Aberrant methylation and associated transcriptional mobilization of *Alu* elements contributes to genomic instability in hypoxia

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Abstract

Hypoxia is an integral part of tumorigenesis and contributes extensively to the neoplastic phenotype including drug resistance and genomic instability. It has also been reported that hypoxia results in global demethylation. Because a majority of the cytosine-phosphate-guanine (CpG) islands are found within the repeat elements of DNA, and are usually methylated under normoxic conditions, we suggested that retrotransposable *Alu* or short interspersed nuclear elements (SINEs) which show altered methylation and associated changes of gene expression during hypoxia, could be associated with genomic instability. U87MG glioblastoma cells were cultured in 0.1% O_2 for 6 weeks and compared with cells cultured in 21% O_2 for the same duration. Real-time PCR analysis showed a significant increase in SINE and reverse transcriptase coding long interspersed nuclear element (LINE) transcripts during hypoxia. Sequencing of bisulphite treated DNA as well as the Combined Bisulphite Restriction Analysis (COBRA) assay showed that the SINE loci studied underwent significant hypomethylation though there was patchy hypermethylation at a few sites. The inter-*alu* PCR profile of DNA from cells cultured under 6-week hypoxia, its 4-week revert back to normoxia and 6-week normoxia showed several changes in the band pattern indicating increased *alu* mediated genomic alteration. Our results show that aberrant methylation leading to increased transcription of SINE and reverse transcriptase associated LINE elements could lead to increased genomic instability in hypoxia. This might be a cause of genetic heterogeneity in tumours especially in variegated hypoxic environment and lead to a development of foci of more aggressive tumour cells.

Keywords: DNA methylation ● CpG islands ● SINE ● U87MG ● bisulphite sequencing ● genetic instability ● long-term hypoxia

Introduction

An unstable tumour genome is likely to generate a significant amount of genetic heterogeneity in solid tumours such as gliomas [1] with heterogeneous microenvironment. Active repeat elements are one of the many factors contributing to genomic instability. Repeat elements such as short interspersed nuclear elements (SINEs) and long interspersed nuclear elements (LINEs) consist of 35% of human genome [2]. In mammals the low basal level of SINE expression has been attributed to extensive methylation resulting in transcriptionally inactive heterochromatin [3]. Also, hypomethylation of LINE1 and SINEs has been associated with the clinicopathological behaviour of the tumours [4, 5]. SINEs are dependent on the reverse transcriptase activity of LINE 1 for retrotransposition and insertional mutagenesis [6]. SINE expression responds to external factors such as heat shock and stress [7]. We have previously shown that *alu* mediated recombination increases when cells are cultured in low doses of chemotherapeutic drug, cisplatin [8]. A recent study by Bennett *et al.* [9] suggests that *Alu* elements pose the largest transposon-based mutagenic threat to the human genome and play an important role in genetic variations in diseases.

Of the 70–80% of methylated CpG sites [10], the repetitive elements and areas of low CpG density are preferential targets of aberrant methylation [11]. DNA methylation is an important epigenetic phenomenon that has a significant role in long-term regulation of gene expression [12]. Although classical models propose that DNA methylation patterns are rigid [13], recent evidence suggests that site-specific DNA methylation is a dynamic process that depends on the interplay of several factors of the methylation machinery [14, 15].

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Alterations in methylation have been linked to tumorigenesis [16] and can further induce phenotypic and gene expression changes associated with targeted *de novo* epigenetic alterations important in tumour progression [17]. There is a general trend of hypomethylation in tumours [18] and the extent of genome wide hypomethylation in tumours parallels the degree of malignancy [19].

As tumour cells proliferate, inadequate blood supply and aberrant neovascularization result in hypoxic tumour interiors [20]. Decreased penetration of drugs and paucity of free radical generation under hypoxic conditions often lead to resistance of the tumour to radiotherapy and chemotherapy [21]. The change in the microenvironment due to hypoxia and other factors like lactic acidosis, decreased supply of nutrients, etc may result in local changes in the epigenetic pattern of the cell genome. Increased genomic instability and mutations under hypoxic conditions and genotoxic stress have also been demonstrated by Rice *et al.* [22], where a transient hypoxia followed by methotrexate exposure in normoxia led to a significant increase in methotrexate resistant cells with DHFR gene amplification.

Yuan *et al.* [23] have attributed the increased genetic instability observed in hypoxic cells to the compromised DNA repair gene activity particularly the MMR gene. They have observed a 5-fold increase in the mutation frequency in hypoxic cells using a λ phage shuttle vector with a reporter gene for identifying mutations [24]. The down-regulation of MMR and increased genetic instability has also been recently observed in stem cells, where reduced transcription activation of MMR genes has been attributed to specific epigenetic events [25]. Hypoxia and its associated factors are also linked to the process of aging [26].

We suggested that long-term hypoxic stress may also cause repeat element mediated genetic instability. We have studied changes in the methylation status of the CpG islands within and around the repetitive SINEs of a glial tumour cell line (U87MG) and an osteosarcoma cell line (SaOS2) after exposure to prolonged hypoxic conditions. Transcription of SINEs and LINEs was studied by real-time PCR and genomic instability was determined by inter-*alu* PCR.

Materials and methods

Cell line and culture

U87MG (glioblastoma cell line, wild-type p53) and SaOS2 (p53 null osteosarcoma cell line) lines have been used for all the experiments (Supporting Information). Experiments on normoxia and hypoxia were initiated simultaneously with similar passage number (12–15). Cells were cultured in 75 cm^2 and 25 cm^2 vented tissue culture flasks.

