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EUELC project: a multi-centre, multipurpose study to investigate early stage NSCLC, and to establish a biobank for ongoing collaboration

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ABSTRACT: The European Early Lung Cancer (EUELC) project aims to determine if specific genetic alterations occurring in lung carcinogenesis are detectable in the respiratory epithelium. In order to pursue this objective, nonsmall cell lung cancer (NSCLC) patients with a very high risk of developing progressive lung cancer were recruited from 12 centres in eight European countries: France, Germany, southern Ireland, Italy, the Netherlands, Poland, Spain and the UK. In addition, NSCLC patients were followed up every 6 months for 36 months. A European Bronchial Tissue Bank was set up at the University of Liverpool (Liverpool, UK) to optimise the use of biological specimens.

The molecular–pathological investigations were subdivided into specific work packages that were delivered by EUELC Partners. The work packages encompassed mutational analysis, genetic instability, methylation profiling, expression profiling utilising immunohistochemistry and chip-based technologies, as well as in-depth analysis of FHIT and RARβ genes, the telomerase catalytic subunit hTERT and genotyping of susceptibility genes in specific pathways.

The EUELC project engendered a tremendous collaborative effort, and it enabled the EUELC Partners to establish protocols for assessing molecular biomarkers in early lung cancer with the view to using such biomarkers for early diagnosis and as intermediate end-points in future chemopreventive programmes.

KEYWORDS: Biobank, case control, European Early Lung Cancer, epidemiology, genetic alterations, nonsmall cell lung cancer

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Lung cancer is the result of multiple molecular changes that occur in the bronchial cells, resulting in the deregulation of pathways which control normal cellular growth, differentiation and apoptosis [1]. The disease is clinically divided into two categories: nonsmall

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cell lung cancer (NSCLC), including squamous cell carcinoma, adenocarcinoma and large cell carcinoma, representing ~80% of all lung cancers, and small cell lung cancer. These two major types of lung cancers are, in essence, two completely different diseases, each of which has its own recommended therapies.

At present, more males than females die each year from lung cancer, but in recent years a rapid increase in lung cancer mortality has been observed among females in developed countries, contrasting with a levelling off or decrease among males in the western world [2]. Although the causes of lung cancer are almost exclusively environmental, it is likely that there is substantial individual variation in susceptibility to respiratory carcinogens [3]. A number of genes have been found to be frequently mutated in lung cancers, and may be candidates to be used as biomarkers of early carcinogenic effect on the lung. In addition, such alterations might be specific to the carcinogens responsible for the development of lung cancer. However, there is a need for knowledge on mechanisms of action of human lung carcinogens. Given that senescent/apoptotic tumour and stromal cells lead to the release of naked DNA into the circulation, which is enriched in plasma and serum of lung cancer patients [4], novel approaches to the detection of early disease can be envisaged. The attraction of using somatic genetic changes as targets for early disease is that they may be detected using highly sensitive molecular techniques, which can potentially be applied not only in tissue biopsy but also in exfoliated material.

The central hypothesis behind early detection is that by detecting and then treating lung cancer at an early stage, patient mortality can be improved [5]. The European Early Lung Cancer (EUELC) project aims to determine whether specific genetic alterations occurring in lung carcinogenesis are detectable in the respiratory epithelium. In order to pursue this objective, NSCLC patients with a very high risk of developing progressive lung cancer were recruited from 12 centres in eight European countries: France, Germany, southern Ireland, Italy, the Netherlands, Poland, Spain and the UK. In addition, NSCLC patients were followed up every 6 months for 36 months. The study was funded under the European Union Framework V Programme, which sought to promote greater research integration, co-ordination and exchange between European research institutions.

PARTICIPANTS

Beginning in 2002 and continuing through to 2006, 1,176 NSCLC patients and 1,584 controls were recruited from participating hospitals. Patients with a surgically resected primary NSCLC, histologically or cytologically confirmed, were recruited and matched with one or two controls. Most centres recruited hospital controls, whereas in the UK population controls were selected. Hospital controls were recruited either in the same hospitals or in general public hospitals serving the same areas as where the lung cancer patients resided. To be eligible, control subjects had to attend the hospital for a disease not attributable to smoking and had to be free from history of cancer or respiratory disease. Population controls were selected from population registers of general practitioners in the UK. Matching criteria were: centre, sex and age ($\pm$5 yrs). The study protocol was approved for each centre by its relevant institutional or national Ethics Committee. Overall, the response rate was 79.4% for lung cancer patients and 89.1% for control subjects.

DATA AND SPECIMEN COLLECTION

After obtaining written, informed consent, all participants completed a 45-min in-person interview, administered by a research interviewer. A standardised lifestyle questionnaire was used to collect detailed information on socioeconomic and demographic characteristics, medical history, family history of cancer, history of tobacco consumption and occupational exposure to asbestos. Data collection was identical for both lung cancer patients and control subjects. Extensive information about tobacco smoking was elicited for all participants including their age at the start and end of all periods of consumption and the number of cigarettes smoked per day. All periods of consumption counted towards total exposure. An individual who had smoked at least 100 cigarettes in their lifetime was defined as an ever-smoker. Ever-smokers included former and current smokers, and recent quitters (those who had quit within the previous year). A former smoker was an individual who had quit smoking at least 1 yr before diagnosis or at least 1 yr before the interview for cancer patients and control subjects respectively. Information on history of cancer among first-degree relatives (*i.e.* parents, brothers and sisters, and biological children) was recorded, including age of diagnosis, site of cancer and relationship to the participant [6]. Characteristics of NSCLC patients and control subjects with complete data are presented in table 1.

Lung cancer patients were followed up every 6 months for the duration of the project. A short questionnaire, including status of tobacco consumption, was completed by each patient during each follow-up visit. Lung cancer patient specimens, including blood, bronchial washing or sputum and tissue samples, were collected at surgery. In addition, during the clinical-recall visits blood and sputum or bronchial washing samples were collected. All collected specimens (from surgery and follow-up visits) were processed and archived.

