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ORIGINAL INVESTIGATION

Promoter methylation study of the *H37/RBM5* tumor suppressor gene from the 3p21.3 human lung cancer tumor suppressor locus

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Abstract Loss of heterozygosity (LOH) at chromosome 3p21.3 is one of the most prevalent genetic disturbances occurring at the earliest stage of tumor development for a wide variety of human cancers, culminated in lung cancer. The 19 genes residing at 3p21.3 have been vigorously characterized for tumor suppressor activity and gene inactivation mechanism because of their potentially significant merits of clinical applications. Many of these 19 genes have been shown to manifest various growth inhibitory properties, however none of them are inactivated by coding mutations in their remaining allele as in the Knudson's *two- hits* hypothesis. Thus far the most prevailing, alternative gene inactivation mechanism known for the 3p21.3 TSGs is epigenetic silencing by promoter hypermethylation. Previously, we have focused our investigation on one of the 19 genes at 3p21.3, *H37/RBM5*, and demonstrated its tumor suppressor activity both in vitro and in vivo as well as its mRNA/protein expression loss from the remaining allele in a majority of the primary lung tumors examined. The current study tested our hypothesis that the H37 inactivation in primary lung tumors may, as seen in most of the other 3p21.3 TSGs, be due to hypermethylation in its promoter CpG islands. Contrary to this most plausible postulation, however, we found no evidence of epigenetic gene silencing for the *H37* TSG. Here we suggest some of the possible, further-alternative means of the *H37* gene expression loss in tumor, including defects in transcription and post-transcriptional/translational modifications as well as mechanisms related to haploinsufficiency.

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Introduction

Loss of Heterozygosity (LOH) at Chromosome 3p are the most frequent and earliest genomic abnormalities in a wide spectrum of human cancers including lung, breast, head and neck, ovarian, cervical/uterus, colorectal, pancreatic, esophageal, and bladder (Ji et al. 2005). In particular, LOH in 3p21.3 region occurs in more than 65% non-small cell lung cancers (NSCLCs) and 95% of small cell lung cancers (SCLCs) and is seen as early as in the histologically normal bronchial epithelium of current and former smokers, suggesting the tumor suppressor genes (TSGs) contained in this region are likely to play important role(s) in tumor initiation (Ji et al. 2005). After two decades of intensive gene "hunts" by multiple groups of investigators worldwide, by 1996, the 19 genes were identified from the 370 kb minimal overlapping deletion region at 3p21.3

(Lerman and Minna 2000). Subsequently, to determine which one(s) of these 19 genes is(are) the long-sought-after, so-called "golden" TSGs at 3p21.3, the classical criteria to discern a gene as a *bone fide* TSG was employed—the Knudson's *two-hit hypothesis* in which the remaining allele is inactivated by mutations in the coding region (Knudson 1971). However, the extensive search for mutations in the 19-gene set did not reveal a single gene with a high mutation rate (>10%) in the analyzed lung tumor samples despite frequent reduced or absent mRNA levels of many of these genes (Lerman and Minna 2000). This raised the possibility that the putative 3p21.3 TSGs, rather than having a second hit (mutation) in the remaining wild type allele in the tumor, may be inactivated by alternative mechanisms such as chromosome instability, dramatic gains and losses of genetic materials (aneuploidy), promoter hypermethylation, haploinsufficiency, and altered RNA splicing, etc.

Over the past decade, we have focused on one of the above 19 genes at 3p21.3, *H37/RBM5/Luca15*. To the present date, we and many others have compiled growing literatures on the *H37* gene which strongly suggests its involvement in apoptosis and cell cycle regulation and converges on its role as a TSG (Sutherland et al. 2005). Using the lung cancer cell model, for example, we showed that H37 clearly inhibits tumor growth both in vitro and in vivo with antitumor mechanisms involving cell cycle (G1) arrest and apoptosis (Oh et al. 2006). Further, the *H37/RBM5* gene was included in the 17 common gene signature associated with metastasis (one of the nine genes down-regulated in metastases) identified in multiple solid tumor types (Ramaswamy et al. 2003). Various types of solid tumors including lung cancer carrying this gene expression signature had high rates of metastasis and poor clinical outcome.

