

***FoxO3a* gene is a target of deletion in mouse lung adenocarcinoma**

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Abstract. Lung adenocarcinomas (LAC) of smokers and never-smokers differ from one another in epidemiology, and clinical and molecular characteristics. The pathogenetic differences between these tumors are potential biomarkers and therapeutic targets. Mouse carcinogenesis models of human LAC are proven tools applicable for the identification of these molecular changes. Allelic loss frequency on human chromosome 6q is higher in LAC of smokers compared with never smokers. We analyzed the orthologous region on mouse chromosome 10 and found this region similarly was a more frequent site of allelic loss in carcinogen-induced LAC compared with non-induced or spontaneous LAC. We then conducted high resolution quantitative PCR-based deletion mapping of this region and identified the *FoxO3a* gene as the focus of bi-allelic or homozygous deletion (HD). HDs were detected in 5 out of 15 (33.3%) LAC cell lines and in 6 out of 75 (8%) carcinogen-induced primary LAC. *FoxO3a* was exclusively affected by HD in 7 of the samples examined, as loss of both alleles did extend to the nearest flanking genes of *FoxO3a*. Deletion of *FoxO3a*, either by HD or subclonal loss was detected in 38 out of 75 (50.7%) of carcinogen-induced LAC in contrast to only 1 out of 10 (10%) of LAC of untreated mice. Several of the samples also were subjected to direct sequence analysis; however, no intragenic mutations were detected. These results implicate *FoxO3a* as a selective target of deletion in mouse LAC. Significant association with carcinogenic induction suggests that deletion of *FoxO3a* contributes to the development of carcinogen-initiated tumors.

Introduction

Lung cancer is the leading cause of cancer death world-wide, and in the US accounts for more deaths annually than breast, colon and prostate cancer combined (1,2). It has been estimated

that ~75% of lung cancer cases result from exposure to carcinogens in cigarette smoke (1). In recent years lung adenocarcinoma (LAC) has become the most prevalent type of lung cancer world-wide, and among all lung cancer types, is also the most prevalent among never-smokers (3). Tumors develop through an abnormal evolutionary process involving the selection of genetic and epigenetic changes that are advantageous for malignant growth (4). With regard to LAC, tumors of smokers and never-smokers have very different oncogenomes, indicating they are developmentally distinct (5-8). For example, whereas tumors in smokers typically have a high frequency of *K-ras* and *TP53* mutations those in never-smokers have high frequencies of *EGFR* mutation and elevated Akt1 expression (9-13). These categories of LAC also are differentially responsive to therapy, which is particularly evident in target therapy where EGFR inhibitors are highly effective in tumors with *EGFR* mutations, but ineffective in tumors with *K-ras* mutations (11,12). Based on differences in pathogenetics, treatment responsiveness and epidemiology, LAC of smokers and never-smokers have been considered separate diseases (14). Further understanding of the underlying genetic changes that differentiate these distinct LAC sub-types is expected to improve the diagnosis and treatment of all LAC.

Previous studies have shown that carcinogen-induced LAC in mice have strikingly similar genetic changes as those seen in human LAC of smokers (15-18). This suggests that mice may be used as a manipulable model to study the underlying molecular events that give rise to the etiologically different types of LAC. To gain a better understanding of the relationship between LAC etiology and pathogenetics we have examined the chromosomal differences attributed to carcinogen exposure in LAC using age and gender-matched C3H/HeJ x A/J and A/J x C3H/HeJ F₁ hybrid mice. In line with the differences observed in LAC of smokers and never-smokers we also observed major oncogenomic differences between the carcinogen-induced and spontaneous tumors (19,20). Chromosome 6q21-qter is a site of frequent allelic loss in LAC of smokers compared with never smokers (4-9). This region also contains a lung candidate susceptibility gene, *RGS17*, based on its linkage with familial lung cancer contains (21). We examined the orthologous region in our mouse model for LAC and have identified the *FoxO3a* gene as the primary target of deletion in this region, implicating a role in LAC development.

