Residential radon, *EGFR* mutations and *ALK* alterations in never-smoking lung cancer cases

Alberto Ruano-Ravina[1,2,3], María Torres-Durán[4], Karl T. Kelsey[3], Isaura Parente-Lamelas[5], Virginia Leiro-Fernández[4], Ihab Abdulkader[6], José Abal-Arca[5], Carmen Montero-Martínez[7], Iria Vidal-García[7], Margarita Amenedo[8], Olalla Castro-Añón[9], Antonio Golpe-Gómez[10], Javier González-Barcala[10], Cristina Martínez[11], Rosirys Guzmán-Taveras[11], Mariano Provencio[12], María José Mejuto-Martí[13], Alberto Fernández-Villar[4] and Juan Miguel Barros-Dios[1,2,14]

Affiliations: [1]Dept of Preventive Medicine and Public Health, University of Santiago de Compostela, Santiago de Compostela, Spain.[2]CIBER de Epidemiología y Salud Pública, CIBERESP, Madrid, Spain. [3]Dept of Epidemiology, Brown School of Public Health, Brown University, Providence, RI, USA. [4]Service of Pneumology, University Hospital Complex of Vigo, Vigo, Spain. [5]Service of Pneumology, Ourense Hospital Complex, Ourense, Spain. [6]Service of Pathology, Santiago de Compostela University Clinic Hospital, Santiago de Compostela, Spain. [7]Service of Pneumology, University Hospital Complex of A Coruña, A Coruña, Spain. [8]Oncologic Hospital, A Coruña, Spain. [9]Service of Pneumology, Hospital Lucus Augusti, Lugo, Spain. [10]Service of Pneumology, Santiago de Compostela University Clinic Hospital, Santiago de Compostela, Spain. [11]National Institute of Silicosis, University Hospital of Asturias, Oviedo, Spain. [12]Service of Oncology, Puerta de Hierro University Hospital, Madrid, Spain. [13]Service of Pneumology, Arquitecto Marcide Hospital, Ferrol, Spain. [14]Service of Preventive Medicine, University Hospital Complex of Santiago de Compostela, Santiago de Compostela, Spain.

Correspondence: Alberto Ruano-Ravina, Dept of Preventive Medicine and Public Health, School of Medicine. C/San Francisco s/n, University of Santiago de Compostela, 15782. Santiago de Compostela, Spain.
E-mail: alberto.ruano@usc.es

ABSTRACT The aim of this study was to assess if residential radon exposure might cause *EGFR* mutations or *ALK* rearrangements in never-smokers.

We designed a multicentre case–control study in a radon-prone area (Galicia, Spain); only lung cancer cases were included in the study. We obtained residential radon measurements and clinical information for all the participants. We compared the median values of residential radon between patients with *EGFR* mutations or *ALK* rearrangements *versus* those without them.

323 patients were included. Median age was 70 years and 19.5% were males. 42 and 15% of patients were *EGFR*- and *ALK*-positive, respectively. The most frequent *EGFR* alterations were exon 19 deletions and exon 21 (L858R) single-point substitution mutations. *ALK*-positive patients were 10 years younger than *ALK*-negative patients. Residential radon levels were two-fold higher in patients with exon 19 deletions compared with patients with exon 21 (L858R) single-point substitution mutations (216 *versus* 118 Bq·m^{-3}; p=0.057). There were no differences in residential radon levels by *EGFR* mutation status. *ALK*-positive patients (n=12) essentially had two-fold residential radon levels compared with *ALK*-negative patients (290 *versus* 164 Bq·m^{-3}, respectively).

Residential radon may have a role in the molecular signature of lung cancer in never-smokers, although more studies with larger sample sizes are needed to support this hypothesis.

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Introduction

Lung cancer is an important public health problem. Tobacco consumption is the main risk factor and the five-year survival is only 13%, though it is slightly better in women compared to men [1]. This low survival rate has been attributed to ~57% of lung cancers being diagnosed at a later stage (stage IV) [2]. In 2012, for the first time, lung cancer mortality was the first cause of cancer death in women in developed countries [3]. The epidemiology of lung cancer is somewhat unknown in never-smokers, but some risk factors are well defined, including exposure to environmental tobacco smoke (ETS) or working in certain occupations. Exposure to residential radon plays an important role in the genesis of lung cancer, although a complete description of the nature of this role is lacking, despite the publication of recent studies [4] and its classification as the first risk factor for lung cancer in never-smokers [5]. Different meta-analysis and pooling studies have shown a dose–response relationship between radon exposure and lung cancer risk [6–8].

