EpiRadBio – Second Periodic Report - Summary

Project context and objectives

The main aim of EpiRadBio is to combine epidemiology and radiobiology to assess cancer risks in the breast, lung, thyroid and digestive tract after exposures to ionizing radiation with cumulated equivalent doses of the order of 100 mSv or below. Such exposures are of central importance for radiation protection, since they correspond to

- the dose limit for occupational exposure (100 mSv in five years)
- exposures currently occurring in the work place (generally below 100 mSv over lifetime)
- exposures currently occurring due to medical diagnostics, e.g., by CT scans causing equivalent doses in the order of 10 mSv per examinations

While most of these exposures are from low-LET radiation, also exposures to high-LET radiation occur. Thus, EpiRadBio will explore cancer risks not only from the important types of low-LET radiation but also from alpha radiation. It is of urgent importance to analyse these cancer risks, because

- recent epidemiological results challenge the assumption of a dose-dose-rate effectiveness factor (DDREF) made presently for radiation protection
- the non-linear dose response of non-targeted effects and the differences in gene expression in the low-dose and medium/high-dose region question the linear-no-threshold dose-effect model used presently for radiation protection.

Due to lack of statistical power, conventional epidemiology has limitations in studying organ specific health effects from exposures with equivalent doses of the order of 100 mSv and below. Radiobiology can provide insights into basic mechanisms of carcinogenesis after radiation exposures but does not give quantitative results for risks of humans. EpiRadBio proposes an innovative approach combining epidemiology and radiobiology in order to address the problem: Incorporation of radiobiological results into the evaluation of epidemiological data on the basis of molecular epidemiology and models of carcinogenesis.

In order to supply a new basis for the risk assessment underlying current radiation protection, the key objectives of EpiRadBio are to

- perform measurements of telomere lengths, array-based comparative genomic hybridisation and other 'omics' with cancer tissue and blood samples from members of outstanding radioepidemiological cohorts in order to characterize key processes of carcinogenesis in humans exposed to low dose radiation
- analyze radiation responses of stem cells and low dose perturbation of intercellular communication in 2D and 3D models using human, normal breast and lung epithelial cells in order to elucidate further key processes of carcinogenesis and supplement the studies of samples from epidemiological cohorts
- integrate the new radiobiological results in models of carcinogenesis in order to include this knowledge in an evaluation of key epidemiological data
- derive cancer risks including individual risk factors after exposures to ionizing radiation with cumulative equivalent doses in the order of 100 mSv or below for supporting radiation protection.

Description of the work performed since the beginning of the project and main results achieved so far

Sub-project 1 Genomic instability and individual susceptibility

WP1.1 *Individual susceptibility to genomic instability: Epidemiology and radiation biology studies in French haemangioma patients*
All authorisations have been obtained to perform studies on the French Haemangioma cohort (FHC). Patients have been contacted and until now (Mai 2014) already 176 samples of 400 donors required in total are present. The results of the cytogenetic analysis after Telomere Centromere staining of the first 60 donors indicate evidence that telomere length varies dose-dependent even 40-60 years after exposure to low dose ionising radiation. This has also been demonstrated in a feasibility study with samples from females exposed (Mayak workers) and non-exposed (Ozyorsk citizen) without having cancer (cooperation between SUBI and CEA). In the next report period, the number of donors analysed will be increased and the following studies will be performed: dose-dependent variation of telomere length distribution and chromosomal instability (especially around 50 mGy) and the study of the bystander effect.

WP 1.2: *Genomic instability in tumoral tissues of radiation-related breast and lung cancer*
The breast and lung cancer tissues of the MAYAK workers cohort provide a unique source of material to study the possible impact of radiation exposure on cancer development. Dose estimates and excellent medical records are available. However, there was a significant delay in delivery of samples from SUBI due to regulatory affairs. Both the lung cancer as well as the FFPE breast cancer samples were not received before 2013.

We now have completed a comparative arrayCGH analysis of closely matched breast cancer samples from exposed workers and non-exposed local controls. Bioinformatic analysis, which is still ongoing, has identified significant differences in numerical genomic aberrations between the two groups, as shown below.