In vitro exposure to hypoxic condition

The vented cap flasks were kept in Anoxomat chambers (Mart® Microbiology, and the Anoxomat™ system) with appropriate O_2 concentrations for hypoxia (0.1%) and normoxia (21.0%). These atmospheres were created in the anaerobic chambers after vacuuming and then flooding the chambers with pure CO_2 and N_2 according to the set partial pressures. We frequently measured the partial pressure of oxygen with the help of dissolved oxygen (DO) meter in one of the control flask after 6 hrs to confirm the level of hypoxia. Culture media were pre-exposed to hypoxia for 2 hrs before feeding the cells. Cells were routinely passaged on attaining 75–80% confluency at a split ratio of 1:3. At the time of passaging, minimum possible time (5–10 min.) was spent for trypsinization and replating, and then the cells were immediately shifted to the hypoxic conditions. With the help of the DO meter we have also observed that atmospheric O_2 is not significantly miscible in media upto 10–15 min. As the duration of culture under hypoxia progressed, the requirement for passaging the cells decreased in frequency as a result of decrease in proliferation, but the media were replenished every 3 days. As a measure of cellular response to hypoxia, we have observed an increase in the induction of HIF-1 protein starting from 4 hrs and in 24 hrs of hypoxia indicating that cells respond to the hypoxic stress given (Fig. S1). Cells were in three groups (*i*) 6-week hypoxia (H) (*ii*) 6-week normoxia (N) and (*iii*) 6-week hypoxia followed by further 4-week reversion to normoxia (HR). DNA and RNA were extracted from cells after appropriate time-points. The experiments were repeated twice.

Cell viability studies

U87MG cells from same passage number were cultured for 6 weeks in hypoxia (0.1% O_2) and normoxia (21% O_2) conditions, respectively. A flask of hypoxic cells was reverted back to normoxia for 4 weeks. The cells from hypoxic, normoxic and hypoxia revertent conditions were plated in a 96-well plate at a density of 5×10^3 cells / 100 μl / well and subsequently cultured in their respective conditions and counted at 2, 4, 6 and 8 days of culture. Viability was measured by Trypan blue dye exclusion.

Morphology and caspase-3 cleavage for apoptosis

Morphological analysis was done for identifying features of apoptosis in U87MG and SaOS2 cells grown in hypoxia. The microphotographs were taken at various magnifications in cells grown under normoxia and hypoxia.U87MG cells subjected to hypoxia for various time-points and normoxia were lysed in a triple detergent buffer (sodium deoxycholate, NP-40 and SDS) and protein inhibitor cocktail (Amresco, Solon, OH, USA). Total protein was quantified using the BCA protein assay kit (Pierce, Rockford, IL, USA) and 20 μg protein was run on a 15% SDS-PAGE and electroblotted on a PVDF membrane (Schleicher and Schuell, Germany) with the Mini-Protean system from Bio-Rad (Hercules, CA, USA). The blots were checked for transfer with Ponceau S (Sigma-Aldrich, St. Louis, MO, USA) and blocked in 4% non-fat milk in TBS (Tris buffered saline). The blots were probed with the monoclonal anti-caspase-3 antibody (BD Pharmingen, San Diego, CA, USA) overnight, goat antimouse alkaline phosphatase linked IgG secondary antibody and developed with BCIP/NBT system (Promega, Madison, WI, USA).

Isolation of RNA and real-time RT-PCR for SINE (*alu*) and LINE expression

Total RNA was isolated from U87MG and SaOS2 cells using TRIZOL® (Invitrogen, Carlsbad, CA, USA) and reverse transcribed with RETROscript® reverse transcriptase kit (Ambion, Foster City, CA, USA). Real-time PCR

was done using intra-*alu* primers for SINE expression; L1-F and L1-R for LINE expression and β-actin primers (IDT, Coralville, IA, USA) as normalization control (Table 1) in RotorGene cycler (Corbett Research, Sydney, Australia) in two biological replicates.

Bisulphite treatment

Genomic DNA was extracted from experimental U87MG and SaOS2 cells. Bisulphite treatment of genomic DNA was done using EpiTect kit (Qiagen GmBH, Hilden, Germany). The DNA after bisulphite treatment was purified with Wizard^{TM} DNA Clean-up System (Promega).

Primers targeting CpG sites of SINE

SINE sequence at chromosome 16 nucleotide 12755020 to 12754683 (NW_926528.1) and chromosome 7 nucleotide 4397659 to 439838 (NT_079595.2) were chosen for their highest homology with the consensus SINE sequence using NCBI BLAST. CpG islands were identified within 500 bp of the above genomic sequence. Nested primers and M13 tagged inner primers were designed from the flanking regions of the sequences of interest (Table 1).

DNA methylation analysis by PCR sequencing

The PCR products were sequenced directly using primers designed specifically against one of the bisulphite-converted strand. The sequencing chromatograms were analysed for change in methylation status of the CpG islands by comparing height of peaks of T (indicating unmethylated cytosine) and the peaks of C (indicating methylated cytosine) at a CpG island (Supporting Information). Twenty-three sites on chromosome 16 and 25 sites on chromosome 7 could be reproducibly analysed.

Global methylation

Global methylation of the bisulphite treated DNA samples was assessed by Line COBRA using LINE 3 and LINE 4 primers (Table 1) as described by Matsuzaki *et al.* in two biological replicates [27]. The IDV of digested (methylated) and undigested (unmethylated) bands was determined by Chemi-Imager gel documentation system (Alpha Innotech Corp., San Leandro, CA, USA) to obtain the percent fractional methylation at the LINE TaqI site (Supporting Information).

HIF-1α and DNMT1 protein expression

U87MG cells exposed to normoxia and hypoxia (various time-points) were lysed in a triple detergent buffer (as described previously), 30 μg of total protein was resolved on a 7.5% SDS-PAGE. The gel was electroblotted onto a PVDF membrane (Schneider and Schuell) and blocked in 4% BSA overnight at 4°C. It was further probed with rabbit polyclonal anti-DNMT1 (Abcam, Cambridge, MA, USA) or mouse monoclonal anti-HIF-1α (BD Biosciences, San Jose, CA, USA), goat anti-rabbit or antimouse alkaline phosphatase linked IgG (Santa Cruz Biotech. Inc.,