Recent evidence has suggested that the molecular profile of lung cancer differs by carcinogen exposure history, such that lung cancer in never-smokers arises *via* a different biological pathway than lung cancer in ever-smokers [9, 10]. Some driver mutations/alterations, including alterations in the *EGFR* and *ALK* genes, have been recently discovered to occur primarily in never-smokers. These observations have facilitated the use of targeted treatments that have an impact on survival [11, 12]. Interestingly, these mutations are mostly present in stage IV adenocarcinoma, which is consistent with what is known of the epidemiology of lung cancer in nonsmokers [13].

Radon is a gas that occurs naturally as a decay product of the uranium present in the earth's crust; it tends to accumulate indoors, mainly in dwellings and workplaces. Radon's most stable isotope, ^{222}Rn, has a half-life of 3.8 days and releases high-energy alpha radiation when decaying into elements. Because of this half-life, ^{222}Rn itself engenders little radiation risk. However, two of its decay products, polonium 218 (^{218}Po) and 214 (^{214}Po), have a very short half-life and release alpha radiation when decaying to other products [5]. Alpha radiation releases a large amount of energy in a very short linear track. Alpha particle interaction with cells in general, including lung epithelial cells, is well known to potentially induce large-scale molecular changes, chiefly DNA double-strand breaks, translocations and gene deletions [14]. It is this type of damage that is thought to ultimately enhance the radon-associated risk for lung cancer [5]. Although a role for radon in the genesis of p53 gene mutations in lung cancer has been suggested, it has not been consistently observed [15]. Similarly, the occurrence of *KRAS* point mutations in radon-exposed miners with lung cancer has also been reported, although this finding has not been confirmed [16].

Additionally, some never-smokers with adenocarcinoma are known to present with signature *EGFR* mutations and *ALK* translocations. There is a paucity of epidemiological investigations into the factors that might be responsible for producing these molecular changes. We suggest that residential radon could have a role in the genesis of one or both of these alterations in this subgroup of lung cancer patients. There is only one research study available on the relationship between residential radon and *EGFR* alterations, and its researchers observed a lack of association. However, this study included only 70 patients and was performed in an area without elevated radon concentrations [17]. In an effort to test our hypothesis, we have studied a region in Spain with high but variable exposure to radon, where the population is stable and has lived for an extended period of time in the same dwellings [18].

Thus, we have sought to find out if there is any association between residential radon exposure and mutations/alterations in the *EGFR* and *ALK* genes.

Material and methods

Design and setting

This study is a case series consisting of lung cancer patients originally recruited for a multicentre, hospital-based, case–control study involving nine Spanish hospitals. The overall objective of this study, termed LCRINS (Lung Cancer Risk Factors in Never Smokers), is to identify and characterise the different risk factors for lung cancer in never-smokers, with special emphasis on residential radon. Patient recruitment started in 2011 and is ongoing. Lung cancer cases are prospectively included as they are being diagnosed at the participating hospitals. At these hospitals, the pulmonologist responsible for diagnosing lung cancer takes part in the study and identifies lung cancer patients who have never smoked. The estimated sample size calculated at the beginning of the study was 250 cases, but this number was increased

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due to a longer recruitment period. The study protocol was approved by the Galician Ethics Committee (2010/295) and all participants signed a written consent before taking part in the research.

Inclusion and exclusion criteria

All cases had a pathologically confirmed lung cancer diagnosis. To be included in the study, patients had to be aged ⩾30 years with no upper age limit and no previous cancer history. Cases were recruited between January 2011 and April 2015.

To be defined as a never-smoker, participants had to fulfil the World Health Organization (WHO) definition for never-smokers. A never-smoker was thus defined as: 1) an individual reporting <100 cigarettes smoked in his/her lifetime; or 2) an individual who had not smoked >1 cigarette per day for >6 months in his/her lifetime. Smoking status was assessed by interviewing each participant and recording their responses to a questionnaire; if the case was an ever-smoker, they were excluded from the study.

