Persistence of γ-H2AX Foci in Irradiated Bronchial Cells Correlates with Susceptibility to Radiation Associated Lung Cancer in Mice

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Running Title: Persistent γ-H2AX Foci and Lung Cancer Incidence

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Abstract

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The risk of developing radiation-induced lung cancer differs between different strains of mice, but the underlying cause of the strain differences is unknown. Strains of mice also differ in their ability to efficiently repair DNA double strand breaks resulting from radiation exposure. We phenotyped mouse strains from the CcS/Dem recombinant congenic strain set for their efficacy in repairing DNA double strand breaks during protracted radiation exposures. We monitored persistent γ-H2AX radiation induced foci (RIF) 24 hours after exposure to chronic gamma-rays as a surrogate marker for repair deficiency in bronchial epithelial cells for 17 of the CcS/Dem strains and the BALB/cHeN founder strain. We observed a very strong correlation R^2 = 79.18%, $P < 0.001$) between the level of persistent RIF and radiogenic lung cancer percent incidence measured in the same strains. Interestingly, spontaneous levels of foci in non-irradiated strains also showed good correlation with lung cancer incidence (R^2=32.74%, P =0.013). These results suggest that genetic differences in DNA repair capacity largely account for differing susceptibilities to radiation-induced lung cancer among CcS/Dem mouse strains and that high levels of spontaneous DNA damage is also a relatively good marker of cancer predisposition. In a smaller pilot study, we found that the repair capacity measured in peripheral blood leucocytes

also correlated well with radiogenic lung cancer susceptibility, raising the possibility that such phenotyping assay could be used to detect radiogenic lung cancer susceptibility in humans.

INTRODUCTION

Radiation exposure carries with it an increased risk for lung cancer. This increased risk has been observed in atomic bomb survivors *(1)* and in cohorts with medical *(2)* (NCRP No 170, section 9.4), occupational, and environmental radiation exposures *(3)*. Radiation-induced lung cancer remains a concern for diagnostic radiology and radiotherapy patients, and the risk of radiogenic lung cancer is a factor in limiting flight times for spaceflight crew members *(4)*. There is some evidence for genetic susceptibility to radiogenic lung cancer in humans *(5, 6)*, but the relative contributions of the pathways through which naturally occurring genomic sequence variants are manifested (e.g., DNA repair, apoptosis, EGFR signaling) are unknown.

Inbred mouse strains differ in their susceptibilities to radiogenic cancers, including lung cancer *(7)*, and these strain differences in susceptibility are likely due to genetic differences between strains. The CcS/Dem recombinant congenic strain set consists of 20 inbred strains (CcS1 through CcS20) derived from the BALB/cHeA and STS/A founder strains *(8, 9)*. Each CcS/Dem strain is genetically distinct and has approximately 87.5 percent of its genome derived from the BALB/c founder and the remainder from STS/A. The strains have been phenotyped for their incidences of radiation-induced lung cancers and hematopoietic malignancies, and vary considerably in their susceptibilities to both *(9)*.

Ionizing radiation induces DNA double strand breaks (DSB), and the misrepair of these lesions can contribute to carcinogenesis. Individuals differ in their capacity to efficiently repair DNA DSB and there are also differences in repair efficiencies between mouse strains. It has been 20

