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ORIGINAL ARTICLE

KEAP1 gene mutations and NRF2 activation are common in pulmonary papillary adenocarcinoma

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Distinctive histological variants of lung cancer are increasingly recognized to have specific genetic changes that affect tumor biology and response to therapy. In this study, we evaluated true papillary adenocarcinoma of the lung, proposed as a distinct diagnostic category with relatively poor response to therapy, to determine whether these tumors also have specific molecular alterations that would affect sensitivity to chemotherapy. Specifically, we measured protein levels of P53, excision repair cross-complementation 1 (ERCC1) and ribonucleotide reductase M1 (RRM1) by immunohistochemistry and evaluated the Kelch-like erythroid cell-derived protein with cap-n-collar homology (ECH)-associated protein 1 (*KEAP1*) gene for mutations, correlating mutations of this gene with total and nuclear expression of the nuclear factor erythroid-2-related factor 2 (NRF2). We found high levels of P53 in 23 of the 55 specimens (41.8%), similar to the rate of *P53* gene mutations observed in general for pulmonary adenocarcinoma, and levels of ERCC1 and RRM1 also showed distributions similar to those reported generally for non-small lung cell cancer (NSCLC). However, *KEAP1* alterations were observed at a significantly higher frequency in papillary adenocarcinoma tumors (60%) than what has been reported previously for NSCLC (3–19%). These mutations of *KEAP1* were associated with increased nuclear accumulation of NRF2 in tumors, as expected for functional alterations. Thus, high rates of *KEAP1* mutations and NRF2 overexpression in true papillary adenocarcinoma could be related to poor prognosis and chemotherapy resistance. Furthermore, this distinctive molecular characteristic supports the recognition of true papillary adenocarcinoma as a diagnostic entity.
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INTRODUCTION

Specific mutations in lung cancer seem to be relatively restricted to particular histologically defined phenotypes. For example, mutations of the *RB* gene are common in small cell cancers but rare in non-small lung cell cancers (NSCLCs), whereas mutations (or methylation) of *P16* are characteristic in the NSCLCs.[1] Among NSCLCs, coding mutations of the *KRAS* and epidermal growth factor receptor (*EGFR*) genes are relatively restricted to adenocarcinoma,[2] with *EGFR* mutations being particular prevalent in adenocarcinoma tumors with the bronchioalveolar histological pattern of growth.[3] Thus, genomic data for lung cancer cannot be generalized across various histological types of the disease.

True papillary adenocarcinoma has been recently recognized as a distinctive histomorphological subtype of lung adenocarcinoma by World Health Organization classification,[4] and the molecular characteristics of this subtype of lung cancer have not yet been defined. This variant seems to have a less favorable prognosis and poorer response to chemotherapeutic agents[5] than other forms of pulmonary adenocarcinoma, and thus recognition of this histological pattern of lung cancer could have clinical significance. However, many pathologists do not routinely recognize the morphological characteristics of papillary adenocarcinoma, and the actual incidence and significance of correctly classifying true papillary cancers is still not known.

Although molecular characteristics of pulmonary papillary adenocarcinoma are undefined, several molecular alterations might be suspected based on associations with poor response of lung cancers to commonly used agents. For example, aberrant P53 expression was found to be associated with resistance to cisplatin-based chemotherapy,[6] and more recently, high expression levels of the excision repair cross-complementation 1 (*ERCC1*) and ribonucleotide reductase M1 (*RRM1*) genes have been associated with poor prognosis and poor response of NSCLCs to cisplatin and gemcitabine.[7–9] In addition, the Kelch-like ECH-associated protein 1 (KEAP1) and the nuclear factor erythroid-2-related factor 2 (NRF2) signaling pathway have been identified to have important roles in the cellular response to oxidative stress, electrophiles and xenobiotics,[10–13] and *KEAP1* mutations lead to constitutively active NRF2 and subsequent protection of cancer cells from chemotherapeutic drugs.[14,15] The frequency of these mutations in lung cancers is still uncertain; our previous study and others have shown that *KEAP1* gene mutations occur in ~20% of NSCLCs

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(particularly in adenocarcinoma),[16,17] whereas a more recent report found only one mutation among 31 cases of NSCLC.[18] Undoubtedly, these variations in reported rates of *KEAP1* mutations are due, at least in part, to differences in lung cancer phenotypes evaluated in the various studies.

