Int. J. Cancer: **119,** 2546–2556 (2006)
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Downregulation of the KIP family members $p27^{KIP1}$ and $p57^{KIP2}$ by SKP2 and the role of methylation in $p57^{KIP2}$ inactivation in nonsmall cell lung cancer

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Knowing the status of molecules involved in cell cycle control in cancer is vital for therapeutic approaches aiming at their restoration. The $p27^{KIP1}$ and $p57^{KIP2}$ cyclin-dependent kinase inhibitors are nodal factors controlling normal cell cycle. Their expression in normal lung raises the question whether they have a mutual exclusive or redundant role in nonsmall cell lung cancer (NSCLC). A comparative comprehensive analysis was performed in a series of 70 NSCLCs. The majority of cases showed significantly reduced expression of both members compared to normal counterparts. Low KIP protein levels correlated with increased proliferation, which seems to be histological subtype preponderant. At mechanistic level, degradation by SKP2 was demonstrated, *in vivo* and *in vitro*, by siRNA-methodology, to be the most important downregulating mechanism of both KIPs in NSCLC. Decreased $p57^{KIP2}$-transcription complements the above procedure in lowering $p57^{KIP2}$-protein levels. Methylation was the main cause of decreased $p57^{KIP2}$-mRNA levels. Allelic loss and imprinting from *LIT1* mRNA contribute also to decreased $p57^{KIP2}$ transcription. *In vitro* recapitulation of the *in vivo* findings, in A549 lung cells ($INK4A\text{-}B^{(-/-)}$), suggested that inhibition of the SKP2-degradation mechanism restores $p27^{KIP1}$ and $p57^{KIP2}$ expression. Double siRNA treatments demonstrated that each KIP is independently capable of restraining cell growth. An additional demethylation step is required for complete reconstitution of $p57^{KIP2}$ expression in NSCLC.
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Key words: $p57^{KIP2}$; $p27^{KIP1}$; CDKN1C; lung tumors; SKP2; methylation; DMR1-LIT1

The information regarding the status of molecules involved in cell cycle control in various types of cancer is vital in view of the development of individual-based therapeutic approaches aiming at their restoration. The suppressive effect of cyclin-dependent kinase inhibitors (CDKIs) on CDK complexes is among the mechanisms that control normal cell cycle.[1]

The KIP family of CDKIs includes $p27^{KIP1}$ and $p57^{KIP2}$, encoded by *CDKN1B* and *CDKN1C* loci, respectively, with $p57^{KIP2}$ being the least studied one. Their role in cell cycle control, differentiation and growth has been shown in animal models.[2–5] In certain normal tissues only one member is expressed, while in others, including lung, expression of both is required.[6]

Low $p27^{KIP1}$ levels in several types of cancers, including lung carcinomas, are associated with increased tumor growth.[7,8] Reduced $p57^{KIP2}$ expression in certain tumors[9–12] is inversely associated with cellular proliferation, suggesting an oncosuppressor activity, whereas in others $p57^{KIP2}$ expression is positively correlated with proliferation indicating an opposite function.[13] In childhood tumors, contradictory $p57^{KIP2}$ expression results have also been reported.[14–16] In lung tumors, to the authors' awareness, there are no data on $p57^{KIP2}$ protein status.

Recent evidence suggest that KIP intracellular levels are actively regulated by S-phase kinase-associated protein 2 (SKP2), an F-box protein required for SCF ubiquitin-mediated degradation.[17,18] In nonsmall cell lung cancer (NSCLC), overexpression of SKP2 has been documented,[19–21] without being associated with both KIP family members.

KIP mutations are rare in tumors.[22,23] While $p27^{KIP1}$ is predominantly downregulated by posttranslational mechanisms,[5] reduced $p57^{KIP2}$ expression may also result from loss of heterozygosity (LOH), increased promoter methylation and epigenetic downregulation from the *DMR1* locus.[22,24–28] In mammals, $p57^{KIP2}$ is expressed mainly from the maternal allele, due to imprinting of the paternal one,[29] whereas maternal *DMR1* is epigenetically silenced and expression of the other allele leads to epigenetical downregulation of various paternal genes, including *CDKN1C*.[30,31] In cell lines deranged epigenetic modifications at *DMR1* promoter are associated with *CDKN1C* silencing.[32] In lung cancer only one report shows aberrant methylation of $p57^{KIP2}$ promoter[33] and another demonstrates selective maternal allele loss,[22] whereas there is no information dealing with *DMR1-CDKN1C* relationship.

The expression of both KIP homologues in normal lung tissue[6] raises the question whether they possess a mutual exclusive or redundant role in this cancer. This information is vital in view of development of drugs mimicking their function.[34] Furthermore, the mechanisms governing their expression may provide new targets for therapeutic intervention.[35] To this end, we performed a comparative study of these molecules in NSCLCs focusing on their expression status, mechanisms involved in their deregulation and impact on tumor kinetics. On the basis of the results, we recapitulated *in vitro*, the *in vivo* findings, and examined the significance of their reconstitution on tumor growth.

Material and methods

Tissue samples

Frozen and formalin-fixed, paraffin-embedded material from 70 primary NSCLC tumors and corresponding normal lung tissue were analyzed (Table I). Of the 70 employed cases, 10 patients have been previously examined for $p27^{KIP1}$ expression status, proliferation and apoptotic indexes.[7] Patients received no chemo-, radio- or immunotherapy prior to surgery. The study was performed following criteria of the revised (1983) Helsinki Declaration of 1975.

Immunohistochemistry and indirect immunofluorescence

Antibodies. The C-20 anti-$p57^{KIP2}$ (Santa Cruz, BioAnalytica, Athens, Greece), SX53G8 anti-$p27^{KIP1}$ (Dako, Kalifronas, Athens, Greece), 1G12E9 anti-SKP2 (Zymed, AntiSel, Athens, Greece), 4D11D8 anti-JAB1 (Zymed, AntiSel, Athens, Greece) and sc-7269 anti-Ki-67 (Dako, Kalifronas, Athens, Greece) antibodies

This article contains supplementary material available via the Internet at http://www.interscience.wiley.com/jpages/0020-7136/suppmat.

Grant sponsor: The European Social Fund and National Resources-(EPEAEK-II), PYTHAGORAS II; Grant number: 7953; Grant sponsor: The SARG, University of Athens; Grant numbers: 73/17/03, 70/4/4792; Grant sponsor: The Roy Castle Lung Cancer Foundation, Liverpool, UK.

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Received 25 January 2006; Accepted 8 June 2006
DOI 10.1002/ijc.22214
Published online 20 September 2006 in Wiley InterScience (www.interscience.wiley.com).

TABLE I – SUMMARY OF CLINICOPATHOLOGICAL AND *IN SITU* DATA OF THE PATIENTS

Number of patients	Sex		Smoking[1]		Histology subtype NSCLCs	Stage[2]			$p57^{KIP2}$ (IHC)	$p27^{KIP1}$ (IHC)	PI (Ki-67) (IHC)	AI (TUNEL)
	M	F	Y	N		I	II	III	MV (%) ± st. dev (range)	MV (%) ± st. dev (range)	MV (%) ± st. dev (range)	MV (%) ± st. dev (range)
39	37	2	35	2	SqCs	22	12	4	24.37 ± 22.09 (0–70)	28.04 ± 21.31 (0–85)	34.23 ± 13.32 (8–60)	1.88 ± 1.34 (0.2–6.0)
30	23	7	26	3	AdCs	18	10	2	36.58 ± 30.69 (5–90)	30.21 ± 17.91 (0–60)	31.74 ± 17.05 (3–65)	1.53 ± 0.67 (0.5–2.6)
1	1		1		LcCs				10	23	20	NA

NSCLC, nonsmall cell lung carcinoma; SqC, squamous carcinoma; AdC, adenocarcinoma; LcC, large cell carcinoma; MV, medium value; St. dev, standard deviation; NA, nonavailable; IHC, immunohistochemistry; PI, proliferation index; AI, apoptotic index.
[1]Nonavailable data in 3 cases.–[2]Nonavailable data in 2 cases.

were used for IHC analysis and the Ab-6 anti-$p57^{KIP2}$ (57P06) (NeoMarkers, BioAnalytica, Athens, Greece) for IF analysis.