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Materials and methods

Lung tumors and cell lines. Age and gender-matched C3H/HeJ x A/J and A/J x C3H/HeJ F1 hybrid mice were used in this study. LAC were induced by VC, AFB1, or NNK or developed spontaneously (i.e. in the absence of carcinogenic induction). All tumors were diagnosed as adenocarcinomas, as previously described (19). VC is a synthetic mouse lung carcinogen; AFB1 is fungal toxin and human carcinogen; NNK is a human lung carcinogen. Previous studies have demonstrated that each is a potent inducer of LAC in mice (22-24). Tumors were induced with 20 or 60 mg VC/kg body weight by a single intraperitoneal (i.p.) injection at 7 weeks of age, with NNK by i.p. injection of 50 mg NNK/kg body weight 3 times per week for 8 weeks, and with AFB1 by i.p. injection with 6.25 mg/kg 3 times per week for 8 weeks. The carcinogen-induced tumors were obtained from 6 to 14 month old mice. Spontaneous tumors developed without carcinogenic induction and had longer latency periods typically occurring after 14 months of age. Genomic DNA was isolated from cell lines and tissues using previously published methods (19).

The Spon cell lines and CL20, CL13, CL25, CL30, MC7 and MC14 were grown in RPMI-1640 medium containing 2% fetal bovine serum. LM1 and LM2 were grown in Minimal Essential Medium containing 10% fetal bovine serum. CMT64 was grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. CL and MC cell lines were derived from methylene chloride-induced lung adenocarcinomas, all other were derived from spontaneous lung adenocarcinomas, as previously described (20). All medium was supplemented with 2 mM glutamine, 100 μ g/ml penicillin and 100 mg/ml streptomycin. Cells were grown in a humidified incubator at 37°C and 5% CO₂.

Loss of heterozygosity (LOH) and deletion analysis. Several informative (heterozygous) DNA markers on chromosome 10 were used to determine allelic loss or LOH frequencies in spontaneous, AFB1-, NNK- and VC-induced lung tumors by quantitative polymerase chain reaction (qPCR). Microsatellite markers used were D10MIT3, D10MIT38, D10MIT194, D10MIT67, D10MIT96 and D10MIT230. PCR reactions of 50 ng of tumor or normal lung DNA were carried out at 1 min. 95°C, 30 sec at 55-60°C and 30 sec at 72°C for 22-25 cycles. Prior to PCR one primer of each pair was 5' end-labeled with γ -³²P-ATP. Heterozygous PCR products were separated by electrophoresis in 8% denaturing polyacrylamide and autoradiographed. LOH is a reduction of one allele relative to the other in tumor vs. normal DNA. To account for genetic and cellular heterogeneity of tumor tissue, significant LOH was scored as a reproducible reduction of one allele by $\geq 40\%$ relative to the other and normalized against the allelic ratios of corresponding normal lung or liver DNA from untreated mice. This approach has been previously described (19).

The templates for deletion analysis of specific genes within the candidate region were synonymous in both parental mouse strains used in this study. This analysis was carried out to both quantify gene loss and to map the location of highest frequency of loss in cell lines and tumors. PCR was carried out essentially as described above with the exception that unlabeled

