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Microsatellite alterations at 3p and 19q in EBC DNA of smokers: are they reversible after smoking cessation?

To the Editors:

Carcinogens in cigarette smoke may leave fingerprints in the bronchial tissue in the form of specific mutations that initiate cancer development [1]. Numerous genetic alterations have been recognised as critical effects of cigarette smoke and studied in the airways of current and former smokers. Microsatellite alterations (MAs) at 3p and 19q are among the most studied, being considered useful markers of genetic susceptibility and genome destabilisation in susceptible smokers [2, 3]. Instability and/or loss of heterozygosity at 3p and 19q have largely been reported in the lung tissue, sputum and blood of smokers and lung tumour patients [2, 4]. These mutations, which are considered an early effect of cigarette smoke, are dose-dependent and related to the number of cigarettes smoked in a lifetime [3]. In addition, short-term exposure to cigarette smoke seems to cause MAs at 3p that are not necessarily a consequence of the development of a neoplastic mass [5].

Our group recently reported MAs at these loci in the exhaled breath condensate (EBC) of smokers and patients affected by nonsmall cell lung cancer, demonstrating that EBC could be a surrogate for tissue in assessing tobacco-induced molecular damage in the lungs [2, 3].

Interestingly, although smoking cessation may reverse the clonal expansion of abnormal cells, the persistence of some genetic alterations in former smokers indicates that a high rate of clonal genetic damage persists even after smoking cessation [6].

Several genetic susceptibility markers have been studied after smoking cessation and classified as rapidly reversible, slowly reversible or irreversible [7]. Indeed, to know whether a gene alteration is reversible could turn out to be important, as rapidly reversible genes seem to have different biological functions than slowly reversible or irreversible genes [7].

The aim of this study was to test the reversibility of MAs at 3p and 19q by analysing these markers for the first time in the EBC and whole blood (WB) of smokers at baseline (T0) and then at 12 months after smoking cessation (T1). The correlations between MAs with sex, pack-yrs, exhaled carbon monoxide and Fagerström nicotine dependence score were also investigated.

The study population consisted of 63 patients (mean±SD age 47±16 yrs; 41 males) who participated in a multidisciplinary smoking cessation programme with a further genetic study at the Smoking Unit of the Dept of Respiratory Disease, University of Foggia (Foggia, Italy). Furthermore, none of the subjects who underwent the spirometry showed any alterations (forced expiratory volume in 1 s 101±3.3% predicted; forced vital capacity 102.1±2.7% predicted).

Of the 63 smokers in the multidisciplinary smoking cessation programme who provided baseline WB and EBC for genetic study, 28 (38%) subjects (age 53±8.5 yrs; 20 males) successfully quit smoking for 12 months and provided new samples. After enrolment, 16 participants received 300 mg·day^{-1} bupropion, six participants received 25-mg Nicorette® Invisi patches (Johnson & Johnson, Stockholm, Sweden), whereas others opted for a motivational programme.

The EBC was collected using the EcoScreen (Jaeger, Wurzburg, Germany), and the DNA was extracted from both WB and EBC by using a QIAamp DNA Mini Kit (Qiagen, New York, NY, USA).

The analysis of MAs was performed by using the following five polymorphic microsatellite markers from chromosomes 3p and two from chromosome 19q: 3p24.2 (D3S2338), 3p23 (D3S1266), 3p14.2 (D3S1300), 3p25–26 (D3S1304), 3p21 (D3S1289), 19q13.2 (D19S393) and 19q13.3 (D19S908). Fisher's exact or Chi-squared tests were used to compare qualitative data, and a Wilcoxon test was used for comparison between categories. Data are presented as mean±SD. Significance was defined as a p-value of <0.05.

A higher success rate for smoking cessation was observed in subjects who provided samples for genotyping, compared with the rate reported in our Smoking Unit for subjects who participated in the usual multidisciplinary smoking cessation programme without genetic studies (38% *versus* 20%).

As regards the MAs, only 18 patients had informative results in all seven of the loci considered in EBC; regarding the total number of analysed loci, 178 (91%) out of 196 the analyses resulted in informative microsatellites. Table 1 shows the results of the microsatellite analysis in EBC DNA for each locus and patient.