Most significantly, using primary NSCLCs, we demonstrated reduced expression of *H37* transcript and protein in the patients' tumor (as compared with their adjacent normal tissue) (Oh et al. 2002). As it was becoming increasingly plausible that *H37* may have good potential to be developed into new cancer therapeutics/diagnostics, in an effort to explore the best strategy of *H37* clinical application, we wished to understand molecular mechanism behind this *H37* gene/protein expression loss in tumor. By this time, accumulating evidences suggested that many of the 3p21.3 TSGs are inactivated by aberrant epigenetic methylation in their promoter region, including *RASSF1A, BLU*, *CACNA2D2*, *HYAL1* and *SEMA3B* with corresponding gene expression loss in various human cancers (Ji et al. 2005). *RASSF1A*, in particular, is known to be one of the most extensively promoter-hypermethylated genes thus far detected in human cancer (Dammann et al. 2003). Taken together, it appeared that epigenetic silencing is the pivotal mechanism for the 3p21.3 TSGs to be inactivated in primary cancers and in tumorigenesis. Therefore, we set out to examine if promoter hypermethylation is also responsible for the observed H37 gene expression loss in lung tumors.

Materials and methods

Extraction of genomic DNA from primary lung tumor/normal pair samples and NSCLC cells lines

Primary NSCLC tumors and adjacent normal tissues used were specimens obtained from various hospitals during 1988–1989. After surgical removal, the samples were immediately snap-frozen in liquid nitrogen and stored at −80°C until genomic DNA was extracted following the routine protocol (Davis 1986). A549, H460, and H520 cells were purchased from ATCC (Manassas, VA, USA) and their genomic DNA was extracted using DNeasy Blood & Tissue kit (Quiagen, Valencia, CA, USA).

Preparation of DNA sample for bisulfite treatment

Two microgram of the extracted DNA from each sample was digested with restriction enzymes that cut just outside of the *H37* CpG island, i.e. DraI, HpaI, AlwI, and BsaXI. This additional step was performed to generate a short DNA fragment which gives bisulfite chemicals easier access to the template for complete (C to T) conversion reaction. The digested samples were treated with 1/9 volume of freshly prepared 3 M sodium hydroxide and incubated at 39°C for 30 min. Subsequently, 30 μg of glycogen was added, samples were extracted once with phenol/chloroform, and DNA was precipitated by adjusting to 3 M NH_4OAc pH 7.0 and adding three volumes of ethanol. After centrifugation, pellets were washed twice with 70% EtOH, dried, and dissolved in 40 μl of water. The purified DNA was denatured by heating at 97°C for 5 min followed by quench on ice. Freshly prepared 6.3 M NaOH (2 μl) was added and samples were incubated at 39°C for 30 min.

Bisulfite treatment and desalting of DNA

Bisulfite solution was made by dissolving 40.5 g of sodium bisulfite (Fisher Scientific, Pittsburgh, PA, USA) in 80 ml of water with slow stirring to avoid aeration. After adjusting pH to 5.1 with freshly prepared 10 M NaOH, 3.3 ml of 20 mM hydroquinone (Sigma-Aldrich, St. Louis, MO, USA) was added and the volume was adjusted to 100 ml with water. Bisulfite solution (416 μl) was added to the denatured DNA and samples were overlaid with three

drops of mineral oil. Samples were incubated in a thermal cycler for five cycles of 55°C for 3 h, 95°C for 5 min. After bisulfite conversion, the mineral oil was removed and samples were desalted with the Wizard DNA Clean-up System (Promega, Madison, WI, USA) following the manufacturer's instructions. NaOH was added to a final concentration of 0.3 M and samples were incubated at 37°C for 15 min. DNA was then precipitated by adding 40 μg of glycogen, adjusting to 3 M NH_4OAc pH 7.0 and adding three volumes of ethanol. After centrifugation, pellets were washed with 70% ethanol and dissolved in nuclease- free water.

PCR amplification

For both primary and nested PCR, platinum Taq PCRx DNA polymerase kit (Invitrogen, Carlsbad, CA, USA) was used. The master mixture for 15 reactions was prepared as follows; 150 μl 10× PCRx enhancer, 75 μl 10× PCRx amplification buffer, 22.5 μl 50 mM $MgSO_4$, 15 μl 10 mM dNTP, 7.5 μl Taq PCRx DNA polymerase, 412 μl H_2O. The master mix. was dispensed into a single reaction volume of 50 μl with subsequent addition of DNA template and forward and reverse primers in standard amounts. PCR reactions were performed in a thermocycler with 94°C 10 min pre-cycle denaturation, 25 cycles of 94°C 30 s, 53°C 30 s, 72°C 45 s and further extension of 72°C 10 min. 1/50 volume of the primary PCR reaction (1 μl) was used as the DNA template for a nested reaction using the same reaction parameters.

Subcloning into TOPO vector and sequencing

PCR product was purified by QIAquick PCR purification kit (Qiagen) according to the manufacturer's protocol. The purified DNA was either directly sequenced by using NF1-NR1 and NF2-NR2 primer pairs (Table 1) for the regions 1 and 2 respectively or was subcloned into TOPO TA cloning vector (Invitrogen) for sequencing of multiple, individual amplicons. The purified plasmid was sequenced using the primer sequences (M13-forward & reverse) available in the TOPO TA vector.

