Noise ratio at the expense of blurring boundaries where copy number state changes. For each marker, the smooth is constructed using a weighted mean of the log2 ratios of surrounding markers with weights proportional to the Gaussian transform of their genomic distance from that marker. The Gaussian transform has standard deviation equal to 50000. The "allele difference value" is the difference of allele A signal and allele B signal each standardized with respect to their median values in the reference HapMap population (Fig 1).

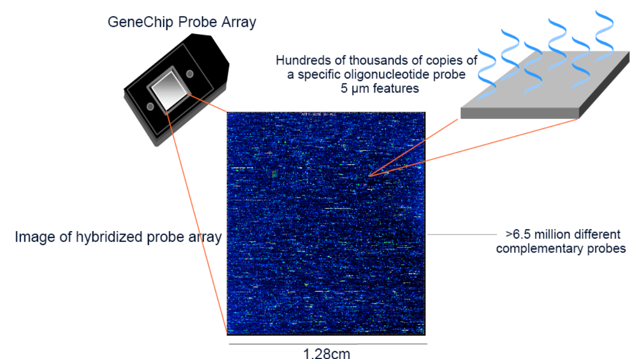

Figure 1: Schema of Affymetrix SNP 6.0 technology

RESULTS AND DISCUSSION

We have performed experiments consisting in irradiation at doses of 2 and 5 Gy of immortalized lymphocyte cell lines using accelerated protons in modulated beams. After irradiation, sensitivity to radiation has been evaluated by standard clonogenic survival assays and growth assays.

Clonal cell cultures have been prepared from surviving clones and genomic DNA was extracted from irradiated and non-irradiated clonal cell cultures for evaluation of stable submicroscopic chromosomal aberrations by DNA arrays. Irradiated and non-irradiated policlonal cultures were used as reference. After 3 weeks the proliferation of cells irradiated at 2 Gy started exponentially (Table 1) and single clones of 50-100 cells were isolated and plated to reach a confluence of 1 x 10^6 cells.

5 Gy irradiation dramatically compromised the cell growth in culture (Fig. 2) and completely blocked clonal growth (Table 1).

The high resolution Affymetrix® Genome-Wide Human SNP Array 6.0 allowed detection of gain and loss of segments smaller than 1 Mb (with a lower size limit of 10 kb). A trend toward a higher number of losses in irradiated samples compared to non irradiated samples was observed in the 2 Gy irradiated cells. More segments in the irradiated cells respect the non irradiated cells revealed the sites of alteration at a level of DNA. In particular loss of thirteen segments on chromosome 6, band q12-q13, was revealed (Fig. 2 A,B). The total size of this alteration was 936 kb. The mean loss size was 72 kb. As shown in Fig. 5 the loss segments at a 2 Gy doses were increased for all chromosomes in the irradiated cells respect no irradiated samples while the number of gains did not alter.

Results obtained from 2 Gy irradiated cultures confirmed the ability of Affymetrix® Genome-Wide Human SNP Array 6.0 to detect submicroscopic chromosomal alterations induced by irradiation.

Clonogenic survival assay					
Cells/ well	**1000**	**100**	**10**	**2**	**Clones isolated 3 weeks after irradiation**
Ctrl	8/96	4/192	1/192	0/384	7/20
2Gy	2/96	1/192	0/192	0/384	9/20
5Gy	0/96	0/192	0/192	0/384	0/20

Table 1 Number of Clones obtained at twenty-one days after irradiation.

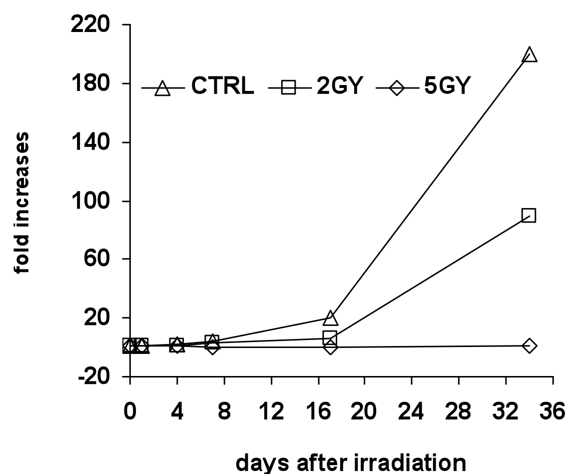

Figure 2: Cell growth in suspension cell cultures non-irradiated (Ctrl) or irradiated with 2 Gy or 5 Gy of accelerated protons. The total cell number is normalized with respect to the initial number of cells in each culture.