TABLE 1 Genetic alterations for each microsatellite marker in exhaled breath condensate DNA from 28 smokers at T0 (baseline) and T1 (12 months smoking cessation)

Patient	D19S393		D19S908		D3S338		D3S1266		D3S1300		D3S1304		D3S1289	
	T0	T1	T0	T1	T0	T1	T0	T1	T0	T1	T0	T1	T0	T1
1	N	N	LOH	LOH	H	H	N	N	LOH	LOH	LOH	LOH	MI	MI
2	H	H	H	H	N	N	H	H	H	H	H	H	N	N
3	LOH	LOH	H	H	MI	MI	MI	MI	LOH	LOH	H	H	H	H
4	LOH	LOH	LOH	LOH	MI	MI	MI	MI	MI	MI	H	H	.H	H
5	LOH	LOH	H	H	MI	MI	MI	MI	LOH	LOH	H	H	H	H
6	N	N	H	H	H	H	LOH	LOH	LOH	LOH	N	N	MI	MI
7	MI	MI	H	H	LOH	LOH	MI	MI	H	H	H	H	MI	MI
8	H	H	H	H	MI	MI	N	N	LOH	LOH	LOH	LOH	N	N
9	H	H	N	N	H	H	H	H	N	N	H	H	H	H
10	MI	MI	H	H	MI	MI	MI	MI	H	H	LOH	LOH	H	H
11	LOH	LOH	H	H	MI	MI	H	H	H	H	H	H	N	N
12	LOH	LOH	LOH	LOH	MI	MI	H	H	MI	MI	H	H	MI	MI
13	LOH	LOH	LOH	LOH	MI	MI	MI	MI	H	H	H	H	N	N
14	H	H	H	H	MI	MI	MI	MI	LOH	LOH	H	H	H	H
15	H	H	N	N	N	N	H	H	H	H	H	H	H	H
16	H	H	H	H	N	N	H	H	H	H	H	H	H	H
17	LOH	LOH	H	H	MI	MI	H	H	MI	MI	LOH	LOH	MI	MI
18	LOH	LOH	LOH	LOH	MI	MI	MI	MI	H	H	LOH	LOH	H	H
19	LOH	LOH	LOH	LOH	MI	MI	LOH	LOH	H	H	LOH	LOH	MI	MI
20	H	H	H	H	LOH	LOH	H	H	H	H	H	H	N	N
21	LOH	LOH	LOH	LOH	LOH	LOH	H	H	MI	MI	H	H	H	H
22	H	H	H	H	LOH	LOH	LOH	LOH	H	H	H	H	H	H
23	LOH	LOH	LOH	LOH	LOH	LOH	MI	MI	MI	MI	H	H	H	H
24	LOH	LOH	LOH	LOH	MI	MI	MI	MI	LOH	LOH	H	H	MI	MI
25	LOH	LOH	H	H	MI	MI	MI	MI	H	H	H	H	H	H
26	LOH	LOH	LOH	LOH	MI	MI	MI	MI	H	H	H	H	H	H
27	H	H	H	H	MI	MI	H	H	LOH	LOH	H	H	H	H
28	H	H	H	H	LOH	LOH	LOH	LOH	H	H	LOH	LOH	H	H

N: noninformative; H: heterozygosity; LOH: loss of heterozygosity; MI: microsatellite instability.

Loss of heterozygosity (LOH) was found in 48 (27%) and microsatellite instability (MI) in 43 (24%) out of the 178 informative loci studied. The most frequently altered microsatellites in EBC DNA were D3S338 (in 75% of the informative DNA samples for that locus), D3S1266 and D19S393 (in 57% of the informative EBC/DNA samples for those loci). All MAs (LOH and MI) observed at T0 (91 out of 178, 51%) were unchanged 12 months after quitting smoking (91 out of 178, 51%) (T1).

When WB DNA was considered, 28 patients had blood samples stored, with 178 (91%) out of the total 196 loci analysed resulting in informative microsatellites. LOH was found in 11 (6%) and MI in 15 (8%) out of the 178 informative loci studied; all MAs (LOH and MI) observed at T0 (26 out of 178, 15%) were unchanged 12 months after quitting smoking (T1).