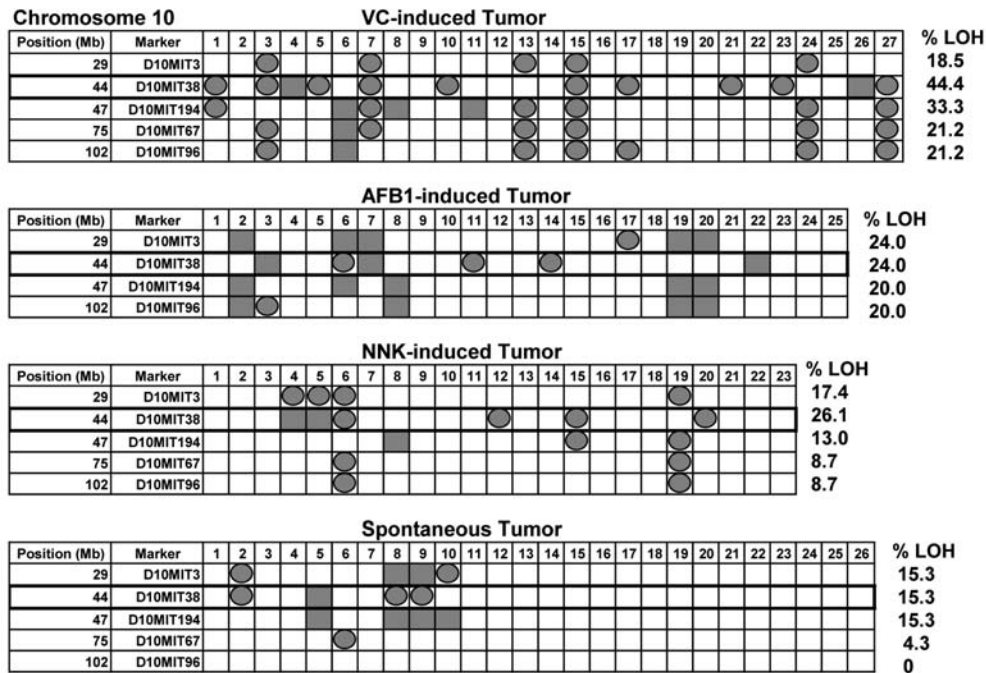
primers were used, after which PCR products were resolved in 2% agarose gels stained with ethidium bromide. DNA dose was determined by calculating target product/control product in the tumors and cell lines divided by that value obtained from normal DNA. *Gapdh* and β -*actin* were used as controls. UVP Imaging and Analysis System and LabWorks software (UVP, Inc., Upland, CA) were used for quantitation. To account for the genetic and cellular heterogeneity of the tumor samples from normal stroma and infiltrate, as previously determined (19), homozygous deletion (HD) or the loss of both alleles was defined as a reproducible gene dose reduction of $\geq 80\%$ relative to control DNA. Sub-HD or deletions not reaching the threshold of HD was a reduction of 40-80% relative to control levels. Because homozygous sequences were used in this analysis, hemizygous losses (loss of one of 2 alleles) and sub-clonal-HDs could not be distinguished apart, and were counted as a single frequency. Clonal HD in cell lines was the reproducible absence of detectable target PCR product relative to the control. Oligonucleotide primers used in this study were designed based on the available gene sequences from NCBI and Ensembl genome browsers using Primerquest software (Integrated DNA Technologies, Coralville, IA). Several primer pairs within *FoxO3a* were used to obtain the qPCR results.

Results

DNA from C3H/HeJ x A/J and A/J x C3H/HeJ F1 hybrid (heterozygous) mouse LAC was subjected to qPCR using informative microsatellite markers positioned on chromosome 10qB2, which contains orthology with human chromosome 6q22-21. Losses on chromosome 10 were detected in 17 out of 27 (63%) VC-induced, 8 out of 23 (34.8%) NNK-induced, 11 out of 25 (44%) AFB1-induced LAC, and in 5 out of 26 (19.2%) spontaneous LAC. LOH frequency was higher in all of the carcinogen-induced groups, which reached significance in the VC ($p < 0.001$) and AFB1 ($p = 0.028$) tumors when compared with the spontaneous tumors by χ^2 analysis (Fig. 1, Table I). Despite a paucity of informative markers between the two closely related parental strains, the markers within chromosome 10qB2 (D10MIT38 and D10MIT194) displayed the highest frequency of LOH compared with distant markers on this chromosome (Fig. 1A). Eleven tumors (VC-induced 4, 5, 10, 21, 23, 26; AFB1-induced 11, 14; NNK-induced 8, 12, 20) displayed LOH exclusively at D10MIT38 (24% of tumors with LOH) and another 3 tumors (VC-induced 8, 11; NNK-induced 8) with losses only at D10MIT194, located ~ 3 Mb telomeric of D10MIT38. This indicated that deletions were likely to be focused on a gene in the vicinity of marker D10MIT38.

Based on this evidence we performed qPCR-based deletion mapping of this region to narrow the site of deletion in an attempt to identify a gene targeted for inactivation in these tumors. This was initially performed on a panel of 15 mouse LAC cell lines sparing limited tumor tissue and DNA. We selected genes/markers positioned every ~ 1 Mb encompassing ~ 10 Mb centered on D10MIT38. Quantitative deletion analysis is described in detail in the Materials and methods section. Briefly, gene loss of 80% in a tumor or cell line relative to normal DNA and normalized with *Gapdh* or β -*actin* (control)