Table 1 Primers used in the study

Primers	Sequence (5′–3′)	Annealing temperature (°C)
Bisulphite sequencing primers		
At chromosome 16		
16 outer F	TTTATAGGGTAGTAGTTTTAGTGGG	50
16 outer R	TATTTACAAAAAACCAAAAATTCAC	
16 inner F	AGTATAGGTGGGGAGGAAGTAGAG	50
16 inner R	TTTACAAAAAACCAAAAATTCACTATC	
At chromosome 7		
7 outer F	TGAATAGAATTTTAGAAATAGGA	46
7 outer R	CTAACAATTTTCATTCCAAC	
7 inner F	TATTAAGATTTATAGAGGTGG	52
7 inner R	ATTATTTAACTTTCAATAATCA	
LINE-COBRA primers		
LINE-3	GYGTAAGGGGTTAGGGAGTTTTT	50
LINE-4	AACRTAAAACCCTCCRAACCAAATATAAA	
Primers for qRT/RT		
Intra-*alu* F	CACGCCTGTAATCCCAGCAC	52
Intra-*alu* R	GGAGTCTCGCTCTGTCG	
L1 F	GCTGGATATGAAATTCTGGGTTGA	60
L1 R	AGGAAATACAGAGAACGCCACAA	
β-actin F	TCATGAAGTGTGACGTTGACATCCGT	52/60
β-actin R	CCTAGAAGCATTTGCGGTGCACGATG	
Inter-*alu* primers		
alu264	CAGAGCGAGACTCC	54
alu267	AGCGAGACTCCG	56

Franklin Lake, NJ, USA) and developed with BCIP/NBT (Promega) as per manufacturers protocol.

Statistical analysis

The change in methylation at site specific CpG at SINE loci on chromosome 16 and 7 was calculated for hypoxia (N − H = percentage methylation in N minus percentage methylation in H), for reverts with respect to normoxia (percentage methylation in N minus percentage methylation in HR) and for revert with respect to hypoxia (percentage methylation in H minus percentage methylation in HR). Statistical differences between test and control for each group were evaluated by paired two-tailed t-test for significance.

Inter-*Alu* PCR

Inter-*alu* primers were taken from Krajinovic *et al.* [28]. The conditions were followed as detailed in Srivastava *et al.* [8] (Table 1). The amplified
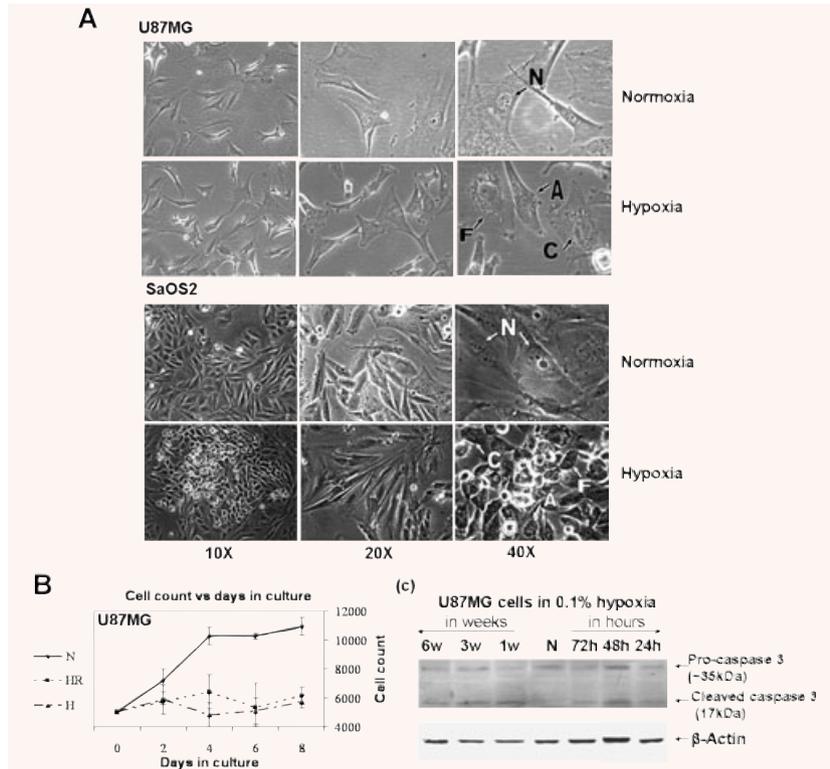
Fig. 1 (**A**) Morphological analysis: Effects of prolonged (6 weeks) normoxia (21% O_2) and hypoxia (0.1% O_2) on the morphology of the U87MG and SaOS2 cells. Characteristic signs of apoptosis like cellular shrinkage, nuclear condensation, blebbing of cell membrane in the form of apoptotic bodies are marked in hypoxic cells. N – Normal, A – Apoptotic bodies, F – Fragmentation, C – Condensation. (**B**) Cell viability assay: Trypan blue stained cell counting was done for measuring survival in hypoxia and normoxia treated U87MG cells with equal number of seeding (5×10^3 cells) per well. The cells cultured initially in hypoxia and normoxia conditions were continued in the same condition (N or H) or reverted back to normoxia (HR). (**C**) Caspase-3 cleavage for apoptosis: Western blot for pro-caspase 3 and cleaved caspase-3 in cell extracts. Cleaved caspase was observed in U87MG cells exposed to 0.1% hypoxia at various time-points including 6-week hypoxia.

products were resolved in native 8% PAGE on Midi protean apparatus (Bio-Rad). Amplification patterns of the samples were obtained and altered bands were identified as either loss/gain or changes in intensity in H and HR samples as compared to N. Each PCR was performed on two biological replicates, repeated at least three times and scoring was done by two independent observers.

PAGE elution, cloning and sequencing

Altered bands were selected and carefully excised from H samples for elution (Supporting Information). They were cloned in PGEMTeasy (Promega) and sequenced in an ABI prism sequencer (Applied Biosystems, Foster City, CA, USA). The sequence analysis was done using MegaBLAST and repeatBLAST and homologies were further analysed for flanking *alu* sequences.