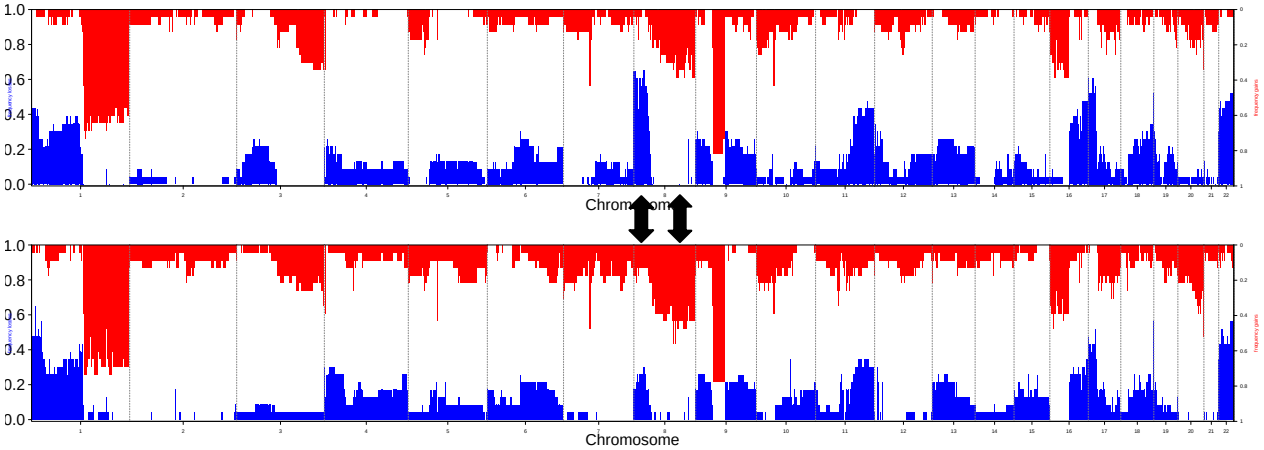

Figure: Comparison of ArrayCGH results for breast cancer for the exposed group (MAYAK, upper panel) and local control group (lower panel). The chromosomes are depicted from left to right in ascending order. Losses are in blue and gains in red. Arrows highlight statistically significant differences between the two groups (P<0.05, FDR<0.2)

FFPE autopsy samples from lung cancer tissues showed very poor DNA quality precluding conventional arrayCGH analysis as performed. We currently are testing options to continue with our NSCLC study using alternative techniques specifically designed for poor quality DNA from FFPE sources.

WP 1.3 *Genomic instability in thyroid cancer*
Work has been carried out to validate the 7q amplification identified as a potential biomarker for radiation induced papillary thyroid cancer under a preceding EU funded project, Genrisk-T. Work to investigate the relationship between protein expression of the CLP2 gene and radiation dose has been completed and submitted for publication. A case-case study on miRNA expression patterns has concluded that there is no correlation between the levels of individual miRNAs and radiation exposure. A further study on methylated DNA patterns in the same cases defined subsets of CpG sites that distinguished papillary carcinomas from patients that were exposed from those who were not exposed to radiation. Statistical analysis also identified differential methylation patterns that correlated with other features, e.g. pathomorphology. However, differences in expression were in most cases very low indicating that technical validation is likely to be difficult. Further validation of the results in a second cohort of patients will need to be carried out, and may reveal markers for the follicular and papillary histological subtypes, as those ones show larger differences in expression levels.

Analysis of the datasets produced by EpiRadBio and Genrisk-T projects using statistical models that permit integration of different types of data (e.g. on mRNA expression and copy number alteration) have suggested that PTCs from patient who were exposed to radiation show a greater perturbation of signalling networks from age-matched patients that were not exposed to radiation. This may suggest a greater degree of instability in the PTCs from patients who were exposed to radiation. This finding needs to be validated in a larger set of patients to increase statistical confidence in the result.