A



B

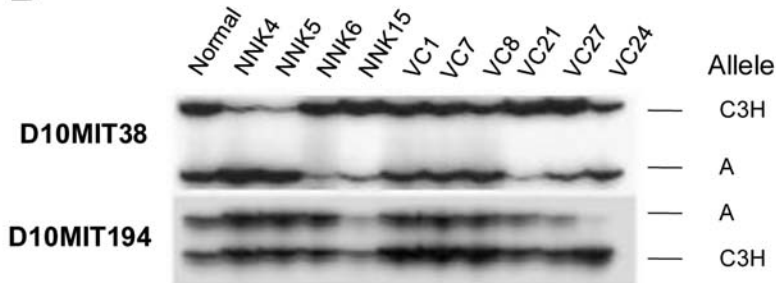


Figure 1. LOH on chromosome 10 in mouse LAC. (A) Summary of LOH in NNK-induced, VC-induced, AFB1-induced and spontaneous LAC. Genetic markers are in order relative to the centromere (at top), with the distance of each marker from the centromere shown in Megabases (Mb). Squares, LOH of C3H/HeJ allele; circles, LOH of A/J allele; white, retained heterozygosity. (B) Representative PCR-LOH analysis of LAC using heterozygous chromosome 10 markers. Markers D10MIT38 and D10MIT194 are the site of highest LOH frequency in the tumors examined. Normal DNA from the experimental mice was used as a control for all quantitative PCR.

was considered an HD. A decrease of ≥ 40 -80% was considered a sub-clonal deletion. These cut-offs were intended to accurately identify deletions of target sequences, but also taking into account the genetic and cellular heterogeneity of the tumors and cell lines. The cell lines used in this study have high degrees of intra-line chromosomal heterogeneity, based on a previous study (25).

This initial analysis indicated that DNA loss frequencies in the cell lines were highest for genes/markers positioned centromeric to D10MIT38. Therefore, we extended our mapping effort in the centromeric direction of this marker. We continued a process of reducing the size of the candidate region based on deletion frequency, and increasing the resolution of the genetic analysis within a more focused candidate region. In the end, all deletions were found to overlap at a single gene, *FoxO3a*, located 41.90 Mb from the centromere (Fig. 2A and B). HD of *FoxO3a* was detected in 5 out of the 15 (33.3%) cell lines. Two of the cell lines (MC14

Table I. Frequency of LOH on chromosome 10 in carcinogen-induced and spontaneous mouse LAC.

Tumor	LOH(%)	P-value
VC	17/27 (63)	5.26E-07
AFB1	11/25 (44)	0.028
NNK	8/23 (34.8)	NS
SP	6/26 (23.1)	

Frequencies are the numbers of tumors with LOH at any tested marker on chromosome 10/number of tumors analyzed. χ^2 test compares the LOH frequencies in carcinogen-induced tumors to the spontaneous tumors (SP). LAC are carcinogen-induced or spontaneously developed as described in the Materials and methods. VC, vinyl carbamate; AFB1, aflatoxin B1; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NS, not significant.