Results

To assess *H37* gene expression loss in lung tumor cells, we performed Northern blot analysis on 11 pairs of primary lung tumor versus adjacent normal tissue samples (Fig. 1). Use of the 3.1 kb full-length *H37* cDNA probe (including 126 bp 5′ untranslated and 520 bp 3′ untranslated regions respectively) generated the expected, main band at ~3.1 kb as well as the two additional bands at 6.5 kb (intermediate signal intensity) and 1.9 kb (very faint) across the entire lanes irrespective of tumor versus normal tissue status. Relative signal intensity of these alternatively spliced transcripts is proportional to (reflective of) the main band's in respective samples, and no other transcript variants were detected which are uniquely present (or absent) in tumor samples, suggesting that *H37* gene silencing in lung tumor is not likely caused by aberrant alternative splicing in tumor cells. When the 3.1 kb main transcripts were quantitatively compared, a majority of the pair samples (except for patients #s 9, 10) show *H37* gene expression decrease in tumor compared with their normal counterpart, and, among these, six pairs show the *H37* gene expression in tumor which falls below 50% of the normal level (i.e. #s 1, 3, 4, 7, 8, 11). In particular, patients # 3, 7, and 8 show near non-existent *H37* expression in their tumor compared to the respective normal tissue counterpart. Although the 3p21.3 LOH status of these tumors is not known, considering that >65% of NSCLCs in general

Table 1 Oligonucleotide primers used in bisulfite genomic PCR

Primer	Sequence[a] (5′ → 3′)	5′–3′ position (bp) from TSS	PCR product size (bp)
PF1	taatttaggtttgtaaaagt(c/t)gat(c/t)gat	−214 to −187	440
PR1	cctaaaataatttaacccctctac	203 to 226	
NF1	tagag(c/t)gttttaagtgataggatg	−149 to −121	204
NR1	ccaaaacctccaacaaaatt	37 to 56	
PF2	taatttttagtttaa(c/t)ggagtagtg	350 to 374	407
PR2	ccaataatctctaaaaacctaatct	732 to 756	
NF2	tttg(c/t)gggtttggaatttttgg	492 to 514	164
NR2	ttcactaatacc(a/g)ccccttat	635 to 655	

P primary, *N* nested, *F* forward, *R* reverse, *1* Region 1, *2* Region 2, *TSS* transcription start site

[a] (c/t), (a/g); Primers were designed to contain degenerate nucleotides to detect both methylated versus non- methylated cytosine at "C"pG positions

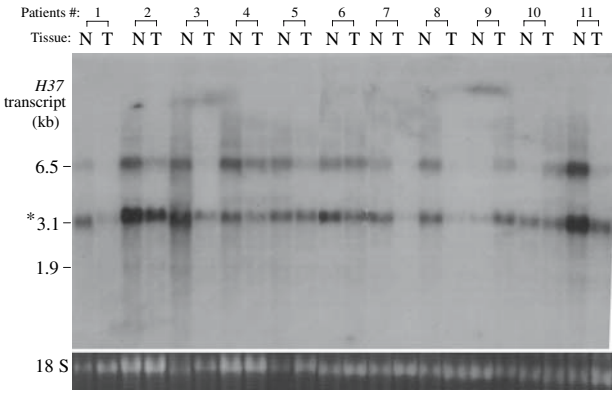

Fig. 1 *H37* mRNA expression level compared in lung tumor (*T*) versus matching normal (*N*) tissue in 11 NSCLC patients. Northern blot shows that *H37* expression in tumor is less than 50% of the corresponding normal lung tissue in 6 of 11 patients (#s 1, 3, 4, 7, 8, 11). The 3.1 kb main transcript is marked with *. Ethidium bromide staining of 18S ribosomal RNA band is shown as a loading control

have heterozygous deletion in the 3p21.3 where *H37* resides, the *H37* transcript level in most of these lung tumor samples may have been expected to be approximately 50% of the level in the respective matching normal tissue. However, further gene expression loss beyond 50% in many of the samples used in our study implied that *H37* gene expression from the remaining allele in tumor may have been (partially) silenced.