Method. IHC was performed by an indirect streptavidin–biotin–peroxidase method, as previously described.[7,36] $p57^{KIP2}$ staining was improved by addition of Triton ×100 (Merck, Athens, Greece) (0.3%) during incubation with the primary antibody. IFC was conducted following previously published protocol.[36]

Controls. Kidney tissues sections were employed as positive controls for $p57^{KIP2}$ IHC and IF analysis, following the manufacturer's instructions. For SKP2 and JAB1 we used previously described positive controls.[21,37,38]

Evaluation. For each antigen, the nuclear IHC signal was scored, respectively. Cytoplasmic $p57^{KIP2}$ staining was also considered, as previously reported.[13] Evaluation of $p27^{KIP1}$ and $p57^{KIP2}$ staining was performed using previously set criteria for oncosuppressor genes,[39] while for SKP2 and JAB1 we employed previously published criteria.[20,21,37] For each antigen, respectively, we considered as labeling index (LI) the percent average of nuclear positive cells from 5 high power fields-HPFs (×400). Interobserver variability was minimal ($p < 0.001$).

Tdt-mediated dUTP nick end labeling assay

Tdt-mediated dUTP nick end labeling assay (TUNEL) assay was performed as described elsewhere.[36]

RNA extraction and cDNA preparation

RNA extraction from cancerous material with 90% tumor cells content and cDNA preparation have been previously published.[36,40]

Comparative reverse-transcription (RT)-PCR and real-time (RT)-PCR

Evaluation of *$p57^{KIP2}$* and *LIT1* mRNA status was performed by a semiquantitative RT-PCR method previously described.[36] Two reference genes, *GAPDH* and *Porphobilinogen Deaminase* (*PBGD*), were used to validate the results. Samples scored increased or decreased for *$p57^{KIP2}$* and *LIT1* mRNA expression, where also confirmed by real-time PCR (MJ-Research DNA-Engine-Opticon) and SYBR Green fluorescence (Platinum SYBR Green qPCR, Invitrogen, AntiSel, Athens, Greece). Primers and annealing temperatures are given in Table II. Tumor samples scoring $\geq \pm 2$-fold difference from the corresponding normal counterpart were characterized as increased or decreased, respectively.

Total protein extraction, cell fractionation and Western blot analysis

Total protein extraction. Total protein extracts were obtained, as previously described.[36]

Nuclear and cytoplasmic extracts. Subcellular fractions consisting of nuclear and cytoplasmic protein extracts were obtained from matched frozen normal tumor tissues following a previously published protocol.[36]

Antibodies and controls. Primary antibodies employed were same as per IHC. Extracts from K562 and normal bronchial epithelial (immortalized nontumorigenic) cell lines were used as positive controls and from HL60 as negative ones for $p57^{KIP2}$ analysis.[24,41] For SKP2 validation extracts from MCF7 and A549 cell lines were used as positive controls.[20,38]

Gel electrophoresis, blotting and evaluation. SDS-PAGE analysis and results evaluation have been previously reported.[36,40]

DNA extraction and LOH analysis

Tissue microdissection, DNA extraction and PCR reactions. These were performed according to protocols previously described.[36,43] The primers and thermal profiles used are given in Table II.

LOH analysis. (*i*) *Conventional LOH Analysis*: PCR products were electrophoretically separated on 10% polyacrylamide gels and stained with silver nitrate as previously reported.[43] (*ii*) *Capil-*

TABLE II – PRIMERS AND ANNEALING TEMPERATURES USED

Locus	Primers (application)	Sequence	Ann. temp. (°C)	Ref.
p57^{KIP2}				
	(Methylation specific PCR)			41
	MSP-U:	5′-TTT GTT TTG TGG TTG TTA ATT AGT TGT-3′	56	
	MSP-M:	5′-CGC GGT CGT TAA TTA GTC GC-3′	63	
	MSP-A:	5′-ACA CAA CGC ACT TAA CCT ATA A-3′	56/63	
	(Pyrosequencing)			
	Fw:	5′-biotin-AAA GAG TGG AGT TGA TT-3′	44	
	Rv:	5′-ACC TAC TAC TAC TAA ACT AAT ATC-3′		
	(Pyrosequencing primer)			
	Seq:	5′-TAC TAA ACT AAT ATC CCT T-3′		
	(LOH PAPA region)			42
	Fw:	5′-CCG GAG CAG CTG CCT AGT G-3′	72	
	Rv:	5′-AAT CCC CGA GTG CAG CTG-3′		
	([real time] RT-PCR)			
	Fw:	5′-CGG CGA TCA AGA AGC TGT CC-3′	59	
	Rv:	5′-CGG GGC TCT TTG GGC TCT-3′		
D11S4088	(LOH)			
	Fw:	5′-GGG CAG AGG CAG TGG AG-3′	54	
	Rv:	5′-GCA TGT TTC GGG GGT G-3′		
LIT1	([real time] RT-PCR)			32
	Fw:	5′-AAG AAA GTG TTG AGT GGT AA-3′	54	
	Rv:	5′-GAT GAT CTG AAA ATG GAA AA-3′		
SKP2	(RT-PCR)			38
	Fw:	5′-TCA ACT ACC TCC AAC ACC TAT CAC-3′	57	
	Rv:	5′-GAC AAC TGG GCT TTT GCA GT-3′		
GAPDH	(RT-PCR)			
	Fw:	5′-CAT CTC TGC CCC CTC TGC TG-3′	54–59	
	Rv:	5′-CGA CGC CTG CTT CAC CAC CT-3′		
PBGD	(RT-PCR)			
	Fw:	5′-TGA ACG GCG GAA GAA AAC A-3′	54	
	Rv:	5′-GCA GAT GGC TCC GAT GGT G-3′		

FW, forward; Rv, reverse; Seq, sequencing; PBGD, porphobilinogen deaminase; LOH, loss of heterozygosity; RT-PCR, reverse transcription-polymerase chain reaction; Ann. temp, Annealing Temperature.

lary Electrophoresis: Heterozygous samples were subsequently analyzed by capillary chip electrophoresis on a 2100 Bioanalyser (Agilent).

Evaluation. LOH estimation was based on previously set criteria.[40]

Methylation analysis

Sodium bisulfite treatment. Sodium bisulfite treatment of DNA was performed with EZ DNA Methylation Kit (Zymo Research, AntiSel, Athens, Greece), as per the manufacturer's protocol.

Methylation-specific PCR. Bisulfite treated DNA was amplified by methylation-specific PCR (MSP) as previously described.[41] The sequence of primers and the annealing temperatures are shown in Table II. HL60 and K562 cell lines were used as positive and negative controls, respectively.[24,41] All results were confirmed twice.

Pyrosequencing analysis. To assess semiquantitative changes in the degree of methylation index (MtI) between normal and tumor counterparts, we employed pyrosequencing analysis of the sodium bisulfite treated DNA of corresponding tissues. The assay was designed to interrogate cytosines at 4 CpGs (positions −714, −712, −710 and −701 relative to the transcription start site) within *CDKN1C* promoter. The percent (%) average of methylated cytosines over all 4 CpG positions was assigned as MtI. Primers for pyrosequencing analysis (Table II) were designed as close as possible to the region of *CDKN1C* promoter that affects transcription in relation to its methylation status.[24]

Briefly, the biotinylated PCR product was bound to streptavidin sepharose beads (Amersham Biosciences), washed in 70% ethanol, denatured in 0.2 M NaOH and washed in neutralisation buffer (Biotage). Beads were released into the plate wells containing 0.33 mM sequencing primer (Table II) in annealing buffer

FIGURE 1 – KIP protein expression in normal and tumor lung tissue. (*a*) and (*b*) Strong positive p57^{KIP2} immunostaining in normal bronchial epithelium (arrows) next to decreased (*a*i, ×400) and absent (*b*i, ×400) p57^{KIP2} expression in cancerous nests. Nuclear p57^{KIP2} immunopositivity in adenocarcinoma (*a*ii, ×400) and squamous cell carcinoma (*b*ii, ×200). Cytoplasmic p57^{KIP2} immunoreactivity in adenocarcinoma (*a*iii, ×630) and squamous cell carcinoma (*b*iii, ×630). Indirect streptavidin–biotin–peroxidase DAB immunohistochemistry on paraffin sections counterstained with haematoxylin. (Black arrowheads depict strong p57^{KIP2} immunopositivity in cancerous regions). (*c*) Cytoplasmic p57^{KIP2} immunoreactivity in a representative case assessed by immunofluorescence, confirms IHC results (see *a*iii). (*d*) Histogram summarizing the findings from KIP Western blot protein expression analysis in the examined NSCLCs. (*e*) Representative Western blot analysis of total protein extracts for p57^{KIP2} and p27^{KIP1} in matched. Normal (N)/Tumor (T) samples (case 36: SqC, case 22: AdC). In the majority of the samples, p57^{KIP2} and p27^{KIP1} protein levels are decreased in tumor *versus* normal tissue. (*f*) Representative Western blot analysis of p57^{KIP2} protein in nuclear and cytoplasmic subcellular compartments of matched Normal (N)/Tumor (T) samples (case 26). p57^{KIP2} is detected both in the cytoplasm and the nucleus of normal and tumor cells.