Histological type was obtained from the pathology records and the same was done for *EGFR* mutations and *ALK* translocations. *EGFR* mutations were first determined systematically in the study hospitals in October 2010, and *ALK* translocations were first determined in January 2011.

Information retrieval

All participants answered a standardised questionnaire regarding lifestyle, with special emphasis on the risk factors for lung cancer. The interviews were performed by the clinicians taking part in the study, who were previously trained to avoid interviewer bias. Each participant provided a total of 3 mL of blood that was collected in an EDTA container with the objective of determining the genetic polymorphisms associated with the susceptibility genes for lung cancer. Samples were stored at −80 °C until analysis. The questionnaire focused on ETS, past and present occupation, diet and performing do-it-yourself activities, such as painting, model-making, furniture refinishing and varnishing, which have been reported to increase the risk of lung cancer in never-smokers [19].

Residential radon measurement

Participants used a radon device in their homes for a minimum of 3 months. The radon detector was of the alpha-track type (CR-39; Radosys Ltd, Budapest, Hungary), which is among the most reliable radon devices. The detector was given out at the hospital with written instructions on how to use it, including pictures on how to correctly position it in the home; 3–4 days after being given the detector, participants were contacted by phone to make sure that the device had been properly positioned, by following the instructions provided. Once the exposure period had finished, participants were called and reminded to send the device back in the sealed envelope given at the hospital. Radon detectors were read at the Galician Radon Laboratory (University of Santiago de Compostela, Santiago de Compostela, Spain). Periodical quality assurance included the use of blanks and double detectors in some of the dwellings. The laboratory has taken part in intercomparison exercises organised by the University of Cantabria (Santander, Spain) and the Nuclear Safety Council of Spain (Madrid, Spain) with excellent results [20, 21]. A seasonal adjustment was considered for all measurements.

Laboratory analysis

The same procedure was used in all participating centres.

EGFR *mutation screening*

Screening for mutations in *EGFR* exons 18, 19, 20 and 21 was performed with the CE-IVD marked cobas *EGFR* Mutation Test kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol.

Fluorescence in situ *hybridisation (FISH) for* ALK

4-µm sections of each sample were collected using pretreated slides (Dako Denmark A/S, Glostrup, Denmark) for each test, and dried at 60 °C for 1 h. Detection of *ALK* rearrangements on 2p23 was performed with the Vysis *ALK* Break Apart FISH Probe Kit (Abbott Molecular, Abbott Park, IL, USA).

Statistical analysis

We initially performed a descriptive analysis followed by a bivariate analysis comparing the age at diagnosis of *EGFR* mutation-positive individuals and did the same for *ALK* translocation-positive individuals. We also compared residential radon concentrations in *EGFR*-mutated *versus* nonmutated individuals, and between patients with and without *ALK* translocations. We also compared radon concentrations within each *EGFR* mutation type using median concentrations between groups. For specific types of *EGFR* mutations, we only compared exon 19 deletion *versus* exon 21 (L858R) single-point substitution mutation because these were the most frequent mutations observed. To perform these comparisons, we used the

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median test, which is the most appropriate nonparametric test to evaluate if median radon concentration differs for different *EGFR* mutations or *ALK* status. We considered p<0.05 as significant. All analyses were performed with IBM SPSS Statistics for Windows, version 22.0 (IBM Corporation, Armonk, NY, USA).

Results

No one refused to participate in the study. Only two patients did not place the radon device in their homes. The return rate of the radon devices was also quite high, with only 38 radon detectors (11.7% of the total number) not returned for various reasons.

The study recruited 323 never-smokers with lung cancer; 80% were females, with a median age of 70 years (interquartile range: 61–78 years). The predominant histological type was adenocarcinoma (78.3%) followed by squamous cell carcinoma (9.3%); 60.4% of all cases were stage IV at diagnosis. Median radon concentration was 182 $Bq{\cdot}m^{-3}$ (interquartile range: 103–333 $Bq{\cdot}m^{-3}$); 44% of participants had lived with a smoker for >20 years. Participants lived a median of 30 years in the dwelling where radon was measured (interquartile range: 15–44 years). A detailed description of their characteristics is shown in table 1.