Therefore, we wanted to investigate the potential molecular mechanism underlying this *H37* expression loss in tumor and test our primary hypothesis that hypermethylation of the *H37* gene promoter is responsible for its gene silencing. To this end, we first identified the *H37* promoter CpG islands (source; http://www.genome.ucsc.edu) (Fig. 2); it contains 69 CpG dinucleotides (GC content 62.1%, observed/expected CpG ratio 0.75) in a 949-bp region. To determine how many, if any, of these cytosines are methylated in primary lung tumor tissue compared with the corresponding normal tissue, bisulfite genomic sequencing was a technique of our choice because the *H37* promoter CpG islands had never been characterized prior to the current study, therefore it was necessary to survey the entire suite of CpG sites to examine which cytosine residues are subject to methylation. We employed 16 pairs of primary NSCLC tumor versus matching normal samples which included the above 11 pairs as well as three NSCLC cell lines, i.e. A549, H460, and H520. In bisulfite genomic sequencing, bisulfite treatment will convert unmethylated cytosines ("C"s) to thymidines ("T"s) while leaving methylated "C"s intact, and subsequent sequencing will distinguish methylated versus unmethylated "C"s. After much tribulations with this rather challenging technique, in part, because the harsh bisulfite chemical can easily destroy the DNA template making it very difficult to PCR-amplify the entire stretch of CpG island, we managed to successfully amplify the two separate regions, each employing primary and nested primer sets (Table 1), region 1, spans 204 bp encompassing transcription start site, 14 CpG sites; region 2, 164 bp, 18 CpG sites (Fig. 2). The detailed description of our rationale in selecting the specific primer sets and the gel pictures showing the generated PCR products were published elsewhere (Oh et al. 2005).

For a half of the samples, we directly sequenced the purified, nested PCR products by using the same (nested) primer sets as the sequencing primers (Table 1). In order to eliminate a possibility of partial bisulfite conversion and/or partial methylation occurred only in some fraction of the initial DNA template (leading to some hybrid or misrepresented sequencing peaks), for the rest of the samples, we subcloned the PCR product into the TOPO-TA sequencing vector and sequenced the multiple (i.e. 4–7) sub-cloned plasmids per sample. Our sequencing data indicted that all the C residues, including "C"s in "C"pGs, have been completely converted to "T" suggesting complete bisulfite conversion but no methylation (Fig. 3a, b). Even in those tumor samples in which *H37* expression seems to be almost completely silenced (i.e. Fig. 1; #s 3, 7, 8), we could not find any evidence of the *H37* promoter methylation. The *RASSF1A* CpG island, known to be heavily methylated in various cancer types including NSCLC, was used as a positive control to show methylation at CpG sites (Dammann et al. 2000) (Fig. 3c). In addition, we found no sequence abnormality (i.e. point mutation, deletion,

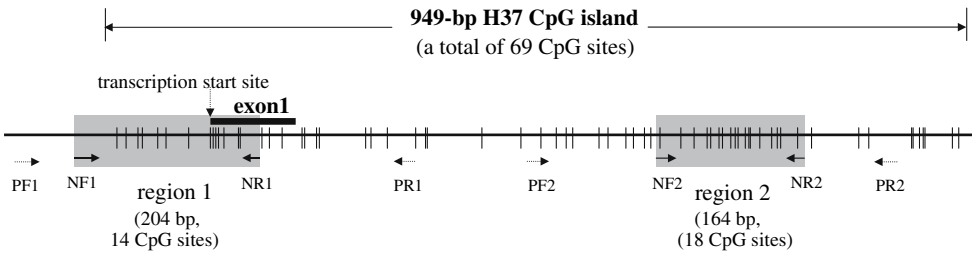

Fig. 2 Genomic map of the *H37* promoter CpG island. Each CpG sites are marked by a *vertical line*; the two adjacent CpG sites located at closer proximity appear as *thicker lines*. The two regions, i.e. region 1 and 2, analyzed in this study are boxed in *grey shadow*. Primers used in bisulfite genomic PCR are located with *arrows*; *P* primary, *N* nested, *F* forward, *R* reverse, *1* region 1, *2* region 2