MANAGEMENT OF THE EUELC PROGRAMME

Standard operating procedures (SOPs) pertaining to each aspect of the research programme were developed and agreed at study inception. A Steering Committee met regularly to measure progress against deliverables, identify issues and suggest solutions. The management and organisation of the programme was enhanced by the use of an interactive web-based database (MACRO; InferMed, London, UK) that provided a secure server for the storage of epidemiological and clinical data.

The database was created in SQL Server 2000 in partnership with InferMed. MACRO 2.2 was installed onto the database server. Interactive access to the central database was created for all of the study centres. A high level of security was essential, including a firewall as well as biometric fingerprint access for all authorised users. This approach also ensured controlled data entry for each centre with data editing being restricted to the data manager and principal investigators.

The EUELC database allowed direct entry of the questionnaire by the research nurses, as well as the clinical data and specimen details. All patients were automatically assigned an EUELC

TABLE 1 Characteristics of lung cancer patients and controls recruited into European Early Lung Cancer project

Characteristic	Patients[#]	Control
Subjects n	922	1524
Sex		
Male	674 (73.2)	1105 (72.6)
Female	247 (26.8)	418 (27.4)
Ethnicity		
White - European	838 (97.4)	1472 (96.8)
White - Other	10 (1.2)	18 (1.2)
Other	12 (1.4)	30 (2.0)
Smoking status		
Never	53 (6.2)	424 (28.1)
Former	466 (54.5)	645 (42.7)
Current	336 (39.3)	440 (29.2)
Occupational asbestos exposure		
No or unsure	667 (79.3)	1343 (89)
Yes	174 (20.7)	166 (11)
Education level		
Low	648 (80)	1033 (68.5)
High[¶]	162 (20)	474 (31.5)
Continuous variables		
Age yrs	64.6±9.2	62.9±9.5
Tobacco consumption pack-yrs	41±25	22.2±24
Total smoking duration yrs	38.5±15.2	23.7±19.5
Histology		
Squamous	392 (42.8)	
Adenocarcinoma	412 (45)	
Large cell	45 (4.9)	
Mixed	11 (1.2)	
NSCLC unspecified	55 (6)	
Tumour stage at presentation		
N undetermined	37 (4.2)	
I	552 (62)	
II	221 (24.8)	
III	81 (9.1)	
IV	0 (0)	
Progressive disease[+]	163 (22.1)	
Length of follow-up months[+]	18	

Data are presented as n (%) or mean±SD, unless otherwise stated. NSCLC: nonsmall cell lung cancer. [#]: includes patients with at least one sample available from tumour, biopsy, bronchial washing, sputum and blood; [¶]: first degree, higher degree, professional qualification or university entrance; [+]: in November 2007.

identifier. No personal identifiers were transferred to the database. The patient's details were retained by the collaborating Partners at each of the centres to ensure local access to necessary information for clinical follow-up and labelling of all biological samples. This mechanism enabled regular review of progress, and identified issues regarding recruitment.

ESTABLISHMENT OF THE EUROPEAN BRONCHIAL TISSUE BANK

To optimise the standardisation, preservation and use of the clinical specimens, the European Bronchial Tissue Bank (EBTB) was set up at the University of Liverpool (Liverpool, UK; figs 1–3). All specimens were processed and archived at the EBTB. The specimens included lung cancer and adjacent normal tissue, biopsies, sputum, bronchial lavage and blood (table 2).

Specimens were collected by a technician employed by each Partner for this purpose. The EBTB developed SOPs to ensure uniformity in the collection and labelling of specimens. Tissue specimens were frozen on dry ice with isopentane and subsequently delivered by courier to the EBTB. All specimens were processed at the EBTB, and a dedicated Laser Capture Dissecting Microscope (Arcturus Pixcell II; Arctur Corporation, Sunnyvale, CA, USA) was available for this project. The EBTB prepared slides from paraffin-embedded tissue in association with EUELC pathologists, as well as isolating DNA/RNA from the above specimens. All specimens were stored at the EBTB in a dedicated -80°C freezer. All specimens were recorded on the MACRO database and had to be signed off as ''consented'' and ''included'', which entailed the Partners holding the consent form in their own laboratory. Specimen details were also recorded in a separate database, linked to MACRO, so the EBTB Manager could undertake an audit of all samples received for UK regulations. All the specimens were stored using an LIMS system (SQL database; Autoscribe Ltd, Riseley, UK), thus the location is known. Specimens were provided to the Partners, as required, to enable procedures relevant to their work package (WP) to be carried out. If a specimen was in short supply, the Task Group Committee prioritised its use.

MOLECULAR–PATHOLOGICAL INVESTIGATIONS OF EUELC SPECIMENS

The molecular–pathological investigations were subdivided into specific WPs, which were delivered by EUELC Partners (fig. 4). The WPs encompassed mutational analysis, genetic instability, methylation profiling, expression profiling utilising immunohistochemistry (IHC) and chip-based technologies, as well as in-depth analysis of FHIT and RARβ genes, the telomerase catalytic subunit hTERT, and genotyping of susceptibility genes in specific pathways.

Ras and p53 mutations

K-Ras mutation analysis was performed for codon 12 with the Point-EXACCT microarray approach [7]. For validation, a subset of lung cancer patients were re-examined in a blind fashion by an independent study centre. p53 mutational status was examined in the EUELC frozen specimens using Arrayed Primer EXtension (APEX), a novel chip-based technology based on incorporation of four dye terminators into oligonucleotide primers by a thermostable DNA polymerase [8]. This can be viewed as a four-colour chip-based sequencing technology where each primer identifies a base in the target sequence. APEX reaction combines both high parallelisation capacity of oligonucleotide array and specificity of molecular recognition by DNA polymerase. Using APEX, oligonucleotides corresponding only to the wild-type sequences are spotted onto the chip, and the identity of the mutation is given by the fluorescence of the incorporated dye terminator.

