

To the Managing Board of the Italian Association for Shwachman Syndrome

Via Pioveghetto, 15

35136 - Padova

Subject: scientific report of the research project “Susceptibility to oxidative stress caused by ionizing radiation exposure in Shwachman Diamond Syndrome affected patients lymphocytes”.

Overall purpose: the aim of the research project “Susceptibility to oxidative stress caused by ionizing radiation exposure in Shwachman Diamond Syndrome affected patients lymphocytes” was to study the effect of ionizing radiation on lymphoblastoid cell lines obtained from SDS patient, focusing on DNA repair pathways which can be deregulated because of the lack of the SBDS gene, leading to a defective DNA repair. In particular, we have focused our attention on the analysis of cell distribution over the cell cycle and on the DNA single strand break repair pathways.

Establishment of lymphoblastoid cell lines and experimental setup: lymphocytes from peripheral blood have been collected from one control and three patients. Lymphocytes were then transformed by incubation with Epstein-Barr virus (EBV), in order to immortalize cells and obtain four lymphoblastoid cell lines. For all the experiments, cell lines have been irradiated with X-rays obtained from a 6MV linear accelerator, commonly used in radiotherapy, at the IRCCS S. Maugeri, Pavia.

DNA content analysis: cell cycle phase distribution has been evaluated by flow cytometry after staining of DNA with propidium iodide (PI). This technique is the one of most common way to characterize cell lines and allows to score cell distribution over the cell cycle. At different time after irradiation with 2Gy and 5Gy of X-rays, cells have been collected and fixed, then underwent staining and scoring. Ten thousand cells were analyzed from each sample.

The results obtained show that patients appear to be more susceptible to radiation compared to the control. Control cells show only a small increase in the G2/M phase (from 15,2% to 19,6% at 48 hours) with a correspondent reduction in the S-phase at 2Gy, and these effects increase after 5Gy; moreover, irradiation with 5Gy increases the effects observed after 2Gy, and causes an increase also in the pre-G1 phase (Fig.1A).

Concerning the three patients analyzed, patient 1 shows a behavior similar to those of control cells after 2Gy (increase in the G2/M population from 17% to 22,7%). Like control cells, the effect is more evident after 5Gy (from 17% to 34%) but the percentage of G2/M cells is larger than for control cells in the same conditions. Interestingly, the amount of apoptotic cells is lower than in the control.

Patient 2 and 3 show a similar behavior: 12h after irradiation, the percentage of cells in G2/M increase from 21,8% to 38% after 2Gy (and from 21,8% to 47% after 5Gy) for patient 2 and from 21,5% to 38,7% after 2Gy (and 21,5% to 47,1% after 5Gy) for patient 3. Both patients 2 and 3 show a recovery in 48h after 2Gy.. Interestingly, only patient 2 shows an increase in the apoptosis both for 2Gy and 5Gy irradiations (Fig. 1B).

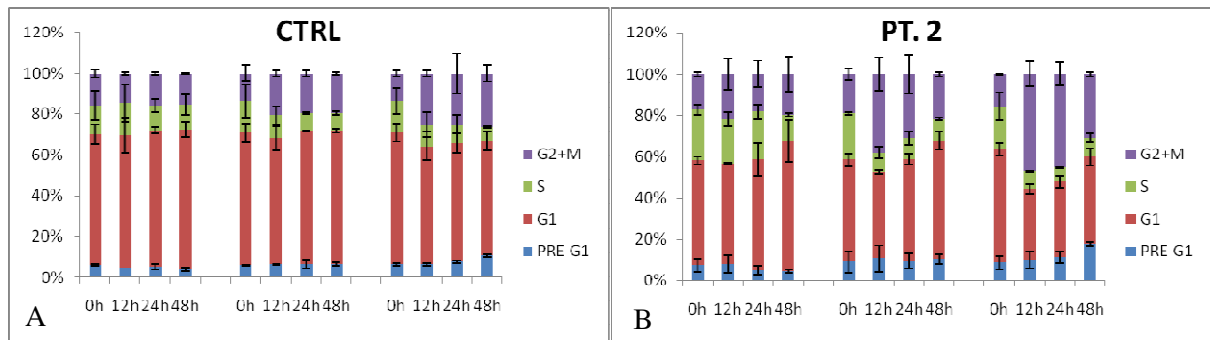


Fig. 1 - Example of cell cycle distributions after staining with PI. A - Control cells; B - Patient 2 cells.

These data suggest that the lack of SBDS causes a slowing down to allow the repair of the radiation-induced damages. Generally, patients seem to need more time to repair the damage, resulting more susceptible to the damage. These could enhance the development of altered cellular population.