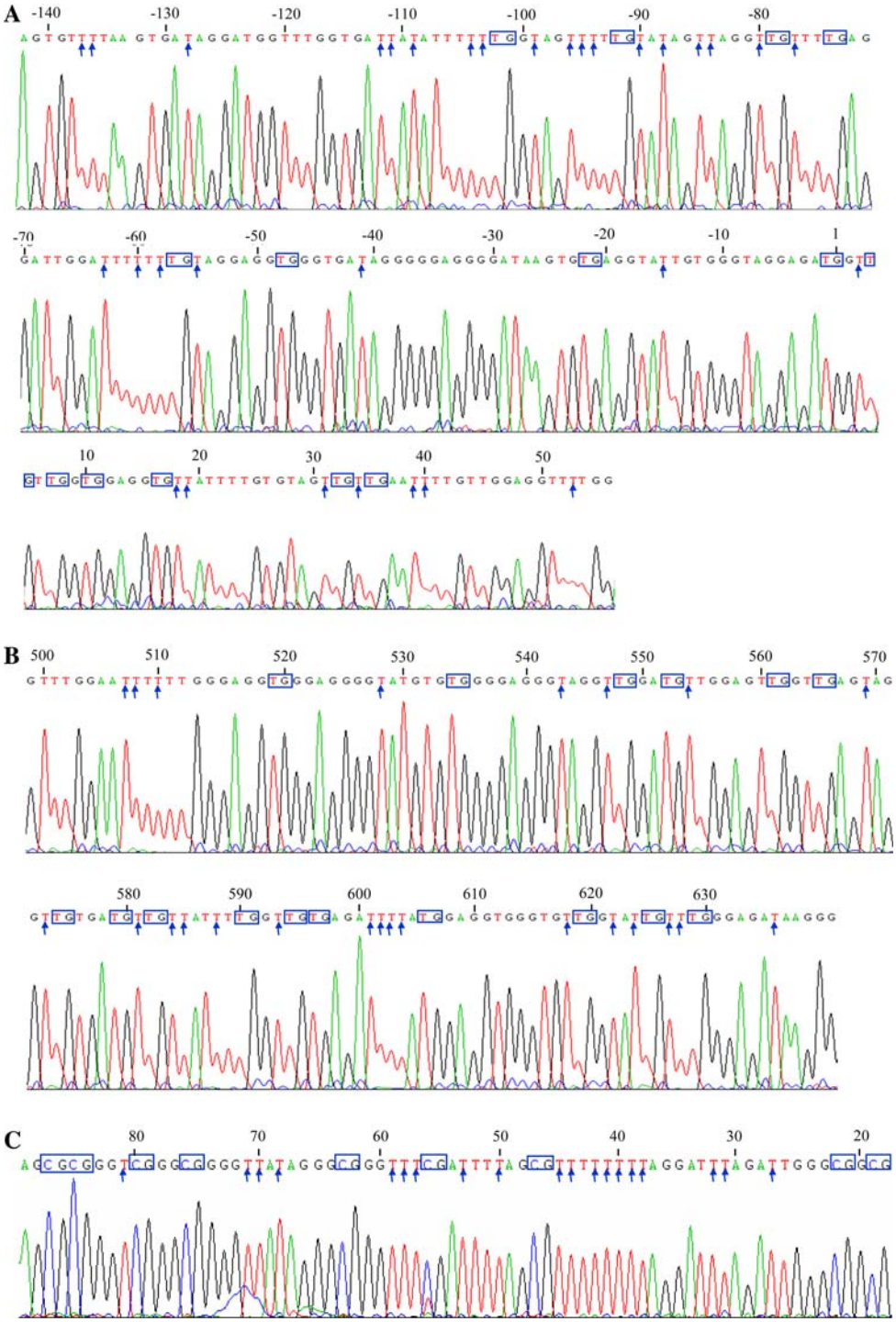

Fig. 3 Sequencing peak profiles of bisulfite genomic PCR. **a** Region 1 (204 bp); **b** Region 2 (164 bp) of the *H37* CpG island. Shown here are the representative sequencing pictograms showing no methylation at any of the CpG sites. **c** Positive control; *RASSF1A* CpG island showing methylation at CpG sites, in A549 cells. Original "C"ytosine residues converted to "T"hymidine after bisulfite treatment are indicated with blue arrows. Individual CpG sites, showing "T" converted from "C" (unmethylated, **a** and **b**) or unconverted "C" (methylated, **c**), are boxed in blue. Numbers above nucleotides indicate base pair (bp) position from the transcription start site at #1. For the *RASSF1A* CpG island analysis, PCR and sequencing primers used are taken from Damman et al. (2000), and shown here is only the 72 bp fragment taken from its entire CpG island as an example

insertion, etc.) in any of the samples examined nor sequence difference between tumor versus matching normal samples.

Discussion

Chromosomal loss and genetic aberrations in the 3p region, 3p21.3 in particular, represent one of the most prevalent as well as the earliest genetic disturbances observed in a wide variety of human cancers (Ji et al. 2005). Many of the 19 genes residing at 3p21.3 demonstrate a wide spectrum of tumor-suppressing activities involved in vital cell physiology such as cell proliferation, apoptosis, cell cycle kinetics, signaling transduction, and ion exchange and transportation (Table 2). Therefore, it has been proposed that the 3p21.3 genes "co-operate" as a "tumor suppressor region" where they interact one another in a network of tumor-suppressor