Genomic instability

Genomic instability was assessed with a previously optimised panel of nine fluorescent-labelled microsatellite markers:

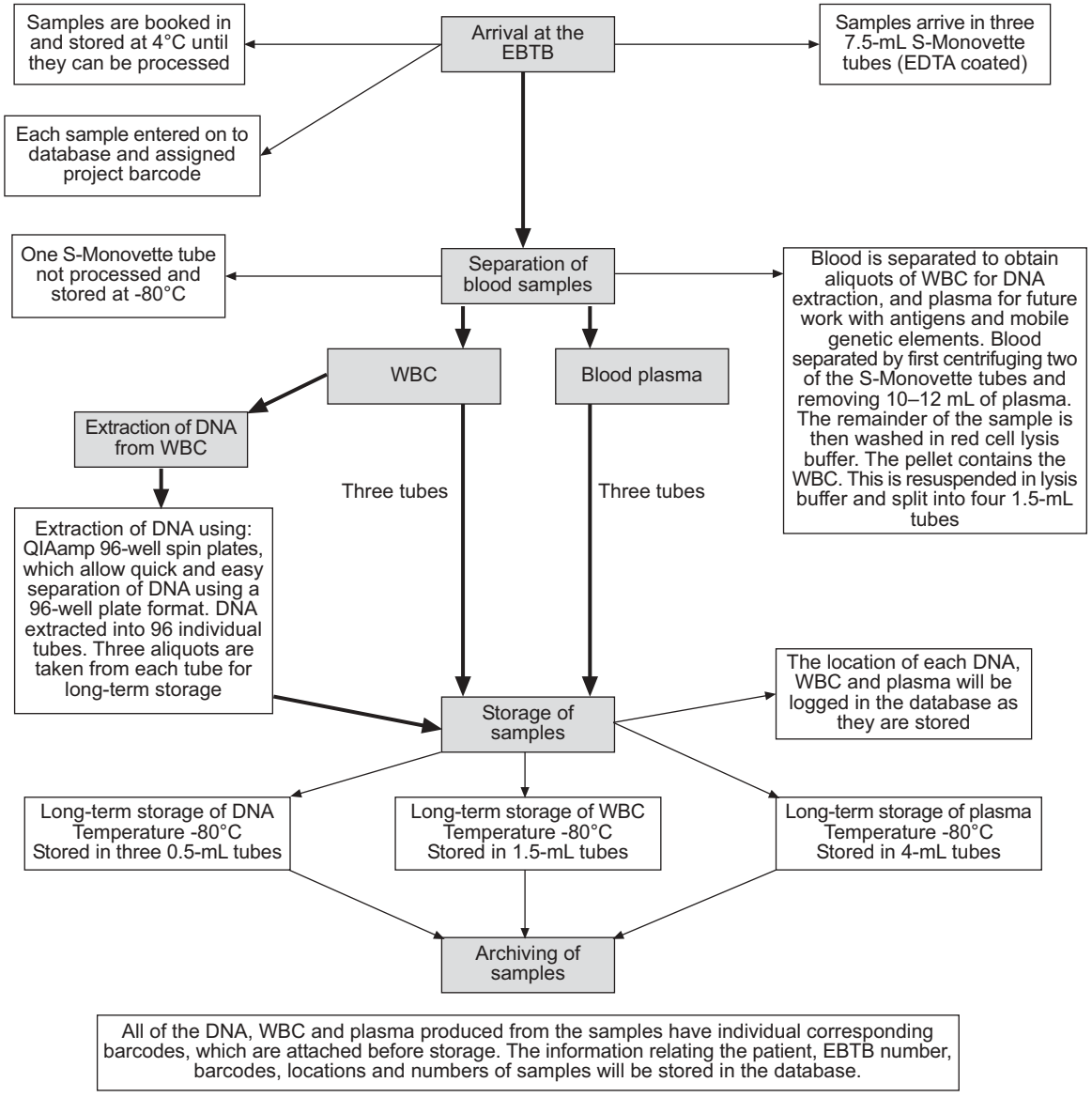

FIGURE 1. European Early Lung Cancer project processing of blood samples. EBTB: European Bronchial Tissue Bank; WBC: white blood cells; EDTA: ethylenediamine tetraacetic acid.

D3S1300, D3S1289, D5S644, D9S161, D9S157, D13S171, D13S153, D17S938 and D17S2179E [9]. The markers comprising the panel were selected as the most informative from a wider selection that were previously tested on a series of 96 lung carcinomas and 164 bronchial lavage specimens [10]. Specificity and sensitivity assays were performed to assess the inter-assay variability and loss of heterzygosity (LOH) threshold with minimal false positives. PCR were run on a 377 ABI PRISM automatic sequencer (Applied Biosystems Inc., Foster City, CA, USA) and the results were analysed using the Genescan™ and Genotyper™ software (Applied Biosystems). The main objectives of this investigation were to detect cancer cells in bronchial lavage/sputum/serum samples by assessing their genomic damage, as well as identifying markers of cancer specific genomic instability, *i.e.* loci that carry damage indicative of carcinogenic processes.

In addition, microsatellite alterations targeting chromosome 3p (including FHIT on 3p14.2) were detected by assaying several genomic loci located on 3p14.1 (D3S1566), 3p14.2 (D3S1300, D3S4103, D3S1234), 3p21 (D3S1289), 3p23–24 (D3S1266), 3p24.2-ter (D3S2338) and 3p25–26 (D3S1304). LOH and the presence of allele shifts indicating genomic instability were recorded in invasive and noninvasive lesions. The aim of this section of the project was to identify the most frequent hot-spot of LOH on chromosome 3 that suggests the presence of tumour suppressor genes (TSG) and the loci more frequently undergoing instability to define the extent of this phenomenon during lung cancer development.

Methylation profiling

DNA methylation of p16 and MGMT promoters were analysed by undertaking a pyrosequencing approach [11]. Assays were