Table 2 describes the mutations observed in the *EGFR* and *ALK* genes. *EGFR* mutation analysis was performed in 209 (64.7% of the total number) participants, while *ALK* analysis was performed in 80 (24.8%) participants. *EGFR* was mutated in 42% of those analysed, and the most frequent mutation was exon 19 deletion (56.3% of all mutations), followed by exon 21 (L858R) single-point substitution mutation (39.1%). The remaining mutation frequency was extremely low. *ALK* was rearranged in 15% of those analysed. The median age of patients with an *EGFR* mutation was 67.5 years compared with 69 years for those without mutations in *EGFR* (p=0.811). Median age at diagnosis of individuals with translocated *ALK* was 57 years, compared with 67.5 years for those without *ALK* translocations (p=0.002).

Table 3 shows the radon concentrations by *EGFR* and *ALK* alteration status. The differences in radon concentrations in those whose tumour had an *EGFR* mutation compared with wild type were small (160 $Bq{\cdot}m^{-3}$ for those with an *EGFR* mutation compared to 174 $Bq{\cdot}m^{-3}$ when *EGFR* was not mutated) (figure 1a). When radon concentrations were analysed by particular *EGFR* mutations, there were no statistically significant differences. When we specifically compared the two most frequent mutations, exon 19 deletion *versus* exon 21 (L858R) single-point substitution mutation, we observed a nearly significant difference (p=0.057). The individuals with exon 19 deletions had a close to two-fold higher radon concentration in their homes (216 $Bq{\cdot}m^{-3}$ for exon 19 deletion compared to 118 $Bq{\cdot}m^{-3}$ for exon 21 (L858R) single-point substitution mutation). Residential radon concentrations for nonmutated *EGFR* participants and those with exon 19 deletions or exon 21 (L858R) single-point substitution mutations are shown in figure 1b and 1c, respectively. When a similar analysis was performed for *ALK* translocations, there were no significant differences in measured radon levels between individuals with *ALK* translocations *versus* ALK-negative patients. Nevertheless, participants with *ALK* translocations had a much higher radon concentration in their homes than those with normal *ALK* (290 *versus* 165 $Bq{\cdot}m^{-3}$, respectively) (figure 2).

Discussion

We sought to determine if residential radon might be associated with alterations in driver genes of never-smokers with lung cancer. We did not observe an association between residential radon level and the overall pattern of *EGFR* mutation. However, since alpha radiation is associated with large-scale genomic alterations, we compared radon levels in never-smokers with regard to known molecular alterations. We found that individuals whose tumours had exon 19 deletions had close to two-fold higher residential radon levels than individuals with exon 21 (L858R) single-point substitution mutations. Residential radon level was also much higher in the home of patients whose tumours harboured an *ALK* translocation compared with individuals who did not have such a translocation. This observation could mean that alpha radiation (through residential radon) might have a role in lung carcinogenesis in never-smokers.

The frequency of *EGFR* mutations in our sample of never-smokers was 42% compared to 66.6% observed in the subsample of never-smokers (n=612) of a large Spanish study by Rosell *et al.* [11]. This difference may be due to the fact that Rosell *et al.* [11] included advanced nonsmall cell lung cancers and a high percentage of adenocarcinomas. *EGFR* mutations are more frequent in these cases [22], whereas we included approximately 20% of early lung cancers. Our mutation rate for *EGFR* would probably be higher if we had had the similar inclusion criteria of Rosell *et al.* [11]. The *EGFR* mutation rate for ever- and never-smokers in the largest published cohort was 11% [22]. Interestingly, 5.6% of all *EGFR* mutations in our study were observed in squamous cell carcinomas and the remaining in adenocarcinomas. A recent review reported the frequency of *EGFR* mutations in never-smokers to approach 45%, which is very similar to our findings [23]. The pattern of *EGFR* gene mutation in our work was very similar to that found by Rosell *et al.* [11]. Exon 19 deletions comprised 56.2% of all mutations in our study compared to