Results

Growth characteristics after exposure to long-term hypoxia

U87MG cells cultured in 0.1% O_2 demonstrated evidence of HIF-1α protein expression (Fig. S1) by 4 hrs. The cells also showed characteristic signs of apoptosis like cellular shrinkage, nuclear condensation and membrane blebbing in the form of apoptotic bodies

even at 6 weeks (Fig. 1A). Similar characteristic changes were also observed in SaOS2 cells. The apoptotic feature of cleaved caspase was also seen in U87MG cell lysates at 1, 2 and 3 days of hypoxia and was found to persist till at least 6 weeks of hypoxic treatment (Fig. 1C).

The growth rate of the U87MG cells was determined on cells cultured for 4 weeks under hypoxic and normoxic conditions and replated in 96-well plates. Maximum growth was noted in cells grown continuously in 21% O_2 (N) (Fig. 1B). When cells were cultured in hypoxia (H), the growth rate was less than in N, but more than the cells reverted to normoxia for 4 weeks after initial culture in hypoxia for 6 weeks (HR). The cells cultured in hypoxia (H) and the revertent HR showed very slow growth rate and remained less than N throughout the assay duration.

By day 8, cell proliferation of N and H were significantly different, demonstrating a decrease in cell growth after long-term hypoxia. HR had a significantly lower growth rate compared to N but not H (Fig. 1B) indicating that the decrease in growth rate may be an irreversible phenomenon.

Expression of SINE and LINE under hypoxia

Real-time PCR and RT-PCR was done with cDNA prepared from total RNA extracted from cells cultured in hypoxia, normoxia and hypoxia of 6 weeks reverted to 4-week normoxia. Average values of two biological replicates are shown. After 6-week hypoxia, there

Fig. 2 SINE (*alu*) and LINE transcript levels: Relative expression of (**A**) SINE and (**B**) LINE in U87MG and SaOS2 cell lines under normoxia, hypoxia and 4-week revert to normoxia by real-time PCR. The SINE and LINE expression are markedly increased in 6-week hypoxia as shown by average values from two biological replicates. Representative photographs of RT-PCR are shown in inset. The values are normalized with respect to β-actin.
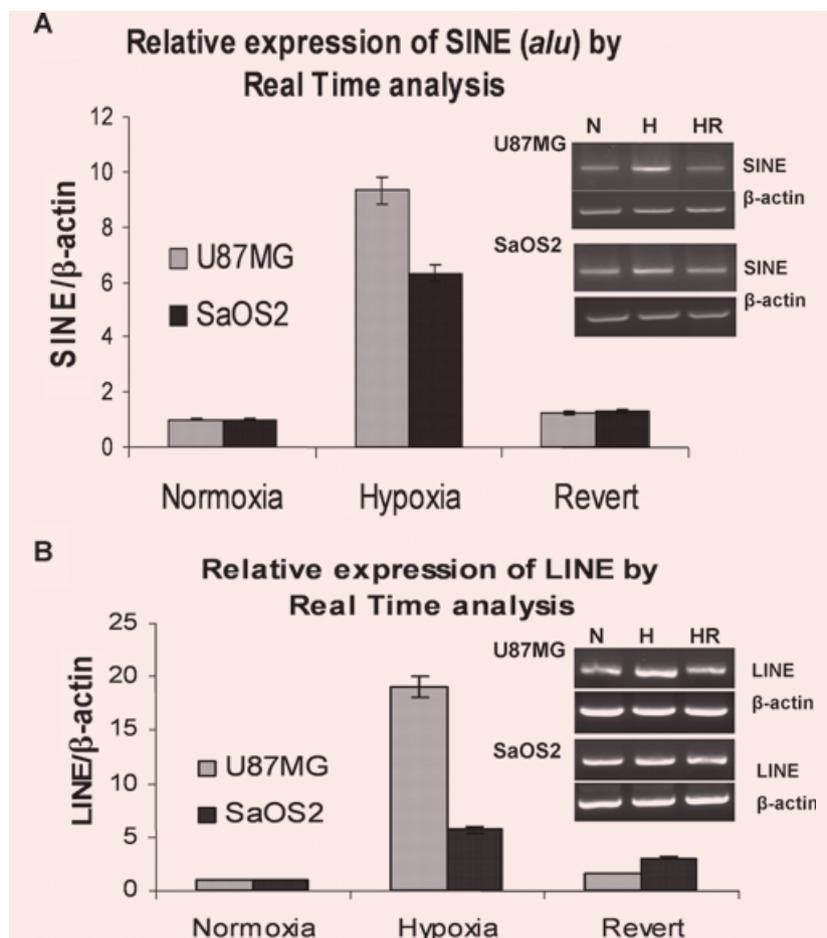
was a 9.57-fold increase in relative expression of SINE in U87MG cells as compared to normoxia ($P = 0.0008$). In case of SaOS2 the increase in the expression level is approximately 6.30-fold in hypoxia ($P = 0.0004$). SINE transcripts levels increased only by 1.29-fold in U87MG and 1.36-fold in SaOS2 revertents (Fig. 2A).

Similarly, the relative expression of LINE 1 also increased by 19.51-fold ($P = 0.0006$) and 5.88-fold ($P = 0.001$) in U87MG and SaOS2 cell exposed to 6-week hypoxia (Fig. 2B). When cells were reverted back to normoxia for 4 weeks, the increase was only 1.68-fold in U87MG and 3.21-fold in SaOS2.

Methylation analysis by bisulphite sequencing

Methylation analysis was done in consensus SINE locus on two chromosomes Chr16 and Chr7 in both U87MG and SaOS2. Tendency for a particular CpG site to show hypo- or hypermethylation was consistent in all the biological replicates for both the cell lines.

From the consensus SINE locus of chromosome 16, 23 CpG sites were chosen for bisulphite PCR sequencing. In U87MG, there was statistically significant decrease in methylation status after exposure to long-term hypoxic conditions which did not change

even after reverting back to normoxic conditions. Seventeen sites showed average decrease in methylation by 9.76% (from 91.37% to 81.63%) ($P = 0.01$) in hypoxia with respect to normoxia (Fig. 3A-*i*) (Fig. S2). Major hypomethylation was seen at two specific CpG sites (site14: from 91.6% to 47.8% and site17: from 77.1% to 34.2%). Of these two sites, site 17 showed no change in methylation on reversion to normoxia, while at site 14 there was some increase in methylation in revertent (62.8%). The average percentage of methylation of these 17 sites in revertents was 81.5%, *i.e.* there was almost no change (0.05%) in methylation in comparison to hypoxia ($P = 0.97$) (Fig. 3A-*i*).