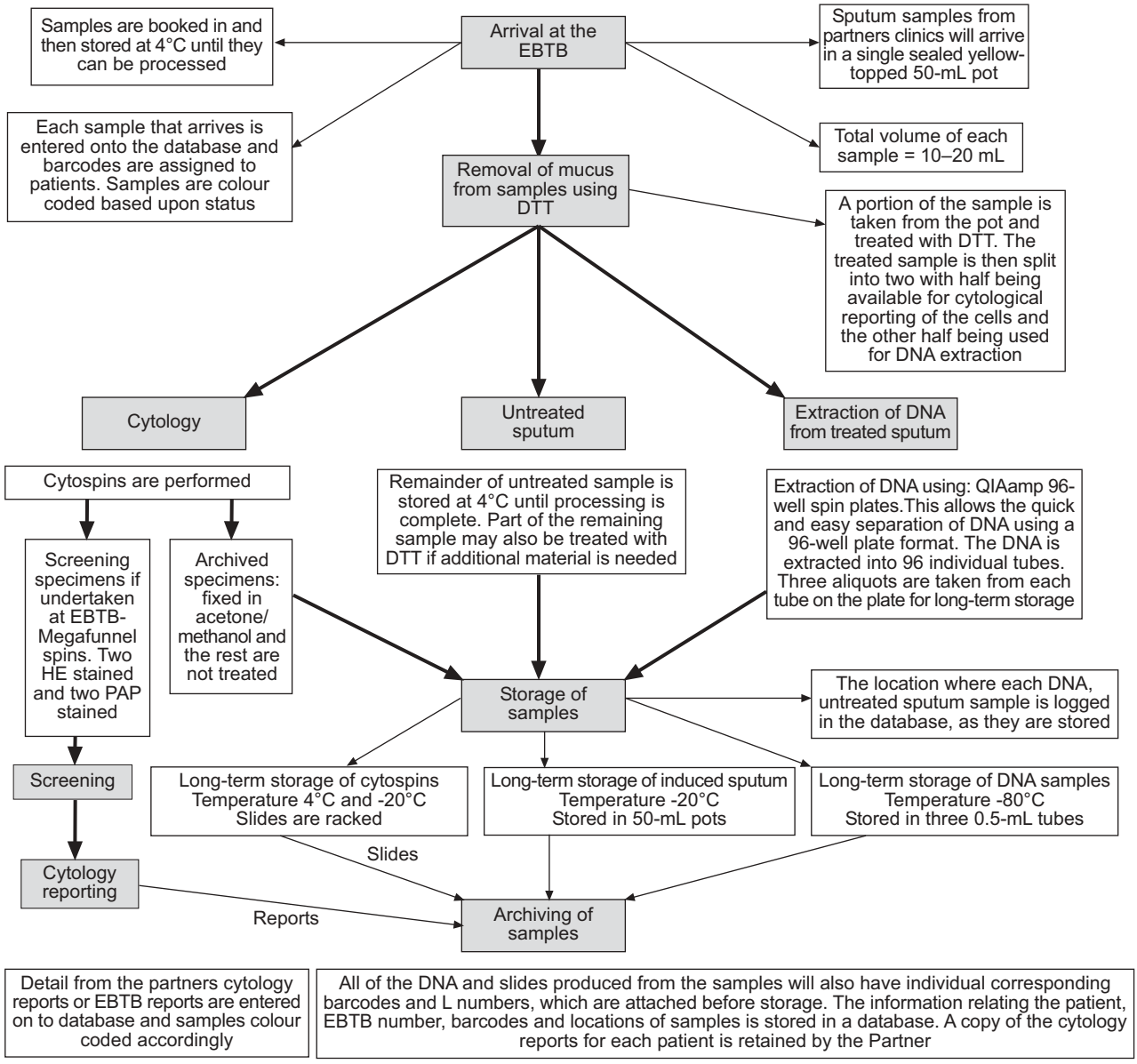

FIGURE 2. European Early Lung Cancer project processing of sputum. EBTB: European Bronchial Tissue Bank; DTT: dithiothreitol; HE: haematoxylin and eosin; PAP: papanicolaou test.

designed using the Assay Design Software V1.0.6 (Biotage, Uppsala, Sweden). The assays examined part of the CpG islands present in the promoters of MGMT and p16. The p16 assay interrogated 7 CpGs and one bisulphite control site, whereas MGMT assay interrogated 12 CpGs and two bisulphite control sites. DNA was bisulphite treated using the EZ Methylation kit Gold (Zymoresearch, Orange, CA, USA) followed by PCR amplification and subsequent pyrosequencing analysis using the SQA reagents (Biotage, Hertford, UK). Analysis was conducted using the PyroQ-CpG software (Biotage, Uppsala).

The FHIT gene is frequently inactivated in lung cancer and other cancers through a variety of genetic and epigenetic mechanisms, including *de novo* promoter methylation [12, 13]. However, review of the literature revealed that the commonly used primers for methylation specific polymerase (MSP) analysis of the FHIT gene are not fully validated and this might explain the lack of correlation between methylation status (as measured by MSP) and FHIT protein expression reported in some studies. Considering the high relevance of the mechanisms of FHIT inactivation, we investigated the methylation pattern of this gene by bisulphite sequencing, after careful bioinformatic analysis (MethPrimer program) of the promoter region, to identify the genomic areas that could represent the best targets for functionally relevant methylation. This identified the region with the highest CpG content in Intron 1 (GenBank accession number AH005169), and specific primers were designed for MSP analysis.

RNA expression profiling

High-quality RNA (Agilent - RIN <6) from selected frozen tumour samples, was profiled by Oxford Gene Technology