FIGURE 2 – Relationship between KIP, Ki-67 and SKP2 expression. p27^{KIP1}, p57^{KIP2}, Ki-67 and SKP2 immunoreactivity on serial sections in a representative SqC case (*a*, ×400 and *b*, ×200). p27^{KIP1} is expressed at the inner sites of tumor nests in contrast to SKP2 which is mainly immunodetected in the external regions (dotted rectangular area). p57^{KIP2} immunopositivity reveals a diffused pattern in tumor regions. Inverse expression between p27^{KIP1} and Ki-67 is also observed in serial cancerous areas. Indirect streptavidin–biotin–peroxidase DAB immunohistochemistry on paraffin sections counterstained with haematoxylin. (*c*) Scatterplots depicting the inverse relationship between p57^{KIP2} IHC expression (LI) and the proliferation index (PI) in the examined NSCLCs (*c*i) and AdCs, where the strongest association was found (*c*ii).

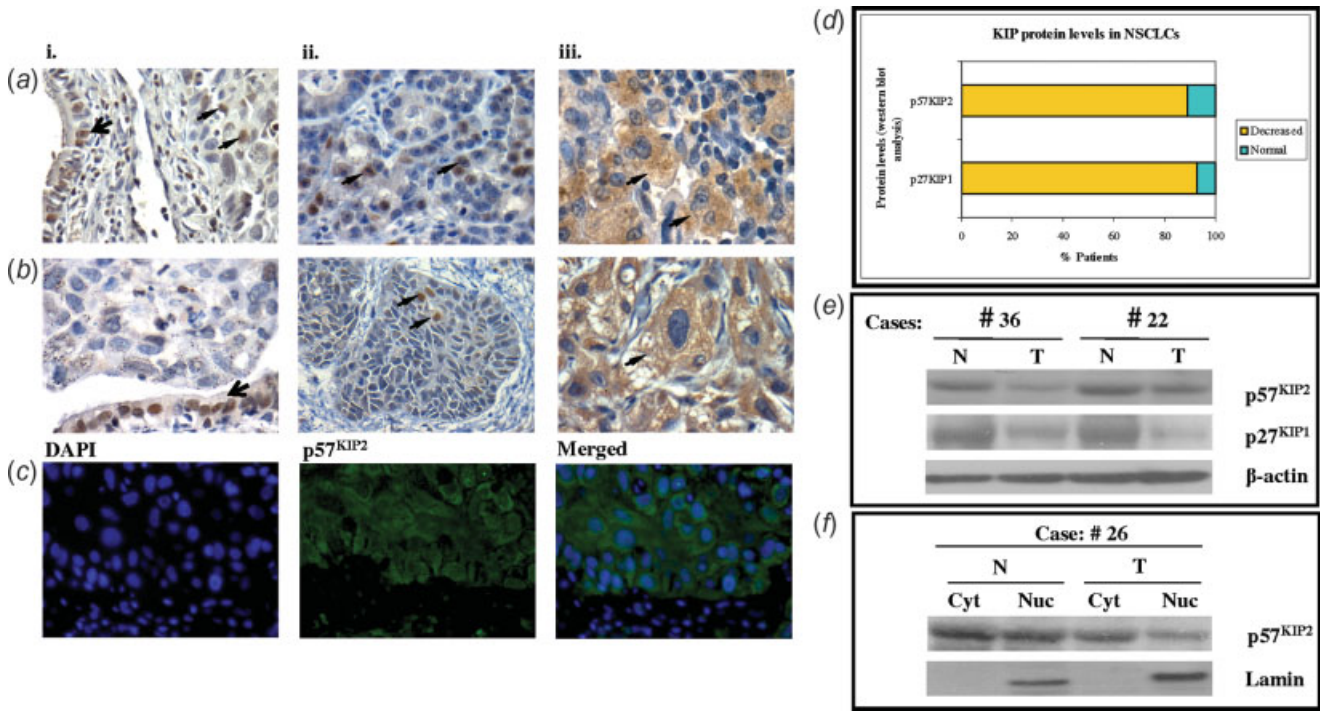

FIGURE 1.

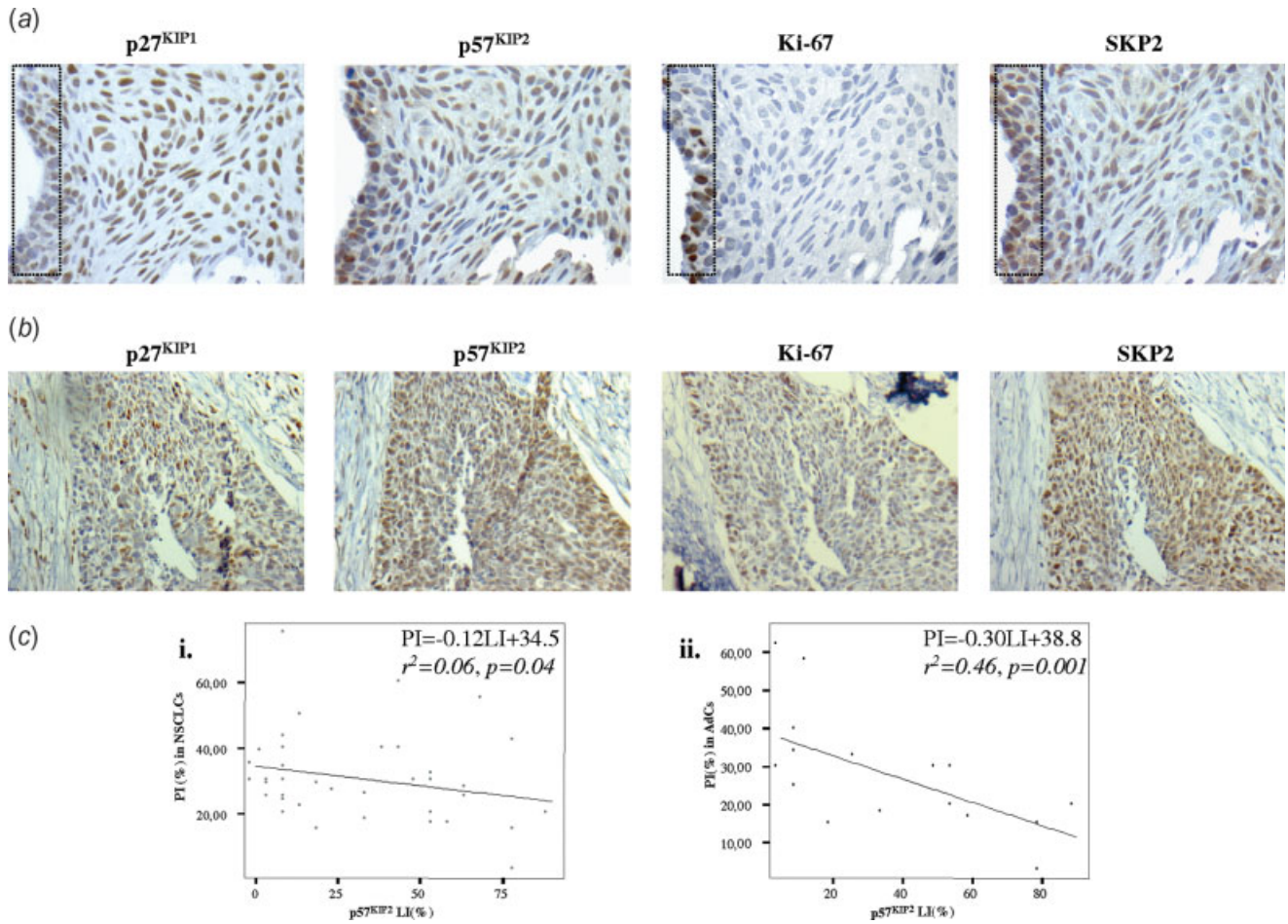

FIGURE 2.