The other six CpG sites at chromosome 16 studied in U87MG behaved differently and showed either increase or no change in the methylation status due to hypoxia in comparison to normoxia (Fig. S2). The average increase in methylation in H at these sites was 5.05% ($P = 0.027$) and in HR there was significant decrease of 11.16% in comparison to H ($P = 0.007$) (Fig. 3 A-*i*). Methylation of reverted cells was even lower than the original normoxic levels ($P = 0.02$). The results point to the reversible nature of these changes.

Bisulphite sequencing of chromosome 16 in SaOS2 showed 13 sites with significant average decrease of methylation in hypoxia by
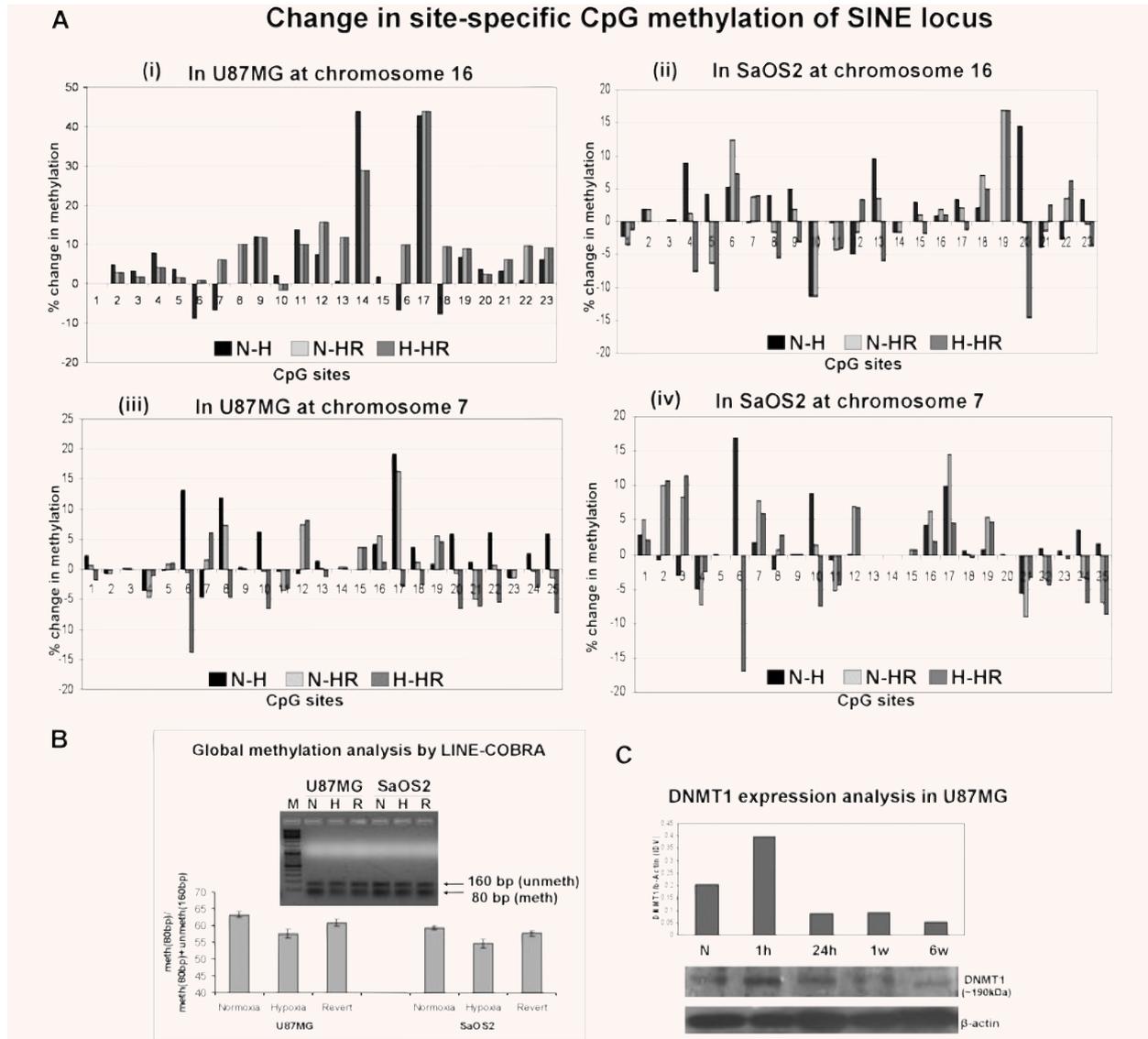
Fig. 3 (**A**) Change in percentage (%) of site-specific CpG methylation at SINE locus (*i*) at chromosome 16 in U87MG, (*ii*) at chromosome 16 in SaOS2, (*iii*) at chromosome 7 in U87MG, (*iv*) at chromosome 7 in SaOS2. Change is calculated for test group as follows: N(-)H: percentage methylation in normal minus percentage methylation in hypoxia; N(-)R: percentage methylation in normal minus percentage methylation in hypoxia; H(-)HR: percentage methylation in hypoxia minus percentage methylation in revert. *x*-axis: CpG sites; *y*-axis: percentage change in methylation. (**B**) Global methylation assay: LINE-COBRA was done in two biological replicates of normoxia (N), hypoxia (H) and reverted samples (HR) of U87MG and SaOS2 cells and the average value is depicted. In the representative picture shown, the band of 160 bp shows the unmethylated fraction and 80 bp is of methylated fraction. Densitometry shows global hypomethylation in hypoxia samples as compared to normoxia controls in both U87MG ($P = 0.033$) and SaOS2 ($P = 0.032$) cells. (**C**) DNMT1 expression: Western blot of U87MG cells exposed to various durations of 0.1% hypoxia showed a marked decrease in DNMT1 levels in 6-week hypoxia (H) as compared to N. The sudden increase in DNMT1 expression in 1 hr hypoxia was followed by reduced expression in 24 hrs, 1 week and 6 weeks. The IDV values are normalized to β-actin levels.