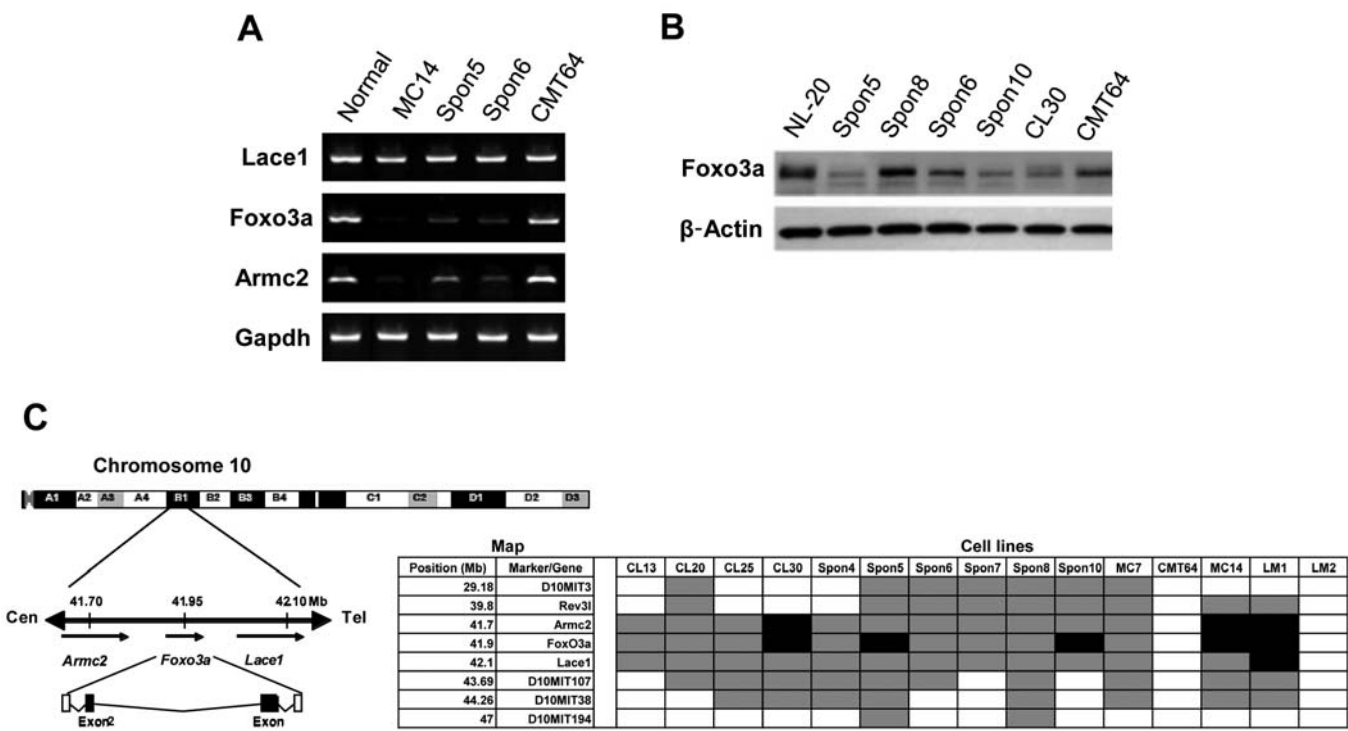


Figure 2. Identification of *FoxO3a* as a target of deletion in mouse LAC cell lines. (A) Experimental results of the deletion mapping identifying *FoxO3a* as the principal target of HD within the candidate region. *Gapdh* is used as a quantitative control gene. (B) Western blot of *FoxO3a* in a representative number of mouse LAC cell lines demonstrating relative concordance between gene and protein levels. β -actin is used as a loading control. NL-20 is a human immortalized lung epithelial cell line that contains both alleles of *FoxO3a* and is used as a comparative control for *FoxO3a* expression in LAC cell lines. (C) Physical map of the *FoxO3a* locus (left), and summary of the deletion mapping results of the candidate region in mouse LAC cell lines. HD (black); sub-clonal deletion (gray); no deletion (white).

Table II. Frequency of *Foxo3a* deletion in mouse LAC.

Tumor	Sub-clonal deletion (%)	Homozygous deletion (%)	Total (%)	P-value
VC	10/27 (37)	2/27 (7.4)	12/27 (44.4)	0.00041
AFB1	12/25 (48)	4/25 (16)	16/25 (64)	0.00018
NNK	10/23 (43.5)	0/23 (0)	10/23 (43.5)	0.00235
SP	1/10 (10)	0/10 (0)	1/10 (10)	

Frequencies are the numbers of tumors with sub-clonal deletion and HD of *Foxo3a*/number of tumors analyzed. χ^2 compares deletion frequency in each carcinogen-induced group vs. spontaneous group. Abbreviations are described in Table I.

and LM1) displayed clonal HDs and 3 cell lines (Spon5, Spon8 and Spon10) had decreases of *FoxO3a* that were $\geq 80\%$ relative to normal DNA indicating that they were sub-clonal HDs (Fig. 2A and B). Deletions not meeting the quantitative threshold ($\geq 40\text{--}80\%$ decreased) of HD were detected in 8 out of 15 (53.3%) of the cell lines, demonstrating that only two cell lines retained a normal copy number of *FoxO3a*. HDs exclusively affected *FoxO3a* in two of the cell lines (Spon5 and Spon10) as HDs did not affect its nearest flanking genes, *Lace1* and *Armc2* (Fig. 2C). This suggested that *FoxO3a* was the primary target of bi-allelic inactivation by deletion in this region. Western blot analysis also was performed on several of these cell lines to demonstrate concordance between *FoxO3a* gene and protein levels (Fig. 2C).