years since the discovery that the histone variant H2AX was specifically modified only at sites of DSBs (1), giving rise to immunofluorescent techniques and a quantitative surrogate marker for radiation-induced DSBs in eukaryotic cells (2). Capitalizing on such discovery, we previously introduced a sensitive assay reflecting the repair efficacy of radiation-induced DNA DSB based on evaluating the persistence of γ-H2AX foci in cells irradiated at low dose rate, the LDR γ-H2AX assay *(10)*. In this latter work, we showed that the assay was able to identify individuals with hypersensitivity radiation induced G(1) arrest and cell killing later linked to DNA repair differences among clinically normal individuals *(10)*. Here, we phenotype the CcS/Dem strains for their DNA DSB break repair efficiencies using the same LDR γ-H2AX assay and compare the results to those previously obtained for their susceptibilities to radiogenic lung cancer as described in the original study *(9)*. We find a strong correlation between the strain differences in susceptibility to radiation associated lung cancer and the persistence of γ-H2AX foci in irradiated bronchial epithelial cells. The correlation extends to the baseline damage in un-irradiated bronchial epithelial cells for all strains as well as in leucocytes from irradiated animals from a smaller cohort conducted at Colorado State University. Within the CcS/Dem strain set, genetically determined differences in DNA DSB repair account for most of the differences between strains in radiogenic lung cancer incidence. This finding suggests that assays to predict individual susceptibility to radiation-induced lung cancers can be developed around the usage of both baseline levels of DNA damage and persistence of damage in irradiated samples.

MATERIALS AND METHODS

Mice

Female CcS/Dem and BALB/cHeA mice were assayed at 11 to 15 weeks of age. All animal work followed American Association for Laboratory Animal Science policies and was approved by the Colorado State University Institutional Animal Care and Use Committee under protocol 09-1582A.

Irradiation

Mice were irradiated with ^{137}Cs γ-rays at a dose rate of 10 cGy/h for 24 h to a cumulative dose of 2.4 Gy. During irradiation the mice were housed in ventilated 5x5x8 cm polystyrene boxes with a cube of Nutra-Gel (Bio Serv, Flemington, NJ) for food and hydration. Sham-irradiated mice that served as controls were treated under similar conditions, but without the ^{137}Cs source being exposed.

Tissue collection

Mice were euthanized immediately following irradiation and their lungs were perfused *in situ*. Fixed lung tissue was paraffin embedded and sectioned for γ–H2AX foci detection by immunofluorescence. For foci detection in peripheral blood mononuclear cells, heparinized blood was collected by cardiac puncture, and erythrocytes lysed with distilled water. The leucocytes were then deposited onto slides, and fixed (detailed methods in Supplemental Methods).

Immunofluorescence staining and quantitation of foci

Paraffin embedded samples were de-waxed and re-hydrated by the standard method followed by antigen retrieval with sodium citrate and microwave *(11)*. Leucocyte samples were treated with

sodium borohydride solution to reduce autofluorescence background signals *(12)*. Slides were stained by anti-gamma-H2AX antibody (Millipore) and Alexa Fluor 594 conjugated secondary antibody, and counter stained by DAPI.

Extended focus images were obtained by Nikon E600 fluorescence motorized microscope with CoolSNAP HQ camera controlled by Optiscan stage system (Prior Photometrics, Tuscon, AZ) and MetaMorph Software (Molecular Devices, Sunnyvale, CA). Visual counting was performed to quantify the foci number per cell. Cells with diffuse γ–H2AX staining suggestive of apoptosis were excluded from counted.

Data analysis

Summary statistics, which included the number of mice (n) per strain and the mean and standard deviation (StDev) of the foci counts weighted by the number of cells counted per mouse by observer 1 or observer 2 (102.2 ± 1.6 and 80.8 ± 39.4, respectively), were calculated using Release 17 of the statistical package Minitab (State College, PA). The Pearson correlation coefficient was calculated for the relationship between DNA repair efficiency (as measured by the mean number of foci per nucleus) and the incidence of radiation associated lung tumors using GraphPad Prism software (San Diego, CA).

RESULTS

It was previously shown that CcS/Dem strains and their BALB/cHeA and STS/A founder strains differ in their susceptibilities to radiogenic lung cancer, lymphomas, and leukemias *(9)*. In that

study, mice of both sexes were irradiated to the whole body with four 1.5 Gy weekly fractions and monitored until they became moribund or reached 450 days of age. The incidences of lung cancer in the strains, originally reported in Szymanska et al *(9)*, are provided in **Table 1**.

