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Review

Are adenosquamous lung carcinomas a simple mix of adenocarcinomas and squamous cell carcinomas, or more complex at the molecular level?

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ABSTRACT

Adenocarcinomas (AC), squamous cell carcinomas (SCC) and adenosquamous carcinomas (ASC) are three histological subtypes of non-small-cell lung carcinomas (NSCLC). ASC are morphologically mixed tumours that contain the two cell components AC and SCC. To understand if they are a "simple" mix of AC and SCC or if they present molecular specificities, as compared with the molecular characterization of both components, we performed a comparative transcriptome analysis on a series of nine ASC, five AC and five SCC induced in rats by radon exposure. We found that 72, 40 and 39 genes were differentially expressed when comparing AC_SCC, ASC_SCC and AC_ASC, respectively. Moreover, when classifying the three histological subtypes, using genes that discriminated AC and SCC, we observed that all ASC were classified as intermediate between the AC and SCC, some being closer to AC, others to SCC. These results indicated that, regarding gene expression, ASC could be considered as a mix of AC and SCC, both in various proportions. However, they also exhibit molecular specificities since we found specific genes discriminating ASC_SCC and AC_ASC. In conclusion, the ASC mixed lung tumours are more complex than simple mixes of AC and SCC components. Neuroendocrine differentiation and ERK proliferation pathways seemed preferentially deregulated in ASC compared to AC and SCC respectively, pathways that are worthy of being explored because they could partially explain the high clinical aggressiveness of ASC.

1. Introduction

Lung cancer is the leading cause of cancer death worldwide [1,2]. The non-small-cell lung carcinomas (NSCLC) are the most frequent type of lung tumours in humans, with two major histological subtypes: adenocarcinomas (AC) and squamous cell carcinomas (SCC). The adenosquamous carcinomas (ASC), a less frequent subtype in human lung cancer, are also classed as NSCLC. ASC form morphologically mixed tumours composed of the two cell components AC and SCC in various proportions, each one representing at least 10% of the whole tumour [3,4]. Although infrequent in humans, this composite tumour is more aggressive than "pure" AC and SCC, with frequent lymph-node metastasis at diagnosis, and a poor prognosis [5–7]. Morphological characterizations of AC and SCC are well described, AC being glandular with alveolar, tubular or papillary structures while SCC are squamous with or without keratin differentiation. Many researches have analyzed genetic and molecular alterations in AC and SCC, but few molecular studies have been conducted on heterogeneous ASC tumours. One paper described identical Tp53 mutations in the two histological components AC and SCC cells of 12 lung ASC [8], suggesting that the two cell components originated from the same clone or genetically related cellular clones. To investigate the molecular characterization of ASC and specifically to determine whether these complex tumours correspond to a simple mix of AC and SCC cells or if they present their own molecular specificities, we used transcriptome analysis to compare gene expression profiles of a series of morphologically well characterized ASC, AC and SCC developed in rats after radon inhalation [9].

2. Materials and methods

2.1. Biological samples

A large series of lifespan experiments carried out in our laboratory on out-bred Sprague–Dawley rats after radon progeny inhalation provided primary rat lung carcinomas [9]. Animals were handled according to the French Legislature and the European Directives regarding the care and use of laboratory animals. After radon exposure, rats were kept until death or were euthanized when moribund. All pulmonary lobes were systematically paraffin-embedded to microscopically detect tumours with a global view and to propose a definitive histological diagnosis according to the European Late Effects Project Group (EULEP) classification [10]. Before embedding, macroscopically detectable tumours were

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punctured and were kept frozen for further RNA extraction and molecular biology studies. For the transcriptomic study, nine ASC, five AC and five SCC were selectively collected in 19 different rats. Normal rat lung, liver, spleen, muscle and brain tissues, kidney epithelial cell line (NRK-52E) and osteosarcoma cell lines (UMR-106 and UMR-108) were also collected to constitute an external rat reference (RR) for microarray hybridization. Total RNA was isolated from each frozen tumour (Exp), and from normal tissues and cell lines, using the RNA-plus lysis solution, according to the manufacturer's protocol (Q-Biogene, Illkirsch, France). To prepare the external rat reference for microarray analysis, RNAs from each normal tissue and cell line were mixed in equimolar quantities.

2.2. cDNA synthesis and labelling

To avoid RNA degradation during microarray hybridization, we developed a technique to hybridize labelled cDNA. For each tumour (Exp) and for the reference (RR), 5 μg of total RNA were amplified using the MessageAmp™ aRNA kit (Ambion). Amplified RNA was reverse transcribed as previously described [11]. The second strand of cDNA was synthesized and labelled after overnight incubation at 37 °C in 1× Hexanucleotide buffer (Roche Diagnostics) containing 5U Klenow DNA polymerase (Roche Diagnostics), unlabelled nucleotides (final concentration 200 μM each dATP, dCTP, dGTP, and 130 μM dTTP), and either Cy3 or Cy5 conjugated dUTP (final concentration 70 μM; Amersham).

