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Frequent epigenetics inactivation of *KEAP1* gene in non-small cell lung cancer

Lucia Anna Muscarella,[1,†,*] Paola Parrella,[1,†] Vito D'Alessandro,[2] Annamaria la Torre,[1] Raffaela Barbano,[1] Andrea Fontana,[3] Antonio Tancredi,[4,5] Vito Guarnieri,[6] Teresa Balsamo,[1] Michelina Coco,[1] Massimiliano Copetti,[3] Fabio Pellegrini,[3,7] Patrizia De Bonis,[6] Michele Bisceglia,[8] Gerardo Scaramuzzi,[4] Evaristo Maiello,[9] Vanna Maria Valori,[9] Giuseppe Merla,[6] Gianluigi Vendemiale[2,10] and Vito Michele Fazio[1,11]

[1]Laboratory of Oncology; [2]Internal Medicine Unit; [3]Unit of Biostatistics; [4]Thoracic Surgery Unit; [8]Department of Pathology and [9]Oncohaematology; IRCCS "Casa Sollievo della Sofferenza" Hospital; San Giovanni Rotondo, FG Italy; [5]Department of Internal Medicine and Medical Therapy; University of Pavia; PV; [6]Medical Genetics Unit; [7]Unit of Biostatistics; Department of Clinical Pharmacology and Epidemiology; Consorzio Mario Negri Sud; Santa Maria Imbaro, Chieti; [10]Department of Medical Sciences; Institute of Geriatrics; University of Foggia; Foggia, Italy; [11]Laboratory of Molecular Medicine and Biotechnology; Interdisciplinary Center for Biomedical Research (CIR); University Campus BioMedico; Rome, Italy

[†]These authors contributed equally to this work.

Key words: NSCLC, *KEAP1*, *NRF2*, methylation, mutation, outcome

The KEAP1/Nrf2 pathway is a master regulator of several redox-sensitive genes implicated in resistance of tumor cells against chemotherapeutic drugs. Recent data suggest that epigenetic mechanisms may play a pivotal role in the regulation of *KEAP1* expression. We performed a comprehensive genetic and epigenetic analysis of the *KEAP1* gene in 47 non-small cell lung cancer tissues and normal specimens. Promoter methylation analysis was performed using a quantitative methylation specific PCR assay in real time. Methylation at the *KEAP1* promoter region was detected in 22 out of the 47 NSCLCs (47%) and in none of the normal tissues analyzed. Somatic mutations were detected in 7 out of the 47 tumors (15%) and loss of heterozygosity (LOH) in 10 out of the 47 (21%) of the cases. Overall, we found at least one molecular alteration in 57% of the cases. Approximately one third of the tumors had two alterations and this feature was associated with higher risk of disease progression in univariate COX regression analysis (HR = 3.62; 95% CI 1.24–10.65, p = 0.02). This result was confirmed by Kaplan-Meier analysis, which demonstrated an association between worst outcome and *KEAP1* double alterations (p = 0.01, Log rank test). Our results further suggest that deregulation of the NRF2/KEAP1 system could play a pivotal role in the cancerogenesis of NSCLC. In addition identifying patients with *KEAP1* genetic and epigenetic abnormalities may contribute to disease progression prediction and response to therapy in lung cancer patients.

Introduction

Lung cancer is the leading cause of cancer related death worldwide.[1] Non-small cell lung cancer (NSCLC) represents 85% of lung cancer and adenocarcinoma (ADC) and squamous cell carcinoma (SCC) are the most common histotypes.[2] Although progress have been made in the treatment of NSCLC patients, the prognosis remains poor with a 5-year overall survival around 15%.[1] While the elective treatment in patients with initial stages of disease is surgery, patients diagnosed at late stages are only treated by chemotherapy with a combination of drugs that includes often platinum derivative.[3,4] However most of the tumor do not respond to treatment or develop drug resistance after an initial response.[4]

The *KEAP1* gene is a negative regulator of the cell adaptative response to radical oxidant species (ROS) and xenobiotics mediated by NRF2 transcription factor. Under physiological condition, KEAP1 functions as an intracellular redox sensor which binds NRF2 and targets it for proteosomal degradation.[5] When cell is exposed to oxidative damage, KEAP1 releases NRF2 which translocates into the nucleus where specifically recognizes an enhancer sequence known as Antioxidant Response Element (ARE) resulting in the activation of redox balancing genes (e.g., heme-oxygenase-1, HO-1), phase II detoxifying gene (e.g., NADP(P)H quinone oxidoreductase-1, NQO1) and drug transporters (e.g., Multidrug Resistant Proteins, MRP).[6-9] Several studies suggested that the activation of NRF2 protects against chronic disease such as cardiovascular diseases, neurodegenerative disorders, lung inflammation and fibrosis diabetes and nephropathy.[7,10-15] However in recent years the dark side of NRF2 has emerged and growing evidences suggest that NRF2 constitutive upregulation is associated with cancer development, progression and resistance to chemotherapy.[10,15-25] Thus, investigating the deregulation of KEAP1/NRF2 pathway may shed new light into the understanding the molecular mechanism of chemoresistance.