We considered the possibility that strain differences in the efficacy of DNA DSB repair might account for the strain differences in radiogenic lung cancer susceptibility. To quantify DNA DSB repair efficacy we used the low dose rate γ-H2AX assay we have described previously *(10, 13, 14)*. This assay measures differences in the DNA DSB repair capacities of cells by irradiating them at low dose rate over 24 hours, a condition which results in the greater accumulation of DSB in those cells that have less efficient DNA DSB repair. The DNA DSB remaining in the cells immediately following the 24 hour exposure are quantified as γ-H2AX foci. Presumably, greater numbers of persistent foci indicate less efficient repair. Our previous work has been focusing on irradiating cells *in vitro* with this assay. However, since the target cells for radiogenic lung cancer are thought to be bronchial epithelial cells, in this work we irradiated live mice from 17 of the CcS/Dem strains and the BALB/cHeN founder strain at low dose rate for 24 hours, instead. Immediately following irradiation, the mice were euthanized, their lungs perfused with fixative to stop further DNA damage-related processing, and the γ-H2AX foci in their bronchial epithelial cells were detected by immunohistochemistry of paraffin embedded sections.

The foci were not uniformly distributed among the cell types in the lung. More γ-H2AX foci formed in the epithelial cells of the conducting airways (comprising the bronchi and bronchioles) than in the respiratory airways (the alveoli). Within the conducting airways more foci formed in

basal calls than in apical cells of the epithelium (**Figure 1**). As previously noted by Rübe and colleagues *(13)*, there were also fewer foci formed in the stromal cell nuclei in the parenchyma of the lung in irradiated mice. We counted foci in bronchial epithelial cells and found considerable differences between the strains (**Figure 2**). Since the bronchial epithelial cells are identified histologically by their positions within the tissue, our computer algorithms (3-5) could not be used here and we had to rely on human counts. As we reviewed extensively in the past (6), manual foci counting can be affected by large inter-observer variability. To account for this, foci were counted by two different individuals, both blinded to the lung cancer incidence in the different strains.

Background levels of foci per nucleus in the bronchial epithelial cells of unirradiated mice (two mice assayed per strain) were low, about 3 for BALB/c and about 1.5 for the CcS strains. The numbers of foci per nucleus were higher in irradiated mice (4 mice assayed per strain, with the exception of CcS/Dem13 for which only 3 mice were available), and differed considerably between strains (**Table 2**). To determine if strains with less efficient repair were at greater risk for radiogenic lung cancer, we computed the Pearson's correlation between average lung cancer incidence for each strain *(9)* with the average number of remaining RIF 24 hour post-exposure. Since the RIF data were from female mice, they were compared to lung cancer incidence in female mice only. However, for some strains relatively few female mice were phenotyped for lung cancer susceptibility, so a comparison was also made to lung cancer incidence in both sexes combined. The caveat with this approach is that for a number of other radiogenic tumors for which larger datasets are available, there are sex differences in incidence. Strong correlations between persistent RIF levels measured in female mice and lung cancer incidence were found by

both observers (R^2 = 60.56%, P < 0.001 for the incidence in female mice and R^2 = 79.18%, P < 0.001 for the incidence in both sexes) (**Figure 3**). However, the RIF counts of observer 1 were more closely correlated with incidence than those of observer 2 (observer 1, R^2 = 95.66%, P < 0.001; observer 2, R^2 = 68.52%, P < 0.001 for the correlations with incidence in both sexes). The inter-observer difference is likely due to the selection of cells in which to count foci. Interestingly, spontaneous levels of foci in non-irradiated strains also showed good correlation with lung cancer incidence in both sexes as measured by observer 1 (R^2=32.74%, P =0.013 – **Figure 3 C**).

