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D 9.127 – Data set on NGS-based miRNomes analysis in control, irradiated and shielded tissues

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Abstract

Radiation effects are not confined to directly irradiated tissues. The contribution of systemic "out-of-target" effects to the risks of long term detriment following exposure to radiation is largely unknown. Indeed, both protective and damaging effects have been described. From the radiation protection standpoint this level of uncertainty of the risk is problematic, as workplace, environmental and medical exposures frequently involve partial body exposure.

The SEPARATE project analysed the changes at the transcriptome, non-coding RNAs, protein and metabolic levels on out-of-field organs known to be impaired by direct ionizing radiation exposure following exposure of the lower third of the body, whilst the target organs are shielded. Deliverable 9.127 of the project describes progress to date in the molecular studies focused on establishing the role of non-coding RNAs in systemic "out-of-target" effects in hippocampus and heart through miRNome analysis based on next generation sequencing (NGS) performed at ENEA.

Overall, our results, showing a high degree of similarity in the changes induced by total and partial body irradiation in miRNA expression profiles of hippocampus and heart, are novel and indicate that in-field and out-of-field irradiation cause nearly identical modification in non-coding RNAs at 15 days post-irradiation.

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Progress summary

INTRODUCTION

MicroRNAs (miRNAs) are short segments (19–25 nucleotides) of nonprotein-coding single-stranded RNA that interfere with target gene expression acting primarily at post-translational level. A single miRNA has the potential to mediate translation of hundreds of targets and, conversely, several miRNAs can regulate the expression of one gene. miRNAs control a wide variety of cellular functions such as apoptosis, cell proliferation, differentiation, metabolism, and stem cell renewal. While most of the miRNA studies are related to cancers, accumulating evidence has demonstrated that miRNAs are also involved in various physiological and pathological other processes, examples being control of adult hippocampal neurogenesis and cardiovascular events. Given the key role of miRNAs in the cellular response to environmental stress, including ionizing radiation, with changes in the expression profiles following radiation exposure, miRNAs are possible mediator candidates of the out-of-target radiation responses. To test miRNA involvement in out-of-field effects we compared the perturbation induced by in-field and out-of-field X-ray irradiation in the hippocampus and heart.

METHODS

C57Bl/6 mice have been whole-body (WB) and partial (PB) irradiated with 2 Gy of X-rays at 10 weeks of age or left untreated. Fifteen days after irradiation, hippocampi, heart and liver were collected and total RNA was extracted using miRNeasy kit according to the manufacturer's instructions. Total RNA (500 ng) obtained by hippocampi and hearts was converted into miRNA NGS libraries using NEBNEXT library generation kit following manufacturer's instructions. Samples were sequenced on the Illumina NextSeq 500 System. All sequencing data analysis was performed using the R platform (http://www.r-project.org/) and the open-source Bioconductor libraries. Data were filtered based on sequence counts (i.e. > 8 reads per million in at least 6 samples) and pairwise comparisons of differential miRNA expression were performed using edgeR package. All miRNAs with P <0.05 were considered. The selected lists of statistically significant miRNAs were analyzed in Cytoscape (Version 3.6.1) through the application ClueGo (V. 2.5.1) and CluePedia (V. 1.5.1). The initial sets of miRNAs were enriched based on the miRTarBase database in order to obtain the top20 predicted target genes for each miRNA. The obtained networks of genes and miRNAs were then analyzed based on the Reactome pathways database to identify the relevant pathways and functions potentially perturbed by the altered miRNAs. In addition, RNA from organs collected 6 month following irradiation with 2 Gy of X-rays or collected 15 days or 6 months following irradiation with 0.1 Gy of X-rays, were employed for evaluation of quantitative expression, through qRT-PCR custom plates, of the subset of miRNAs found perturbed after NGS analysis. Data reported in this document are relative to the hippocampus and heart while liver samples have not processed yet.