The number of MAs present in EBC DNA and an increase in tobacco consumption were directly related in the smokers studied. In particular, the frequency of MAs increased from smokers with <20 pack-yrs compared with those with >50 packs-yrs smoking exposure, with a mean $\pm$SD number of MAs of 1.6 ± 1.5 *versus* 4.7 ± 1.3, respectively ($p<0.05$). Further, no correlation between MAs and sex, exhaled carbon monoxide and Fagerström nicotine dependence score were observed.

In this study, we investigated the reversibility of MAs at 3p and 19q 12 months after smoking cessation. Although several markers of genetic susceptibility have already been analysed in relation to smoking cessation and therefore classified as reversible or irreversible genes, to our knowledge, no-one has yet analysed the MAS at 3p and 19q, although they are largely recognised as being an important target for cigarette smoke. In accordance with other previous protocols in animals and humans, we analysed MAs at 3p and 19q (ERCC-1, ERCC- 2, fragile histidine triad protein and transforming growth factor-β receptor) in DNA from EBC and WB of a group of smokers who participated in a multidisciplinary smoking cessation programme with a genetic study, both at baseline (T0) and 12 months after smoking cessation (T1).

As expected, at baseline, we found high percentages of exhaled MAs at loci studied in smokers; indeed, these values were comparable to those previously reported by our group. Furthermore, we confirmed that MAs at 3p and 19q are dose-dependent, as they increased proportionally to the increase in pack-years of cigarette smoking. In agreement with SOZZI *et al.* [8], our data further suggested that these loci are the most sensitive markers of smoking status.

In accordance with the observations of SIAFAKAS *et al.* [9] in DNA from sputum cells, we also observed that in the DNA from EBC, MAs are not reduced after short-term smoking cessation. This indicates that MAs at these loci belong to the 21% of the genes expressed in the airway epithelium of smokers that are irreversible [7]. In agreement with BEANE *et al.* [7], we believe that, given the rather rapid turnover of airway epithelial cells, the persistence of these changes following smoking cessation may result from a clonal growth advantage to epithelial cells in the airway harbouring these changes.

It will be necessary to follow these subjects and analyse the alterations still present after further time from smoking cessation in order to conclusively demonstrate the irreversibility of MAs at 3p and 19q and better classify them. Indeed, the correct classification of genetic alterations is very important, as it allows one to recognise their different biological functions. The rapidly reversible genes permit one to distinguish between acute responses to tobacco smoke-induced epithelial cell damage [7], in contrast to those that are slowly reversible for a slower response. However, the irreversible genes are likely to be a useful tool for assessing any past exposure to tobacco smoke; and what is more, these genes provide an insight into why former smokers still run a risk of developing lung cancer [7].

A further result of this study regards the fact that the subjects who were aware of presenting some MAs in their genome, an expression of susceptibility to cancer, stopped smoking with ease, as they were more driven by the fear of developing lung cancer arising from this knowledge than the subjects who stopped smoking when not participating in the genetic studies. As previously demonstrated with other genetic alterations, non-cancer subjects who are informed of their genotype are more likely to quit smoking. Our results are perfectly consistent with the recognised approach to improving smoking cessation that utilises emerging information from molecular genetic studies [10].

In conclusion, we can be said not only to have further confirmed that MAs at 3p and 19q are an early target for cigarette smoke but also to have shown that they are irreversible in the short term after smoking cessation. We believe that it is necessary to follow up on these genetic alterations to determine whether they are still present in ex-smokers after a further period of time from smoking cessation before it can be definitively stated that MAs at 3p and 19q are irreversible and can be employed as markers of past exposure to tobacco smoke.

The further finding of this study, that of a greater percentage of success in smoking cessation among those subjects informed of the presence of MAs in their EBC/DNA and of its susceptibility significance, leads us to support the usefulness of smoking cessation programmes based on genotype information and for its potential ethical consideration.

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EBUS-TBNA in the differential diagnosis of pulmonary artery sarcoma and thromboembolism

To the Editors:

Pulmonary artery sarcoma is a rare tumour of the cardiovascular system. It is often misdiagnosed as acute or chronic pulmonary thromboembolism because its clinical presentation and radiological findings are similar to those of thromboembolism. The diagnosis of pulmonary artery sarcoma by endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) has not been reported. Herein, we report two cases with mass-like lesion in the pulmonary artery. The lesions were safely approached by EBUS-TBNA, and the tissues obtained by EBUS-TBNA were sufficient to diagnose pulmonary artery sarcoma and thromboembolism.