To determine if strain differences in DNA DSB repair efficiencies in peripheral blood leukocytes also correlated with radiogenic lung cancer susceptibility, we undertook a more limited experiment in which we irradiated additional BALB/c mice and mice from 4 CcS/Dem strains that had high, low and intermediate repair efficiencies in their bronchial epithelial cells. The foci were counted by observer 1 and the results are presented in Table 3. Although the observer attempted to count foci only in lymphocytes based on nuclear morphology, monocytes could not be reliably excluded from the counts. However, monocytes comprise only about 5% percent of murine peripheral blood leucocytes compared to about 80% for lymphocytes. The foci counts correlated with lung cancer incidence in female mice (R^2 = 86.354%, P = 0.022), though the correlation with lung cancer incidence in both sexes was not quite significant (R^2 = 75.69%, P = 0.055) (**Figure 4**). The results indicate that the repair capacity in peripheral blood leucocytes is a good predictor of radiogenic lung cancer susceptibility in these strains.

DISCUSSION

The link between cancer and deficient DNA repair has been extensively documented by others (7-10). For example, individuals suffering from the hereditary cancer-prone disease xeroderma pigmentosum (XP) were found to have defective DNA Single Stand Break repairs after exposing their cells to UV light (11). With respect to the γH2AX assay used in this work, one can list several studies where the same assay was critical to identify individuals with repair defects such as patients with genetic diseases like ataxia telangiectasia (AT), Nijmegen breakage syndrome (NBS) (12), xeroderma pigmentosum (XP) (13), Fanconi anemia (FA) (14), and radiation sensitive severe combined immunodeficiency (RS-SCID) (15). The most well-known factor linking DNA repair to cancer is probably the mutation in breast cancer susceptibility proteins type 1 and 2 (BRCA1 and BRCA2) as these two genes are known to play a critical role in DNA DSB repair (16). Note that ATM defects that result in persistent RIF (4, 17) are associated with radiation-induced carcinogenesis in mice (18), and increased toxicity from radiotherapy in ATM heterozygous patients (19, 20). Finally, DNA repair deficiencies are considered risk factors for both acute radiation toxicity and cancer, independently of the type of radiation (21-25). The sizeable body of work linking DNA repair defects to cancer risks is beyond the scope of this work, but one can summarize it with this simple paradigm: defective DNA repair leads to genomic instability, which in turn lead to accumulated mutations and potentially cancer. We previously proposed mathematical formalism linking DNA repair deficiency to carcinogenesis and a more detail review of the literature on the topic can be found there as well (26).

A major finding which emerges from this study is that most of the variance in susceptibility to radiogenic lung cancer in the CcS/Dem strains can be attributed to strain differences in DNA DSB repair efficiencies detectable by the LDR γ-H2AX assay. The R^2 for the combined foci counts of both observers in both sexes was 79%, suggesting 79% of the variance in lung cancer susceptibility is explained by differences in repair capacity. Since the

mice that comprise the CcS/Dem strain set ultimately trace back to wild populations, an implication of this finding is that within these populations naturally occurring differences in DNA DSB repair efficiency might underlie interindividual differences in radiogenic lung cancer susceptibility. Might this also be the case for humans? We have reported differences in repair efficiencies in human fibroblasts from different normal donors detected by the LDR γ-H2AX assay, with about a 40% of those screened having relatively inefficient repair. The magnitudes of the strain or interindividual differences are comparable between mice and humans (the mean foci count in fibroblasts from 15 normal human donors ranged from 4.5 to 11.3 per nucleus; for bronchial epithelial cells in the 17 CcS/Dem strains assayed the range was 8.2 to 23.1 foci per nucleus) *(10)*. Another finding worth noting is the 33% correlation between baseline γ-H2AX foci level in the bronchial epithelial cells from the 20 CcS/Dem strains and their reported cancer incidence. This correlation was found to be statistically significant and confirms the concept that baseline damage levels in an individual is the net balance between spontaneous damage being generated in the tissue and the efficiency of the repair machinery constantly clearing them (26). Higher foci level in an individual either reflect exposure to agents damaging DNA and/or slower repair.