(Biotage). The mix was denatured at 80°C for 2 min and 0.25 μg of single strand DNA binding protein (Amersham Biosciences) was added prior to the pyrosequencing reaction, which was undertaken on a PSQ 96MA machine (Biotage) following the supplier's protocol. Samples were assessed in triplicates. If tumor MtIs scored a 3-fold difference from the standard deviation of the corresponding normal counterpart they were characterized as increased or decreased, respectively.

Cell culture, siRNA and 5′-azacytidine treatment

Human lung adenocarcinoma derived A549 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, AntiSel, Athens, Greece) containing 10% fetal bovine serum at 37°C in 5% CO_2. All following experiments were performed in triplicates.

siRNA transfection. One day before transfection, cells were seeded at 2.25×10^5 cells per cell culture dish of 60 mm × 15 mm at a confluency of 60%. For dual downregulation of $p27^{KIP1}$ and SKP2 or $p57^{KIP2}$ and SKP2, siRNAs (Stealth™ RNAi, Invitrogen, AntiSel, Athens, Greece) were transfected simultaneously at a final concentration of 0.125 μM for each one in the presence of 5 μg/ml lipofectamine (Lipofectamine 2000, Invitrogen, AntiSel, Athens, Greece) 24 hr after cell seeding. Additionally, SKP2 siRNA was cotransfected with negative control duplex siRNAi of medium GC content (Stealth RNAi, Invitrogen, AntiSel, Athens, Greece) and transfection of the nonsilencing siRNA at 2-fold concentration (0.250 μM) was used as control (mock) for all the above double transfections. Both siRNAs and Lipofectamine were diluted in Opti-MEM I (Invitrogen, AntiSel, Athens, Greece) according to the manufacturer's protocol. Cells were incubated for 4 hr with the corresponding siRNAs in Lipofectamine reagent. Subsequently, medium was replaced. Cells were harvested 72 hr after transfection. Gene silencing results were examined with Western blot analysis.

5′-azacytidine treatment. Treatment with 5′azacytidine (Fluka, Sigma-Hellas, Athens, Greece) was performed at a final concentration of 2 and 4 μM,[44] respectively, for 72 hr. Medium with fresh 5′-azacytidine of the corresponding concentration was changed every 24 hr. After the 72 hr 5′-azacytidine treatment, cells were grown for additional 24 hr without 5′-azacytidine and then harvested.

Combined siRNA transfection and 5′-azacytidine treatment. Cells were seeded at 2.25×10^5 cells per cell culture dish of 60 mm × 15 mm at a confluency of 60%. 5′-azacytidine was added 24 hr later at a final concentration of 4 μM. Next day, single transfection with SKP2-siRNA was carried out as referred above in the presence of 2.5 μg/ml lipofectamine, omitting 5′-azacytidine during the first 4 hr of transfection. Subsequently, the cells were treated with 5′-azacytidine (4 μM) for another 48 hr. Finally the cells were grown for additional 24 hr without 5′-azacytidine and then harvested.

A549 growth curve analysis. Untreated A549 cells and cells treated with siRNA (anti-SKP2 Stealth RNAi or Stealth RNAi negative control) were seeded in culture dish of 60 mm × 15 mm following the previously described procedure. Cells were trypsinized and viable cells were counted under the microscope everyday at the same time points of the above respective experiments. Growth curves were plotted with data obtained from 3 independent experiments.

5-Bromo-2-deoxyuridine incorporation. A549 cells were incubated with 5-bromo-2-deoxyuridine (BrdU) labeling reagent of 10 μM for 60 min after each treatment.[45] Cells with incorporated BrdU were visualised by indirect immunofluorescence as described in previous section.

Analysis of apoptosis. After trypsinization, A549 cells were diluted in the supernatant medium to obtain the whole cellular population (viable cells and suspended apoptotic cells). Cells were spread on poly-L-lysine coated microscope slides after cytospin procedure. Apoptotic cells were assessed by the TUNEL assay as previously described.[36]

Statistical analysis

Molecular and clinicopathological explorations. Analysis of variance, Spearman bivariate analysis and Kendal's τ were used to explore molecular results and the correlations with the clinicopathological data, correspondingly. Statistical analysis was performed with the SPSS v11.0 package. Differences were significant if $p < 0.05$.

Survival analysis. Because of recent collection of tumors, survival analysis was not possible.

Results

KIP-members are downregulated in NSCLC

$p57^{KIP2}$ analysis. Immunohistochemical (IHC) analysis demonstrated $p57^{KIP2}$ expression in normal and tumor cells (Figs. 1*a* and 1*b*). LI values are presented in Table I. Staining was nuclear or cytoplasmic.

In normal parenchyma intense staining was observed, in the differentiated cells of the bronchial epithelium (Figs. 1*a*i and 1*b*i), alveoli, chondrocytes, occasionally fibroblasts and endothelial cells.

The nuclear staining in cancerous nests was mostly weaker (Fig. 1*a*i) or absent (Fig. 1*b*i) compared with adjacent normal bronchial epithelium. In the majority of the cases very few cells displayed strong nuclear signal (Figs. 1*a*ii and 1*b*ii). There was no specific $p57^{KIP2}$ topological distribution within cancerous areas of the NSCLC histological subtypes. Cytoplasmic staining was observed in certain cases (Figs. 1*a*iii, 1*b*iii and *c*). Interestingly, we noticed $p57^{KIP2}$ immunostaining in mitotic cells (Suppl. Fig. 1), which is in accordance with its role in regulating mitotic exit.[46]

The *in situ* observations were confirmed by SDS-PAGE analysis showing that 89% of the examined NSCLCs had decreased $p57^{KIP2}$ protein levels compared to normal counterparts (Figs. 1*d* and 1*e*). The specificity of $p57^{KIP2}$ cytoplasmic signal was investigated by analysis of cytoplasmic and nuclear extracts from matched normal tumor lung tissues, respectively. Employing lamin as nuclear fraction control[47] we observed as shown in Figure 1*f* that (*i*) $p57^{KIP2}$ was located in both cytoplasm and nucleus of normal and tumor cells, and (*ii*) levels of $p57^{KIP2}$ in tumor nuclei were lower than those in the cytoplasm, implying impairment of its antiproliferative activity.[4]

$p27^{KIP1}$ analysis. $p27^{KIP1}$ IHC expression was found in both normal and tumor cells, as we have previously described.[7] LI values are presented in Table I. Interestingly, a specific topological distribution was observed in the main NSCLC subtypes. Particu-

Figure 3 – Downregulating KIP-protein mechanisms. (*a*) The histogram shows cumulative results of *$p57^{KIP2}$* mRNA analysis in the whole spectrum of examined NSCLCs. (*b*) Cases with reduced, similar and elevated *$p57^{KIP2}$* mRNA levels are observed in tumors in comparison to their corresponding normal counterparts by semiquantitative RT-PCR analysis. *PBGD*: reference gene Porphobilinogen deaminase. (*c*) Diagram summarizing data from $p57^{KIP2}$ protein expression analysis and mechanisms associated with decreased $p57^{KIP2}$ expression in the whole spectrum of examined NSCLCs. (↑: increased, ↓: decreased.) (*d*) Expression analysis of $p57^{KIP2}$ and $p27^{KIP1}$ in relation to SKP2 in NSCLCs (cases 4, 6 and 12) and their normal counterparts. $p57^{KIP2}$ protein levels are decreased in tumors (i). The same cases show decreased $p27^{KIP1}$ (ii) and increased SKP2 protein levels (iii). SKP2 is detected as a doublet, with the lower mobility band representing the SKP2B-reported isoform. Equal protein loading for both counterparts is estimated by actin presence (iv).

Figure 4 – Mechanisms affecting *$p57^{KIP2}$* transcription. (*a*) Cumulative results of *$p57^{KIP2}$* mRNA status in the examined NSCLCs. Genetic and epigenetic mechanisms involved in regulation of *$p57^{KIP2}$* mRNA expression. (↑: increased, ↓: decreased). (*b*) Semiquantitative pyrosequencing analysis for estimation of the MtI of *$p57^{KIP2}$* promoter in matched Normal (N)/Tumor (T) tissues (case 30) and the corresponding histogram showing increased degree of methylation. (*c*) LOH analysis at *CDKN1C* locus (PAPA region) in normal (blue line) and tumor (red line) tissue. Arrowhead indicates the presence of LOH in a heterozygous case (36). (*d*) Representative cases (5, 14) depicting an inverse *$p57^{KIP2}$* and *LIT1* mRNA expression. *GAPDH*: reference gene.

