SCIENTIFIC PROJECT

RADON FRANCE sub-study (RADON-RATS): PATHOLOGICAL AND MOLECULAR CHARACTERIZATION OF RADON-INDUCED LUNG CANCER IN RATS

1. BACKGROUND

1.1. Radon gas

Radon is an odorless, colorless and tasteless radioactive gas, dissolved in the air. It is the most important source of natural radiation (50%), mainly found on rocky areas, and it tends to accumulate inside homes. Radon gas is considered as a human carcinogen group A, as well as tobacco and its derivatives, by the International Agency for Research on Cancer (IARC), and it is considered the 2nd cause of lung cancer after smoking, and the first one in non-smokers (1,2).

In a collaborative analysis from 13 European case-control studies, indoor radon showed a linear increase of 16% (5% to 31%) of lung cancer risk per 100 Bq/m3 (3), but most recent evidence has shown an non-linear risk of lung cancer even with lower concentrations (4). The World Health Organization (WHO) recommends radon concentration <100 Bq/m^3 (1). The European Commission for Atomic Energy (2013/59/Euratom) has established a non-regulatory recommendation, not to exceed 300 Bq/m3 in European homes (5).

In Europe there are several radon-prone areas, mainly the granitic areas of the Bohemian Massif, the Iberian Peninsula, the Massif Central, the Fennoscandian shield, Corsica, Cornwall and the Vosges Mountains, in the crystalline rocks of the Central Alps and karst rocks of the Swiss Jura and the Dinarides, the black shales in North Estonia and in certain volcanic structures in central Italy. In all countries participating in the European Indoor Radon Map (EIRM), including France, more than 30 % of the area have a median concentration of >100 Bq/3m and 4.2 % above 300 Bq/m3 (5).

However, to date indoor radon exposure is not regulated in our continent, considered a European public health problem. The latest update of the European Cancer Prevention Code includes, in section 9, the control of radon exposure as one of the 12 ways of cancer prevention (6).

1.2. Radon gas and lung cancer

Radon is the leading cause of lung cancer in non-smokers, but the carcinogenesis mechanism remains unknown. Radon emits ionizing radiation type alpha that have been linked to wide variety of cytotoxic and genotoxic effects in preclinical studies, which could favor the cancer carcinogenesis (7–9). When alpha particles are inhaled, impact on the respiratory epithelium, causing deoxyribonucleic acid (DNA) damage by their high-energy transfer capacity. A wide variety of secondary cytogenetic and molecular alterations have been associated with the radioactive alpha particles, among which genetic mutations have been described, including point deletions or substitutions, chromosomal rearrangements, etc., generally studied in peripheral blood lymphocytes (8,9). Radon can also generate high chromosomal instability however until now no chromosomal alteration and other molecular alterations have been studied in tumor tissue in exposure-population (9). To date, the biological or molecular carcinogenic mechanism of radon gas and lung cancer is still unknown.

1.3. Molecular alteration and lung cancer

Over the last years, several oncogenic molecular alterations have been described, basically somatic mutations (EGFR, BRAF, HER2, MET, etc.) or chromosomal rearrangements (ALK, ROS1, RET, NTRK), responsible of lung cancer mainly in non-smoking population, but any risk factor has been identified yet.

Our hypothesis is that certain molecular alterations could be related to cellular damage induce by the indoor radon exposure. So far, three preliminary studies have assessed this hypothesis, showing high median concentrations in ALK-rearranged, EGFR and BRAF-mutated patients, but with poor statistical power for small sample sizes (10–12).

Study	N	Molecular alt	Area	Radon median	Sig
Taga *et al* *(10)*	**N=24**, prospective Non-smokers women	EGFR mut. (n=24)	Non radon-prone area	EGFR mut. **46,5 Bq/m3**	Non sig.
Mezquita *et al* *(12)*	**N=48**, prospective	EGFR mut. (n=38) ALK rearrang. (n=10) BRAF mut. (n=2)	Intermediate area	EGFR mut. **96 Bq/m3** ALK rearrang.**116 Bq/m3** BRAF mut. **125 Bq/m3**	Non sig.
Ruano *et al* *(11)*	**N=95**, retrospective Non-smokers	EGFR mut. (n=83) ALK rearrang. (n=12)	Radon prone area	EGFR exon 19 **216 Bq/m3** EGFR exon 21 **118 Bq/m3** ALK rearrang. **290 Bq/m3**	Non sig.

Table 1. Studies reported about the indoor radon concentration and molecular alterations in NSCLC patients.

In addition to this study, Mezquita et al will present the preliminary data of Radon France study at WCLC 2018, in Toronto. We assessed the correlation between the radon exposure areas in France and the molecular alterations, nationally registered in NSCLC patients. We evaluated retrospectively 116.424 NSCLC cases with molecular assessment (*EGFR, BRAF, HER2* and *KRAS* mutations (m) and *ALK* and *ROS1* rearrangements (r)) from the French Platform led by *INCa* (French National Cancer Institute). The risk of indoor radon exposure by region in France was based on the official French Radon map from the *Institut de Radioprotection et de Sûreté Nucléaire* (INSN, France). The prevalence of driver alterations (*EGFR, BRAF, HER2* and *ROS1* were significantly higher in high exposure area. The prevalence of *KRAS* mutations was significantly higher in low exposure area. Until now, this is the largest study that has evaluated the indoor radon estimation and molecular defined lung cancer subpopulation.