Several studies have reported mutations of the interacting domain between KEAP1 and NRF2 leading to a permanent

*Correspondence to: Lucia Anna Muscarella; Email: l.muscarella@operapadrepio.it
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NRF2 activation in NSCLC.[18-20] Somatic mutations of the *KEAP1* gene were also reported in patients affected by gall bladder tumors and in a breast cancer cell line.[21,22] Although recent studies demonstrated low or absent expression of the *KEAP1* in more than half of NSCLCs, only one paper investigated the epigenetic alterations of *KEAP1* in NSCLC. Wang et al. found aberrant hypermethylation at the *KEAP1* gene promoter in lung cancer cell lines, negative for *KEAP1* mRNA expression, and in five lung cancer tissues.[26] We have recently reported in malignant gliomas that promoter methylation modulates *KEAP1* mRNA expression in cell lines and primary tumors further suggesting that epigenetic mechanism may play a pivotal role in the regulation of *KEAP1* expression.[27]

In this study we performed a comprehensive genetic and epigenetic analysis of the *KEAP1* gene and we found at least one molecular alteration in 57% of the cases. Aberrant hypermethylation at the *KEAP1* gene promoter was the most common alteration found in 47% of the cases. Mutations were detected in 15% of the cases whereas LOH at the gene locus was demonstrated in 21% of the tumors. Overall one third of the tumors showed two alterations in *KEAP1* gene suggesting that both copies of the gene might be inactivated.

Results

KEAP1 aberrant methylation is a frequent event in NSCLC. To determine *KEAP1* methylation status in NSCLC we used a primers/probe set designed to amplify the CpG region containing putative transcriptional regulatory elements as described previously.[26,27] *KEAP1* methylation was determined on DNA obtained from 47 NSCLC and 12 normal lung tissues (NLT). Methylation levels in tumor samples ranged from 0–484.80 (Median 0; IQR 0–2.65) whereas methylation was not detected in the normal lung tissues analyzed (Median 0; IQR 0-0) (p = 0.002 Mann Whitney Test), (**Sup. Figs. 1–3**). Overall methylation at the *KEAP1* promoter region was detected in 22 out of the 47 NSCLC (47%) (**Table 1**). Statistically significant differences in methylation frequencies were demonstrated for both ADC and SCC as compared with NLT. No differences were found between ADC and SCC.

Somatic alterations of the _KEAP1_ gene in NSCLC. To determine the presence of mutations in the *KEAP1* gene we analyzed the 47 NSCLC cases by fluorescence based direct sequencing. Mutational analysis of the *KEAP1* coding region and intron/exon boundaries identified mutations in 7 out of the 47 NSCLC samples (15%) and in all cases they were only detected in tumor tissue. A deletion in exon 4 (c.1074del22bp) was found in one ADK sample (LC-23), (**Fig. 1**), with a predicted stop codon at 392 position (L358LfsX392). Other variants were classified as follows: 5 missense (D294Y, V369A, A466P, P322L, V167F) and one intronic substitution (c.640-12delC). Only one of the missense variants (V167F) was previously described in reference 19. Finally, two silent variations (rs1048287 and rs1048290) were found respectively in 10% and 38% of lung tumors.

The functional impact of the five non conservative missense mutations and the intronic variant detected in lung tissues was evaluated performing an in silico analysis using bioinformatics tools.[28] The already known V167F mutation falls in the BTB domain, whereas three out of the four novel identified mutations, the P322L, V369A and A466P are localized in the Kelch repeat domain (or DGR domain); and the remaining mutation D294Y is located in the IVR region (**Fig. 3**). The multialignment analysis showed that each of the mutated residues is strongly conserved among species even among different organisms along the philogenetic tree, suggesting that any variation could lead to a deleterious effect[29,30] (**Fig. 4**). To determine whether the newly identified mutations may impact the function of the KEAP1 protein, we interrogated the publicly available SIFT (sift.jcvi.org/www/SIFT_seq_submit2.html) [31] and Polyphen (genetics.bwh.harvard.edu/pph), softwares for the prediction of the functional outcome of aminoacidic changes. The two softwares gave similar results, with a predicted deleterious effect for 2 variants out of 5.

The deleterious effect of the intronic variant c.640-12delC on the canonical splicing process was investigated by using three publicly available softwares. Softberry (linux1.softberry.com/berry.phtml?topic=fsplice&group=programs&subgroup=gfind), BDGP (www.fruitfly.org/seq_tools/splice.html) and ESE finder (rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home).[32-34] When the wild type sequence was compared to the mutated sequences, the first two tools assigned a slightly different but significant score for the predicted acceptor splice site. Moreover the ESEfinder software detected the lack of a SF2/ASF binding site for the mutated sequence.

To ascertain the status of the *KEAP1* locus we genotyped the NSCLC and their corresponding peripheral blood sample using the following microsatellite markers D19S865, DM1, D19S906 and D19S2840 (**Fig. 2**). Of the 47 NSCLC 10 tumors (21%) demonstrated LOH for at least one of the markers (**Table 1**).

Genetic and epigenetic alterations of the _KEAP1_ gene are associated with worst patient's outcome. Genetic and epigenetic analysis of the *KEAP1* gene demonstrated at least one alteration in 27 out of the 47 NSCLC (57%). Twelve tumors (26%) showed two alterations in *KEAP1*, suggesting that both copies of the gene may be inactivated (**Table 1**). In particular, of the seven tumors bearing a somatic mutation, 4 displayed promoter hypermethylation while 2 showed LOH. In one case LOH analysis could not be performed and tumor was not methylated. In tumors without somatic mutations, 5 cases showed both methylation and LOH. Of the remaining cases, 11 displayed only methylation and 2 showed only LOH. In two cases LOH analysis could not be performed.