A 79-yr-old female with hypertension and atrial fibrillation presented with sudden-onset left chest and shoulder pain. She had taken warfarin for atrial fibrillation, but the warfarin had been discontinued for 1 week because of a scheduled endoscopy. We performed chest computed tomography (CT), which revealed an extensive intraluminal low-attenuated mass- like lesion involving the entire luminal diameter of the left main and left lower lobar pulmonary artery (fig. 1a). D-dimer was 0.94 $\mu g \cdot mL^{-1}$ (reference value <0.4 $\mu g \cdot mL^{-1}$). Positron emission tomography (PET)-CT with ^{18}F-fluorodeoxyglucose (FDG) showed increased FDG uptake, with a maximum standardised uptake value (SUV max) of 18.6 in the left main pulmonary artery (fig. 1c). For a tissue diagnosis, EBUS-TBNA was performed targeting the mass-like lesion encasing the pulmonary artery (fig. 1d). The sonograph revealed a round heterogeneous mass with distinct margin that include some necrotic area. The cytopathological examination confirmed spindle cell malignancy with vimentin and CD31 expression by immunohistochemistry (fig. 1e and f); this was compatible with pulmonary artery sarcoma. She underwent a left pneumonectomy (fig. 1b) and final pathology revealed pulmonary artery sarcoma $4.5 \times 2.0 \times 2.0$ cm in size with marked nuclear pleomorphism and mitotic count of 15/10 high-power fields. 6 months after surgery she is alive without complications or relapse.

A 68-yr-old female with hypertension presented with dyspnoea and syncope. Chest CT revealed a 2.6×3.1 cm mass-like density in the left main pulmonary artery trunk occupying the entire luminal diameter and extending to the left lower lobe pulmonary artery (fig. 2a). The possibility of pulmonary artery sarcoma was not excluded because the mass-like density in the pulmonary artery was distributed asymmetrically and the left lower lobe pulmonary artery bulged out by the mass. Although the D-dimer level was 11.83 $\mu g \cdot mL^{-1}$ and only mildly increased FDG uptake (SUV max 3.5) was noted, D-dimer can be elevated in

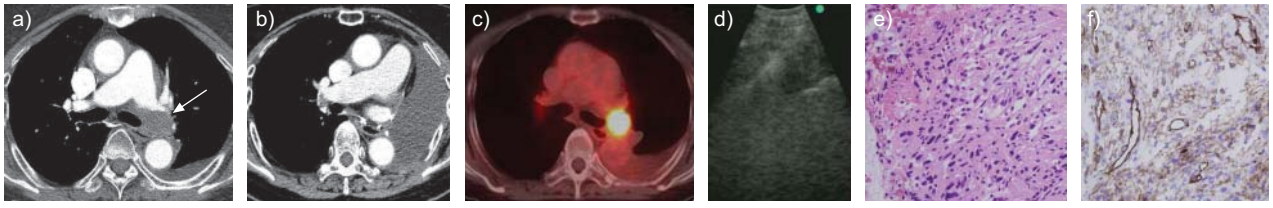

FIGURE 1. Chest computed tomography (CT), positron emission tomography (PET)-CT with ^{18}F-fluorodeoxyglucose (FDG), endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) and pathological specimens from EBUS-TBNA in case 1. a) CT scan showing an intraluminal low-attenuation lesion in the left main pulmonary artery. b) The lesion (sarcoma) was resected completely after a left pneumonectomy. c) PET-CT showing increased FDG uptake (maximum standardised uptake value of 18.6) in the intraluminal lesion of the left main pulmonary artery. d) EBUS showing a heterogeneous mass near the left pulmonary artery with the needle inserted in the mass. e) The histological examination revealed poorly differentiated spindle-cell malignancy (haematoxylin and eosin stain $\times 100$). f) Tumour cells show focal but definite immunoreacitivity for CD31 (immunostaining $\times 200$).