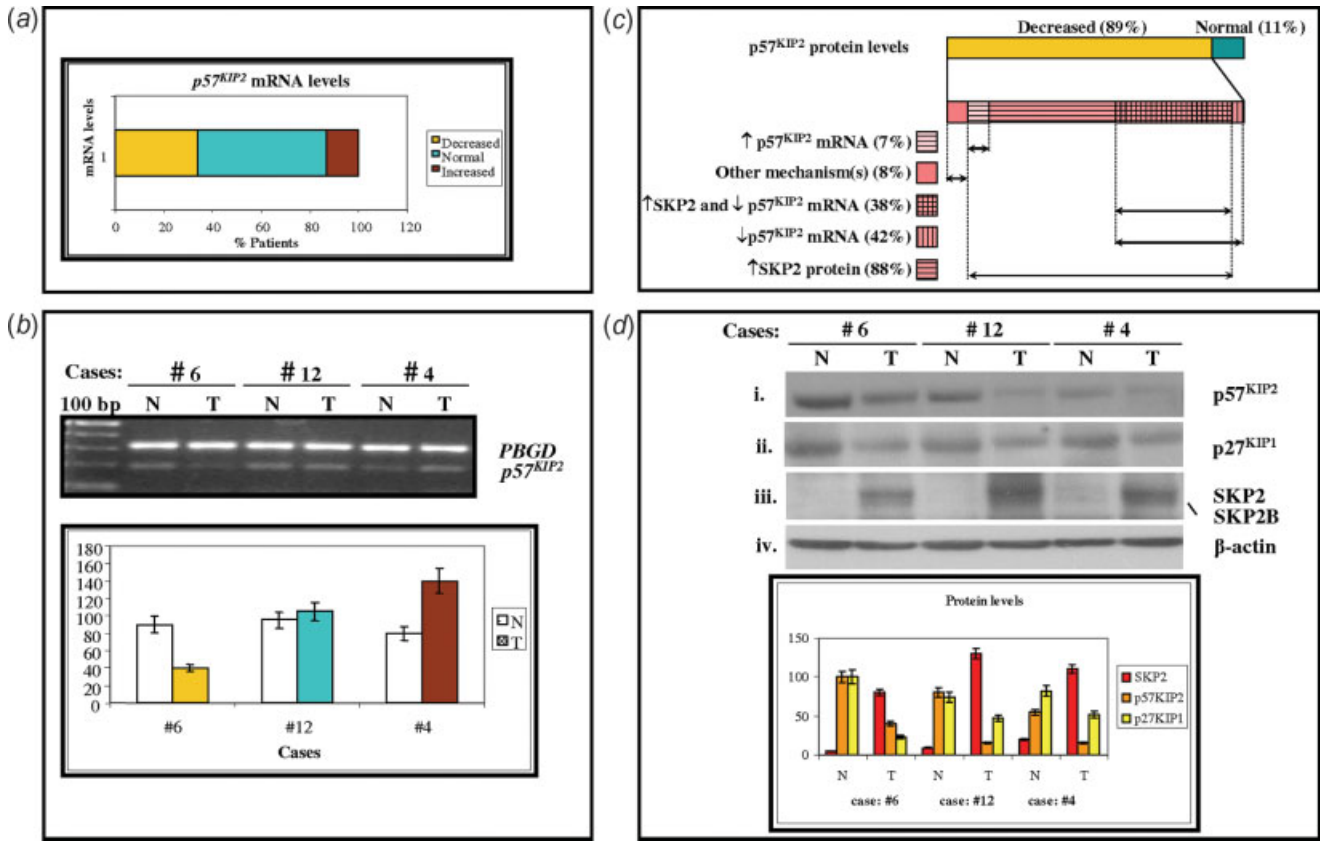

FIGURE 3.

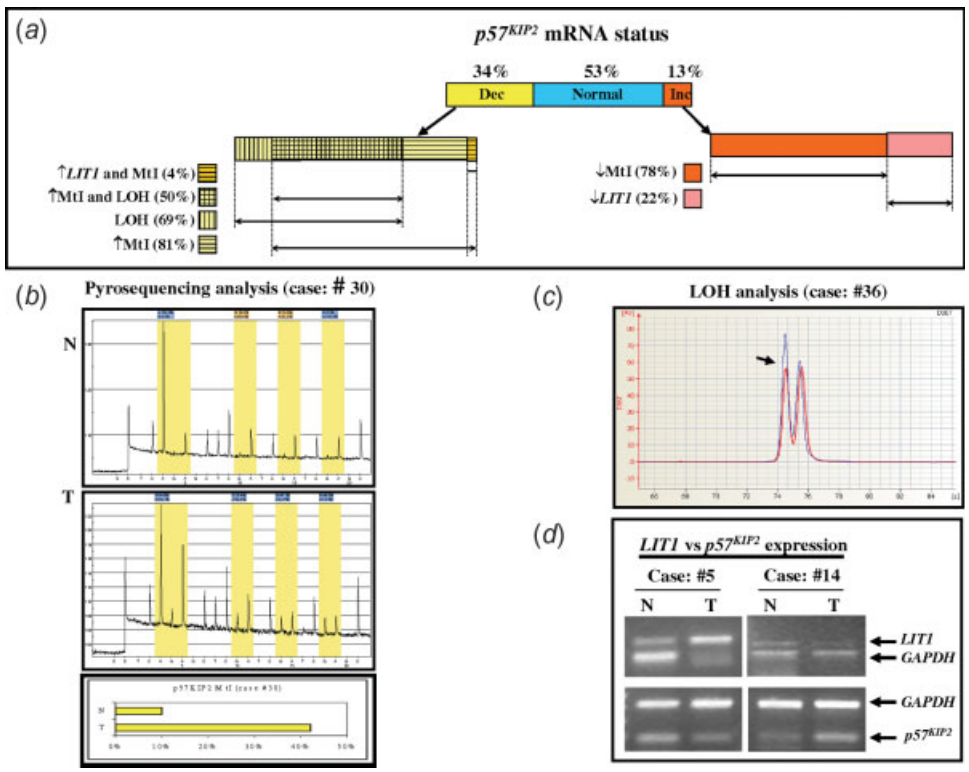

FIGURE 4.

larly, in SqCs $p27^{KIP1}$ nuclear staining was localized in the inner layers of tumor nests (Figs. 2*a* and 2*b*), whereas this distribution was not found in AdCs.

Intense staining of $p27^{KIP1}$ in cancer cells may be misleading as to whether this molecule is indeed downregulated. Nevertheless, Western blot analysis showed in 92.6% of our series (25 representative cases) decreased levels in tumor counterparts as compared to corresponding normal tissue (Figs. 1*d* and 1*e*).

In situ relationship between $p27^{KIP1}$ and $p57^{KIP2}$. Staining for both KIPs was performed in serial sections, but there was no specific mutual pattern of expression in any histological NSCLC subtype (Figs. 2*a* and 2*b*).

Relationship between $p27^{KIP1}$ and $p57^{KIP2}$ status and patients' stage. No association was found. The high frequency of KIP downregulation suggests a relatively early involvement in NSCLC development.

Low KIP expression is associated with proliferation

An inverse statistical association was found between $p57^{KIP2}$-LI and the PI in NSCLCs (Spearman test $r = -0.245$, $p = 0.04$), which was more significant in AdCs (Spearman test $r = -0.679$, $p = 0.001$) (Figs. 2*c*i and 2*c*ii, respectively) (Table I). No correlation was observed between $p57^{KIP2}$ expression and the apoptotic index of the tumors (Table I).

In an IHC analysis of serial section there was an inverse localization between $p27^{KIP1}$ and Ki-67 positive cells (Figs. 2*a* and 2*b*), which is consistent with our previous report.[7] Specifically, $p27^{KIP1}$ immunopositivity was observed centrally in cancerous nests, while Ki-67 was distributed at the frontline. This topological distribution was stronger in SqCs than in AdCs.

$p57^{KIP2}$ protein downregulation is associated with decreased transcription and increased SKP2 expression

Putative mechanisms leading to decreased $p57^{KIP2}$ protein expression could involve alterations at the transcriptional or posttranslational level.

Alterations of $p57^{KIP2}$ transcription. Decreased (2- to 5-fold) $p57^{KIP2}$ mRNA was observed in 34% of cases, while 13% displayed elevated (2- to 5-fold) levels (Figs. 3*a* and *b*). In a large portion of cases with reduced $p57^{KIP2}$ protein expression low mRNA levels were found (Fig. 3*c*). Interestingly, a few samples from the group of patients with reduced $p57^{KIP2}$ protein expression showed increased mRNA (Fig. 3*c*).