Follow up data were available for 35 patients (**Table 2**). In univariate time to event analyses the only factor associated with patient's outcome was the presence in the *KEAP1* gene of two genetic or epigenetic abnormalities. This features was associated with increased risk of disease progression (HR = 3.62; 95% CI 1.24–10.65; p = 0.02) in univariate Cox regression prediction analysis and was confirmed by Kaplan-Meier analysis (**Fig. 5**) which demonstrated an association between worst outcome and the double alterations (p = 0.01). In multivariate Cox regression analysis including age, sex, tumor dimension, lymph node status, methylation status, tumor histotype and presence of synchronous distant metastases as confounder variables, only the

Table 1. *KEAP1* molecular alterations detected in non-small cell lung cancer (NSCLC)

	Histotype*		D1S865[∫]	DM1[∫]	D19S906[∫]	D1S2840[∫]	Methylation	DA[§]
LC-4	ADK	A466P	nd	nd	nd	NI	yes	yes
LC-5	ADK	D294Y	L	L	L	R	no	yes
LC-13	SCC	V167F	nd	nd	nd	nd	no	-
LC-15	ADK	IVS3-12	R	R	NI	NI	yes	yes
LC-17	ADK	V369A	R	R	R	NI	yes	yes
LC-19	SCC	P322L	nd	nd	nd	nd	yes	yes
LC-23	ADK	L358LfsX392	L	L	L	L	no	yes
LC-37	SCC	-	nd	nd	L	nd	yes	yes
LC-1	ADK	-	nd	NI	R	R	yes	no
LC-6	ADK	-	R	NI	L	L	yes	yes
LC-7	SCC	-	R	R	NI	NI	yes	no
LC-9	SCC	-	nd	NI	NI	R	yes	no
LC-10	ADK	-	nd	R	NI	NI	yes	no
LC-12	SCC	-	NI	R	R	R	yes	no
LC-14	mixed	-	R	nd	nd	R	yes	-
LC-18	SCC	-	nd	R	R	NI	yes	no
LC-22	SCC	-	R	L	R	R	yes	yes
LC-27	SCC	-	R	NI	R	R	yes	no
LC-26	SCC	-	R	R	R	NI	yes	no
LC-29	SCC	-	R	L	L	NI	no	no
LC-34	SCC	-	R	R	R	R	yes	no
LC-38	SCC	-	R	R	L	L	yes	yes
LC-39	ADK	-	NI	R	L	L	no	no
LC-41	SCC	-	L	L	L	L	yes	yes
LC-43	SCC	-	nd	nd	nd	nd	yes	-
LC-46	ADK	-	R	R	R	NI	yes	no
LC-47	ADK	-	nd	R	R	L	yes	no

*ADK, lung adenocarcinomas; SCC, lung squamous carcinomas; mixed, lung adenosquamous carcinomas. [∫]LOH (L) was defined as allelic imbalance ≤ 0.5; NI, not informative; R, retention of heterozygosity; nd, not determined. [§]DA, double abnormalities.

latter was associated with a high risk of disease progression (HR 15.22; 95% CI 1.23–187.7; p = 0.03). No association was found between *KEAP1* alterations in overall survival analysis, although this lack of association might be due to the low number of patients included in the study and the short median follow up time.

Discussion

A growing number of evidences suggest that increased expression of NRF2 due to genetic alteration of the repressor *KEAP1* may play a pivotal role in the pathogenesis of lung cancers. Increased NRF2 expression could facilitate cell growth and survival through the activation of cytoprotective factors.[20,25-27,35] Moreover NRF2 directly regulate the expression of phase II detoxifying gene and drug transporters; thus it is not surprising that chemoresistance to doxorubicin, cisplatin, etoposide, paclitaxel administration has been recently reported in cell lines derived by different tumor types including lung cancer.[8,9,16,17,23,24,26]

We have performed a comprehensive genetic and epigenetic analysis in 47 surgical resected NSCLCs. Aberrant promoter hypermethylation was the most common abnormality identified in 47% of the NSCLCs, mutations were detected in 15% of the cases and LOH was demonstrated in 21% of the tumors. Overall 57% of the NSCLC analyzed showed one genetic or epigenetic abnormalities. Approximately one third of the tumors had two alterations suggesting that both copies of the gene are inactivated and this feature was associated with higher risk of disease progression in univariate analysis.

This is the first report analyzing *KEAP1* methylation status in a large series of lung cancers. Wang et al.[26] found *KEAP1* promoter hypermethylation in three lung cancer cell lines and 5 primary tumors. We used a real time based semiquantitative method to determine methylation status in the promoter region showing the higher density of methylated CpGs.[26,27] Methylation was detected in approximately half of the NSCLCs but in none of the normal lung tissue analyzed confirming that methylation is a cancer specific event. The relationship between promoter hypermethylation and gene expression was previously demonstrated by treating the cell line with 5-deoxy-azacytidine which was indeed able to restore mRNA levels.[26] Interestingly, Solis et al. recently reported reduced or absent KEAP1 protein expression by immunohistochemical analysis in 56% of the NSCLCs. Thus it is tempting to speculate that promoter aberrant hypermethylation may represent a major mechanism for *KEAP1* downregulation.