pathways. Elucidating molecular mechanisms for inactivation of the 3p21.3 TSGs in human tumorigenesis will be helpful in designing future translational strategy for using them as anticancer molecular therapeutic agents and targets. Here we summarized the 3p21.3 genes' inactivation mechanism and other genomic aberrances thus far observed in human cancers (Table 2). Mutations are rarely present in 3p21.3 genes, i.e. only a few mutations altering amino acid sequences found in *SEMA3B, FUS2, HYAL1, FUS1, BLU*, and *NPRL2*. In contrast, promoter hypermethylation appears to be the prevailing mechanism for inactivating 3p21.3 gene expression, as observed in a majority of tumor samples tested for *SEMA3F, GNAT1, SEMA3B, HYAL2, RASSF1A, BLU*, and *CACNA2D2* (Table 2). Targeting promoter hypermethylation holds particular promises for developing new therapeutics and diagnostics for lung cancer because DNA methylation can be easily detected using body fluids such as serum, urine, bronchioalveolar lavage and sputum and be easily reversed by demethylating drugs. Therefore, our hypothesis that the *H37* 3p21.3 TSG may also be inactivated in tumor by its promoter hypermethylation was certainly appealing.

Although we made hard efforts to analyze the entire *H37* CpG island of 949 bp, which tops the typical CpG island length spanning 0.2 to 1 kb, the technical difficulty only allow us to successfully amplify the two regions, i.e. Region 1 and 2 (Fig. 2). According to the two best recognized mechanisms for de novo methylation in TSG promoter CpG islands in cancer, (1) methylation spreads as "waves" from the adjacent, normal methylated flanks (i.e. Alu region) to encompass the entire CpG island (Graff et al. 1997); (2) random "seeding" of methylation at certain CpG sites within the island expands across the CpG island (Song et al. 2002). In support, most of the TSG CpG islands analyzed by bisulfite genomic sequencing for its entirety demonstrate methylation across the entire island with the area closest to the transcription start site most heavily methylated (Melki et al. 1999, 2000). Taken together, though we await improved techniques for confirmation on complete lack of methylation in the entire *H37* promoter CpG sites, it seems unlikely that the *H37* CpG island is methylated exclusively in those CpG sites outside of the two regions (which include the area encompassing the transcription start site) tested in the current study.

H37 gene/protein silencing demonstrated in primary lung tumors, therefore, is likely to be rendered by still another alternative mechanism of gene inactivation yet to be uncovered. Here we postulate on some of the possible ways the *H37* gene expression may have become partially lost in lung tumor cells;

1. Transcriptional defects; (a) Mutations within the *H37* promoter region, which can be extended further distant upstream of the area tested in the current study, interfere with transcription factor binding thus decreased transcription efficiency; (b) Transcriptional activator(s) or transcription factor(s) required for *H37* gene transcription are defected/inactivated due to certain genetic/biochemical disturbances occurred upstream of the putative, *H37* gene activation cascade; (c) Binding of aberrant transcriptional repressors (gain of function) in the *H37* promoter interferes with gene transcription.
2. Deficiency in post- transcriptional mRNA regulation; Malfunctions in any of the various multi-protein complexes involved in maturation of *H37* pre-mRNA, as well as aberration in *cis*-acting elements in its 3′ untranslated region, may lead to deficiency in *H37* mRNA available for protein translation. Alternatively, faster mRNA turnover caused by defects in transcript decay machinery may also contribute to the same effects. Post-transcriptional defect is particularly intriguing to us given that H37 is an mRNA binding protein, and several *H37* alternative splice variants are found in hematopoetic cells (Sutherland et al. 2005).
3. Defects in post-translational modification; Although this possibility is not likely to be the sole mechanism for H37 expression loss in tumor cells, which begins to occur at a mRNA level, it is conceivable that post-translational H37 protein inactivation may further contribute to its loss of function in tumor cells. In support, it has been suggested that reversible phosphorylation of H37 protein may be a mechanism underlying H37-modulated apoptosis (Shu et al. 2007). Interestingly, the one 3p21.3 gene already advanced to the phase I/II clinical trial, *Fus1*, is known to be inactivated in tumor solely by defects in post-translational modification, i.e. myristoylation (Uno et al. 2004).
4. Haploinsufficiency-related gene expression loss; The growing evidence suggests that haploinsufficiency [predisposition to cancer in hemizygous (+/−) state] may play a critical role in further inactivating the remaining wild type allele, especially, for the genes localized in the chromosomal region frequently undergoing heterozygous deletion, i.e. 3p21.3 (Uno et al. 2004). For example, loss of one allele due to chromosomal deletion is likely to cause decreased genomic stability, unbalanced chromosomal spatial symmetry, increased susceptibility to stochastic delays of gene initiation, altered transcriptional and translational stoichiometry, and/or interrupted gene expression, leading to decreased gene expression from a remaining allele (Uno et al. 2004). Therefore, it is possible that one allele loss caused by LOH of 3p21.3 may interfere with normal gene transcription from the remaining