TABLE 1 Description of the study cohort

Variable	
Patients n	323
Sex	
Male	63 (19.5)
Female	260 (80.5)
Age years	
Mean	69
Median	70
Percentile 25–75	61–78
Minimum	35
Maximum	94
Education (completed studies)	
No formal studies	143 (44.3)
Primary studies	120 (37.2)
Secondary studies	29 (9.0)
University degree	26 (8.0)
Unknown	5 (1.5)
Histological type	
Squamous cell carcinoma	30 (9.3)
Adenocarcinoma	253 (78.3)
Small cell lung cancer	19 (5.9)
Large cell lung cancer	8 (2.5)
Other types	12 (3.7)
Unknown	1 (0.3)
Stage at diagnosis#	
I	35 (14.3)
II	14 (5.7)
IIIA	30 (12.2)
IIIB	18 (7.3)
IV	148 (60.4)
Small cell lung cancer	19
Unknown	59
Residential radon exposure Bq·m^{-3}	
Mean	271
Median	182
Percentile 25–75	103.5–333
Minimum	11
Maximum	2350
Unknown	38 (11.7)
Years living with a smoker	
<20	180 (55.7)
⩾20	143 (44.3)
Years living in the measured dwelling	
Mean	31.4
Median	30
Percentile 25–75	14–44

Data are presented as n (%) unless otherwise stated. #: percentages calculated excluding unknown stages and small cell lung cancer.

62.2% in the study by Rosell *et al.* [11] and exon 21 (L858R) single-point substitution mutation was 39.1% in our work compared to 37.8% in the study by Rosell *et al.* [11]

15% of *ALK*-tested patients had rearrangements in the present study. This agrees with other studies that report 5–11% of *ALK* rearrangements in never-smokers [23]. The study of Kwak *et al.* [12] observed a frequency of 5.5%, although this included only 24% of patients who were ever-smokers. Other studies have reported rearrangement frequencies of 6.9% in the USA, although with a frequency of ever-smokers close to 40% [24]. The positive rate for *ALK* in the largest cohort study including ever- and never-smokers was 5% [22].

Similar to other reports, we observed that patients with *EGFR* mutations have a similar age than patients with no mutations [11]. For *ALK* translocations, translocation-positive patients are usually diagnosed at younger ages, approximately 7–10 years earlier than never-smokers without *ALK* translocations [25].

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TABLE 2 Description of mutations and translocations in *EGFR* and *ALK*

Gene	
EGFR	
Analysis performed	209 (64.7)
Not analysed	114 (35.3)
Mutated[#]	90 (42.0)
Nonmutated[#]	119 (58.0)
Mutation type[¶]	
Exon 18 (G719X)	0 (0)
Exon 19 deletion	49 (56.2)
Exon 20 (S768I)	0 (0)
Exon 20 (T790M)	2 (2.3)
Exon 20 insertion	3 (3.4)
Exon 21 (L858R)	34 (39.1)
ALK	
Analysis performed	80 (24.8)
Not analysed	243 (75.2)
Translocated	12 (15.0)
Nontranslocated	68 (85.0)

Data are presented as n (%) unless otherwise stated. [#]: three participants had *EGFR* mutations but mutation type was not recorded; [¶]: two participants displayed more than one mutation.

In our case, *ALK*-translocated patients were 10 years younger and the difference was statistically significant. Ren *et al.* [26] observed a similar result in a cohort of Chinese women.

The possible effect of residential radon on mutations/translocations in the driver genes *EGFR* and *ALK* is intriguing and provides a plausible new explanation for the different biological characteristics of lung cancer in never-smokers compared to ever-smokers. The only available studies were published by Taga *et al.* [17] and Mezquita *et al.* [27] in 2012 and 2015, respectively. Taga *et al.* [17] observed no association between residential radon and *EGFR* mutations. Nevertheless, this study included only 70 patients and exposure to radon was low. Only 27% of participants had radon concentrations >83 $Bq \cdot m^{-3}$ whereas in the present study 75% of participants had radon concentrations >100 $Bq \cdot m^{-3}$. This high radon exposure provides a plausible explanation for its influence on the alterations of driver genes since high radon exposure is the most evident difference between the study by Taga *et al.* [17] and the present one. The study by Mezquita *et al.* [27] included only 31 patients from Madrid (Spain), generally a low radon area. They observed that *ALK* rearrangements were associated with levels higher than the action levels recommended by WHO.