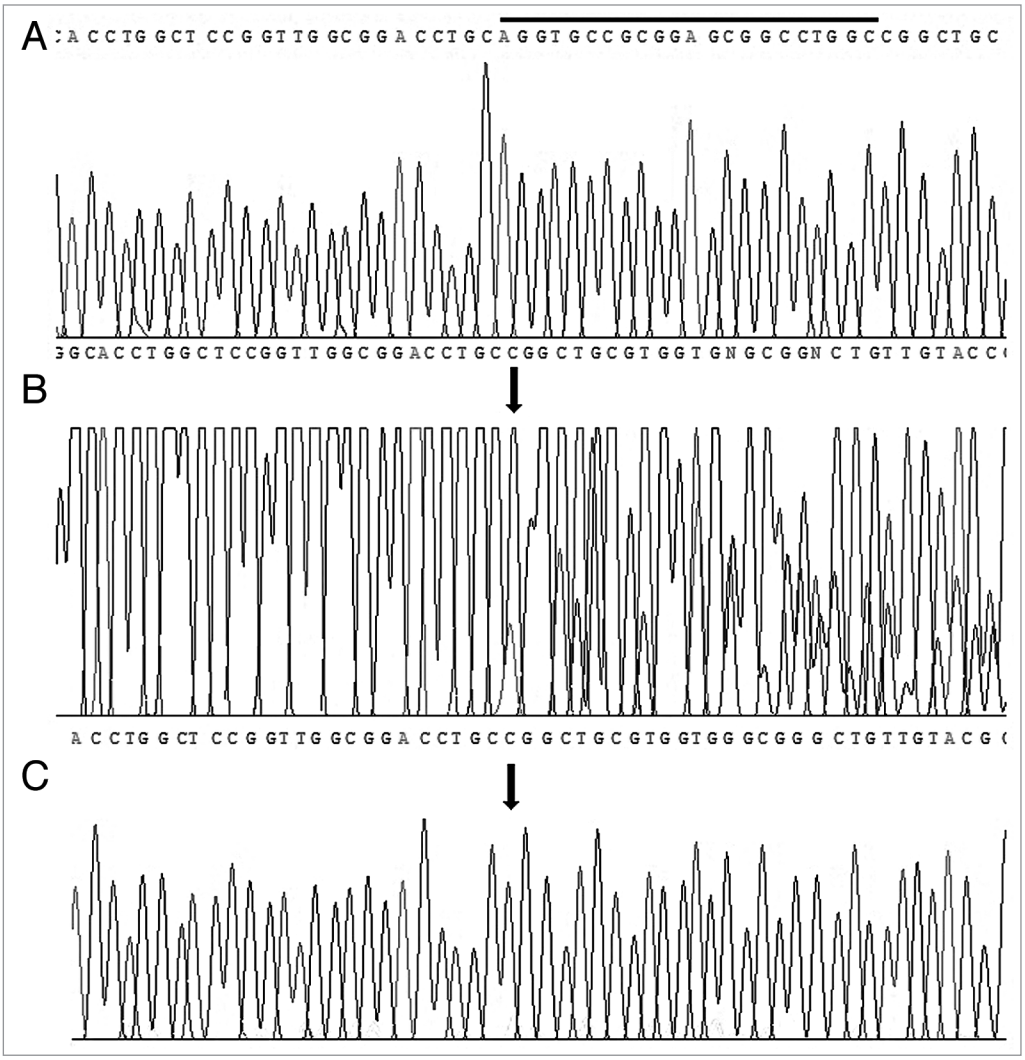

Figure 1. Electropherograms showing the c.1074del22bp mutation (black arrow) identified within *KEAP1* exon 4 in ADK patient LC-23. (A) WT sequences of the exon 4 regions; (B) mutant sequences with the c.1074del22bp in heterozygosis; (C) mutant sequence cloned in pSCA vector. The black line indicates the deleted region; the arrows indicate the position of the first nucleotide deleted in mutant sequences.

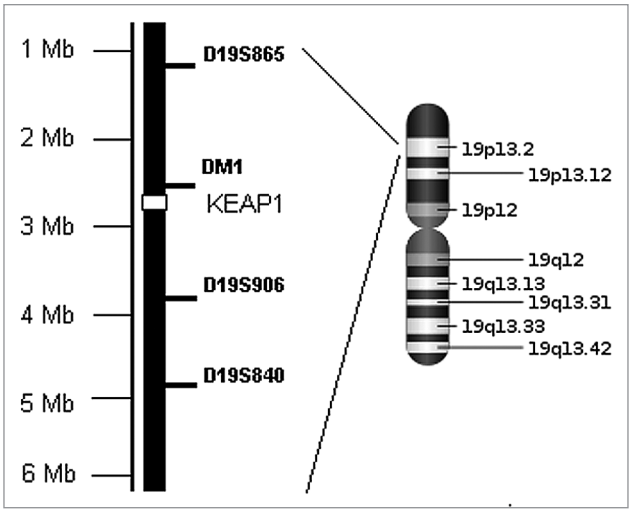

Figure 2. Integrated map of the 19p13.2 region spanning the *KEAP1* gene, showing the ideogram of chromosome 17 and the relative position of microsatellite markers used in the allelic loss study.

KEAP1 mutation in NSCLCs was previously reported in 50% of lung cancer cell lines and in 8–20% of primary lung tumors.[5,19,21] In our series we detected seven mutations in seven different NSCLC; one of those mutations resulted in a truncated protein whereas the other six consist in a single base substitutions (**Table 3**). The KEAP1 protein consists of three major domains: an NH_2-terminal domain Broad complex, Tramtrack and Bric-a-Brac (BTB) domain; a central intervening region (IVR); and a series of six COOH-terminal Kelch repeats. While the BTB and IVR domains are required for the redox-sensitive regulation of keap1, the Kelch repeats domain directly binds the NRF2 protein.[37-41] All the mutations found in this study fall into these different domains (**Fig. 3**). While the effect of truncating mutation on protein function is obvious, for the 5 missense and the intronic variations, we evaluated whether these alterations could interfere with the proper activity of the protein by performing in silico analysis. Either conservation and software prediction analyses suggests that the detected missense and intronic mutations may indeed affect protein functions (**Figs. 3 and 4**).