However, a couple of caveats need to be considered in extrapolating our findings to populations and to humans. The first is, obviously, that there are likely to be differences between the pathogenesis of radiation-induced lung cancers in mice and humans, so further study will be necessary to link repair proficiency with radiogenic lung cancer in humans. The second is in extrapolating the magnitude of an effect determined in a strain set to a general population. There is limited genetic diversity in the CcS/Dem strains. Since they derive from two inbred founder strains there can be at most two alleles for each locus. So, while differences in susceptibility between BALB/c and CcS/Dem strains can be largely or entirely explained by their differing repair capacities, there may be other collections of inbred strains that differ in susceptibility due to other causes. By extension to humans, differences in DNA DSB repair efficiencies in a general population may explain only part of the variance in lung cancer susceptibility.

The finding that strain differences in repair efficiency measured in peripheral blood leucocytes mirrors those in bronchial epithelial cells and consequently correlate with radiogenic lung cancer is not surprising. We demonstrated that the LDR γ-H2AX assay distinguished individuals with one defective copy of *ATM* from individuals with two defective copies or two normal copies, regardless of whether the assay employed fibroblasts or lymphoblasts *(14)*. This work also reflects that LDR γ-H2AX assay may be able to identify new genetic variance involved in DNA repair and potential cancer risk. A previous study had already suggested such findings with RIF repair kinetic assay being sensitive enough to detect subtle genetic differences in primary human fibroblast derived from 25 apparently healthy individuals and 10 patients with DNA repair-defective syndromes (27). In this work, authors showed clear kinetic differences with sick patients but even within the healthy donor, a wide variation in RIF levels and kinetics was observed suggesting other unknown factors influencing DNA repair. In mice, Rübe and colleagues *(15)* followed the kinetics of DNA DSB repair in two traditional laboratory mouse strains (BALB/c and C57BL/6), a strain engineered with a knockout allele of *Atm*, and a SCID strain with a spontaneous mutation in the gene encoding the catalytic subunit of DNA dependent protein kinase (*Prkdc*). The latter two strains have profound defects in DNA DSB repair. They observed that the rank order of repair efficiencies between the four strains of mice was the same regardless of the tissues assayed (lung, brain, heart or intestine irradiated *in vivo* and peripheral blood lymphocytes irradiated *ex vivo*). One way to compare directly human and mouse data is the usage of blood samples. For example in our study, the baseline level damage for figure 4 was XXX which is comparable to the reported 0.5 to 1 foci/cell in adult healthy donors PBL (28, 29). Unlike bronchial epithelial cells, peripheral blood cells are readily accessible; so, it should be possible to test for a correlation between repair efficiency of peripheral blood lymphocytes irradiated *ex vivo* and radiogenic cancer susceptibility in humans if a suitable cohort could be identified.

SUPPLEMENTARY INFORMATION

Detailed methods or tissue collection, immunofluorescent staining, and foci quantification are available as Supplementary Information.

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Figure 1. Section of irradiated lung showing many radiation-induced foci formed in the basal epithelial cells compared to the apical cells or stromal cells.