4.98% ($P = 0.0005$) (Fig. 3A-*ii*) from 92.23% to 87.26% (Fig. S3). Ten sites (which showed either increase or no change in methylation) showed significant ($P = 0.03$) average increase of 2.71% in methylation (from 89.61% to 92.32%) as compared to normoxia (Fig. 3A-*ii*).

Similarly, the locus on chromosome 7 showed significant average decrease by 5.35% (Fig. 3A-*iii*) from 94.43% to 89.08% in a major group of 16 CpG sites (Fig. S4) in hypoxic U87MG cells ($P = 0.001$). The other minor group of nine sites showed

average increase by 1.24% from 94.03 to 95.28% ($P = 0.052$) (Fig. 3A-*iii*).

The corresponding locus in SaOS2 also showed a major group of 16 sites undergoing average decrease of methylation by 3.33% from 97.42% to 94.08% ($P = 0.012$) in hypoxia as compared to normoxia (Fig. 3A-*iv*). The other group of nine sites showed average increase by 1.89% from 93.42 to 95.32% ($P = 0.03$) (Fig. 3A-*iv*) (Fig. S5).

Global methylation assay

Normoxic (N), hypoxic (H) and reverted (HR) U87MG cells showed an average global methylation from two biological replicates of 63.35%, 57.7% and 60.95%, respectively (H < N, $P = 0.033$), while the methylation of SaOS2 cells showed 59.45%, 54.7% and 57.75%, respectively (H< N, $P = 0.032$) (Fig. 3B). Representative photograph of a LINE-COBRA is shown in Fig. 3B [23].

DNMT1 protein expression

The DNMT1 expression was markedly reduced in U87MG cells exposed to 6-week hypoxia. Although the DNMT1 expression of U87MG cells showed a steep rise in expression immediately on exposure to hypoxia, the levels reduced in 24 hrs and subsequent time-points. This phenomenon was observed in two biological replicates and a representative Western blot is shown (Fig. 3C).

Inter-*alu* PCR for genetic instability

Inter-*alu* PCR is a modified fingerprinting technique to study genome instability [8, 28–30]. PCR product amplified from DNA extracted from H and HR were compared with N. A number of changes in intensity were observed, while clear gains or losses of bands were few and difficult to analyse. We were able to carefully extract and sequence four alterations which were gain of intensity (marked a, b, c and d in Fig. 4).

Although three of the sequenced bands showed homology to sequences flanked by inverted *alu* elements, one of the smaller bands (400 bp, 'd') showed homology to chr 14 (AL355834.4) and *alu* at only one end indicating that a single priming event had taken place. Two altered bands of size ~1 kb ('a') and 500 bp ('c') showed a change in intensity. The 1 kb (loss of intensity in revert) band showed homology to 963 bp on chromosome 8 (nt5375089–5376042) and is flanked by Zn finger motif and BTB domain 214048 bp on its 5′ side and by a hypothetical protein 41,263 bp at 3 side. The ~500 bp band ('c') showed homology to chromosome 9 (NT_024000.16) and encodes a pseudogene similar to part of translocation protein (myeloid leukaemia associated protein, SET), the 5 end of the gene codes for euchromatic histone methyltransferase1 and it houses two CpG islands. One of the bands visualized as gain of intensity ~500 bp (marked 'b') showed homology to homo sapiens F-box protein 27 (FBXO27),
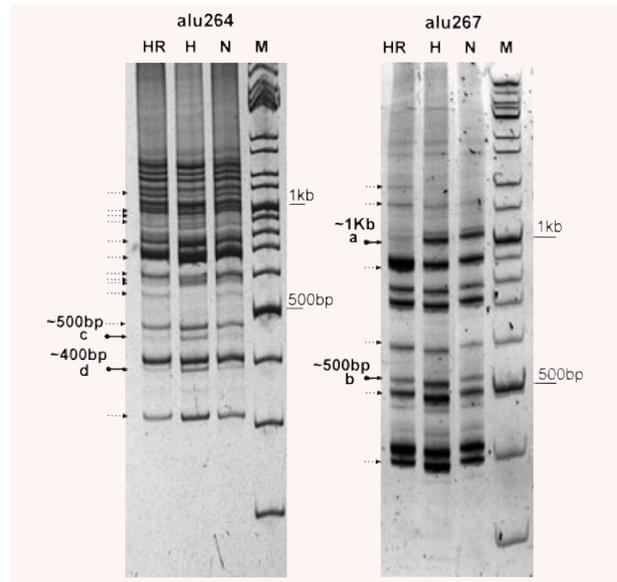

Fig. 4 Inter-*alu* PCR for detecting genomic instability: Representative inter-*Alu* PCR profile generated for normoxia, hypoxia and revert with primers alu264 and alu267 in U87MG cells. Photographs have been inverted for better visualization. A number of alterations are observed, mostly as changes in intensity (·····▶). The alterations which were cloned and sequenced are marked differently (●──▶), and these changes were consistent among biological replicates.

mRNA (NM_178820) which forms a part of the SCF complexes acting as protein-ubiquitin ligases. F-box proteins interact with S-phase kinase protein (SKP-1), through the F box.

Discussion

In solid tumours; microenvironment, hypoxia, increased genomic instability and mutation have been demonstrated to be associated in numerous studies [31]. Cross-talk between tumorigenesis, hypoxia and methylation pattern may determine the pathophysiology of oncogenesis and tumour metastasis [32–34]. Increase in radio and chemo resistance with increase in intra-tumour hypoxia may also be correlated with the change in methylation pattern [35, 36]. However not much is known about the transcription and methylation status of repeat elements under long-term hypoxia and consequent effects on genomic instability.