Sub-project 2 Radiation induced perturbation and activation of intercellular communication involved in carcinogenesis

WP2.1 *Low-dose induction of proliferation, differentiation and genome instability in stem cells*
Routine procedures are now in place for collecting, isolating and cryopreserving epithelial and fibroblast cells from primary breast tissue obtained from reduction mammoplasty's at the University of Rostock and Queen's University Belfast. Isolated cells from tissue are allowed to form mammospheres in defined medium. Different sorting strategies including magnetic activated cell sorting have been used and are being further optimised. A range of markers including EpCAM, MUC1, CD49f, CD44, and CD24 have been used. The aldefluor assay is under establishment as an additional marker of stem cells and preliminary experiments have been done in primary cells.

Preliminary irradiation studies have been initiated with primary tissue cells from individual patient samples and have included cell growth, colony forming ability, cell surface marker expression and differentiation markers.

3D acini structures have been generated from the MCF10A cell line in matrigel. Dose response relationships were obtained for acini size and shape, cytokeratin staining

(CK14/18), E-cahedrion, GM103 (golgi), Laminin V, cleaved caspase-3, phosphor-ezrin, Ki67, gammaH2AX, MUC1 and DAPI. Quantitative PCR is also being used to analyse dose response relationships at doses from 0.01 – 2Gy. Dose dependent changes in growth and circularity are observed up to 12 days after irradiation.

WP2.2 *Molecular mechanisms of radiation-induced carcinogenesis – role of tissue-environment and stress responses*

The recent works performed in this task were focused on the effect of dose rate at the levels of epithelial-mesenchymal transition (EMT) markers, telomeric repeat-containing RNA (TERRA) expression, gene amplification, and adaptive response in term of mutation induction.

EMT induction after TGFβ treatment, alpha particle irradiation and acute gamma irradiation were studied in human bronchial epithelial cell lines BEAS-2B and HBEC-3KT. No induction of EMT was observed in the cells exposed to 100 mGy or 1 Gy alpha- or acute gamma radiation. Further, we investigated exposed BEAS-2B and HBEC-3KT cell lines chronically (1.4 and 14 mGy/h). Our results suggest that the higher dose-rate of gamma radiation with a dose of 1 Gy enhances the level of EMT and the expression of myofibroblast marker (α-SMA). In co-culture with fibroblasts, EMT was enhanced as well.

UNIPV showed enhanced levels of TERRA expression and gene amplification in HeLa cells when exposed to acute gamma radiation. We wanted to study the effect of dose rate on the TERRA expression and gene amplification in HeLa and MCF10A cells. Three experiments were performed. The results are inconclusive due to a great standard deviation between the experiments. A detailed analysis of all the results showed that the reproducibility problem may be related to the fact that the cells were irradiated, frozen by partner SU and then shipped to UNIPV where the experiments were carried out after thawing of the cells. This procedure may have affected cell viability and cell cycle progression differently in different samples.

To study the dose rate effects on the level of oxidative stress, MRC5 and MCF10a cells were exposed to different doses of gamma radiation (0.1-100 mGy) delivered at different dose rates (ranging from 1.4- 15 mGy/h and acute). Preliminary results show that the dose of 50 and 100 mGy delivered at higher dose rate result in an increased levels of oxidative stress marker (8-oxo-dG) in MRC5 cells. In MCF10A cells, higher level of oxidative stress was observed at 100 mGy with lowest dose rate. Further we studied the effect of dose rate on adaptive response. These initial results indicate that cells that are pre-exposed to 50 mGy at low dose rate show lower numbers of mutation to a challenging dose compared to when the cells have been pre-exposed to an acute dose of 50 mGy.

WP2.3 *Interactions of transformed and healthy cells – pro- and anti-carcinogenic effects*

The generality of the previously observed perturbation of intercellular signalling and increase in oxidative stress levels following the irradiation of normal cells has been confirmed with the use of human lung and breast epithelial cells, along with demonstrating the importance of the POD/HOCl and $NO^{\cdot}/ONOO^{\cdot-}$ pathways. The role of TGF-β and its intracellular signalling through $p38^{MAPK}$ contributes in part to regulation of the intercellular signalling involving enhancing oxidative stress.