Having identified *FoxO3a* as the apparent principal target of deletion in the region of frequent allele loss on chromosome 10qB2 in LAC cell lines, we next directly examined *FoxO3a* and its nearest flanking genes for deletions in the primary LAC. *FoxO3a* HDs were clearly evident in 4 out of 25 (16%) AFB1-induced tumors and in 2 out of 27 (7.4%) VC-induced (Fig. 3A and B), as determined by qPCR. Among these, AFB1-1, AFB1-4, AFB1-10, VC-14 and VC-16 had HDs exclusively affecting *FoxO3a* (Fig. 3B). Also, several tumors displayed deletions of *FoxO3a* not reaching the quantitative level of HD. This level of deletion was detected in 10 out of 27 (37%) VC-induced LAC, 12 out of 25 (48%) AFB1-induced LAC, 10 out of 23 (43.5%) NNK-induced LAC, and in only 1 out of 10 (10%) spontaneous tumors (Table II) indicating that loss of *FoxO3a* was a very frequent occurrence

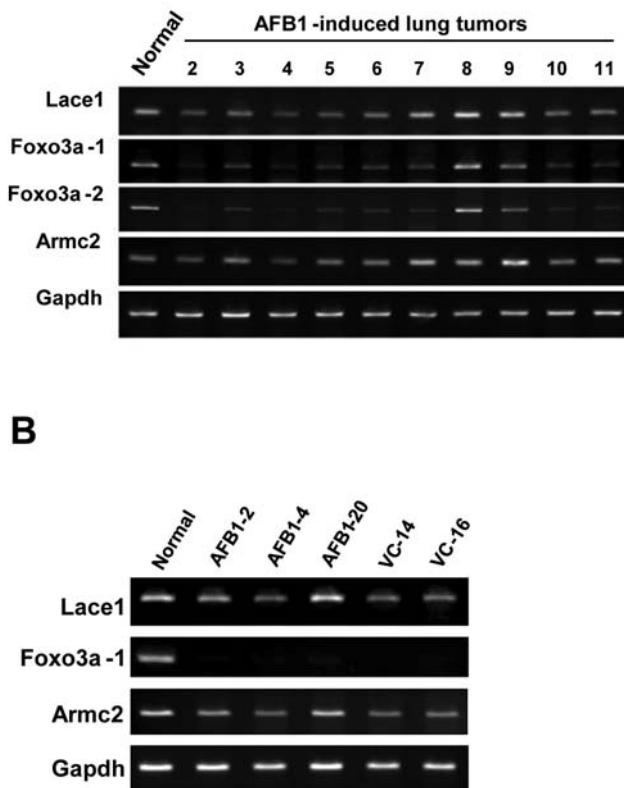


Figure 3. Deletion analysis of *FoxO3a* in mouse primary LAC. (A) Deletion analysis of *FoxO3a* and neighboring genes in AFB1-induced mouse primary LAC. *FoxO3a*-1 and -2 are generated from separate primer pairs of non-overlapping regions of the *FoxO3a* exon 2. (B) Representative qPCR results showing HDs exclusively of the *FoxO3a* gene in primary LAC. Genomic information was obtained from the Ensembl genome browser. χ^2 test of each carcinogen-induced group vs. spontaneous group.

in carcinogen-induced LAC compared with spontaneous tumors ($p \leq 0.00235$).

Discussion

Genes that contribute to the repression of tumor development are selectively inactivated in cancer. DNA loss is a common mode of gene inactivation occurring as a result of many different chromosome aberrations such as translocations, rearrangements and deletions (26). Regional DNA losses can also be mapped to identify novel cancer genes selectively inactivated in a given cancer type. This is accomplished by using a high resolution genetic map to define foci of DNA loss in tumors of a given type relative to normal DNA. In this study, we mapped a focus of HD to *FoxO3a* at 41.90 MB on chromosome 10 in mouse LAC. Although reports have shown that LOH in the region of *FoxO3a* is common in several human cancer types including LAC of smokers, this is the first report that this gene is the target of genetic inactivation in sporadic cancer (5-8,27,28). *FoxO3a* is located 2.3 MB centromeric of the orthologue of the candidate human lung cancer susceptibility gene, *Rsg17* (21). Human *RSG17* also is over-represented in sporadic lung cancer. Consistent with this role, we observed no deletions of *Rsg17* in mouse LAC (data not shown).