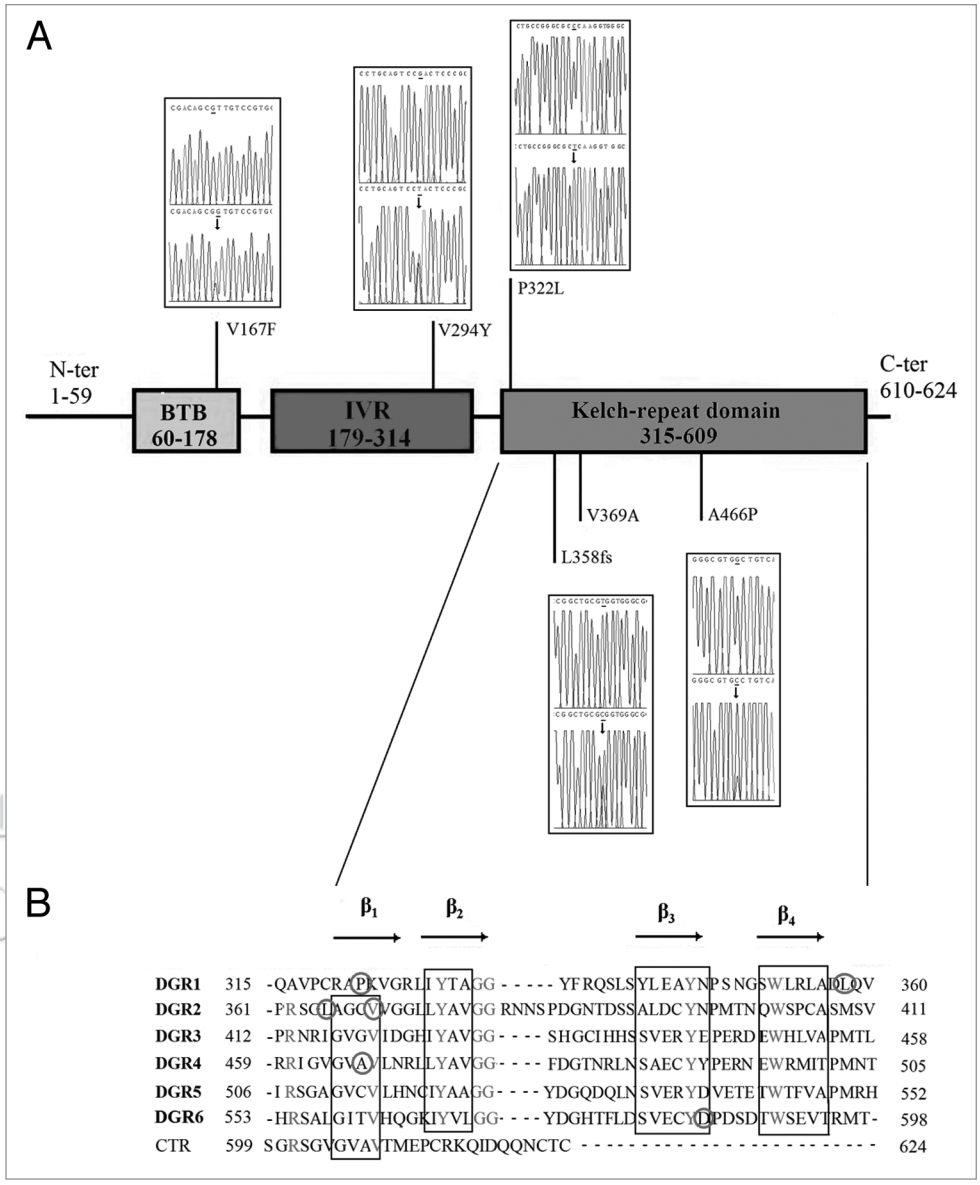

Figure 3. Functional effects of missense mutations and intronic variant. (A) The aminoacidic changes and relative electropherograms showing the sequence variations are reported across the structure of keap1 protein: the three functional BTB, IVR and KELCH-repeat domains are indicated. (B) Sequence conservation of the KEAP1 protein along the philogenetic tree. Fragment of the whole KEAP1 protein sequence (residues 93–468) in Homo is compared with the orthologues in macaca, pan, mus, rattus, tetraodon, danio, xenopus and drosophila. Boxes indicated the mutated residues and the conservation values (scaled 0–10) is indicated by the histograms below the alignments. The imaging editor has been made by the Jalview online software (www.jalview.org). The four *KEAP1* domains and their corresponding functions are shown with different colors. The positions of the mutations are reported. To view color, see online version.

Deregulation of the NRF2/KEAP1 system has been linked to chemoresistance to a variety of anticancer drug. Indeed NRF2 directly regulates the expression of phase II drug metabolism enzymes and phase III drug transporters including the Multidrug Resistance-associated Protein transporters (MRPs).[8] Indeed, several studies on cell lines derived from lung cancer but also neuroblastoma, breast, ovarian and endometrial tumors have reported chemoresistance to doxorubicin, cisplatin, etoposide and paclitaxel.[16,19,23,26] In addition, Solis et al. have recently reported that nuclear NRF2 expression and low or absent KEAP1 expression in patients with NSCLC were associated with poor outcome. In our study, follow up data were available for only a subset of NSCLC cases (n = 35), nevertheless, patients bearing two genetic and epigenetic abnormalities showed a three time higher risk to progress. These evidences further suggest that *KEAP1* inactivation plays a pivotal role in NSCLC development and progression.

A main reason of the high mortality due to lung cancer is that in the majority of the cases, NSCLC is diagnosed already when it is at an advanced stage.[42] It has been estimated that early detection could potentially increase survival rates by 10- to 50-fold.[42] Current screening techniques include chest X-ray and spiral computed tomography, sputum cytology but they have not been