In this investigation, we first evaluated levels of P53, ERCC1 and RRM1 using immunohistochemistry (IHC), then, evaluated the frequency and types of *KEAP1* mutations using direct sequencing methods, and correlated *KEAP1* mutation status with patterns of NRF2 expression in these tumors. These studies are intended to both identify mechanisms that might affect the clinical behavior of these cancers, and also to determine whether these cancer do have distinctive molecular characteristics that would support the recognition of this category of lung cancer.

MATERIALS AND METHODS

Pulmonary papillary adenocarcinoma samples

We identified a total of 58 cases of true pulmonary papillary adenocarcinoma in the surgical pathology archives of The Johns Hopkins Hospital using the diagnostic criteria adapted by the World Health Organization,[1,2] and a tissue microarray was prepared using core samples (1 mm diameter, three cores per case) of paraffin tissue blocks from individual cases. Adequately preserved DNA for sequencing was available from tissue samples from 20 of these cases. Use of human tissue and clinical information was reviewed and approved by the Institutional Review Board for Human Subjects.

Immunohistochemistry

For P53, a primary polyclonal antibody of goat anti-human C-terminal peptide (C-19, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at 1:50 dilution. A primary rabbit anti-human polyclonal antibody raised against a C-terminal RRM1 peptide and a mouse anti-human monoclonal antibody raised against the full length ERCC1 protein (Abcam, Cambridge, MA, USA) were both used at 1:100 dilutions. To stain for NRF2, a rabbit polyclonal antibody (C-20, Santa Cruz Biotechnology) was used at 1:250 dilution. For all antibodies, after washing, a secondary antibody conjugated with peroxidase was applied to detect and visualize the specific antigen-antibody complexes using LASB System-HRP assay kit (Dako, CA, USA). Both positive and negative controls were included in the procedures.

DNA purification, amplification and sequence analysis

Primers for PCR amplification and sequencing were designed in the area of exon 3 of *KEAP1*[16] and synthesized by Invitrogen (Carlsbad, CA, USA), and amplification of DNA from early passage A549 cells (American Type Culture Collection, Manassas, VA, USA) and from primary tumor samples was performed using Taq polymerase (Qiagen, CA, USA) as previously described.[16]