We have assessed the increase in transcript levels of *alu* (SINE) and of LINE, which is a source of reverse transcriptase. We have also observed genomic instability with the help of inter-*alu* PCR, possibly as a consequence of mobilization of the increased SINE transcripts. Our results have correlated this increase in transcript to aberrant methylation of the CpG islands, particularly at the SINE loci, under hypoxic conditions.

In our system, the cells lost their proliferative capability to a significant extent after being cultured in hypoxic conditions for 3 weeks, which could not be regained even after reverting back to normoxia. Hypoxia and HIF are known to modulate a number of cell cycle regulators and metabolic pathways which may result in a decrease in proliferating capability [37].

Our results show that global demethylation with site specific changes in the SINE loci were accompanied by increase in SINE expression. The additional increase in LINE1 transcript enhances the chance of SINEs being actively retrotransposed. *Alu* mediated recombination contributing to genetic instability in hypoxic cells was observed with the help of inter-*alu* PCR. Inter-*alu* PCR provides a good 'non-invasive' analysis of the cellular microsystem, which is highly responsive to stress. Cells with acquired genetic instability under hypoxic conditions may be selected over its normal counterpart and may or may not persist with same genetic signature even after reversal to normoxia depending on whether such instability gives a potential growth/survival advantage. Alteration of a band can occur if the particular fragment has duplicated and/or transposed to other chromosomal location. Inter-*alu* PCR also identifies specific sequences which are associated with instability. *Alu* elements are largely present in the non-coding part of the genome, and detecting an alteration associated with flanking *alu* within an mRNA is of considerable interest.

Previously ignored as junk, repetitive *Alu* elements are now known to modulate gene expression by alternate splicing, RNA editing and translation regulation [38–40] and may generate new enhancers, promoters and polyadenylation signals to many genes. Inverted *alu* are particularly unstable and are potential targets of RNA editing. Athanasiadis *et al.* [40] demonstrated favoured A-I editing when two inverted *Alu* element are present in close proximity (<2 kb). Such aberrant editing is found in several neurological disorders [41] and many such undiscovered potential transcriptomes may hold great physiological or pathological importance [42].

In this study we have shown data for four alterations, one of which is within an mRNA sequence of F-box protein 27. Sequence analysis shows that the F-box mRNA is interspersed with *alu* elements including two in reverse orientation. Incidentally its associate, SKP-1, is known to be regulated by hypoxia and HIF-1 [43, 44], and in light of this, the functional relevance of our observations is being studied. The mechanism of retrotransposition or the chromosomal localization of the recombined fragments flanked by SINE elements is yet to be addressed.

To the best of our knowledge, our observations on long-term hypoxia resulting in increased expression of SINE elements, possibly as a result of hypomethylation and subsequent *alu* mediated genetic instability, are a novel finding. Although *alu* mediated recombination and instability has been associated with a number of diseases, the importance of the methylation status of repeat elements and their expression in hypoxic regions of solid tumours needs to be further studied. This genomic instability may lead to evolution of a low-grade tumour to a high-grade one and hence needs to be addressed in order to counter the adverse phenotype associated with hypoxia.

Acknowledgements

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 HIF-1 induction in U87MG cells exposed to 0.1% hypoxia.

Fig. S2 U87MG site specific CpG methylation at SINE locus on chromosome 16.

Fig. S3 SaOS2 site specific CpG methylation at SINE locus on chromosome 16.

Fig. S4 U87MG site specific CpG methlation at SINE locus on chromosome 7.

Fig. S5 SaoS2 site specific CpG methylation at SINE locus on chromosome 7.

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References

1. **Misra A, Chattopadhyay P, Dinda AK, et al.** Extensive intra-tumor heterogeneity in primary human glial tumors as a result of locus non-specific genomic alterations. *J Neurooncol.* 2000; 48: 1–12.

2. **Smit AF**. The origin of interspersed repeats in the human genome. *Curr Opin Genet Dev*. 1996; 6: 743–8.

3. **Arnaud P, Goubely C, Pélissier T *et al.*** SINE retroposons can be used in vivo as nucleation centers for de novo methylation. *Mol Cell Biol.* 2000; 20: 3434–41.

4. **Cho N-Y, Kim B-H, Choi M *et al.*** Hypermethylation of CpG island loci and hypomethylation of LINE-1 and Alu repeats

in prostate adenocarcinoma and their relationship to clinicopathological features. *J Pathol.* 2007; 211: 269–77.

5. **Choi I-S, Estecio MRH, Nagano Y *et al.*** Hypomethylation of LINE-1 and Alu in well-differentiated neuroendocrine tumors (pancreatic endocrine tumors and carcinoid tumors). *Mod Pathol.* 2007; 20: 802–10.

6. **Boeke JD.** LINEs and Alu-the polyA connection. *Nat Genet.* 1997; 16: 6–7.

7. **Liu W-M, Chu W-M, Choudary PV *et al.*** Cell stress and translational inhibitors transiently increase the abundance of mammalian SINE transcripts. *Nucleic Acids Res.* 1995; 23: 1758–65.

8. **Srivastava T, Seth A, Datta K *et al.*** Inter-alu PCR detects high frequency of genetic alterations in glioma cells exposed to sub-lethal cisplatin. *Int J Cancer.* 2005; 117: 683–9.

9. **Bennett EA, Keller H, Mills RE *et al.*** Active Alu retrotransposons in the human genome. *Genome Res.* 2008; 18: 1875–83.

10. **Ehrlich M, Gama-Sosa MA, Huang LH *et al.*** Amount and distribution of 5-methyl-cytosine in human DNA from different types of tissues of cells. *Nucleic Acids Res.* 1982; 10: 2709–21.

11. **Gardiner-Garden M, Frommer M.** CpG islands in vertebrate genomes. *J Mol Biol.* 1987; 196: 261–82.

12. **Razin A, Cedar H.** DNA methylation and gene expression. *Microbiol Rev.* 1991; 55: 451–8.

13. **Costello JF, Plass C.** Methylation matters. *J Med Genet.* 2001; 38: 285–303.