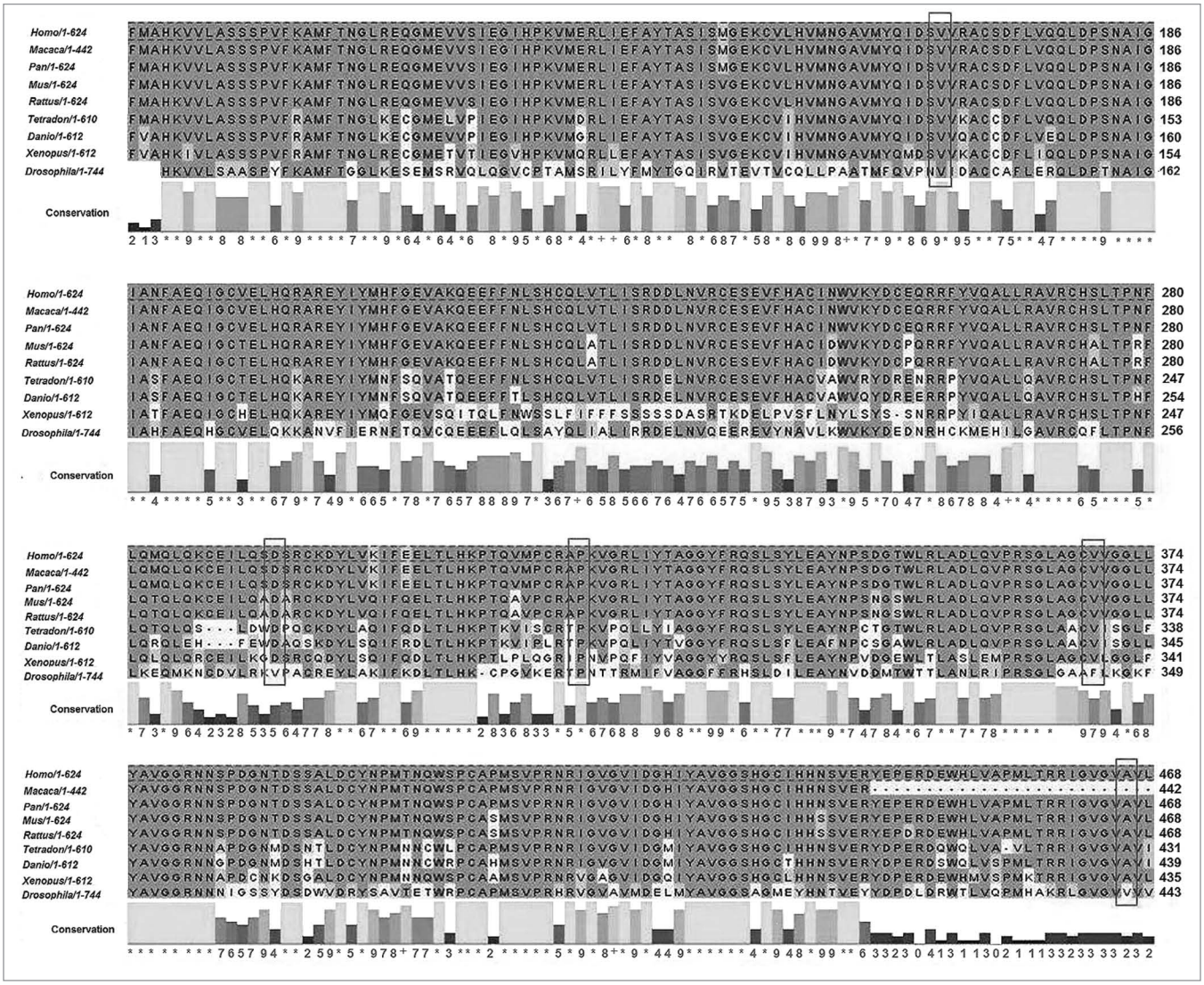

Figure 4. Alignment of the six DGR repeats and the CTR domain. Boxes indicated the four beta sheets structures; arrows indicate conserved amino-acid residues shown in red, and the mutations reported in the text are indicated by the circle.[18] To view color, see online version.

yet proven effective in increasing patient survival.[43,44] The possibility to detect cancer cells by analyzing genetic and epigenetic abnormalities in lung cancer patients has been demonstrated in various bodily fluids including plasma, sputum and bronchoalveloar lavage DNA of lung cancer patients.[45-49] In particular promoter hypermethylation can be detected with a very high degree of specificity even in the presence of an excess of unmethylated DNA. Thus the high frequency and cancer specificity of *KEAP1* epigenetic alterations detected in our study also suggest that the determination of *KEAP1* methylation status may represent a novel cancer-specific marker able to augment our ability to detect NSCLCs in early stages.

In conclusion, we show that genetic and epigenetic abnormalities of *KEAP1* are frequent events in NSCLC, thus suggesting that deregulation of the NRF2/KEAP1 system could play a pivotal role in the cancerogenesis of NSCLC. Identifying patients with *KEAP1* genetic and epigenetic abnormalities may be important for the selection of chemotherapy treatment. In addition, *KEAP1* alterations may be used to detect cancer cells in cytological specimen, sputum or bronco alveolar lavage (BAL) providing a potential powerful tool for early detection.

Materials and Methods

Patients and samples. We analyzed non-small cell lung cancer tissues obtained as fresh frozen specimens from 47 patients surgically treated at Thoracic Surgery Department, IRCCS Casa Sollievo della Sofferenza, San Giovanni Rotondo. As control, 12 histologically confirmed normal lung tissues distant from tumor were analyzed. Peripheral blood leukocyte (PBL) samples were collected for each of the surgically treated patients. All human materials used in the study were collected according to the guidelines of the Local Ethical Committee. Prior written and informed consent was obtained from each patient accordingly

with Institutional guidelines. Patient's clinicopathological characteristics are shown in **Table 2**.