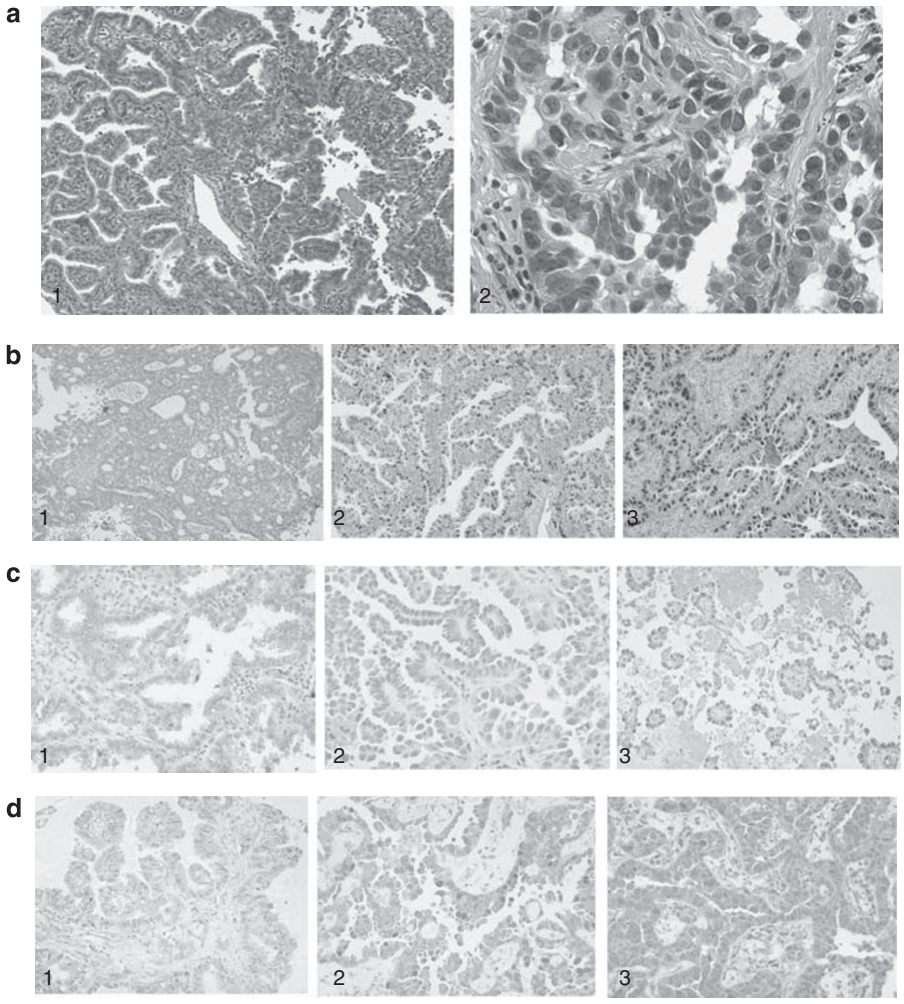

Figure 1 (**a**) The surgical resection specimen of pulmonary papillary adenocarcinoma. (**b**–**d**) immunohistochemical studies of P53, RRM1 and ERCC1. (1) Background staining; (2) weakly staining; and (3) strongly staining.

All potential genetic alterations were confirmed by bi-directional sequencing and testing a second, independent DNA sample from the tumor. All chromatogram alterations of *KEAP1* sequences were validated by a repeat sequencing and re-analyzed by manual review.

RESULTS

Characteristics of papillary adenocarcinomas

All cases selected for this study were characterized morphologically by numerous papillary structures with central vascular cores constituting at least 75% of the tumor volume (Figure 1a1). The papillary structures were further complicated by tertiary branches and tufts, and consisted of individual tumor cells with high nuclear to cytoplasm ratio and markedly atypical nuclei (Figure 1a2). Among patients with available clinical information, 46 of 49 (93.9%) were current or former smokers. Cases included 15 cases (27%) stage pT1, 28 cases (51%) stage pT2, two cases (4%) stage pT3 and 10 cases (18%) stage pT4.

Expression of P53, ERCC1 and RRM1 in papillary adenocarcinoma

Using IHC to estimate the frequency of *P53* mutations in pulmonary papillary adenocarcinoma, we observed high levels of P53 expression (2+ or 3+) in 23 of 55 (41.8%) of the cases of pulmonary papillary adenocarcinoma (Figure 1b). This represents a frequency generally similar to the frequency of P53 mutations/overexpression reported previously for pulmonary adenocarcinoma in general. For RRM1 expression, 5/28 cases (17.6%) were negative, 15/28 cases (53.6%) stained weakly positive and 8/28 cases (28.5%) stained strongly positive (Figure 1c). For ERCC1, 5/28 cases (17.6%) of tumors were negative, 18/28 cases (64.2%) stained weakly positive and 5/28 cases (17.6%) stained strongly positive (Figure 1d). Although these data are only semi-quantitative, it is evident that the distributions of staining intensities for these two proteins are not significantly different than those reported for NSCLC in general.[5]

Mutations of *KEAP1* in papillary adenocarcinoma

To determine the frequency of mutations in *KEAP1* in pulmonary papillary adenocarcinoma, we amplified and sequenced exon 3 of the *KEAP1* gene in DNA derived from the 20 tumor samples with suitable frozen tissue. In all, 12 of the 20 cases (60%) showed mutations of *KEAP1*, which is significantly more frequent than the 18.5% of cases (10 of 54 cases) with *KEAP1* mutations that we observed in non-selected cases of pulmonary non-small cell carcinomas ($P<0.05$) and strikingly more frequent than the one of 31 cases more recently reported.

Representative electropherograms are shown in Figure 2a. Overall, four of the 12 mutations (cases P4, P5, P6 and P8) were characterized