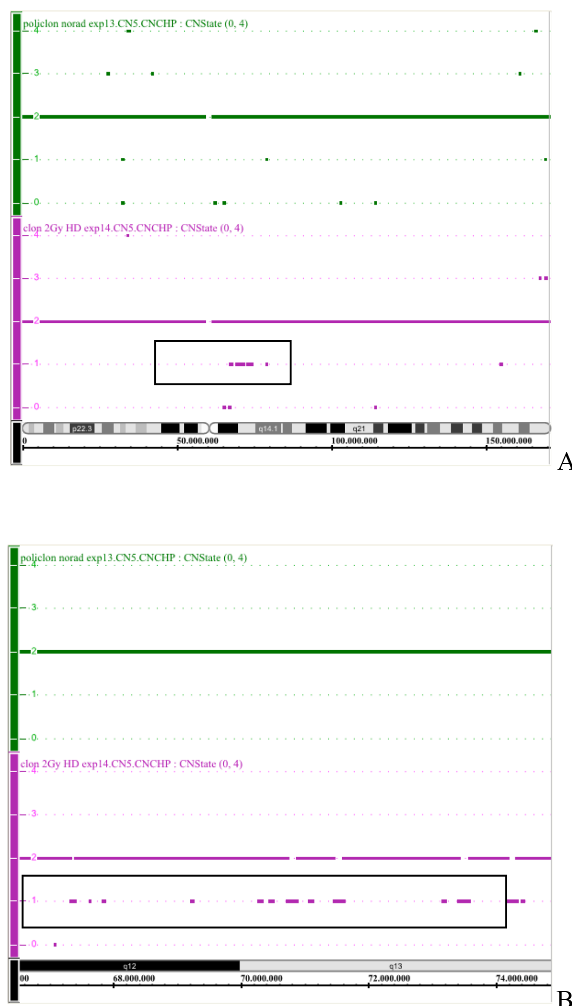

Figure 3: View of whole chromosome 6 (A). Zoomed view of 6q (q12-q13)(B).The rectangle in A and B shows the loss zone**.** Copy Number (CN) states indicate homozygous deletion (CN=0), heterozygous deletion (CN =1), diploid state (CN=2), single copy gain (CN=3), amplification $\geq$ 4 (CN=4)

Inhibition of cell proliferation was correlated with a cell cycle arrest in the G_2/M phase observed at 24 h and 48 h after irradiation with both doses (Fig. 3), indicating the presence of DNA damage and activation of the G_2/M checkpoint. Irradiation with 2 Gy but not 5 Gy increased significantly the apoptotic cell fraction, suggesting that the higher dose (5 Gy) of radiation produced also damage to the apoptotic machinery. The non-irradiated (control) cell cultures presented a proliferation index of 20% (i.e., fraction of cells with >2n DNA content, found in the S and G_2/M phases of the cell cycle), which is closely similar to previous reports on both normal and leukemic human B lymphoid cells [12-14]. In our experiments, in both 2 Gy and 5 Gy irradiated cell cultures this index doubled (data not shown) due to the G_2/M cell cycle arrest. Similar effects on G_2/M arrest and the proliferation

index were also described for a human B lymphoblastoid cell line exposed to 6 Gy of gamma radiation [12].

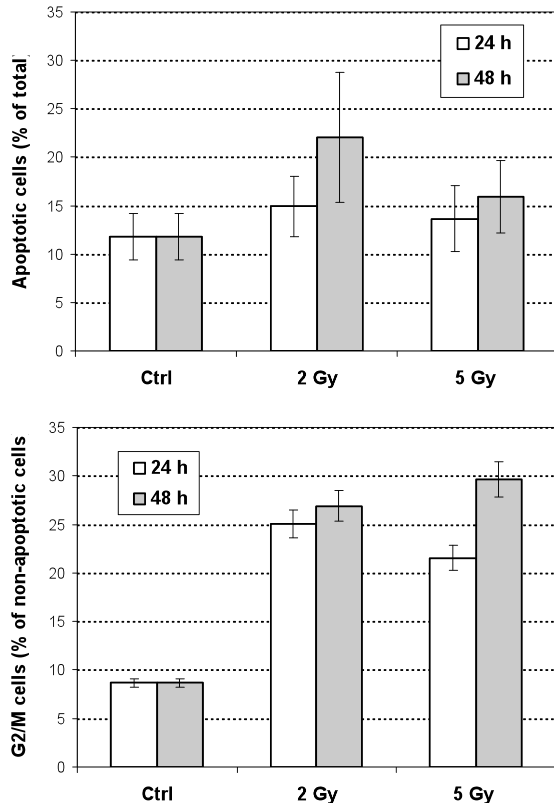

Figure 4: Apoptotic and G_2/M cell fractions assessed by flow-cytometry at 24 h and 48 h in non-irradiated cultures or after irradiation with 2 Gy or 5 Gy of accelerated protons.

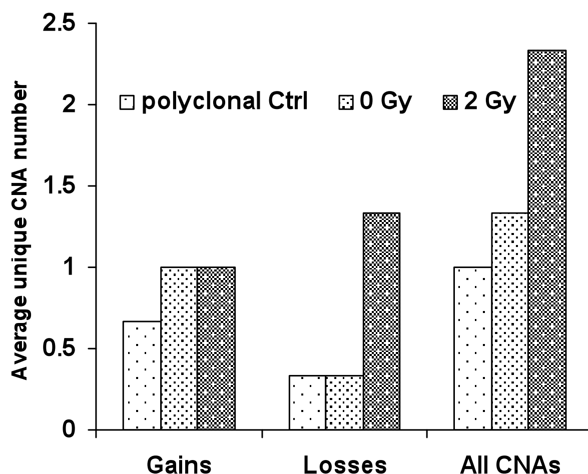

Figure 5: Mean number of gains and losses assessed by SNP array in non irradiated cells and irradiated cell at 0 - 2 Gy doses of accelerated protons.

In conclusion, the results in the present study demonstrate that DNA whole genome scanning technology using SNP6.0 DNA arrays is a promising approach for genome-wide identification of DNA copy number alterations in cells exposed to accelerated protons.

ACKNOWLEDGEMENTS

This work was partially supported by the Romanian Ministry of Education and Research under the CNCSIS Grant PNII-Ideas No. 1138/2009 and CNMP Grant PNII-Partnerships No. 71-073/2007 – PROPETHAD.

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