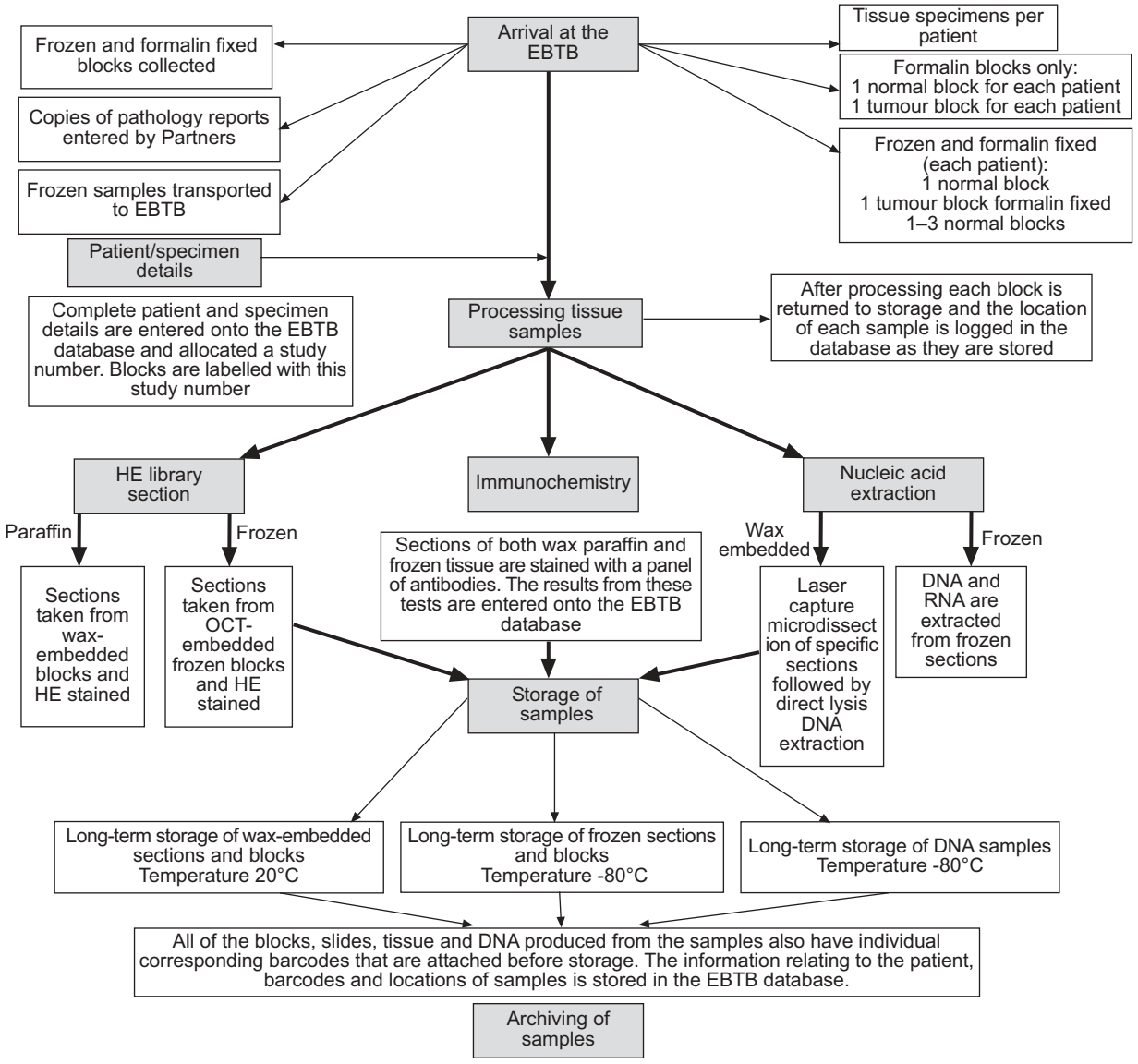

FIGURE 3. European Early Lung Cancer project processing of lung tissue. EBTB: European Bronchial Tissue Bank; HE: haematoxylin and eosin.

(Oxford, UK). One microgram of total RNA was labelled using a low-input RNA Amp kit (Agilent 5184-3523; Agilent Technologies, Palo Alto, CA, USA). Lung cancer RNAs were labelled with Cy3 and were hybridised with a Cy5 reference sample labelled from RNA consisting of a 50:50 mix of Universal human reference (Stratagene 740000; Agilent Technologies) and a Human Lung cell line (Ambion AM7864; Applied Biosystems). The labelled samples were then hybridised to Agilent 44k human whole genome oligo microarrays (Agilent G4112A; Agilent Technologies).

Protein expression of genes within the cell-cycle control pathway

We have optimised and validated automatised immunohistochemical procedures for evaluation of p16, cyclin D1and cyclin E expression in paraffin embedded lung cancer samples. Immunostaining and scoring protocols were set up for each marker. Reciprocal validation of the results was carried out between two different laboratories by running a subset of samples in parallel. High concordance was found in all cases. We have also set up protocols for automatic immunohistochemical procedures for hnRNP A1, ASF/SF2 and alpha CP4 [14–16]. In addition, a cross-laboratory validation was performed for these markers.

In order to evaluate the expression of cyclin D1 and E, hnRNP A1and ASF/SF2 in exfoliated material from lung cancer patients, we optimised and validated the immunocytochemical procedures. Due to the limited number of cells and small volume of bronchial washing and sputum available in some cases, the optimisation of the technique was performed in cytospin spreads of normal sputum spiked with a known number of lung cancer cells (from a cultured cell line). In addition, we have also optimised other techniques for exfoliated material from lung cancer patients, such as multiFISH for lung cancer associated loci with the LaVysion set of probes [17].

TABLE 2 Biological specimens archived in the European Bronchial Tissue Bank

Specimen type	n
Blood	
Plasma	3524
White blood cells	2672
Sputum	
Frozen	386
Preserved	691
Bronchial lavage	
Frozen	463
Preserved	556
Primary lung cancer normal tissue	
Frozen	715
Preserved	724
Primary lung cancer tumour tissue	
Frozen	730
Preserved	806
Total	11267

Protein expression of angiogenic and invasive phenotypic markers

Following the same strategy as described previously, automatic immunochemical and quantification protocols have been optimised and validated for vascular endothealial growth factor (VEGF), VEGF R1 and R2 [18]. To test the specificity of the antibodies, a variety of additional controls including: Western blot, absorption controls, recognition of an antigen by different antibodies, siRNAs inhibition, immunohistochemical detection of VEGF in hypoxic cells *versus* normoxic cells, and quantative PCR for VEGFR2 in human lung cancer cell xenografts were performed. After these analyses we concluded that the tested antibodies were reliable and specific. A new score, VEGF signalling score, was calculated as the sum of VEGF, VEGFR1 and VEGFR2 scores. The combination of the expression of VEGF, VEGFR1 and VEGFR2 has helped to understand the relevance of this pathway in the lung tumours.

In addition, immunohistochemical protocols to analyse microvessel density (MVD; CD-34) and lymphatic vessel density (LVD; podoplanin) were optimised in paraffin embedded lung cancer samples. To analyse the MVD and LVD, we have set up a scoring protocol using a 25-point Chalkley eye-piece graticule. We have also optimised and validated the immunocytochemical procedures for VEGF and its receptors in cytospin spreads of normal sputum spiked with lung cancer cells (from a cultured cell line).