Table 2 19 putative tumor suppressor genes at 3p21.3 and mechanisms of their gene/protein inactivation

Gene	Function	Tumor suppressor activity	Gene/protein inactivation mechanism and other genomic aberrance	References
RBM6	RNA-binding protein 6; Binds poly(G) RNA homopolymers in vitro	Unknown	Missense mutation in 1/39 LCCL[a]; Truncated protein caused by deletion of exon 5; Fusion to the CSF1R gene caused by t(3;5)(p21;q33) translocation	Gu et al. (2007), Sutherland et al. (2005)
H37/RBM5	RNA-binding protein 5; Binds poly(G) RNA homopolymers in vitro	Reduces cell proliferation and promotes apoptosis and cell cycle arrest in various cancer cells	No mutation found in 18 LCCL; Several alternative spliced variants; Antisense transcription	Sutherland et al. (2005)
SEMA3F	Secreted and membrane-associated proteins involved in nerve growth cone migration and cell motility and adhesion	Reduction in colony formation upon transfection into H1299 cells; Suppresses mouse fibrosarcoma line A9 xenograft growth; Inhibit tumor angiogenesis	No mutation found in 30 LCCL; Promoter methylation (in 206 bp immediate 3′ outside of CpG island) found in 8/8 LCCL, 4/4 melanoma cell lines, and 3/10 breast cancer cell lines	Kusy et al. (2005); Zabarovsky et al. (2002)
GNAT1	Guanine nucleotide-binding protein G(t), alpha-1 subunit; Encodes transducin protein isolated from the eye	Expressed stably in 15/15 chronic nasopharyngitis tissues, while absent or down-regulated in 24/33 specimens of NPC	No mutation found in 35 LCCL; promoter hypermethylation found in 33/33 primary NPC tissues and in 12/15 chronic nasopharyngitis tissues	Yi et al. (2007)
SLC38A3	Solute carrier family 38, member 3; involves H+ exchange and Na+ cotransport; mediates glutamine efflux/uptake	Downregulated after passage of microcell hybrid (human chromosome 3-mouse fibrosarcoma A9) in SCID mice	No mutation found in 38 LCCL	Kholodnyuk et al. (2006)
GNAI2	Guanine nucleotide-binding protein G(i), alpha-2 subunit; Inhibit adenylate cyclase in response to beta-adrenergic stimuli	Gαi2-deficient mice develop colonic adenocarcinoma	No mutation found in 34 LCCL; Missense mutations (Arg179Cys/His/Gly) reported in human endocrine tumors	Zhang et al. (2000)
SEMA3B	Extracellular secreted protein; Guide growth cones during neuronal development	Not expressed in many LCCL; Colony formation reduced in NSCLC upon gene transfection; Inhibit tumor angiogenesis	Three missense mutations found in 39 LCCL; promoter hypermethylation found in 4/8 NSCLC cell line, 11/27 NSCLC primary tumors, 29/35 HCCs and 15/15 patients with CC; methylation status correlated with 3p loss in neuroblastoma	Nair et al. (2007); Tischoff et al. (2005); Zabarovsky et al. (2002)
IFRD2	Interferon-related developmental regulator 2; predicted to be a soluble nuclear protein by PSORT	Unknown	No mutation found in 63 LCCL	Lerman and Minna (2000)
HYAL3	Hyaluronidase; Degrades hyaluronic acid which regulates cell proliferation, migration and differentiation	Unknown	No mutation found in 40 LCCL; Alternative splice variants result in enzymatically inactive protein; Frequent N-glycosylation sites (post-translational)	Lokeshwar et al. (2002)
FUS2/Nat6	Cytoplasmic *N*-acetyl transferase	Unknown	Four missense mutation found in 78 LCCL; Alternatively spliced variants (intronless vs. one intron present); SNP c767A/T (R222W) prevalent in Asian populations	Lerman and Minna (2000)