	Low risk	Intermediate	High	P
EGFR mutation	1962 (10%)	4338 (11%)	**4176 (11.4%)**	**<0.0001**
ALK rearrangement	577 (3.3%)	1019 (3%)	896 (3%)	0.35
BRAF mutation	327 (1.8%)	**830 (2.4%)**	**692 (2.4%)**	**0.0001**
HER2 mutation	109 (0.6%)	**266 (0.9%)**	**252 (0.8%)**	**0.01**
ROS1 rearrangement	61 (0.9%)	133 (0.9%)	**126 (1.3%)**	**0.005**
KRAS mutation	**4717 (29.8%)**	9215 (28.2%)	7895 (27%)	**<0.0001**
Molecular drivers*	3037 (3.9%)	**6587 (4.4%)**	**6142 (4.4%)**	**<0.0001**
* *EGFR, BRAF & HER2* mutations, *ALK & ROS1* rearrangements; *KRAS* mutation excluded.				

Table 2: Prevalence of molecular alteration by radon risk area in France.

Currently, there is ongoing a collaborative study with the IFCT-Biomarqueurs France to enrich the clinical data of Radon France as well as the assessment of the hypothesis radon-molecular alteration.
All these studies together have established an interesting rational for developing studies in preclinical models in order to study the pathological and molecular pathways of the mechanism of carcinogenesis of the radon gas.

1.4. Radon and preclinical models: rats

To date, no causality preclinical models have been specifically evaluated in molecular defined lung cancer subpopulations. A few studies have evaluated the role of radon gas in animal models, mainly inhaling radon gas in radon-exposure chamber (13). However so far, no pathological and molecular characterization has been assessed in radon-exposed animal model.

Between 1972 and 1992, the *Laboratoire Expérimental du Carcinologie* led by Dr. Sylvie Chevillard from the *Commission d'Enérgie Atomique* (France) worked on a research program with more than 3.580 rats exposed to radon in a radon exposure chamber, in which a total of 70 rats (2-3%) developed lung cancer, that were archived in the Pathology Biobanking, from the CEA, Fontenay-aux-Roses (France). After 1992, the research program with the radon exposure chamber was stopped and no radon exposure chamber is currently available in France for performing prospective experiments in preclinical models.

Thus, we propose a study for assesing the pathological and molecular characteristics of this retrospective cohort of 70 radon-induced lung cancers in rats, to study the carcinogenesis mechanisms, and to be compared to the carcinogenesis in patients.

2. OBJECTIVE

2.1. Primary Specific Endpoints

- To characterize the molecular and pathological phenotype of radon-induced lung cancers in an animal model (non-genetically modified rats)

2.2. Secondary Specific Endpoints

- To compared the molecular alterations found on rats to the human's
- To compared the pathological characteristics found on rats to the human's
- To establish potential causality hypothesis on humans based on pathways altered in rats

3. METHODS

The global project RADON FRANCE is currently involving 3 different cohort of research:

- Cohort 1: Radon France study, in collaboration with IRSN and the INCa
- Cohort 2: Radon- IFCT- Biomarkers France study, with the support of the IFCT
- **Cohort 3**: Radon-Rats, in collaboration with the CEA

The "Alain Depierre Award" will be used exclusively for the cohort 3. This will be a retrospective study for the pathological and molecular assessment of radon-induced lung cancers in a cohort of 70 rats from a research program performed for the CEA between 1972 and 1992 in France.

3.1. Sample collection

Dr. Laura Mezquita will collect the samples in the Pathology Banking, at CEA, Fontenay-aux-Roses (France) (Samples from N=3.580 rats archived, n=70 lung cancer).
We are working in collaboration with the Dr. Sylvie Chevillard, head of the *Laboratoire Expérimental du Carcinologie*, from the CEA, giving the agreement for the transfer of samples for analysis to Gustave Roussy.

3.2. Pathological assessment

The pathological assessment will be performed for a pathologist specialist in Thoracic Oncology in archived Formalin-fixed, Paraffin-embedded (FFPE) including: morphology, histological pattern, grade of differentiation, etc. and other tumor and microenvironment characteristics. A quality control will be performed before immunohistochemistry (IHC) staining, considering the age of the samples. The IHC staining will include: the standard IHC in lung cancer customized for tissue in rats (TTF1, P63, neuroendocrine markers, etc) and specific molecular IHC (*ALK, ROS1, BRAFV600E, EGFR exon 19, EGFR exon 21, HER2*).