FHIT gene analysis

We validated the use of sensitive technologies including genomic DNA PCR with fluorescent primers for the amplification of microsatellites to detect LOH, as well as immunohisto/cytochemistry with specific antibodies to evaluate abnormalities in the FHIT suppressor gene [19]. FHIT gene abnormalities were evaluated at a genomic level by LOH using fluorescent DNA PCR technique and analysis with automated DNA sequencer ABI Prism 377 (Perkin Elmer, Waltham MA, USA). FHIT protein expression was analysed by IHC on frozen/paraffin embedded sections and on cytological specimens (bronchial washings and brushings) and sputum smears using a rabbit polyclonal antiserum against the FHIT protein. The occurrence of cells with absent/reduced expression of the FHIT protein was recorded. The specificity of the rabbit polyclonal anti-FHIT antibody had been previously established by Western blot analysis of cell lines and FHIT transfectant clones. Reproducibility of IHC analysis was demonstrated by independent testing and blind scoring in two different pathology laboratories from the participating centres. In order to dissect the contribution of the different biological mechanisms leading to FHIT gene inactivation, we investigated at the association among molecular alterations (LOH and promoter methylation) and FHIT protein expression in the EUELC patients. Hypermethylation of the FHIT promoter was also evaluated in normal lung of these patients and the association between FHIT alterations in tumours and normal lung and clinical outcome of the patients was studied in an attempt to provide a molecular tool useful for identification of individuals at higher risk of relapse or second primary tumours [20].

Telomerase activity in early lung cancer

We validated the commercially available LightCycler TeloTAGGGh TERT quantification kit (Roche Diagnostics, Manheim, Germany) for hTERT (encoding the catalytic subunit of telomerase enzyme) mRNA detection and quantification using dilution series of telomerase-positive cells of a cancer cell line into telomerase-negative normal human epithelial cells. A further comparison was performed with in-house hTERT RT-PCR and TRAP assays, as previously described on a series of lung cancer specimens and corresponding distant normal lung tissue, showing good concordance [21]. Further validation involving tissue specimens was performed using parallel hTERT IHC. HTERT mRNA analysis by the LightCycler TeloTAGGGh TERT quantification kit and IHC also revealed good concordance, indicating that the commercial assay provides an sufficiently accurate representation of hTERT mRNA status, as a surrogate of telomerase activity.

Retanoic acid receptors and retinoid X receptor expression

The aim of this WP was to assess the status of pre-invasive lesions for retinoic acid (RA) receptors (RAR) and retinoid X receptors (RXR) expression using specific antibodies, and riboprobes [22]. This study was a prerequisite for treatments of RA-sensitive lung cancers with retinoid derivatives. Direct aerosolisation of the retinoids on bronchial epithelium, providing the required concentration of the drug to activate receptors, with reduced toxicity, may possibly improve the chance of controlling early lung cancer. In this way, IHC using biopsy specimens from pre-neoplastic lesions is a simple and reliable procedure to identify patients expressing RARβ at normal levels and, thus, the most susceptible to benefit from RA treatments. In addition, determining the relative levels of expression of the different RAR and RXR in RA-resistant patients would be of great importance for the use of new RXR and/or RAR selective ligands.

We tested many antibodies available from commercial companies; however, only the RXRγ antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) was considered