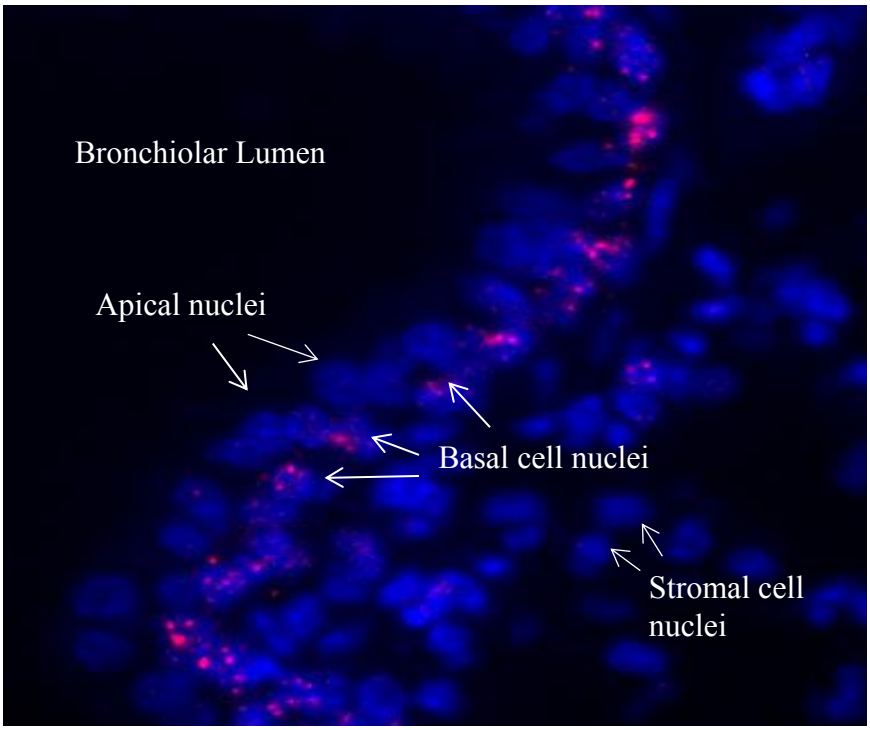

Figure 2. Frequency histograms of the number of γ-H2AX foci per cell in lung basal epithelial cells in CcS/Dem strains and BALB/c mice. The more efficient the strain in DSB repair the more its histogram will be skewed to the left.