Experimental techniques were developed, and data obtained, on the variation of response as a function of time and distance (along with information contribution of the various signaling pathways) providing data that has enabled the development of a semi

empirical mathematical model. In parallel, a mechanism based model has been developed following both the POD/HOCl and $NO^{\cdot}/ONOO^{\cdot-}$ pathways and their role in inducing apoptosis in transformed cells as a result of intercellular signalling with normal cells. Although the rate of apoptosis is found to increase with the increase of these primary species, it was noted that if there is an increase in the level of superoxide produced following the irradiation of transformed cells, and the $NO^{\cdot}/ONOO^{\cdot-}$ pathway dominates, then the efficiency of apoptosis may reduce following irradiation. Therefore depending on parameters chosen for the model, the long term consequence may either be pro or anti-carcinogenic. Experiments will be performed in the next period to start testing this.

The release dynamics of signalling proteins have been further investigated (TGF-β, LAP, IL-6, IL-8 etc) as a function of dose and time in order to provide additional experimental data to aid modelling. In parallel a more general exploratory study has been performed at the gene expression level suggesting that other pathways might need to be explored at the released protein and receptor level (e.g. PDGF, wnt pathways).

Sub-project 3 Epidemiology, carcinogenesis and risk

WP 3.1 *Breast cancer risk*

The follow-up of female Swedish haemangioma patients, their mothers and sisters have been extended to the end of 2009. New absorbed doses to the right and left breast anlage have been calculated for every treatment and the total breast doses for each individual have been estimated. The result shows that the new dose estimations are lower than the old ones. An analysis of breast cancer in the Swedish haemangioma patients with the new dosimentry system has been performed with descriptive ERR and mechanistic models. The reduction in dose lead to a significant increase in risk estimates (about a factor of 2).The mechanistic models included models with an explicit path of genomic instability.

Work has commenced to analyse breast cancer risk in the Swedish haemangioma cohort taking into account familial relations.

For the study of breast cancer among thyroid cancer survivors, the French part of the has been updated by access to the medical records of all the centres. Dosimetry for treatment with iodine 131 and with external radiation has been improved.

Breast cancer mortality and incidence follow-up among Mayak workers have been completed. A descriptive risk analysis has been performed indicating a linear external-dose response with an ERR per unit dose of 0.14/Gy (non-significant).

WP3.2 *Lung cancer risk*

Lung cancer among Mayak workers after plutonium exposure has been analyzed with mechanistic models of carcinogenesis, both with two and three stages. In both types of models the dose response of the clonal expansion rate on plutonium dose rate had a levelling form, i.e. linear with a larger slope at low dose rates with a levelling at higher dose rates.

An analysis of the Wismut miners with mechanistic models is in progress. The Wismut miners cohort is the worldwide largest study for lung cancer risk after radon exposure. Preliminary results show a similar radon exposure rate dependence of the clonal expansion rate as in the case of plutonium with a levelling form. Such a levelling dose response might indicate cell-cell signalling and a potential bystander effect. These

results are consistent with the findings from the Eldorado cohort (Eidemüller et al, PloS One, 2012) and indicate similar underlying mechanisms for different alpha-particle exposures.