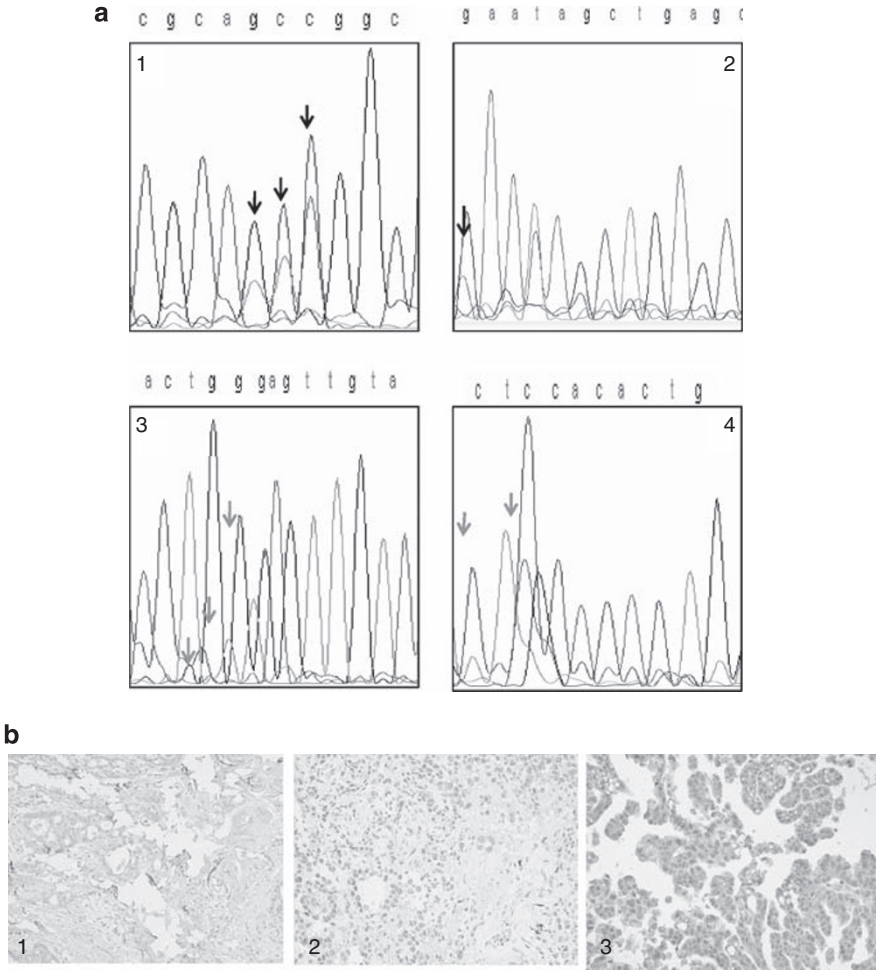

Figure 2 (**a**) Electropherograms of *KEAP1* mutations. (1) The replacement of GCC to TTA at 367th amino acid in the Kelch domain. (2) The replacement of 334th amino acid of tyrosine with phenylalanine, (3) the insertion of a stop-codon at 346th amino acid, (4) the insertion of A and G. (**b**) immunohistochemical studies of NRF2. (1) The normal lung parenchyma. (2) The tumor cells with wild-type *KEAP*. (3) The tumor cells with *KEAP1* mutations.

by GCC replacing TTA at the codon corresponding to the 367th amino acid in the Kelch domain of *KEAP1*, which leads to premature termination and truncated KEAP1 protein (Figure 2a1). Three tumors (P17, P18 and P19) showed mutations that replace tyrosine with phenylalanine in the 334th amino acid, resulting in a frameshift in the Kelch domain of *KEAP1* (Figure 2a2). Three tumors (P2, P10 and P14) showed insertion of a stop codon at the position of the 346th amino acid, leading to premature termination and truncated KEAP1 protein (Figure 2a3). Two tumors (P3 and P15) showed an insertion of A and G, that results in premature termination and truncated KEAP1 protein (Figure 2a4). Finally, three tumors (P5, P6 and P8) showed amino acid substitution of glycine at 333th to cysteine in the Kelch domains of *KEAP1*. Interestingly, 9 of the 12 mutations would result in premature termination and truncated KEAP1 protein, and 3 of the 12 tumors had more than one mutation affecting the *KEAP1* gene.

Abnormal expression and distribution of NRF2 protein in papillary adenocarcinoma

To evaluate expression and cellular localization of NRF2 protein in pulmonary papillary adenocarcinoma tumors, we performed IHC using a polyclonal anti-NRF2 antibody. The overall expression of NRF2 protein for 55 pulmonary papillary adenocarcinoma tumors is summarized in Table 1. Normal lung parenchyma shows weak, and predominantly cytoplasmic, staining of NRF2 protein (Figure 2b1), and eight tumor tissues with wild-type *KEAP1* also demonstrated predominantly cytoplasmic staining of NRF2 (Figure 2b2). By contrast, all 12 tumor tissues harboring *KEAP1* mutations showed an increased level of NRF2 staining both in the nucleus and cytoplasm (Figure 2b3), suggesting that there is increased nuclear accumulation and activation of NRF2 in tumor cells in the setting of *KEAP1* mutation.

Table 1 Summary of NRF2 and P53 expressions in the pulmonary papillary adenocarcinomas

		P53 stain	*NRF2 stain*
Stage (cases)	*Score*	*Case numbers*	*Case numbers*
pT1 (*n*=15)			
	0	6	5
	1	4	3
	2	5	7
pT2 (*n*=28)			
	0	8	3
	1	10	16
	2	10	9
pT3 (*n*=2)			
	0	1	0
	1	1	1
	2	0	1
pT4 (*n*=10)			
	0	0	0
	1	2	3
	2	8	7

Abbreviation: NRF2, nuclear factor erythroid-2-related factor 2.
Note: staining was scored semiquantitatively using a three tier system: 0, undetectable; 1+, weakly positive; 2+, moderately to intensely positive.