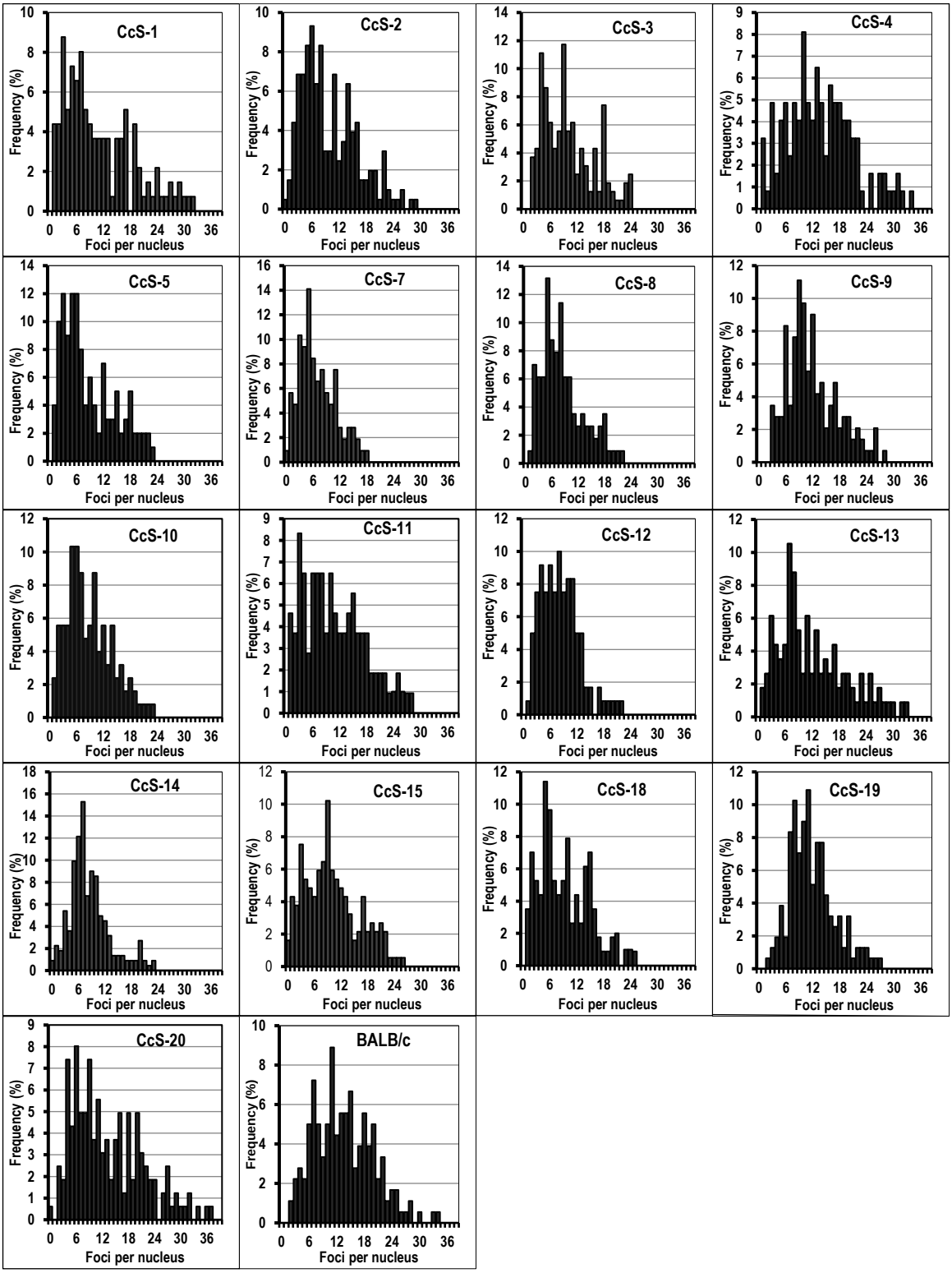

Figure 3. Correlation between repair efficiencies and radiogenic lung cancer incidences in BALB/c and CcS/Dem strains. Repair efficiencies are expressed as foci counts per nucleus in the LDR γ-H2AX assay. To account for inter-observer variability, the foci counts for both observers are plotted (■ observer 1, ● observer 2) and the correlation of foci to incidence are calculated using all of the data points.

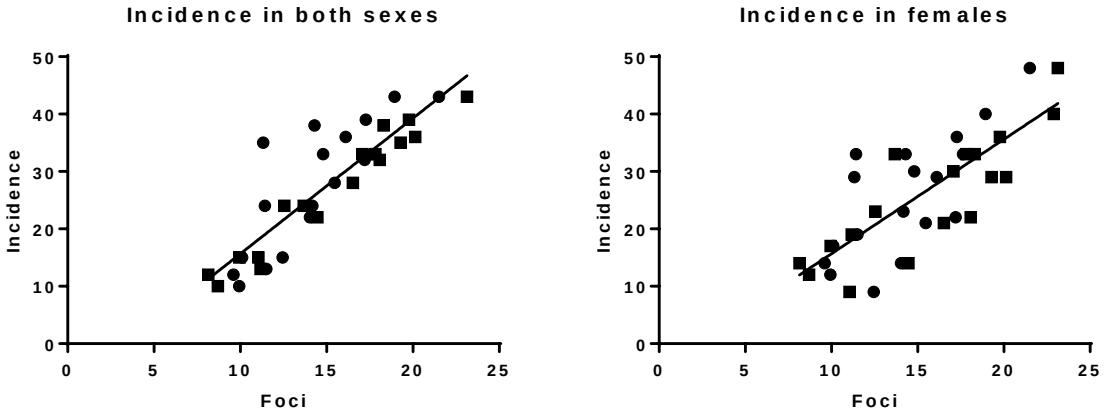

Figure 4. Correlation between repair efficiencies in peripheral blood leucocytes and radiogenic lung cancer incidences in BALB/c and CcS/Dem strains, CcS2, CcS3, CcS4 and CcS10.