2.3. Microarrays

The rat 10K 50 mers Oligo Set (6100 genes of known function, 3560 ESTs, 100 replicas and 169 Arabidoposis negative controls) (Ocimum Biosolutions) was spotted on the CEA genomic platform (CEA, Evry, France) on GAPS coated slides (Corning) and exposed to UV radiation as previously described [11]. Gene annotation was updated monthly from the database of Stanford University (http://source.stanford.edu) and functional grouping of genes was performed by using key words of the NCBI Entrez Gene database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene) and specific bibliographic data.

2.4. Hybridization

For each tumour, competitive hybridization was performed using equal quantities of Cy3-labelled cDNA from Exp and Cy5-labelled cDNA from RR. After alcohol precipitation with 20 μg each of poly(A), yeast tRNA and rat Cot-1 DNA (Rat Hybloc, Applied Genetics Laboratories), the cDNA pellet was dissolved in 50 μl of hybridization solution (50% formamide, 10× SSC and 0.2% SDS) and heated for 5 min at 95 °C. The preparation was dropped onto pre-hybridized arrays (incubated 1 h at 42 °C in a buffer 1% BSA, 5× SSC, 0.1% SDS). After overnight incubation at 42 °C in a humidified chamber, slides were washed for 4 min in 1× SSC–0.2% SDS at 42 °C, 0.1× SSC–0.2% SDS, 0.1× SSC and distilled water at room temperature, and were dried by centrifugation. Competitive hybridization was performed for each tumour in duplicated dye-swap experiments.

2.5. Microarray scanning and data analysis

Microarrays were scanned using a confocal laser scanner (ScanArray Express, PerkinElmer). Independent images were acquired for Cy3 (532 nm) and Cy5 (635 nm). The spot coordinates were determined by automatic positioning of a grid using an image-analysis spot-tracking software (patent US 10/173,672 June, 19, 2002; CA 2,389,901 June, 20, 2002). The signal intensity of each spot was defined as previously described [11,12].

Data normalization and expression quantification were also performed as described [11], expression ratios being log-transformed (base 2) and median-centred. To identify genes that were differentially expressed between two groups of tumours (AC versus SCC, AC versus ASC or ASC versus SCC), we used a supervised microarray analysis. We did a permutation *t*-test to select a first list of genes. Then, to avoid choosing an arbitrary cut-off of *p*-value for the *t*-test, an EM algorithm was used to precisely calculate, for each tumour, the probability of over- and under-expression for each of these genes. We finally selected genes with a difference of more than 95% between these two probabilities, showing no contradiction in the sense of gene expression deregulation when comparing all the tumours of a given subtype, and showing a difference in the sense of deregulation when comparing the two subtypes of tumours. For example, to discriminate AC from ASC, we retained genes over-expressed in all AC and under-expressed in all ASC, and conversely, under-expressed in all AC and over-expressed in all ASC. Finally, using the selected genes, tumours were classified using the ascendant hierarchical classification algorithm of the web-available TIGR MultiExperiment Viewer (MeV) software (http://www.tm4.org/mev.html).

2.6. Quantitative real-time reverse transcription-PCR

For the first strand cDNA synthesis, 100 ng of amplified RNA was reverse transcribed using random hexamers (pd(N)6; Boehringer Mannheim) and the reverse transcriptase MMLV (Invitrogen). PCR was performed in duplicate using the 7300 real-time PCR system (Applied Biosystems) by using the ABsolute QPCR ROX Mix (Abgene). Quantification of each gene expression was calibrated as previously described [13–15], using a reference standard curve obtained by serial dilutions of a PCR product prepared from RR. The geometric mean expression of three housekeeping genes, Actb, Gusb, and Hmbs, allowed to normalize the expression of the interesting genes by using the web-available geNorm software (http://medgen.ugent.be/~jvdesomp/genorm) [16]. The PCR primers and probes were purchased from Applied Biosystems for Hp, Fgg, Krt19, Cdk2, Dsp, Prkaa2, Gng12, Gnas, Notch2, Rab13, Sftpa1, Amph1, Muc1, Mst1, Actb, Gusb, and Hmbs genes (TaqMan Gene Expression Assays: assay ID Rn00561393_m1, Rn00561196_m1, Rn01496867_m1, Rn01529542_m1, Rn01434652_m1, Rn00576935_m1, Rn01425123_m1, Rn00569454_m1, Rn00577522-m1, Rn00709515_m1, Rn00824545_m1, Rn00824616_m1, Rn01462585_m1, Rn00577395_m1, Rn00667869_m1, Rn00566655-m1 and Rn00565886-m1 respectively).

2.7. K-Ras gene mutation

On the series of tumours, K-Ras mutations at codons 12, 13 and 61 were screened for on PCR-amplified cDNA using the forward primer GCCTGCTGAAAATGACTGAGTAT and the reverse primer AAAGAAAGCCCTCCCCAGTT (1.7 mM $MgCl_2$—Tm 60 °C). Sequencing reactions were carried out on both strands by the Premium Read service of Cogenics (Meylan, France) and compared with the referential sequence NM_031515 from GenBank database (NCBI).

3. Results

The lifespan experiments carried out in our laboratory after radon progeny inhalation in rat provided three types of primary lung carcinomas [9]: squamous cell carcinomas (SCC), adenocarcinomas (AC) and adenosquamous carcinomas (ASC), which present molecular similarities with human non-small cell lung cancers [17]. Each tumour type could have heterogeneous microscopic phenotypes (Fig. 1). SCC could have different stages of squamous