Table 2 continued

Gene	Function	Tumor suppressor activity	Gene/protein inactivation mechanism and other genomic aberrance	References
HYAL1	Hyaluronidase; Degrades hyaluronic acid which regulates cell proliferation, migration and differentiation	Functions as a tumor promoter and a suppressor, depending on the concentration, in prostate cancer; oncogenetic activity in bladder cancer	Two missense mutations found in 40 LCCL; two mutations identified in a patient with lysosomal disorder, mucopolysaccharidosis IX; Retention of 5′ end intron prevents translation in head and neck squamous cell carcinoma; Alternatively spliced variants resulting in enzymatically inactive protein in bladder/prostate cancer	Frost et al. (2000); Lokeshwar et al. (2005a); Lokeshwar et al. (2005b); Lokeshwar et al. (2002)
HYAL2	Hyaluronidase; serve as a receptor for the oncogenic virus Jaagsiekte sheep retrovirus	Adnoviral transduction reduces metastatic foci on the lung surface by 70%	No mutation (but one complete gene deletion) found in 40 LCCL; Loss of expression in adult brain due to promoter methylation; Sequestration by viral protein leads to release of oncogenic factor negatively controlled by Hyal2	Hesson et al. (2007); Zabarovsky et al. (2002)
FUS1	Unknown, Functional motifs predicts member of the novel cAMP dependent protein kinase A and A kinase-anchoring protein families	Overexpression suppresses lung tumor growth, inhibits lung metastases in mouse models, induces apoptosis and alters cell cycle kinetics	Three (deletion) mutations found in 79 LCCL leading to truncated protein; No evidence for hypermethylation of promoter; Lack of myristolation in fus1 protein in tumor (post translational defects)	Ji et al. (2002); Uno et al. (2004)
RASSF1A	Similar to the RAS effector proteins; Regulates apoptosis, microtubule dynamics, and cell-cycle progression via multiple effector proteins	Knockout mice have an increased susceptibility to a variety of cancers, including lung and gastrointestinal adenocarcinomas, particularly when exposed to carcinogens such as urethane, ionizing radiation and benzo(a)pyrene	No mutation found in 39 LCCL; Promoter hypermethylation observed in SCLC (80%), NSCLC (30%), breast (60%), bladder (35–62%), glioma (57%), hepatocellular (up to 100%), medulloblastoma (79%), NPC (67%), neuroblastoma (55%), testicular (up to 83%), most of these associated with loss of expression	Dammann et al. (2003); Hesson et al. (2007)
BLU/ZMYND10	Zinc finger MYND domain-containing protein; function unknown—most likely transcriptional regulator	Overexpression resulted in decreased colony and tumor formation in neuroblastoma, NSCLC and NPC tumor cells	Three missense mutations found in 61 LCCL; Hypermethylation and downregulation observed in glioma (80%), cervical squamous cell carcinomas (77%), NPC (66%), neuroblastoma (41–70%) and NSCLC (19–43%), gall bladder carcinomas (26%), ependymomas (13.6%) and SCLC (14%)	Hesson et al. (2007); Zabarovsky et al. (2002)
NPRL2/G21	Unknown	Overexpression inhibits growth and induces apoptosis in various tumor cell lines	Three mutations (1 stop, 2 missense) found in 38 LCCLs; Non-sense deletions and missense mutations found in ESTs from multiple tumor types; 3′ homozygous deletions possibly leading to improper splicing; No methylation detected in renal cancers	Hesson et al. (2007); Li et al. (2004)

Table 2 continued

Gene	Function	Tumor suppressor activity	Gene/protein inactivation mechanism and other genomic aberrance	References
101F6	Exact function unknown; Contains cytochrome b-561 ferric reductase transmembrane domain	Forced expression in NSCLC reduced cell viability by induction of apoptosis; systemic delivery via adenoviral vector reduces lung metastases in mice	No mutations found in 78 LCCL	Hesson et al. (2007); Ji et al. (2002)
PL6/TMEM115	Unknown; predicted to be an integral membrane protein	Not tested	No mutations found in 78 LCCL; No methylation found in NPC cell lines	Chow et al. (2004)
CACNA2D2	α-2-δ regulatory subunit of a voltage gated Ca2+ channel gene	Adenoviral transfection inhibits lung cancer cell growth both in vitro (with apoptosis) and in vivo	No mutations found in 100 LCCL; Promoter hypermethylation found frequent in NPC and glioma, but rare in respective primary tumors	Carboni et al. (2003); Hesson et al. (2007)

LCCL lung cancer cell lines, *NPC* nasopharyngeal carcinoma, *SCID* severe combined immunodeficiency, *SNP* single nucleotide polymorphism, *SCLC* small cell lung cancer, *NSCLC* non- small cell lung cancer

[a] Source of mutation analysis in LCCL listed at first for each gene: Lerman and Minna (2000)

H37 allele, and that *H37* gene/protein dosage in a tumor cell thus reduced below ~50% of the normal level may be sufficient to induce H37 mediated growth inhibition.

As it is becoming increasingly evident that loss of *H37* gene expression may, in part, contribute to lung tumorigenesis, understanding the mechanism of *H37* gene expression loss in tumor is important and warrants further investigation. Such knowledge may aid to develop a strategy of re-introducing the gene for future clinical application. It is hoped that our current research efforts to elucidate general cellular function(s) of H37 by identifying its protein interaction partners and downstream target genes may shed us new lights for understanding potential mechanism for *H37* gene loss in tumor.

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