WP3.3 *Thyroid cancer risk*

Two versions of the UkrAm cohort have been made available in April 2013 and in April 2014 to HMGU by IEM which contain data sets that were used in Tronko et al. (2006) and Brenner at al. (2011). The Data analysis has started with both descriptive models and mechanistic models with individual likelihood regression. Compared to Poisson regression, this method uses individual data rather than data in person year tables for calculation of risk estimates. It allows combining incidence data for the first (prevalence) screening period and the second to fourth (incidence) screening periods. In the analysis only cases of papillary thyroid cancer (PTC) have been used because measurements of molecular data are performed only for this cancer type. Mechanistic risks models provide risk estimates based on the phenomenological description of cell-based processes. Deregulated MAPK is involved in the majority of PTCs and active MAPK induces cell proliferation, which is a prerequisite for growth of a lesion. Disturbed MAPK signaling in differentiated non-malignant thyroid tissue may indicate early molecular changes on the pathway to cancer. From microarray expression data (Abend et al. 2012) on normal tissue (corresponding to tumor tissue from UkrAm PTC patients) ten most informative genes have been identified by HMGU (ZYTO) and, depending on their cellular location, a MAPK activation score has been defined. The score depends on sex, age at exposure, age attained and radiation dose and is associated in the mechanistic model with clonal expansion of cells carrying early molecular changes, i.e. disturbed proliferation. The CLIP2 gene represents a surrogate marker of genomic instability and de-regulated gene expression is preferentially found in patients exposed at young age (Heß et al. 2011). The role of CLIP2 for radiation-induced genomic instability is explored with mechanistic models. Data on MAPK activation and CLIP2 expression will be available for the integration into mechanistic models in June 2014. Risk predictions from standard descriptive models and from simpler mechanistic models (without integrated molecular data) have been compared for the UkrAm cohort. General agreement of risk estimates has been observed.

WP3.4 *Risk evaluation for radiation protection*

A mechanistic model for colon cancer carcinogenesis and radiation risk has been developed at HMGU based on the incidence data from 1958-1998 of the Japanese a-bomb survivors. The model explicitly considers the main molecular pathway of colon cancer either with micro-satellite instability (MSI, 15-20% of cases) or with chromosomal instability (CIN, 80-85% of cases). The model predicts radiation risks in the MSI and CIN pathways separately. By combining the cases of both pathways the conventional risk estimates for colon cancer incidence can be calculated and a direct comparison of the risk estimates from descriptive models is possible.

The software to calculate the measures of lifetime risk that will be used in the evaluation process has been completed. This was a challenging process which involved combining two existing pieces of software written in different computer languages.

Expected results and their potential impact and use

The innovative approach of combining epidemiology and radiobiology of EpiRadBio will contribute to radiation protection by delivering quantitative cancer estimates for exposure scenarios of relevance in our society. The shape of the dose response will be elucidated for exposures to organs with equivalent doses in the order of 100 mSv or below. Tissue sensitivity will be quantified by studying cancer risks in the breast, lung, thyroid and digestive tract. Individual variability in radiation sensitivity will be analysed by studying genomic instability in peripheral lymphocytes of individuals with and without cancer, and by taking account of familial factors (e.g., breast cancer among close relatives or influence of the number of children and age at birth of first child on breast cancer risk) and lifestyle factors (e.g., smoking and alcohol consumption). Cancer risks of different radiation quality types will be assessed by comparing lung cancer risks from plutonium and external radiation of Mayak workers, and breast and thyroid cancer risks from incorporated iodine and external radiation. These studies will also supply information on cancer risks from internal versus external exposure to radiation. By bringing together pathologists, radiobiologists, epidemiologists, statisticians, mathematical modellers and dosimetrists and by including non- radiation research communities, EpiRadBio applies a multi-disciplinary approach to solve the central problem of radiation protection.

The address of the project public website: www.epiradbio.eu

Project objectives, work progress and achievements, project management

In order to supply a new basis for the risk assessment underlying current radiation protection, the key objectives of EpiRadBio are to

• perform measurements of telomere lengths, array-based comparative genomic hybridisation and other 'omics' with cancer tissue and blood samples from members of outstanding radioepidemiological cohorts in order to characterize key processes of carcinogenesis in humans exposed to low dose radiation

• analyse radiation responses of stem cells and low dose perturbation of intercellular communication in 2D and 3D models using human, normal breast and lung epithelial cells in order to elucidate further key processes of carcinogenesis and supplement the studies of samples from epidemiological cohorts

• integrate the new radiobiological results in models of carcinogenesis in order to include this knowledge in an evaluation of key epidemiological data

• derive cancer risks including individual risk factors after exposures with cumulative equivalent doses of the order of 100 mSv or below for supporting radiation protection.