DNA extraction. Sections, 5-μm-thick, were cut from OCT embedded fresh tissues to ensure that tumor samples contained at least 70% cancer cells. Tumor specimens were then carefully dissected, under a microscope from six to ten 12 μm-thick sections to enrich for areas that contained tumor cells. DNA was subsequently extracted from frozen fresh tissues and paraffin-embedded specimen as previously described. DNA from peripheral blood leukocyte (PBL) samples was extracted using the standard phenol-chloroform procedure.[35]

Quantitative methylation specific PCR analysis (qMS-PCR). DNA extracted from tumor samples was subjected to bisulphite treatment and DNA purification using the Epitect Bisulphite kit (Qiagen Sci, MD) according to manufacturer's instruction. Bisulphite-modified DNA was used as template for quantitative Methylation Specific PCR (qMS-PCR). Primer/probe sets for the *KEAP1* promoter region and for the unmethylated promoter region of the *ACTB* as reference gene were previously described in reference 27. Calibration curves for both target and reference genes were construed using serial dilutions (90-0.009 ng) of a commercially available fully methylated DNA (CpGenome Universal Methylated DNA, Millipore, Chemicon, cat#S7821). Amplification reactions were carried out in triplicate in a volume of 10 μL that contained 50 ng bisulphite-modified DNA, 600 nM forward and reverse primers, 200 nM probe, 0.6 U of Platinum Taq polymerase (Invitrogen, Inc., Rockville, MD), 200 μM each of dATP, dCTP, dGTP, dTTP and 2 μl of PCR buffer.[24] PCR conditions were as follows: one step at 95°C for 3 min, 50 cycles at 95°C for 15 seconds and 60°C to 62°C for 1 min. PCR reactions were performed in 384-well plates on a ABI PRISM 7900 Sequence detection system (Applied Biosystems, Carlsband CA) and were analyzed by SDS 2.1.1 software (Applied Biosystems, Foster City, CA). Each plate included calibration curves for the *ACTB* and *KEAP1* genes, patient DNA samples, positive (CpGenome™ Universal Methylated DNA, Millipore, Chemicon, cat#S7821) and negative (Universal Unmethylated DNA, Millipore, Chemicon, cat#S7821) controls and multiple water blanks. Methylation levels were calculated as the ratio of *KEAP1* to *ACTB* and then multiplied by 1,000 for easier tabulation (average value of triplicates of gene of interest/average value of triplicates of *ACTB* x 1,000).

Mutation analysis. DNA obtained from the 47 NSCLC tissues was analyzed by fluorescence based direct sequencing for the entire *KEAP1* gene coding region, including exon-intron boundaries (**Sup. Table 1**). For each mutated case paired normal tissues was also analyzed to exclude that the mutation occurred in the germline.

Amplification reactions were carried out in a final reaction volume of 25 μl containing 100 ng of genomic DNA template, 0.25 nM dNTPs, 20 pmol of each primers, 1 U HotMaster Taq polymerase (Eppendorf AG, Hamburg, Germany), in 1x PCR Reaction Buffer. PCR cycling conditions consisted of an initial denaturation step at 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, annealing for 30 sec, extension at 72°C for 30 sec and ending with a final elongation step at 72°C for 7 min.

Table 2. Patient's clinicopathological characteristics

Median Age (IQR)	66 (59–72)	
Gender		
Male	32	91%
Female	3	9%
Smoke habit		
No	1	3%
Yes	29	83%
Unknown	5	14%
Histotype		
Adenocarcinomas	13	37%
Squamous Cell Carcinomas	19	54%
Mixed Type	3	9%
Tumor dimension		
T1	9	26%
T2	15	43%
T3	6	17%
T4	5	14%
Lymph node status		
N0	24	69%
N1	10	29%
Nx	1	3%
Disease Progression		
no	19	54%
yes	15	43%
unknown	1	3%
Status		
alive	21	60%
dead	14	40%
Median months Follow up (IQR)	33 (20–44)	

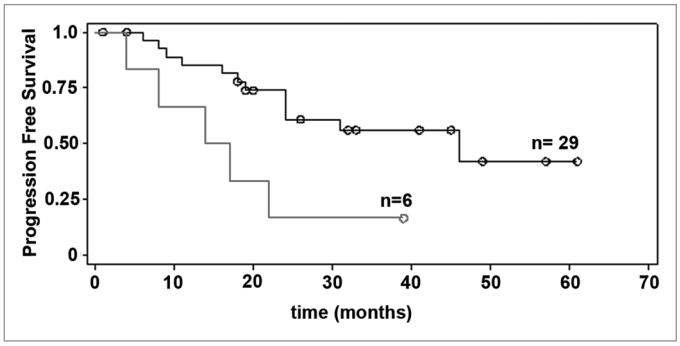

Figure 5. Kaplan-Meier curve showing the association between *KEAP1* double abnormalities and worst progression free survival (PFS) p = 0.01 Long rank Test.

PCR products were purified using GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK) and sequenced. Sequencing reactions were performed in 10 μl of final volume using 3 pmol of primer, 4–6 ng of DNA template and 1 μl of Big Dye Terminator Ready Reaction mix v.

Table 3. Changes in Keap1 protein arising from somatic mutations[a,b]