RESULTS

MiRNome analysis after direct or bystander radiation exposure of the hippocampus

Since the discovery of the first microRNA 25 years ago, microRNAs have emerged as critical regulators of gene expression within the mammalian brain and, in particular, as a crucial part of the gene regulatory networks governing adult neurogenesis, where several miRNAs converge on the same transcriptional regulator forming a feedback loop to fine-tune gene expression. Deficit in miRNA biogenesis disrupt neuronal development, function and survival. MiRNA levels in the brain are sensitive to a variety of environmental stimuli, including radiation exposure. To test miRNA involvement in out-of-field effects we compared the perturbation induced by in-field and out-of-field X-ray irradiation in the hippocampus. To this aim, hippocampi were dissected from the brain of C57BL6 females unexposed or 15 days after WBI and PBI with 2 Gy of X-rays. As a criteria for up-regulation we assumed a p-value of < 0.1 and a log2FC > 3 and for down-regulation a p-value of < 0.1 and a log2FC <-3. Compared to unirradiated mice, miRNome analysis reveals 25 differentially expressed miRNAs in PBI and 19 in WBI mice (Figure 1A).

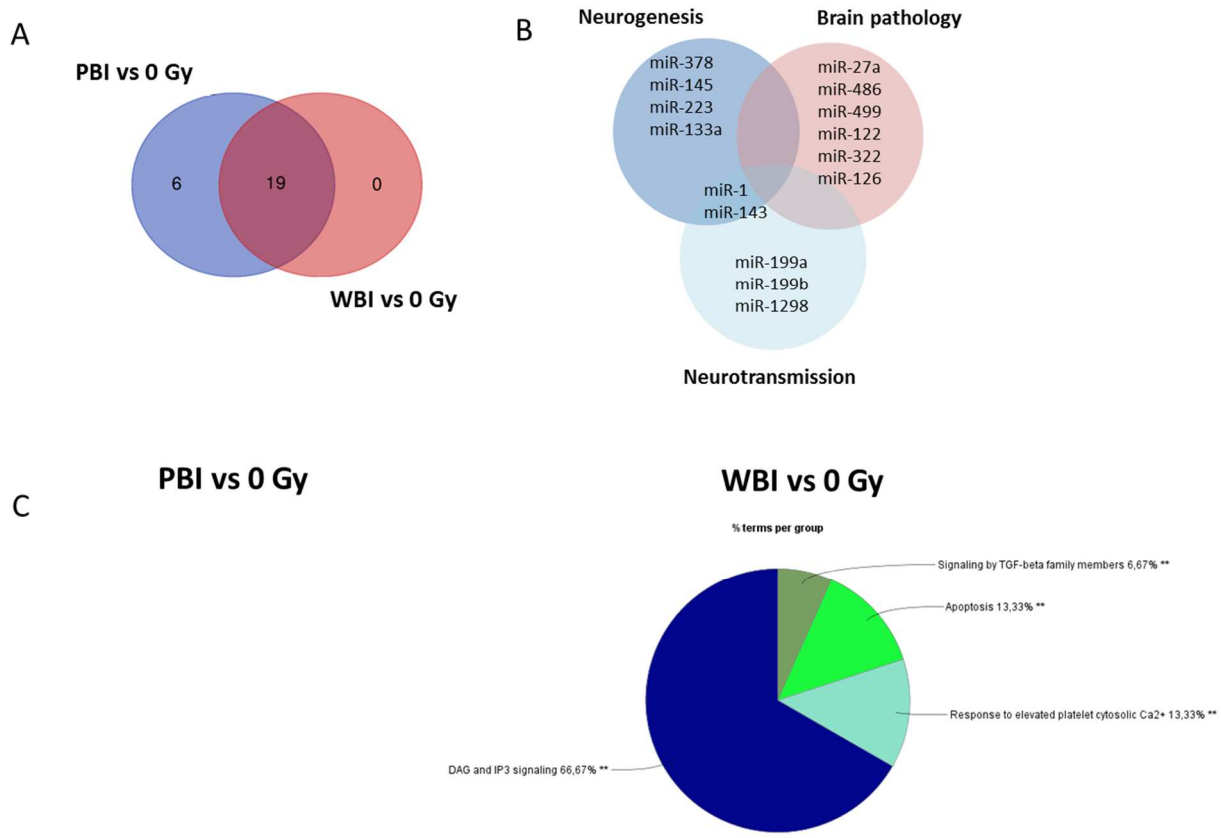

Fig. 1. (**A**) Venn diagram of the significantly deregulated miRNAs (|log2FC| > 3 and p-value of < 0.1) shared in the hippocampus of PBI and WBI mice *vs* unirradiated mice. (**B**) miRNAs known to be involved in brain-related functions. (**C**) Pathway enrichment analysis of the significantly altered miRNAs in PBI and WBI C57Bl6 mice compared to unirradiated mice**.** Focus on some of the predicted genes and corresponding pathways related to the deregulated miRNAs in PBI (left) and WBI (right).