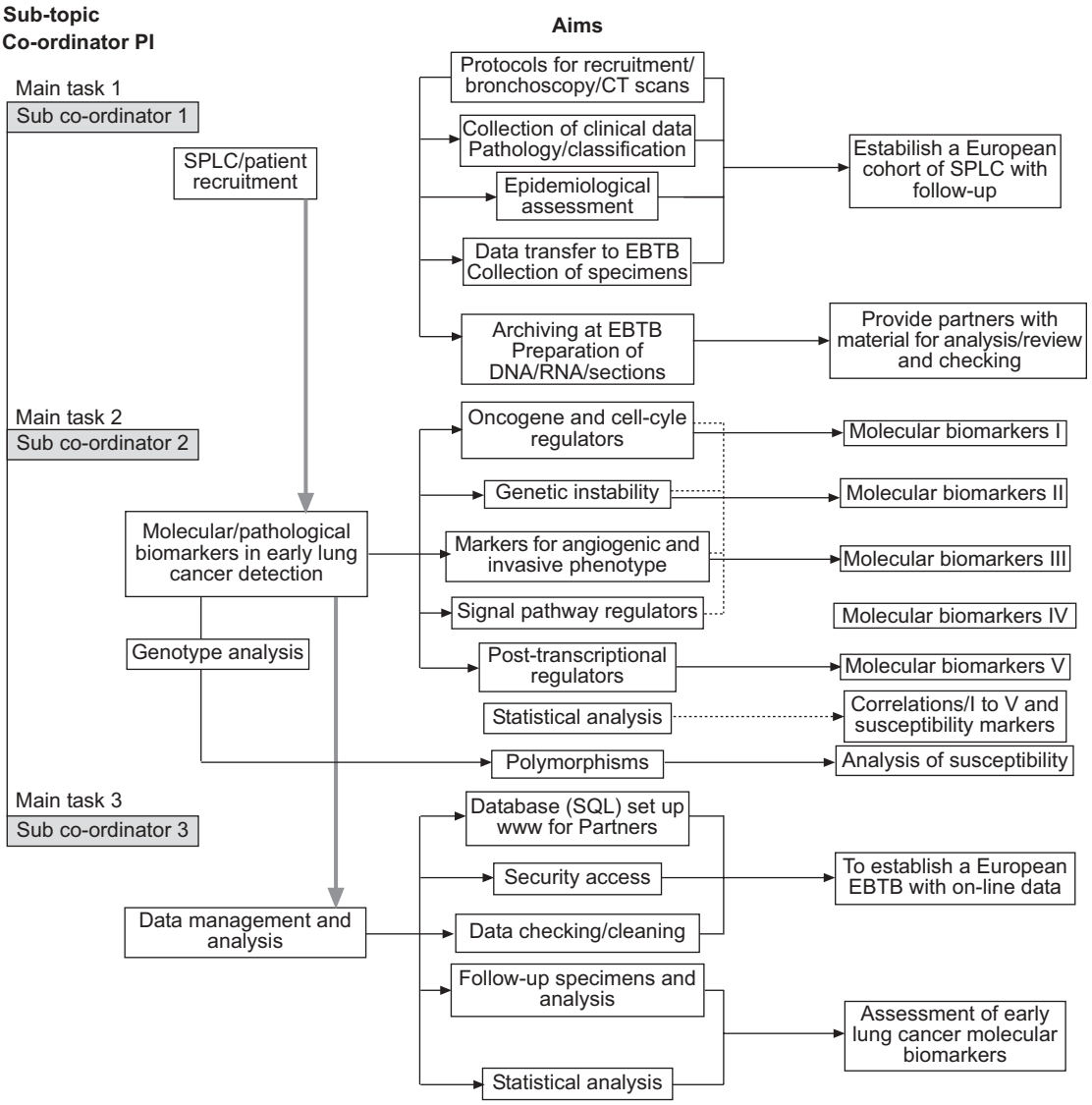

FIGURE 4. Flow-chart showing the management of the European Early Lung Cancer project. The thick grey lines indicate the linkage of the main tasks. SPLC: second primary lung cancer; CT: computed tomography; EBTB: European Bronchial Tissue Bank.

useful on paraffin embedded tissue. We set up and optimised a semi-quantitative IHC procedure based on a tyramine signal amplification using RARα, RARβ, RXRα, RXRβ and RXRγ antibodies. The relative level of expression of each receptor in pre-invasive bronchial cells compared with the normal surrounding mucosa or more distant lung tissue cells was assessed.

Genotyping

The overall aim of this WP was to gain a greater understanding of the molecular basis for inter-individual variation in susceptibility to lung carcinogenesis. Therefore, we focused on the characterisation of genetic polymorphisms in xenobiotic metabolising-enzymes and DNA-repair enzymes that influence the susceptibility of developing lung cancer. Genotyping analyses was carried out on xenobiotic metabolising enzymes, with known genetic polymorphisms involved in the metabolism of environmental or tobacco carcinogens, and on DNA-repair enzymes involved in repairing damage resulting from endogenous and exogenous mutagens, with the aim of improving our understanding of the molecular basis of lung carcinogenesis. Pathway-based approaches have been spectacularly overtaken by powerful genome-wide association studies, which very recently mapped major lung cancer susceptibility loci to chromosome 15q25 [23–26], 6p21 [26], and 5p15 [26, 27].

STATISTICAL ANALYSIS

During patient recruitment, a statistician regularly retrieved missing or aberrant values in clinical data and follow-up status of patients. Reports were regularly prepared and sent to centres to correct or confirm patient information to ensure good quality to the EUELC database. A second step was to exclude individuals with missing information on a set of major variables from analyses when data gathering efforts were exhausted or were unrealisable.

Two parallel approaches were used to assess the epidemiological and molecular risk factors of disease progression after

primary lung cancer surgery. The first was a proportional hazard model in the setting of the competing risk developed by KIM [28], including stratification by centre. Indeed, the standard Cox proportional hazard model assumes that a censored patient has the same chance of occurrence of disease progression than an uncensored patient (an assumption which is not true when patients die from other causes than cancer). Although this model is perfectly suited to follow-up data, the number of patients lost to follow-up may introduce a potential bias due to informative censoring. Therefore, we designed a nested case–control study as an alternative. In the second model, disease-progression patients were matched to disease-free patients based on length of follow-up (at least as long as the event time of disease progression subjects), centre, sex, age ($\pm$6 yrs), and histology and nodal stage. The main advantage of this second model was the ability to control for potential biases due to length of follow-up. However, it leads to a slight decrease in the power of the analysis as strict adherence to matching criteria reduces the study sample size.

The multiplicity of measures and comparison raised a multiple comparisons issue that results in a dramatic increase in the type I and type II errors. Therefore, we validated results on another subsample of the database and/or on external databases and samples.

DISCUSSION

To date this was one of the largest planned, early lung cancer projects in Europe and it enabled the EUELC Partners to establish protocols for assessing molecular biomarkers in early lung cancer with the view to using such biomarkers for early diagnosis and as intermediate end-points in future chemo-preventive programmes.

The EUELC Partners undertook a pan-European approach to this objective and decided to recruit patients with a history of a completely resected primary NSCLC. Lung cancer patients were followed up every 6 months for the duration of the project and specimens were collected at each clinical-recall visit. The EBTB was set up at the University of Liverpool to optimise the use of biological specimens. The EBTB facilitated the validation of sensitive technologies such as RT-PCR, genomic DNA PCR with fluorescent primers for the amplification of microsatellites in order to detect either LOH or microsatellite instability, as well as immunohisto/cytochemistry with specific antibodies to evaluate abnormalities in the TSG(s)/oncogenes, and a range of molecular markers considered to be frequently involved in invasive lung cancer. Moreover, chip technologies were used for p53 mutational analysis and also for expression profiling.

We undertook a full genetic analysis on a subset of specimens collected in this project. We were particularly interested to identify specific molecular–pathological mechanisms that determine which individuals are at risk of developing second primary lung cancers, metastasis and/or recurrences. Given the relative rarity of second primary lung cancer, we identified a subset of individuals who fall into two categories: those with progressive disease and those who are considered disease free post-surgery.

The earliest discovery of progressive disease prompted a repeat of bronchoscopy and computed tomography to discover malignant nodules in the lung. The genetic lesions identified in resected or biopsied lung cancer, pre-neoplastic lesions and normal bronchial mucosa from lung cancer patients may, in future, prompt a search for advanced disease by bronchoscopy (bronchial brushings and washings), transthoracic needle aspiration and cytological (sputum) specimens as well as utilising plasma/serum samples of lung cancer patients during follow-up and in those individuals who develop progressive disease.

This study has engendered a tremendous collaborative effort, and the Partners have agreed to an extended period of clinical follow-up for lung cancer patients. In future, it may be possible to employ specific treatment methods for chemoprevention or selective endobronchial lesion ablation (such as photodynamic therapy) or very early treatment, and this will be evaluated based on the occurrence of such genetic changes and risk modelling. The eventual understanding of the genetic and environmental basis of lung cancers will enable the identification of high-risk populations for which effective prevention, early detection and chemoprevention strategies will be developed [29, 30].

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STATEMENT OF INTEREST

None declared.