We previously reported that chromosome instability (CIN) is more prominent in genotoxic carcinogen-induced mouse LAC than in spontaneous (non-induced) LAC suggesting that carcinogen-induced DNA damage is an underlying cause of CIN in LAC (19,20). Our results from the same set of tumors indicate that *FoxO3a* deletions occur mainly in carcinogen-induced tumors and derivative cell lines with extensive CIN. CIN is an enhanced rate of chromosomal defects that serves as a source of genetic variability in cancer cells and seems to be mechanistically linked with *FoxO3a* loss in this study (29-31). Interestingly, the human FRA6F fragile site is located within the human *FoxO3a* gene (32). A characteristic of fragile sites is that they are relatively sensitive to breakage induced by carcinogens and CIN (33). They also are conserved within mammalian species (34). Therefore, this raises the possibility that the destabilization of the mouse ortholog of FRA6F by carcinogenic damage or CIN may contribute to the loss of *FoxO3a* in LAC. The absence of point mutations within *FoxO3a* as an alternative mechanism of inactivation in any of the tumors and cell lines examined in this study tends also to support this possibility (data not shown).

It was recently shown that germline *FoxO3a* deficiency does not predispose mice to lung adenoma or adenocarcinoma; however it does increase susceptibility to ovarian and pituitary tumors (35). Together with our findings this suggests that *FoxO3a* inactivation alone is insufficient for lung tumor predisposition in mice, but may contribute to the progression of these tumors. As such, *FoxO3a* inactivation may exert its effect in a context that includes prior selection of other tumor-advantageous genetic or epigenetic changes. Such an effect was recently reported for the *Lkb1* tumor suppressor gene, which was shown to cooperate with *K-ras* mutations in germline predisposition to NSCLC in mice (36).

The cellular role of *FoxO3a* is consistent with that of a classical tumor suppressor (37,38). It is a transcription factor activated by various cellular stresses, many of which are highly relevant to carcinogenesis, such as DNA damage, oxidative stress and hypoxia (39-42). *FoxO3a* mitigates an anti-oncogenic response of either growth inhibition or apoptosis when stress-activated (37,38). As it relates to this study we recently demonstrated that *FoxO3a* activates pro-apoptotic transcription program in response to NNK (43). In the absence of stress, *FoxO3a* is functionally inactivated by Akt and IKK, which by phosphorylating specific serine and threonine residues promote 14-3-3 binding and inactivation by nuclear exclusion (44-46). Upon activation by specific biochemical mechanisms that are currently unresolved, *FoxO3a* is free to localize to the nucleus and to elicit its stress-response as a transcription factor (37,38). Hence, stress-activated *FoxO3a* overrides the proliferative and oncogenic signaling of Akt and I κ B kinase (IKK) (37,38). In this way, *FoxO3a* may protect against the transformative effects of carcinogen-induced DNA damage while diminishing oncogenic signaling through Akt or IKK in LAC.

Akt is a component of the EGFR/PI3K signaling network, which is of considerable relevance in LAC (47-49). Akt is functionally activated by PI3K in the transduction of growth and oncogenic stimuli from EGFR (47). PI3K is directly antagonized by the PTEN tumor suppressor (50). Although alterations of PTEN are rare in LAC, Akt1 overexpression is

relatively common in LAC of never-smokers (13). Along with this change, *EGFR* mutations also are typical of LAC of never-smokers (10-12). In contrast, *K-ras* mutations are common in LAC of smokers, which also constitutively activate both Akt and MAPK branches of the EGFR network (9,51). It is tempting to speculate that FoxO3a may be more prone to selection pressure in carcinogen-induced tumors, rather than other constituents of the EGFR/PI3K/Akt pathway, because FoxO3a also plays a significant role as an anti-carcinogenic transcription factor. Consistent with this, allelic losses are more frequent in the region of human *FoxO3a* in LAC of smokers compared with never-smokers (5-7). It is of interest then to investigate whether FoxO3a inactivation is more specific to the carcinogenic etiology of smoking in human LAC.

Our investigation into this potential role in human LAC thus far has shown that *FoxO3a* HDs are relatively common in human LAC of smokers, and significantly less so in squamous cell carcinoma of the lung (unpublished). Additional LAC of never-smokers need be examined to determine whether or not its inactivation is specific to tobacco carcinogen-induced LAC. The results reported here suggest that *FoxO3a* inactivation is recurrent in carcinogen-induced LAC. Its deletion may be a consequence of the action of carcinogens and CIN on *FoxO3a* gene structure in combination with oncogenic selection pressure exerted on its function as an anti-carcinogenic transcription factor and negative regulator of EGFR/PI3K/Akt signaling. Further study is needed to more precisely elucidate the etiologic and mechanistic factors driving the selective inactivation of *FoxO3a* in LAC.

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