As shown in the Venn diagram (Figure 1A), we detected a marked overlap in miRNAs expression profiles induced by PBI and WBI, all the 19 deregulated miRNAs after WBI also found after PBI. Other 5 miRNAs, namely, miR-27a-3p, miR-322-3p, miR-322-5p, miR-126-3p, miR-126-5p over-expressed in PBI group also showed a trend toward increased expression in WBI group but did not fully meet the criteria we have set for deregulation. As shown in Figure 1B, many of the deregulated miRNAs are biomarkers of brain injuries (miR-27a, miR-486, miR-499 and miR-122) or associated with neurodegenerative diseases (Parkinson and Alzheimer diseases; miR-322 and miR-126), others are involved in the control of NSCs homeostasis (miR-378, miR-145, miR-223, miR-133a, miR-143), neuronal function and connectivity (miR-1, miR-133, miR-199, miR-1298) or have neuroprotective functions (miR-155). To further investigate the perturbation induced by PBI and WBI, because miRNAs function through silencing their target genes, we searched targets of differentially expressed miRNAs, using the miRNA enrichment function of Cytoscape plugin CluePedia, selecting the top 20 genes with a miRTarBase Score > 0.6. To comprehensively study the interaction between miRNAs and their predicted targets, we performed analyses of pathway (Clupedia) for the miRNA-target pairs and top 20 predicted target genes. Not surprisingly, given the high degree of overlapping in deregulated miRNA following WBI and PBI, results of the pathway analysis in the hippocampus 15 days after WBI and PBI converged on the same perturbed regulatory pathways, ie DAG and iP3 signaling, the apoptotic signaling and the TGF-beta signalling (Fig. 1C).

We also investigated whether after irradiation with a low dose (0.1 Gy) miRNAs found differentially expressed in PBI and WBI hippocampi at 2 Gy were still perturbed. Quantitative expression of the subset of miRNAs (n=25) was evaluated by qRT-PCR custom plates. The analysis revealed only 3 differentially expressed miRNAs in WBI hippocampi (miR-143-5p, miR-378a-3p, miR-378a-5p; *p*-value = 0.04) and no statistically significant deregulation after PBI (Fig. 3), suggesting a dose-dependence in perturbation of miRNAs both in in-field and out-of-field hippocampus.

miRNA	0,1 Gy PBI vs 0 Gy (LogFC)	*P*-value	0,1 GyWBI vs 0 Gy (LogFC)	*P*-value
mmu-miR-378a-3p	0,02*	0.87	0,28	0,04
mmu-miR-378a-5p	0,06*	0.54	0,17	0.04
mmu-miR-143-5p	-0,28*	0,67	0,66	0.04

Fig. 2. Differentially expressed miRNAs in PBI and WBI mice at 0.1 Gy compared to unirradiated mice. *Asterisks indicate those samples not meeting the differential expression criteria.

MiRNome analysis after direct or bystander radiation exposure of the heart

After irradiation with 2 Gy of X-rays, miRNnome analysis from 15 days-irradiated heart samples show 39 differentially expressed miRNAs after PBI (PBI *vs* 0Gy; p-value < 0.05) and 208 differentially expressed miRNAs after WBI (WBI *vs* 0Gy), with a high number of miRNAs in common (n = 39; Figure 3A). Pathway analysis of commonly deregulated miRNAs highlights the perturbation of regulatory pathways with a critical role for the cardiac function (Figure 3B). Of note, the deregulation of miR-1a, miR-499, miR-133, miR-223 and miR-155 converges on the activation of PKA-mediated phosphorylation of CREB, whose activation is associated with two well-recognized radiation-induced heart diseases, i.e. cardiac hypertrophy and heart failure. In addition, miR-122 and miR-27 controls the activation of the TGF-β signalling, the master regulator of fibrosis, thus their deregulation may point toward myocardial fibrosis, another frequent after irradiation, is promoted by the secretion of extracellular matrix (ECM) into the interstitial space. Finally, due to the capability to mediate translation of multiple targets, the deregulation of miR-1a, miR-133 and miR-127 is also correlated to the apoptosis activation.