We then explored the possibility of an association between mutations of *KEAP1* and *P53*. Among the eight tumor tissues with wild-type *KEAP1*, three cases (37.5%) demonstrated high expression of P53 protein. In contrast, 8 of 12 (67%) cases with *KEAP1* mutations showed high expression of P53 protein, consistent with mutations of the *P53* gene. Thus, occurrences of mutations of *P53* and *KEAP1* are positively correlated (difference between groups significant by χ^2-test, P=0.003).

DISCUSSION

We evaluated protective pathways in pulmonary papillary adenocarcinoma, a histomorphologically distinctive subtype of lung adenocarcinoma. We found that the frequency of high expression levels of P53, ERCC1 and RRM1 are similar in cases of pulmonary papillary adenocarcinoma to those observed previously in other types of pulmonary adenocarcinoma. However, we found a remarkably high *KEAP1* mutation frequency (12/20 cases, 60%) in pulmonary papillary adenocarcinoma, which is significantly greater than our previously reported rates of 18% for pulmonary non-small cell carcinoma in general and 28% for adenocarcinoma,[16] and strikingly higher than the frequency of ~3% recently reported for NSCLC.[18] As expected, *KEAP1* mutations were associated with increased nuclear expression of the NRF2 transcription factor in these papillary cancers, but some cases without *KEAP1* mutations also displayed increased NRF2 expression and nuclear localization. These high rates of *KEAP1* mutations and NRF2 overexpression in true papillary adenocarcinoma of the lung could be related to the reported poor prognosis and chemotherapy resistance of these cancers, and we propose that this distinctively high frequency of *KEAP1* mutations supports the recognition of true papillary adenocarcinoma as a diagnostic entity.

The *KEAP1* alterations found in pulmonary papillary adenocarcinoma tumors included insertions, deletions and frameshifts, with the most frequent type of mutation in pulmonary papillary adenocarcinoma (involving 58.3% (7/12) of the mutations) consisting of a single base insertion that would result in a premature stop codon and thus a truncated KEAP1 protein. Remarkably, 25% (3/12) of the cases with mutations were found to have more than one mutation affecting the *KEAP1* gene. Taken together with our previous data and others, our present study indicates that *KEAP1* gene alterations are frequent in pulmonary adenocarcinomas, particularly in papillary adenocarcinomas.

We also found that cancer cells of pulmonary papillary adenocarcinoma showed an increased accumulation of NRF2 by IHC staining, and in tumors with *KEAP1* mutations, both nuclear and cytoplasmic accumulations of NRF2 are markedly increased. These data suggest that frequent mutations of *KEAP1* in pulmonary papillary adenocarcinoma could significantly reduce the sensitivity of these cancers to chemotherapy. Increased NRF2 staining was also demonstrated in several cases of pulmonary papillary adenocarcinoma with wild-type *KEAP1*, suggesting that there are other mechanisms that could contribute to stabilization and induction of NRF2. Possible mechanisms for this include: (1) increased levels of the nuclear oncoprotein prothymosin, which regulates the intranuclear dissociation of KEAP1–NRF2 complex;[19] (2) somatic mutations in *Nrf2* gene which impair its binding with Keap1;[19,20] (3) alternative splicing of *KEAP1*, resulting in a non-functional KEAP1 protein in cancer cells;[21] (4) methylation of CpG-rich regions of *KEAP1* promoter, resulting in decreased expression of this protein;[22] and (5) phosphorylation of NRF2 by protein kinase(s) associated with the mitogen-activated protein kinase/extracellular signal-regulated kinase signaling cascade, leading to stabilization of NRF2.[23] Evidently, mechanisms for regulation of

KEAP1–NRF2 pathway are complex, and further studies are needed to understand exaggerated NRF2 responses in cancer cells even in the absence of *KEAP1* mutations.

In summary, our finding of frequent *KEAP1* mutations in pulmonary papillary adenocarcinoma and the association between loss of functional *KEAP1* and increased NRF2 activity suggest important roles for the KEAP1–NRF2 pathway in the regulation of antioxidants, detoxification enzymes and drug transporters activity in this subset of pulmonary adenocarcinoma. The relatively disproportionate frequency of *KEAP1* mutations in pulmonary papillary adenocarcinoma supports the recognition of these cancers as a unique subset of lung adenocarcinoma.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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