3.3. Molecular analysis: Sample collection, processing and analysis

The molecular analysis will be perfomed exclusively if the quality control of the samples is ensured. Tumor DNA (tDNA) will be extracted from the Archived FFPE biopsy sample using the Maxwell® RSC DNA FFPE Kit according to the manufacturer's instructions (Promega.
Targeted sequencing libraries will be generated using the Ion AmpliSeq Library kit 2.0 according to the manufacturer's instructions (Life Technologies). Tumor samples will be analyzed with a customized targeted panel specifically designed for rats (Life Technologies).

The primers used for amplification will be partially digested by FuPa enzyme. The digested product will be then ligated with adapters and barcodes, amplified and purified using Agencourt beads. The quantity of the libraries will be assessed using the Qubit 2.0 Fluorometer. An equal amount of each library will be pooled and amplified with the Ion OneTouch 2 system by the emulsion PCR with the Ion PGM Template OT2 200 Kit (Life Technologies). The enrichment will be then performed with the Ion One Touch ES (Enrichment System). The enriched Ion Spheres will be loaded into a 316v.2 Ion Sequencing Chip. Sequencing will be made using Ion Personal Genome Machine (PGM, Life Technologies) using real-time measurement of the hydrogen ions produced during DNA replication. The sequencing data will be analyzed with the Torrent Suite Variant Caller 4.2 software and reported somatic variants will be compared with the reference genome.

3.4. Data collection

A case report form (CRF) will be developed specifically for this project, including all the variables related to the exposure experiment as well as the pathological and molecular characteristics analysed in this study.

3.5. Statistical Analysis

The analyses will be performed with SAS software. To assess the objectives of this study, we will perform a descriptive analysis, correlation and comparison study and bivariate analysis. We will consider statistical significance when p-value is <0.05 (bilateral).

4. POTENTIAL RESULTS

Our study, for first time could potentially demonstrate the causality of radon gas in molecular defined lung cancer (*EGFR, ALK, BRAF, ROS1, HER2*, etc).
In this experiment, we will study the phenotype of lung cancer exposed to radon in rats; and subsequently we could evaluate if the pathological and molecular characteristics in lung cancer in rats are similar to the characteristics in humans with lung cancer harboring molecular alterations.
Additional, we will study the mechanism of pathogenesis and carcinogenesis (including the molecular pathways) in rats and to compare our findings to the carcinogenesis mechanism already known in lung cancer patients.

5. RELEVANCE

Radon gas represents the second cause of lung cancer after smoking and the first-one in non-smoking population. Radon gas causes 20,000 deaths from lung cancer in Europe each year and 21,000 in United States (14).

This study, together with Radon France and the prior evidence reported (table 1), will develop a innovate field of research based on prevention in lung cancer.

For first time, we could establish the carcinogenesis mechanism related to molecular driver alterations in an animal model.
The significance of our work includes:

- Providing valuable information on radon exposure in a preclinical model.
- Establishing, for first time, the potential causality between radon and the molecular-defined lung cancer
- Define the pathological/molecular phenotype of radon-induced lung cancer in rats
- This can be the first step for establishing a future carcinogenesis model for radon-induced lung cancer.
- Important impact in the near future on lung cancer prevention, taking into consideration that radon gas is an environmental and potentially preventable risk factor
- Raising the awareness about the radon-related lung cancer, making this “invisible” risk factor more real

6. TIMETABLE

Sample collection
Period: 2 months.
Work group: Dr. Laura Mezquita will collect the samples in the Pathology Banking, at CEA, Fontenay-aux-Roses (France). Collection of the samples available for analysis of the rats cohort (out of 3.580 samples archived, n=70 lung cancer samples).

Quality control
Period: 2 months.
Work group: Pathological Department, at Gustave Roussy, France.

Pathological analysis
Period: 4 months.
Work group: Dr. Julien Adam from the Pathological Department, at Gustave Roussy, France.

Molecular analysis
Period: 4 months.
Work group: Dr. Ludovic Lacroix, Dr. Etienne Rouleau, Dr. Cecile Jovelet, from the Genomic Platform-Molecular Biopathology Unit and Biological Resource Center, at Gustave Roussy (France).

Data collection
Period: 11 months.
Work group: Dr. Laura Mezquita, from the Thoracic Oncology Group, at Gustave Roussy (France).

Statistical analysis
Period: 2 months.
Center: Radiation Epidemiology Unit, at Gustave Roussy (France).

Communications of findings
Period: Since October 2019.
Data will be submitted for presentation at international meetings and the results may be submitted for publication in Journals (Q1) in Clinical or Molecular Oncology and/or Epidemiology.

Duration: 12 months	NOV 2018	DEC 2018	JAN 2019	FEB 2019	MAR 2019	APR 2019	MAY 2019	JUN 2019	JULY 2019	AUG 2019	SEP 2019	OCT 2019
Sample collection												
Tissue quality control*												
Pathological analysis												
Molecular analysis**												
Data collection												
Statistical analysis												
Congress Paper												

** inclunding the preparation of samples for analysis ** molecular analysis will be performed exclusively if the quality control of samples is ensured*

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