Data analysis of miRNAs deregulation after low dose exposure with 0.1 Gy is still in progress.

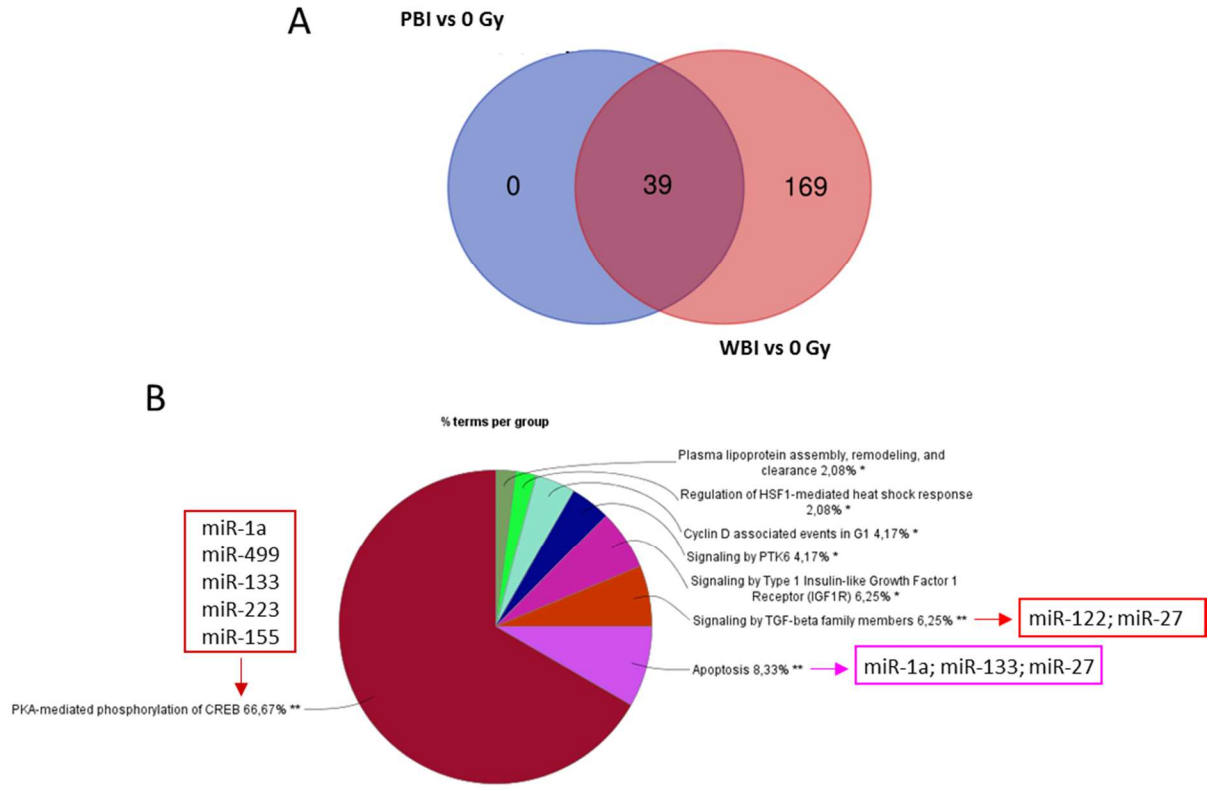

Fig. 3. Venn diagram of the significantly deregulated and shared miRNAs in the heart of PBI and WBI mice *vs* unirradiated mice (**A**). Pathway analysis predicted by the significantly altered miRNAs in common after partial and total body irradiation (**B**).

miRNAs commonly deregulated in the out-of-field hippocampus and heart following partial-body exposure

In the attempt to identify miRNAs regulating the abscopal effect in a tissue-independent manner, we compared the miRNAs differentially expressed after PBI with 2 Gy in the hippocampus (n = 25) and heart (n = 39). The Venn diagram shows a remarkable high number of commonly deregulated miRNAs (n=21; Fig. 4A). The predicted pathway analysis reveals the involvement of many intracellular signals transduction and second messengers (Figure 4B) that play an important role in the cell response to damage controlling proliferation, differentiation, apoptosis, and survival, both in heart and brain. These molecular alterations detected 15 days post-irradiation might represent early stages in the progression of non-cancer effects in nervous and cardiac tissue.