Posttranslational regulation of $p57^{KIP2}$. Prompted by recent studies showing that $p57^{KIP2}$ and $p27^{KIP1}$ are targeted for ubiquitin-mediated degradation by SKP2-dependent,[17,18] we examined the contribution of this putative mechanism in our set of NSCLCs. In the majority of cases with reduced $p57^{KIP2}$ protein, we observed a striking association with increased SKP2 levels (Figs. 3*c* and 3*d*). Notably, SKP2 was detected as a doublet, with the lower band probably representing the SKP2B reported isoform (Fig. 3*d*).[38] This is the first report describing its presence in human lung tumors.

There was no particular histological subtype preponderance regarding the above examined mechanisms.

Decreased $p57^{KIP2}$ transcription is associated with increased CDKN1C promoter degree of methylation and complemented by 11p15.5 LOH

Potential genetic and epigenetic processes affecting $p57^{KIP2}$ transcription could include the following.

Methylation. MSP demonstrated promoter methylation in normal and tumor tissues (Suppl. Fig. 3). Since the paternal allele is known to be imprinted,[29] we employed a semiquantitative pyrosequencing assay to measure the *CDKN1C* promoter degree of methylation (MtI) (Figs. 4*a* and 4*b*). In 38% of tumors we found increased MtI, while 22% exhibited reduced MtI. Juxtaposing the pyrosequencing and mRNA results we noticed that the degree of methylation is inversely related with $p57^{KIP2}$-mRNA levels ($p = 0.001$, ANOVA, Suppl. Fig. 3), indicating that promoter methylation is an important modulator of $p57^{KIP2}$ transcription (Fig. 4*a*). Interestingly, the majority of cases with increased mRNA but low $p57^{KIP2}$ protein, mentioned above (Fig. 3*c*), fell into the group of carcinomas with reduced methylation (Fig. 4*a*).

LOH of 11p15.5 region. We examined two polymorphisms located within the central coding region of $p57^{KIP2}$ locus [*PAPA* region,[42]] and within intron 10 of *KCNQ1* gene (*D11S4088*). We observed LOH in 69% of cases with decreased $p57^{KIP2}$ mRNA for at least one of the markers (Figs. 4*a* and 4*c*). As shown in Figure 4*a*, LOH complements methylation-dependent $p57^{KIP2}$ silencing and contributes to $p57^{KIP2}$ mRNA downregulation.

Epigenetic silencing by LIT1-mRNA. *DMR1* functions as a promoter sequence for the expression of the *LIT1* antisense transcript.[30,48] Given that *LIT1* transcript has been associated with suppression of *CDKN1C*,[26,32] we investigated its relationship with $p57^{KIP2}$ mRNA levels. *LIT1* levels were found low in 48.27% and high in 6.9% of our samples. The anticipated inverse relationship between *LIT1* and $p57^{KIP2}$ mRNA was found only in 3 cases (Figs. 4*a* and 4*d*), denoting the insignificant contribution of this mechanism in $p57^{KIP2}$ regulation.

$p27^{KIP1}$ protein downregulation is associated mainly with increased SKP2 expression

Since there are no reports referring to $p27^{KIP1}$ alterations at the mRNA level, we focused on posttranslational mechanisms involved in its downregulation.[5] Particularly, serial section analysis of $p27^{KIP1}$ and SKP2 revealed an interesting expression pattern in the main NSCLC subtypes. In SqCs an inverse localization was observed with $p27^{KIP1}$ staining in the center of cancerous nests and SKP2 almost exclusively at the frontline of nests (Figs. 2*a* and 2*b*). In AdCs, this pattern was not clear, although in a few cases the inverse $p27^{KIP1}$/SKP2 relationship was observed. Nevertheless, irrespectively of NSCLC subtype, Western blot analysis showed that decreased $p27^{KIP1}$ expression levels were accompanied by increased SKP2 (Fig. 3*d*).

In a few cases normal SKP2 was accompanied by low p27KIP1 protein levels. In these patients an alternative SKP2-independent degradation mechanism could function. Recent reports present evidence that Jun activation domain-binding protein 1 (JAB1) may link $p27^{KIP1}$ degradation to a SKP2-independent proteolysis pathway.[49] Indeed in these samples JAB1 IHC expression was found increased (Suppl. Fig. 2).

Restoration of KIP expression, by inhibition of SKP2 degradation pathway, restrains cell growth

Having in mind our *in vivo* findings we considered their potential exploitation for the design of therapeutic strategies aiming at restoration of KIP levels in NSCLCs. Such an intervention would be meaningful only if it results in retardation of tumor growth in this type of tumors. To examine this issue and recapitulate our *in vivo* findings, we manipulated the A549 lung cell line by blocking SKP2 expression using siRNA technology and demethylating *CDKN1C* gene, independently or together. The A549 cells were employed for the following reasons: (*i*) they express high SKP2 levels,[20] (*ii*) they exhibit low $p57^{KIP2}$ and $p27^{KIP1}$ levels, as assessed in the current study and (*iii*) they are *INK4A-B*$^{(-/-)}$,[50] thus there would be no interference from these nodal cell cycle regulatory molecules in the course of our analysis.

KIP protein levels are regulated in a SKP2-dependent manner in A549 cell line. Treatment with siRNA resulted in a significant reduction of endogenous SKP2 mRNA and protein levels, respectively (Figs. 5*a*i and 5*a*ii). Diminution of SKP2 expression led to a significant increase of both $p57^{KIP2}$ and $p27^{KIP1}$ protein levels (Fig. 5*a*ii), providing for the first time evidence that in lung cancer KIP protein levels are downregulated by the same proteasome-degradation mechanism.

Silencing of SKP2 expression and CDKN1C promoter demethylation highly restores $p57^{KIP2}$ levels. Pyrosequencing revealed that in

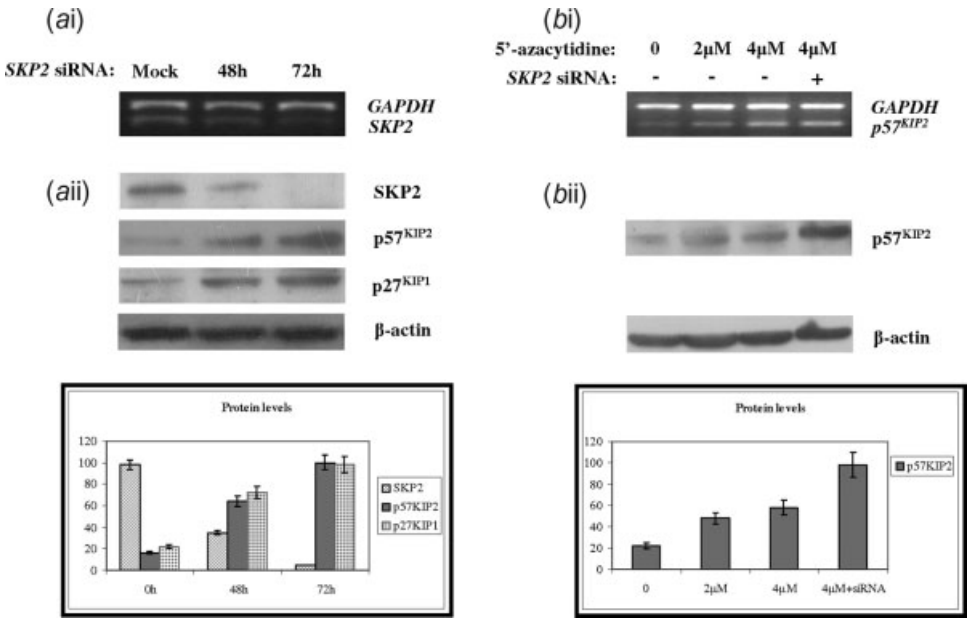

FIGURE 5 – Skp2 silencing along with $p57^{KIP2}$ promoter demethylation restore KIP expression levels in A549 cells. Significant reduction of endogenous SKP2 mRNA (*a*i) and protein levels (*a*ii) at 48 and 72 hr after SKP2 silencing with siRNA. Diminution of SKP2 expression results in a significant increase of $p57^{KIP2}$ and $p27^{KIP1}$ protein levels at these time points (*a*ii). Elevated $p57^{KIP2}$ mRNA levels correlate with increased 5′-azacytidine concentration (*b*i). The $p57^{KIP2}$ protein levels increase nonsignificantly and independently from 5′-azacytidine concentration, since high SKP2 levels overcome increased $p57^{KIP2}$ transcription. Only heavy demethylation along with SKP2 silencing leads to a marked increase of $p57^{KIP2}$ protein expression (*b*ii). Equal protein loading for both counterparts is estimated by actin presence. *GAPDH*: reference gene.