14. **Métivier R, Gallais R, Tiffoche C *et al.*** Cyclical DNA methylation of a transcriptionally active promoter. *Nature.* 2008; 452: 45–50.

15. **Kangaspeska S, Stride B, Métivier R *et al.*** Transient cyclical methylation of promoter DNA. *Nature.* 2008; 452: 112–5.

16. **Watson RE, Curtin GM, Doolittle DJ *et al.*** Progressive alterations in global and GC-Rich DNA methylation during tumorigenesis. *Toxicol Sci.* 2003; 75: 289–99.

17. **Dumont N, Wilson MB, Crawford YG *et al.*** Sustained induction of epithelial to mesenchymal transition activates DNA methylation of genes silenced in basal-like breast cancers. *Proc Natl Acad Sci USA.* 2008; 105: 14867–72.

18. **Shahrzad S, Bertrand K, Minhas K *et al.*** Induction of DNA hypomethylation by tumor hypoxia. *Epigenetics.* 2007; 2: 119–25.

19. **Dunn BK.** Hypomethylation: One side of a larger picture. *Ann N Y Acad Sci.* 2003; 983: 28–42.

20. **Brahimi-Horn C, Chiche J, Pouysségur J.** Hypoxia signalling controls metabolic demand. *Curr Opin Cell Biol.* 2007; 19: 223–9.

21. **Fanga JS, Gilliesb RD, Gatenbyb RA.** Adaptation to hypoxia and acidosis in carcinogenesis and tumor progression. *Semin Cancer Biol.* 2008; 18: 330–7.

22. **Rice GC, Hoy C, Schimke RT.** Transient hypoxia enhances the frequency of dihydrofolate reductase gene amplification in Chinese hamster ovary cells. *Proc Natl Acad Sci USA.* 1986; 83: 5978–82.

23. **Yuan J, Narayanan L, Rockwell S *et al.*** Diminished DNA repair and elevated mutagenesis in mammalian cells exposed to hypoxia and low pH. *Cancer Res.* 2000; 60: 4372–6.

24. **Reynolds TY, Rockwell S, Glazer PM.** Genetic instability induced by the tumor microenvironment. *Cancer Res.* 1996; 56: 5754–7.

25. **Rodríguez-Jiménez FJ, Moreno-Manzano V, Lucas-Dominguez R *et al.*** Hypoxia causes downregulation of mismatch repair system and genomic instability in stem cells. *Stem Cells.* 2008; 26: 2052–62.

26. **Katschinski DM.** Is there a molecular connection between hypoxia and aging? *Exp Gerontol.* 2006; 41: 482–4.

27. **Matsuzaki K, Deng G, Tanaka H *et al.*** The relationship between global methylation level, loss of heterozygosity, and microsatellite instability in sporadic colorectal cancer. *Clin Cancer Res.* 2005; 11: 8564–9.

28. **Krajinovic M, Richer C, Labuda D *et al.*** Detection of a mutator phenotype in cancer cells by inter-alu polymerase chain reaction. *Cancer Res.* 1996; 56: 2733–7.

29. **Kaku H, Ito S, Ebara S *et al.*** Positive correlation between allelic loss at chromosome 14q24–31 and poor prognosis of patients with renal cell carcinoma. *Urology.* 2004; 64: 176–81.

30. **Bonafé M, Cardelli M, Marchegiani F *et al.*** Increase of homozygosity in centenarians revealed by a new inter-Alu PCR technique. *Exp Gerontol.* 2001; 36: 1063–73.

31. **Bristow RG, Hill RP.** Hypoxia, DNA repair and genetic instability. *Nat Rev Cancer.* 2008; 8: 180–92.

32. **Wainfan E, Poirier LA.** Methyl groups in carcinogenesis: effects on DNA methylation and gene expression. *Cancer Res.* 1992; 52: 2071–7.

33. **Herman JG, Baylin SB.** Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med.* 2003; 349: 2042–54.

34. **Jones PA, Baylin SB.** The fundamental role of epigenetic events in cancer. *Nat Rev Genet.* 2002; 3: 415–28.

35. **Vaupel P, Schlenger K, Knoop C *et al.*** Oxygenation of human tumors: evaluation of tissue oxygen distribution in breast cancers by computerized O_2 tension measurements. *Cancer Res.* 1991; 51: 3316–22.

36. **Höckel M, Vaupel P.** Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. *J Natl Cancer Inst.* 2001; 93: 266–76.

37. **Gardner LB, Corn PG.** Hypoxic regulation of mRNA expression. *Cell Cycle.* 2008; 7: 1916–24.

38. **Stuart JJ, Egry LA, Wong GH *et al.*** The 3'-UTR of human MnSOD mRNA hybridizes to a small cytoplasmic RNA and inhibits gene expression. *Biochem Biophys Res Commun.* 2000; 274: 641–8.

39. **Landry JR, Medstrand P, Mager DL.** Repetitive elements in the 5'-untranslated region of a human zinc-finger gene modulate transcription and translation efficiency. *Genomics.* 2001; 76: 110–6.

40. **Athanasiadis A, Rich A, Maas S.** Widespread A-to-I RNA editing of Alu-containing mRNAs in the human transcriptome. *PLoS Biol.* 2004; 2: e391.

41. **Gurevich I, Tamir H, Arango V *et al.*** Altered editing of serotonin 2C receptor pre-mRNA in the prefrontal cortex of depressed suicide victims. *Neuron.* 2002; 34: 349–56.

42. **Hasler J, Strub K.** Alu elements as regulators of gene expression. *Nucleic Acid Res.* 2006; 34: 5491–7.

43. **Tan M, Gu Q, He H *et al.*** SAG/ROC2/RBX2 is a HIF-1 target gene that promotes HIF-1 alpha ubiquitination and degradation. *Oncogene.* 2008; 27: 1404–11.

44. **Paltoglou S, Roberts BJ.** HIF-1alpha and EPAS ubiquitination mediated by the VHL tumour suppressor involves flexibility in the ubiquitination mechanism, similar to other RING E3 ligases. *Oncogene.* 2007; 26: 604–9.