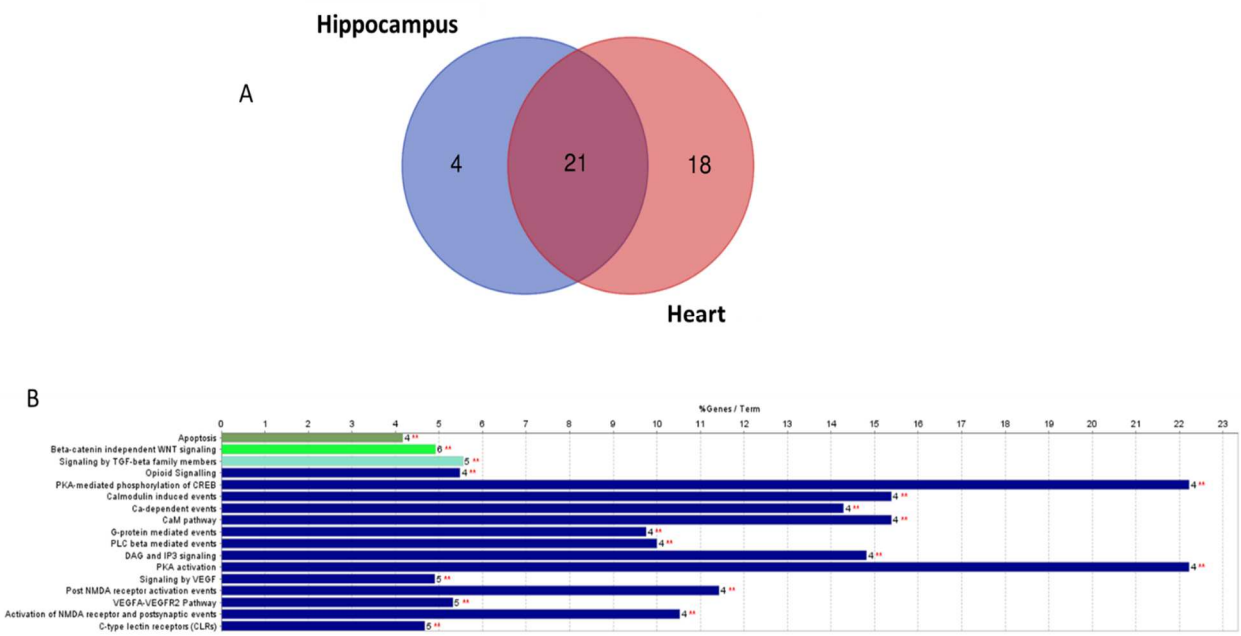

Fig. 4. Venn diagram of the significantly deregulated and shared miRNAs in the hippocampus and heart of PBI mice (**A**). Pathway analysis predicted by the commonly deregulated miRNAs (**B**).

CONCLUSIONS

One of the aims of the SEPARATE project is to elucidate the molecular mechanism through which low/moderate radiation exposure may cause non-cancer tissue reactions in out-of-field organs. We here report changes in miRNAs in out-of-field hippocampus and heart 15 days after partial body exposure that were also detected in WB exposed corresponding tissue. MiRNome analysis reveals that compared to the heart, the hippocampus shows a higher quantity of deregulated miRNAs in common between in-field and out-of-field tissues, suggesting a peculiar sensitivity to radiation-induced abscopal effects. However, over 54% of the deregulated miRNAs were in common between out-of-field hippocampus and heart, suggesting that PBI, through a miRNA-mediated common molecular mechanism, has the potential to induce specific tissue-dependent reactions in shielded tissue. Noteworthy, many of these perturbed miRNAs and predicted target pathways are known to be involved in brain and heart pathology, deserving further investigations as possible early biomarkers of pathology for radiation-induced changes leading to non-cancer diseases.