A549 cells *CDKN1C* promoter is highly methylated (MtI: 80%). Gradual demethylation of *CDKN1C* promoter showed a significant transcriptional enhancement of $p57^{KIP2}$-mRNA expression correlating with increased 5′-azacytidine concentration (Fig. 5*b*i). Although $p57^{KIP2}$ protein expression was elevated in comparison to untreated cells (Fig. 5*b*ii), this increase did not correlate with transcription levels (Fig. 5*b*i). Combined heavy demethylation along with SKP2 silencing led to a marked increase of $p57^{KIP2}$ protein expression (Figs. 5*b*i and 5*b*ii). Particularly, the $p57^{KIP2}$ levels were restored at similar to those observed in a normal bronchial cell line (data not shown). These findings prove that SKP2 overexpression overcomes increased $p57^{KIP2}$ transcriptional expression and suggest that reconstitution of $p57^{KIP2}$ in NSCLC requires promoter demethylation along with downregulation of KIP degradation machinery.

Restrained growth of A549 cells in response to SKP2-siRNA treatment. The significance of our interventions in the A549 cells was investigated in relation to A549 cell growth. Treatment of A549 cells was performed only with SKP2-siRNA, since 5′-azacytidine is a global-genome demethylating agent, leading to a wide range of gene activation.[51] This may interfere with the effect from the reconstituted KIP expression on A549 growth curve analysis.

SKP2 silencing was accompanied not only by restoration of KIP protein expression but also resulted in a significant reduction of cellular growth ($p = 0.0001$, ANOVA) (Fig. 6*a*). This finding suggests that restoration of KIP levels may be associated with inhibition of tumor growth in NSCLCs.

Each KIP member has a significant independent effect on restrained A549 growth in response to SKP2-siRNA treatment. The SCF^{SKP2} mechanism controls the cellular abundance of a variety of important factors involved in cell cycle control ($p21^{WAF1/CIP1}$, $p27^{KIP1}$, $p57^{KIP2}$, cyclin E, p130),[52] DNA replication control (Cdt1, ORC1)[52] and gene transcription control (E2F-1, c-Myc, b-Myb),[52] that may also participate in A549 growth control. Therefore, we proceeded to determine the specific effect of each KIP separately on the growth retardation of A549 cells after SKP2 siRNA silencing. Double siRNA knock-out experiments consisting of SKP2/$p27^{KIP1}$ and SKP2/$p57^{KIP2}$ were performed and compared to SKP2/mock treated A549 cells (Fig. 6). Once again treatment of A549 cells was performed only with siRNAs without 5′-azacytidine.

Silencing of $p27^{KIP1}$ or $p57^{KIP2}$ along with SKP2, respectively, resulted in a significant increase of A549 cell growth ($p = 0.001$ and $p = 0.003$, respectively, ANOVA, Fig. 6*a*), although not at similar levels to mock treated cells. The effect of $p57^{KIP2}$ on restraining cell growth was less intense in comparison to that of $p27^{KIP1}$. Nevertheless, this result may be an underestimation given that in A549 cells the $p57^{KIP2}$ expression levels are also under methylation-dependent transcriptional control, with which we did not interfere. The difference of SKP2/$p27^{KIP1}$ and SKP2/$p57^{KIP2}$ treated cells from mock ones may be attributed to restoration of other factors down regulated by SKP2.[52] Collectively, the above experiment indicates that in a SKP2 downregulation oriented treatment of nonsmall lung cancer cells with overexpression of SKP2, restoration of KIP protein levels play a major role in inhibition of tumor growth, despite the functional involvement of other factors.

KIP-dependent A549 growth inhibition results from reduced proliferation. To explore the kinetic parameters (proliferation and apoptosis) responsible for A549 growth inhibition, we determined cells undergoing DNA synthesis and apoptosis after double siRNA transfections, SKP2/$p27^{KIP1}$ and SKP2/$p57^{KIP2}$, in comparison to SKP2 treated cells.

Inhibition of A549 cell growth in the SKP2 transfected cells (Fig. 6*a*) was due to decreased proliferation (Fig. 6*b*) and increased apoptosis (Fig. 6*c*), as previously described.[53,54] The increased growth in SKP2/$p27^{KIP1}$ and SKP2/$p57^{KIP2}$ treated cells was accompanied by a significant increase of DNA synthesis determined by BrdU incorporation, as compared to SKP2 siRNA transfected A549 cells ($p > 0.001$ and $p = 0.027$, respectively, ANOVA, Fig. 6*b*). On the other, apoptosis was independent of KIP protein levels modulation ($p = 0.09$ and $p = 0.27$, ANOVA, Fig. 6*c*). These findings support the *in vivo* observed association between decreased KIP levels and proliferation, but not apoptosis, in our NSCLC database.

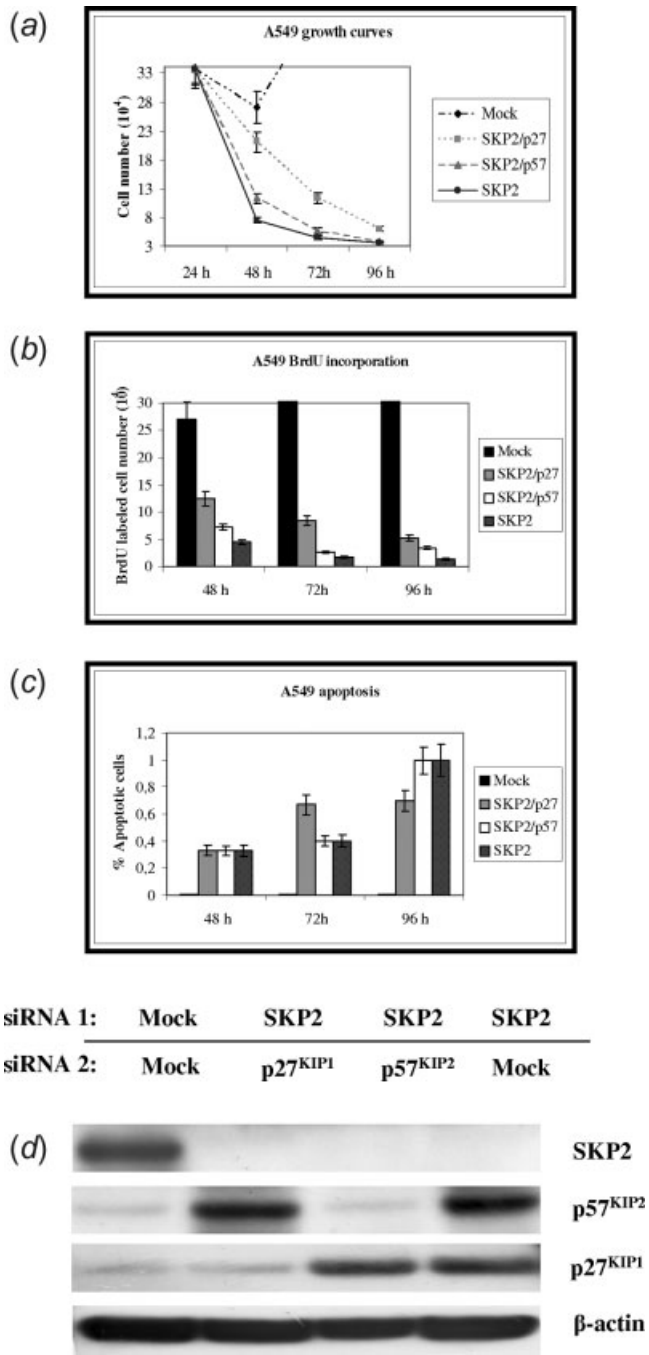

FIGURE 6 – KIP restoration restrains cell growth in A549 cells due to decreased cell proliferation. Diagrams representing the impact of combined treatment with different siRNAs on cellular growth (*a*), cell proliferation (*b*) and apoptosis (*c*) in the A549 cell line. (*d*) SDS-PAGE analysis shows SKP2, $p27^{KIP1}$ and $p57^{KIP2}$ protein levels upon combined siRNAs treatment after 96 hr. β-actin served as a loading control. The reduced growth of mock treated A549 cells at 48 hr was attributed to a temporal decrease in cell viability due to the double transfection. (Mock: A549 cells transfected with Stealth RNAi negative control).