TABLE 3 Residential radon concentration, *EGFR* mutations and *ALK* translocations

Gene	Patients n	Residential radon concentration $Bq \cdot m^{-3}$	p-value
EGFR			
Mutated	90	160 (100–306)	0.932
Nonmutated	119	174 (96–295)	
***EGFR* mutation type**			
Exon 18 (G719X)			
Exon 19 deletion	49	216 (103–412)	
Exon 20 (S768I)			
Exon 20 (T790M)	2	70	0.329[#]
Exon 20 insertion	3	150	0.057[¶]
Exon 21 (L858R)	34	118 (98–266)	
Two mutations	2	224	
ALK			
Rearranged	12	290 (63–352)	0.958
Not rearranged	68	164.5 (105–295)	

Data are presented as median, p25–p75. [#]median test comparing all *EGFR* mutations; [¶]: median test comparing only exon 19 deletions with exon 21 (L858R) single-point substitution mutations.

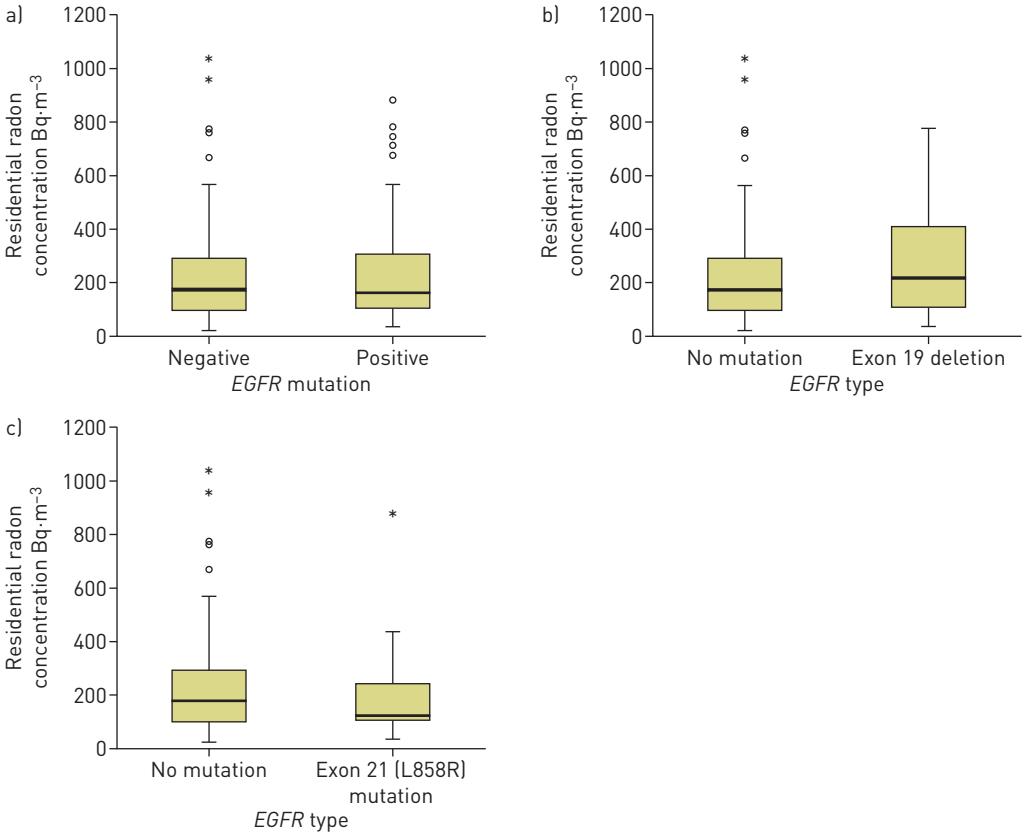

FIGURE 1 Relationship between residential radon exposure and a) *EGFR* mutations, b) exon 19 deletions and c) exon 21 (L858R) single-point substitution mutations. Asterisks represent outliers, *i.e.* values outside the 95% percentile.

Mutations in the genes that encode tyrosine kinase receptors are frequent in lung adenocarcinoma but infrequent in squamous cell carcinoma [13]. Various studies have observed that the molecular signatures of both lung cancer types are different, with more (mutated) genes playing a role in the lung carcinomas of ever-smokers compared to never-smokers [13]. An explanation may be that tobacco, with thousands of chemical substances and dozens of demonstrated carcinogens, induces a spectrum of DNA lesions, which is dominated by small alterations in signalling molecules. In the case of the lung carcinomas in never-smokers, it may be that ETS plays a role, but a recent paper has shown that there are no statistically significant differences in the mutation patterns of *EGFR* and *ALK* of never-smokers exposed to ETS compared to those not exposed to it [28]. The lack of association between exposure to ETS and alterations in driver genes leaves the door open for a possible effect of alpha radiation on these genes. There is evidence for different molecular pathways for lung cancer in never- compared to ever-smokers, even in tumours of the same histological type, such as adenocarcinoma [27, 29].