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REFERENCES

1 Herbst RS, Heymach JV, Lippman SM. Lung cancer. *N Engl J Med* 2008; 359: 1367–1380.
2 Parkin DM, Bray F, Ferlay J, *et al.* Global cancer statistics, 2002. *CA Cancer J Clin* 2005; 55: 74–108.
3 Alberg AJ, Samet JM. Epidemiology of lung cancer. *Chest* 2003; 123: Suppl. 1, 21S–49S.
4 Sozzi G, Conte D, Leon M, *et al.* Quantification of free circulating DNA as a diagnostic marker in lung cancer. *J Clin Oncol* 2003; 21: 3902–3908.
5 Wardwell NR, Massion PP. Novel strategies for the early detection and prevention of lung cancer. *Semin Oncol* 2005; 32: 259–268.
6 Cassidy A, Balsan J, Vesin A, *et al.* Cancer diagnosis in first-degree relatives and non-small cell lung cancer risk: results from a multicentre case-control study in Europe. *Eur J Cancer* 2009; [Epub ahead of print DOI: 10.1016/j.ejca.2009.05.006].
7 Somers VA, Thunnissen FB. Detection of K-ras point mutations in sputum from patients with adenocarcinoma of the lung by point-EXACCT. *Methods Mol Med* 2003; 75: 305–323.
8 Hernandez-Boussard TM, Hainaut P. A specific spectrum of p53 mutations in lung cancer from smokers: review of mutations compiled in the IARC p53 database. *Environ Health Perspect* 1998; 106: 385–91.
9 Field JK, Liloglou T, Xinarianos G, *et al.* Genetic alterations in bronchial lavage as a potential marker for individuals with a high risk of developing lung cancer. *Cancer Res* 1999; 59: 2690–2695.
10 Liloglou T, Maloney P, Xinarianos G, *et al.* Cancer-specific genomic instability in bronchial lavage: a molecular tool for lung cancer detection. *Cancer Res* 2001; 61: 1624–1628.
11 Shaw RJ, Hall GL, Woolgar JA, *et al.* Quantitative methylation analysis of resection margins and lymph nodes in oral squamous cell carcinoma. *Br J Oral Maxillofac Surg* 2007; 45: 617–622.
12 Pekarsky Y, Palamarchuk A, Huebner K, *et al.* FHIT as tumor suppressor: mechanisms and therapeutic opportunities. *Cancer Biol Ther* 2002; 1: 232–236.
13 Iliopoulos D, Guler G, Han SY, *et al.* Fragile genes as biomarkers: epigenetic control of WWOX and FHIT in lung, breast and bladder cancer. *Oncogene* 2005; 24: 1625–1633.
14 Pajares MJ, Zudaire I, Lozano MD, *et al.* Molecular profiling of computed tomography screen-detected lung nodules shows multiple malignant features. *Cancer Epidemiol Biomarkers Prev* 2006; 15: 373–380.
15 Pio R, Zudaire I, Pino I, *et al.* Alpha CP-4, encoded by a putative tumor suppressor gene at 3p21, but not its alternative splice variant alpha CP-4a, is underexpressed in lung cancer. *Cancer Res* 2004; 64: 4171–4179.
16 Zerbe LK, Pino I, Pio R, *et al.* Relative amounts of antagonistic splicing factors, hnRNP A1 and ASF/SF2, change during neoplastic lung growth: implications for pre-mRNA processing. *Mol Carcinog* 2004; 41: 187–196.
17 Zudaire I, Lozano MD, Vazquez MF, *et al.* Molecular characterization of small peripheral lung tumors based on the analysis of fine needle aspirates. *Histol Histopathol* 2008; 23: 33–40.
18 Lantuéjoul S, Constantin B, Drabkin H, *et al.* Expression of VEGF, semaphorin SEMA3F, and their common receptors neuropilins NP1 and NP2 in preinvasive bronchial lesions, lung tumours, and cell lines. *J Pathol* 2003; 200: 336–347.
19 Sozzi G, Veronese ML, Negrini M, *et al.* The FHIT gene 3p14.2 is abnormal in lung cancer. *Cell* 1996; 85: 17–26.
20 Verri C, Roz L, Conte D, *et al.* Fragile histidine triad gene inactivation in lung cancer: the European Early Lung Cancer project. *Am J Respir Crit Care Med* 2009; 179: 396–401.
21 Snijders PJ, Breuer RH, Sutedja TG, *et al.* Elevated hTERT mRNA levels: a potential determinant of bronchial squamous cell carcinoma (*in situ*). *Int J Cancer* 2004; 109: 412–417.
22 Martinet N, Alla F, Farré G, *et al.* Retinoic acid receptor and retinoid X receptor alterations in lung cancer precursor lesions. *Cancer Res* 2000; 60: 2869–2875.
23 Amos CI, Wu X, Broderick P, *et al.* Genome-wide association scan of tag SNPs identifies a susceptibility locus for lung cancer at 15q25.1. *Nat Genet* 2008; 40: 616–622.
24 Hung RJ, McKay JD, Gaborieau V, *et al.* A susceptibility locus for lung cancer maps to nicotinic acetylcholine receptor subunit genes on 15q25. *Nature* 2008; 452: 633–637.
25 Thorgeirsson TE, Geller F, Sulem P, *et al.* A variant associated with nicotine dependence, lung cancer and peripheral arterial disease. *Nature* 2008; 452: 638–642.
26 Wang Y, Broderick P, Webb E, *et al.* Common 5p15.33 and 6p21.33 variants influence lung cancer risk. *Nat Genet* 2008; 40: 1407–1409.
27 McKay JD, Hung RJ, Gaborieau V, *et al.* Lung cancer susceptibility locus at 5p15.33. *Nat Genet* 2008; 40: 1404–1406.
28 Kim HT. Cumulative incidence in competing risks data and competing risks regression analysis. *Clin Cancer Res* 2007; 13: 559–565.
29 Cassidy A, Myles JP, van Tongeren M, *et al.* The LLP risk model: an individual risk prediction model for lung cancer. *Br J Cancer* 2008; 98: 270–276.
30 Smith RA, Field JK, Duffy SW. A global approach to cancer-screening trials. *Lancet Oncol* 2008; 9: 908–909.