Discussion

Our comparative study regarding the KIP members, $p57^{KIP2}$ and $p27^{KIP1}$, in NSCLCs, revealed interesting findings that could be used in future therapeutic interventions.

In the majority of cases both members showed significantly reduced expression levels compared to their adjacent normal counterparts. This along with absence of correlation with clinical stage of patients suggests their relatively early involvement in NSCLC development. Reduced nuclear expression of $p57^{KIP2}$ implies an antiproliferative effect.[4] Indeed, an inverse association with PI in NSCLCs was observed ($p = 0.04$, Fig. 2*c*i), which was more significant in AdCs ($p = 0.001$, Fig. 2*c*ii). A similar effect has been denoted in certain tumor types.[9–12] On the other, $p27^{KIP1}$ expression was inversely related to proliferation mainly in SqCs, suggesting a histological subtype preponderance regarding KIP relationship with proliferation. Recently, it was shown that $p57^{KIP2}$ was required for p73β-mediated apoptosis.[55] However, in our series no association with apoptosis was found. This is in accordance with our previous findings showing that only proliferation and not apoptosis was influenced by decreased CDKI expression,[7,56] and was also confirmed by our *in vitro* siRNA experimental procedure (Figs. 6*b* and 6*c*).

Cellular fractionation analysis demonstrated the $p57^{KIP2}$ cytoplasmic immunostaining specificity. An analogous IHC finding was reported in esophageal carcinomas, but was not further investigated.[13] Cytoplasmic localization of $p57^{KIP2}$ may reflect inactivation of LIM-domain containing protein kinase-1 (LIMK-1), a downstream-effector of the Rho-pathway,[57] which blocks cofilin, an actin-cytoskeleton destabilizing factor.[58] Such an interaction implies an oncogene-like behavior.[59] However, more studies are needed to clarify the functions of cytoplasmic $p57^{KIP2}$ since a recent study has associated elevated LIMK1 activity with invasive growth.[60] On the other, this signal could represent a cytoplasmic retention, and consequently a functional inactivation, similar to that observed in other tumor suppressor genes, including $p16^{INK4A}$.[61]

At the mechanistic level, degradation by SKP2 was observed *in vivo* and demonstrated *in vitro* by siRNA methodology (Fig. 5), to be the most important downregulating mechanism of both KIPs in NSCLCs. Although increased SKP2 has been documented in lung tumors,[19–21] this is the first report displaying its association with codecreased KIP expression in a primary malignancy. We also observed cases with decreased KIP expression but normal SKP2. These cases were found to exhibit increased JAB1 expression, indicating the parallel function of a second KIP-protein degradation mechanism.[49] In addition, decreased $p57^{KIP2}$ transcription appears to complement the above procedures in lowering the levels of $p57^{KIP2}$ protein in tumors (Fig. 3*c*). Unexpectedly, cases with elevated $p57^{KIP2}$ mRNA were also observed (Figs. 3*a* and 3*b*). Although this setting should lead to increased protein production, SKP2 overexpression seems to overcome $p57^{KIP2}$ transcriptional upregulation by degrading the newly produced protein.

The presence of cases with increased $p57^{KIP2}$ mRNA levels suggests the function of a transcriptional counterbalance mechanism. Given that $p57^{KIP2}$ promoter methylation modulates *CDKN1C* transcription,[24] we hypothesized that its demethylation could account for this counterbalance mechanism. Indeed, pyrosequencing analysis demonstrated that in most cases $p57^{KIP2}$-mRNA levels were inversely related with MtI ($p = 0.001$, Fig. 4*a*; Suppl. Fig. 3). This mechanism may also explain the increased $p57^{KIP2}$-mRNA found in childhood tumors, suggesting an oncogenic role for $p57^{KIP2}$, but protein expression was not examined.[16] A potential cause for decreased *CDKN1C* methylation could be increased activity of DNA demethylases, which has been seen in certain types of cancer.[62] Notably, compared to other malignancies, the frequency of $p57^{KIP2}$ regional promoter hypermethylation in lung cancer is the highest reported.[24] Finally, MSP analysis confirmed the reported imprinting of paternal allele,[29] contradicting a recent study showing rare methylation of *CDKN1C* in normal lung tissues.[33]

Although methylation analysis could explain the majority of cases with abnormal $p57^{KIP2}$ mRNA levels, there were several samples that could not be interpreted by the above associations (Fig. 4*a*). Putative mechanisms that could explain these outliers, include LOH and abnormal imprinting from *DMR-LIT1*.[22,25] Both mechanisms suggested were found in NSCLCs (Figs. 4*c* and 4*d*).

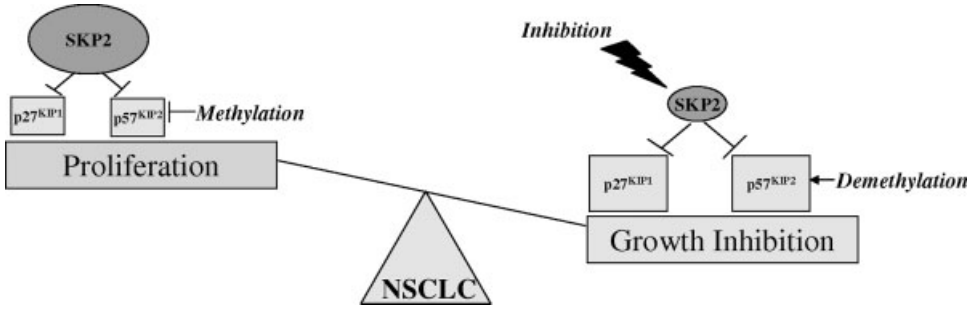

FIGURE 7 – Diagram summarizing regulation of KIP expression in NSCLC. The *in vivo* findings show that SKP2 overexpression downregulates KIP protein levels in NSCLC, enhancing tumor proliferation. In addition promoter methylation of $p57^{KIP2}$ complements this effect. According to our *in vitro* findings, inhibition of SKP2 leads to restoration of KIP level thus conferring to growth inhibition. A further demethylation step of $p57^{KIP2}$ promoter enhances its expressional reconstitution.

DMR1 promoter aberrations associated with silencing of *CDKN1C* are frequently found in certain human tumors and in patients with Beckwith-Wiedemann syndrome.[26,32] Despite the high frequency of abnormal *LIT1* expression found in our series, only 3 cases showed an inverse *p57KIP2-LIT1* mRNA relationship (Fig. 4*a*). On the other hand, LOH was a far more frequent event, found in 69% of cases with decreased *p57KIP2* mRNA (Fig. 4*a*), denoting its significance in *p57KIP2* transcriptional downregulation in lung cancer.

The above information, dealing with downregulating mechanisms of $p57^{KIP2}$ or $p27^{KIP1}$, could be used for therapeutic purposes. To prove this case we attempted to recapitulate our *in vivo* findings by manipulating the A549 lung carcinoma cell line. Gradual demethylation of *CDKN1C* promoter resulted in elevated *p57KIP2* transcription, confirming that methylation is a significant *p57KIP2* transcriptional modulator. In this setting $p57^{KIP2}$ protein increase was nonsignificant, resembling the *in vivo* patterns with increased mRNA levels and nonaffected or even decreased protein levels. Inhibition of SKP2, by siRNA, along with heavy demethylation resulted in highly augmented $p57^{KIP2}$ protein expression, indicating that posttranslational modification and epigenetic silencing cooperate in $p57^{KIP2}$ downregulation. In the above experiment $p27^{KIP1}$ expression was recovered only by SKP2 inhibition. Using double siRNA knock-out methodology we demonstrated that each KIP independently had a significant restrain on A549 cellular proliferation, but no impact on apoptosis, despite that SKP2 silencing influences the expression status of many nodal cell cycle regulating factors.[52] The above findings are in agreement with our *in vivo* observations. The significance of this result is underscored by the fact that A549 cells are *INK4A-B*$^{(-/-)}$.[50] It should be stressed out that LOH does not represent a disadvantage in putative therapeutic strategies based on the observed downregulating mechanisms, since it affects mainly the maternal allele[22] and demethylation would reactivate the silenced paternal one.

In conclusion, we propose that future individual-based therapeutic strategies aiming at functional restoration of these CDKIs should take into consideration the corresponding status and mechanisms leading to their deregulation (Fig. 7) so that combined molecular treatment approaches could be applied.

Acknowledgements

ISP is a fellowship recipient from the Harokopou-Petroutsi Foundation. We acknowledge the support of the Hellenic Anticancer Institute.

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