DNA single strand breaks (SSB) and base oxidation analysis: the amount of DNA SSBs and the base oxidation levels have been evaluated through the COMET assay, a very flexible assay largely used to study DNA damage. After radiation exposure, cells underwent the disruption of the nuclear membrane: afterwards, samples were exposed to high pH for DNA unwinding, then underwent electrophoresis and staining with DAPI. Oxidation of purine and pyrimidine was evaluated by incubating samples with the enzymes formamidopyrimidine-DNA glycosylase (FPG) and endonuclease III (Endo III) before electrophoresis. Enzymes recognize oxidation of bases and create SSBs, that are scored in the same way as “normal” SSBs.

Analysis of one patient and one patient’s parents has been performed. The results show some differences in the amount of SSBs in the patient compared to his parent. Analysis of parent’s cells shows that 0.5, 2, 4, 8 and 24 hours after irradiation the effect of radiation is not observable. Oxidation of purines appears slightly increased in irradiated cells, with the exception of the 4-hours time-point, in which the value for irradiated cells is two times higher than for sham cells. Also the oxidation levels of pyrimidines appears to be slightly increased at 2, 4, and 8 hours, but these modulations are smaller than those measured for purines.

In the patient, the SSB values after irradiation are higher than sham cells at every time-point. The major differences can be seen at 0.5, 4 and 8 hours (Fig. 2A). In this case, the results obtained by analyzing base oxidation are different when compared to the parent’s cells. Regarding purine oxidation, levels of irradiated cells are higher at every time-point but differences at 0.5, 4, 8 and 24 hours after radiation exposure are particularly evident. It is also possible to note a time-dependent reduction in the differences between sham and 2Gy-irradiated cells (Fig. 2B). Pyrimidine oxidation seems not to be modulated by radiation, although the overall oxidation levels are very high.

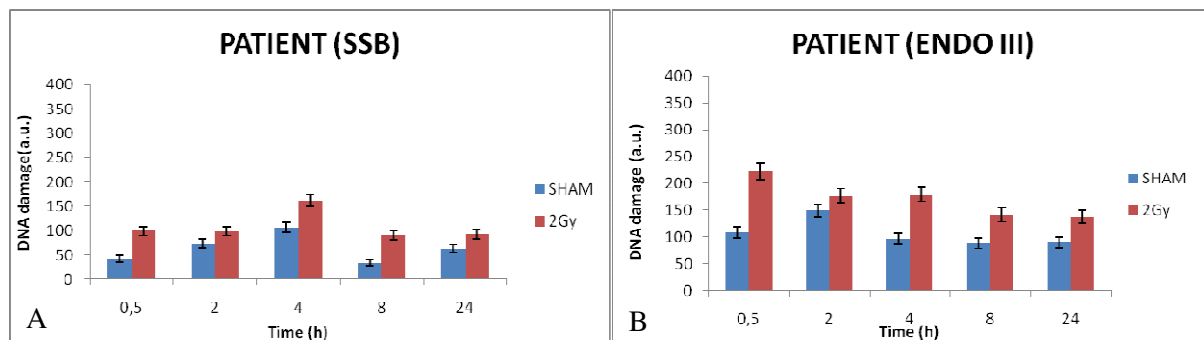


Fig. 2 - Comparison between sham-irradiated and 2Gy-irradiated patient cells. A - Analysis of DNA single strand breaks (SSBs); B - Analysis of purine oxidation.

The results obtained from the COMET analysis suggest that, besides the DNA SSB repair, also the cellular response to the oxidative stress may be altered.

**Conclusions:** an investigation of radio-induced damage caused by exposure to different doses of X-rays has been performed for lymphoblastoid cell lines obtained from controls, patients and patient's parents. In SDS-cells, the lack of SBDS gene influences the response to X-ray exposure. The results obtained analyzing cell cycle distributions suggest that the homologous recombination (HR) pathway might be affected by the absence of SBDS. Moreover, ionizing radiation seems to cause a larger amount of SSBs and of pyrimidine oxidation in the patient's cells compared to the parent. All these data suggest that the lack of SBDS can affect several DNA repair pathways besides HR, not only related to DNA single and double strand break repair but also to DNA damage caused by oxidative stress.

**Publication of results:** the results obtained have been presented at the 40<sup>th</sup> Annual Meeting of the European Radiation Research Society ("Effect of X-rays on DNA repair pathways in lymphoblastoid cell lines derived from Shwachman-Diamond Syndrome patients") and at the MICROS 2013 - 16<sup>th</sup> International Symposium on Microdosimetry ("Radiosensitivity and DNA damage repair in lymphoblastoid cell lines derived from Shwachman-Diamond syndrome patients").

Furthermore, a paper ("Radiosensitivity in lymphoblastoid cell lines derived from Shwachman-Diamond syndrome patients") has been submitted to the peer-reviewed journal "Radiation Protection Dosimetry" as a proceeding of the MICROS 2013 Symposium.