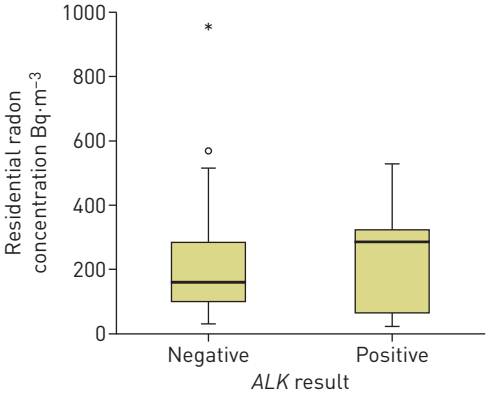

FIGURE 2 Relationship between residential radon and *ALK* rearrangements. Asterisks represent outliers, *i.e.* values outside the 95% percentile.

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While it is not possible to completely explain the differences in residential radon level of patients whose tumours had an *EGFR* exon 19 deletion compared with those whose tumours had an exon 21 (L858R) single-point substitution mutation, alpha radiation likely induces cluster damage, leading to deletion events [30]. The difference in the character of lesions associated with alpha exposure is also consistent with the differences in radon levels we observed for *ALK* translocations. Although we did not find significant differences in radon levels associated with translocation events, figure 2 suggests that the small number of *ALK* translocations (only 12) may have affected the statistical power of our findings. Our cohort was also small and *ALK* rearrangements had a low frequency, making it more difficult to find any association. The fact that most of our participants were females who tend to spend more time at home [4], and the fact that the Galician population has low mobility (participants in our study have lived a median of 30 years in the dwellings where radon was measured) strengthens the hypothesis that radon induces this profile of mutations.

Although some studies have assessed the role of alpha radiation in tumour treatment in animal models [31], there is a paucity of studies investigating how alpha radiation might cause specific alterations in the *EGFR* or *ALK* genes. Recent research suggested that ionising radiation might be involved in the activation of the *EGFR* extracellular signal and might have a role in DNA repair [32]. Alpha radiation might also be involved in cellular signalling without having a direct impact on lung epithelial cells, just through being present in the cell surroundings. This is known as the radiation-induced bystander effect, and results in genomic instability [33].

The present study has four important strengths. First, it has been performed exclusively in never-smokers; therefore, potential confounders related to active tobacco consumption are not present. Second, the study area has been previously defined as a radon-prone area and our population has lived in the same dwellings for a long time (median residence time: 30 years). Third, the high variability of indoor exposure allowed us to study possible dose–response associations not found in other settings. Furthermore, the multicentre design of the study produced a moderately large sample size. Finally, 89% of participants returned the radon detector. This is a very high return rate if we take into account the high mortality of lung cancer and the fact that most of the participants who entered study had stage IV lung cancer.

The present research has some limitations. Only 65 and 25% of all patients were tested for *EGFR* and *ALK*, respectively. *EGFR* testing is usually performed in stage IV adenocarcinoma; the analysed percentage corresponds approximately to the expected figure. The current Spanish guidelines recommend *EGFR* screening in these individuals [34]. For *ALK*, we have to discount those cases with small cell lung cancer and histological types other than adenocarcinoma, stages I–III and patients who were already *EGFR*-positive. Once these patients are eliminated, because they do not fulfil the criteria for *ALK* testing, the remaining number approximately coincides with the number of patients tested for *ALK*. Therefore, we think that there is no selection bias regarding the testing performed in our participants. Nevertheless, we recognise that the sample size is small, especially for *ALK* rearrangement. A further limitation, related to sample size, is that we had too few males to perform a separate analysis in this subgroup.

To conclude, the present findings might suggest that residential radon exposure could have a role in causing genetic mutations in driver genes in never-smokers with lung cancer, although the results of the present study are not statistically significant. Despite being the second risk factor for lung cancer after tobacco consumption, radon exposure has been neglected by clinicians when considering risk factors for lung cancer. Further study on the effects of radon on nonsmokers is urgently needed, but it should be remembered that radon increases the risk of all lung cancers and health authorities should protect the entire population from this risk.

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