	Original amino acid	Effect on coding	Reference	Position and comment
Cell line Q293	Cys23	Tyr	Nioi et al.[22]	N-terminal domain
Patient PT-29	Val167	Phe	Singh et al.[24]	BTB
Patient LC-13	Val167	Phe	This study	BTB
BTC patient	Pro181	Frameshift	Shibata et al.[21]	IVR
Patient P9	His200	Pro	Ohta et al.[20]	IVR
Cell line H460	Asp236	His	Singh et al.[24]	IVR
Patient PT-18	Leu237	Frameshift	Singh et al.[24]	IVR
BTC patient	Cys249	Tyr	Shibata et al.[21]	IVR
Patient PF-3	Ile264	Val	Singh et al.[24]	IVR
Cell line H2126	Arg272	Cys	Ohta et al.[20]	IVR, adjacent to Cys273
Patient PT-23	Gln284	Leu	Singh et al. [24]	IVR
Patient PT-35	Gln284	Leu	Singh et al. [24]	IVR
Patient LC-5	Asp294	Tyr	This study	IVR
Patient LC-19	Pro322	Leu	This study	KR1, within b1
BTC patient	Gly332	Frameshift	Shibata et al.[21]	KR1, after b2-within GG motif
Pt.1	Gly332	Cys	Takahashi et al.[52]	KR1, after b2-within GG motif
Cell line A549	Gly333	Cys	Singh et al. [24]	KR1, after b2-within GG motif
BTC patient	Ser338	Leu	Shibata et al. [21]	KR1, before b3
Patient PF-4	Ser348	Frameshift	Singh et al. [24]	KR1, between b3 and b4
Cell line H1395	Gly350	Ser	Singh et al. [24]	KR1, before b4
Cell line H1993	Gly350	Ser	Singh et al. [24]	KR1, before b4
Cell line H1648	Gly364	Cys	Padmanabhan et al.[18]	KR2, before b1
Cell line H1184	Gly364	Cys	Padmanabhan et al. [18]	KR2, before b1
Patient LC-23	Leu365	Frameshift	This study	KR2, before b1
Patient LC-17	Val369	Ala	This study	KR2, within b1, conserved
BTC patient	Gly379	Asp	Shibata et al. [21]	KR2, after b2-within GG motif
Cell line H1435	Arg413	Leu	Singh et al. [24]	KR3, before b1, conserved
Patient PT-17	Arg413	Frameshift	Singh et al. [24]	KR3, before b1, conserved
Patient P23	Arg415	Gly	Ohta et al. [20]	KR3, before b1
Patient P1	Ala427	Val	Ohta et al. [20]	KR3, within b2
Patient P12	Gly430	Cys	Otha et al. [20]	KR3, after b2-within GG motif
Cell line H838	Tyr443	Frameshift	Singh et al. [24]	KR3, within b3
Patient PT-31	Leu457	Frameshift	Singh et al. [24]	KR3, after b4
Patient LC-4	Ala466	Pro	This study	KR4, within b1
Patient P65	Gly476	Arg	Ohta et al. [20]	KR4, after b2-within GG motif
Pt.4	Arg483	His	Takahashi et al.[52]	KR4, between b2 and b3
Pt.3	Glu493	Gln	Takahashi et al.[52]	KR4, between b3 and b4
BTC patient	Trp544	stop codon	Shibata et al.[21]	KR5, within b4
Lung cancer patient	Tyr537	stop codon	Solis et al.[36]	KR5, within b3, conserved
Patient PF-8	Ser555	Deletion (to Thr560)	Singh et al. [24]	KR6, before b1
Patient PF-9	Ser555	Deletion (to Thr560)	Singh et al. [24]	KR6, before b1
Pt.2	Gly570	Val	Takahashi et al.[52]	KR6, after b2-within GG motif

[a]Human keap1 comprises 624 amino acids. This Table lists residues that are altered in tumor samples and cell lines compiled from Reference 18, 20–22 and 24. [b]Abbreviations: BTC, biliary tract cancer; LC, lunc cancer; KR, Kelch-repeat; b1, first b-strand within b-sheet; b2, second b-strand within b-sheet; b3, third b-strand within b-sheet; b4, fourth b strand within b-sheet.

1.1 (Applied Biosystems, Foster City, CA). Sequencing reactions were loaded on an ABI 3100 sequence detection system (Applied Biosystems) and analyzed using the Sequencing Analysis software v.3.7 (Applied Biosystems).

Molecular cloning of mutations. PCR products from mutated samples were cloned into StrataClone™ PCR Cloning Vector pSC-A (Stratagene, Milan, Italy). Ten clones from each sample were sequenced and mutations were confirmed in at least two of the ten clones analysed. Amplifications were performed directly from crude lysates of 10 single bacterial colonies by means of a Gene Amp PCR System 9700 (Perkin Elmer, Foster City, CA) in a final reaction volume of 25 μl containing clone vector template, 0.25 nM dNTPs, 20 pmol of each primers, 1 U HotMaster Taq polymerase (Eppendorf), in 1x PCR Reaction Buffer. PCR cycling conditions were the same as indicated above.

Loss of heterozygosity analysis. Fluorescence based loss of heterozygosity (LOH) analysis was performed using genomic DNA from paired normal and tumor tissues for four microsatellite markers flanking the *KEAP1* gene: D19S865, DM1,[19] D19S906, D19S840 (**Sup. Table 2**). PCR was performed in a 50 μL reaction volume containing 5 μL 10x PCR Buffer (Eppendorf), 0.25 nM dNTPs, 20 pmol each primer, 1 U HotMaster Taq (Eppendorf) and 100 ng of DNA. Amplification reactions were performed using the LMS2 (ABI Prism™ Linkage Mapping Set version 2) program (GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA) for all markers. Microsatellite analysis was performed on ABI 3100 sequence detection system (Applied Biosystems, Foster City, CA), using ABI Genescan and Genotyper Software 3.7 (Applied Biosystems, Foster City, CA). LOH or allelic imbalance (AI) values were calculated using the following formula: (peak 1 height/peak2 height in tumor DNA)/(peak 1 height/peak 2 height in normal DNA) using a value of 0.5 to define LOH/AI.[51] LOH/AI was confirmed on each positive sample at least twice for each marker.

Statistical methods. Patients baseline characteristics were reported as median and interquartile range (IQR) or frequencies and percentages for continuous and categorical variables, respectively. Baseline comparisons were made using a chi-square test for categorical variables and the Mann-Whitney U-test for continuous variables.

Time-to-event analyses were performed using the Kaplan-Meier method and significance was assessed with a log-rank test. Furthermore, univariate and multivariate Cox proportional hazards regression models were estimated. Risks were reported as hazards ratios (HR) along with their 95% CI. Time to progression was defined as the time between surgery and the first progression event. Overall Survival Time was defined as the time between surgery and death.

A p value < 0.05 was considered for statistical significance. All analyses were performed using SAS Release 9.1 (SAS Institute, Cary, NC).

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Note

Supplemental materials can be found at:
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