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Functional role of the *KLF6* tumour suppressor gene in gastric cancer

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ABSTRACT

Gastric cancer is the second most common cancer and a leading cause of cancer-related death worldwide. The Kruppel-like factor 6 (*KLF6*) tumour suppressor gene had been previously shown to be inactivated in a number of human cancers through loss of heterozygosity (LOH), somatic mutation, decreased expression and increased alternative splicing into a dominant negative oncogenic splice variant, *KLF6*-SV1. In the present study, 37 gastric cancer samples were analysed for the presence of loss of heterozygosity (LOH) of the *KLF6* locus and somatic mutation. In total, 18 of 34 (53%) of the gastric cancer samples analysed demonstrated *KLF6* locus specific loss. Four missense mutations, such as T179I, R198G, R71Q and S180L, were detected. Interestingly, two of these mutations R71Q and S180L have been identified independently by several groups in various malignancies including prostate, colorectal and gastric cancers. In addition, decreased wild-type KLF6 (wtKLF6) expression was associated with loss of the *KLF6* locus and was present in 48% of primary gastric tumour samples analysed. Functional studies confirmed that wtKLF6 suppressed proliferation of gastric cancer cells via transcriptional regulation of the cyclin-dependent kinase inhibitor p21 and the oncogene c-myc. Functional characterisation of the common tumour-derived mutants demonstrated that the mutant proteins fail to suppress proliferation and function as dominant negative regulators of wtKLF6 function. Furthermore, stable overexpression of the R71Q and S180L tumour-derived mutants in the gastric cancer cell line, Hs746T, resulted in an increased tumourigenicity *in vivo*. Combined, these findings suggest an important role for the *KLF6* tumour suppressor gene in gastric cancer development and progression and identify several highly cancer-relevant signalling pathways regulated by the *KLF6* tumour suppressor gene.

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1. Introduction

Gastric cancer is the second most common cancer in the world and one of the leading causes of cancer death worldwide.[1] Many well-characterised tumour suppressor and oncogenes have been analysed to better define their respective roles in gastric cancer development and progression, but only a few consistent and frequent genetic alterations have been identified.[2] Germline mutations in the *E-cadherin* tumour suppressor gene, loss and somatic mutation of the *p53* tumour suppressor gene, oncogenic activation of *β-catenin* and *K-ras* mutations have been found in a subset of gastric cancers.[2]

Kruppel-like factor 6 (*KLF6*) belongs to the Kruppel-like family of transcription factors, which have been shown to play roles in the regulation of diverse cellular processes including development, differentiation, proliferation and apoptosis.[3] Functional inactivation of the *KLF6* gene has been implicated in a number of human cancers including prostate,[4,5] colorectal,[6,7] non-small cell lung,[8,9] gastric,[10] glioma,[11,12] nasopharyngeal,[13] hepatocellular,[14–17] pancreatic[18] and ovarian carcinomas.[19] In contrast to these studies, several groups have failed to identify somatic mutations in the *KLF6* gene.[20–22] These reported differences in mutational frequency highlight important differences in sample selection, numbers of samples, tissue isolation and the analytic techniques used. Further evidence supporting a role for *KLF6* in tumour development is the reported association between reduced *KLF6* expression and decreased patient survival in prostate[23–25] and lung cancers.[26] Depending on cell type and context, *KLF6*'s growth suppressive properties have been associated with a number of highly relevant cancer pathways, including p53-independent upregulation of *p21*,[4] *E-cadherin*,[27] disruption of *cyclin D1* and *CDK4* interaction,[28] induction of apoptosis[8] and *c-jun* inhibition.[29] Most recently, we have shown that a *KLF6* single nucleotide polymorphism (SNP) is associated with an increased prostate cancer risk.[30] Previous studies in gastric cancer[10] had described LOH and mutation in the *KLF6* gene in sporadic gastric cancer, mainly in the intestinal type. Interestingly, the *KLF6* mutations were prevalent in advanced patient samples with an evidence of lymph node metastases.[10] In the present study, we sought to determine the *KLF6* LOH, mutation status and expression levels in a cohort of gastric cancer patient samples, and to determine the biological role of the *KLF6* tumour suppressor gene in gastric cancer cells.

2. Material and methods

2.1. Tumour samples, preparation and DNA isolation

Tumour specimens were collected and analysed under IRB approval. Human gastric cancer and adjacent non-cancer tissues were obtained from gastric cancer patients in Prince of Wales Hospital of Hong Kong during endoscopy or surgery. Matched non-cancer gastric samples were obtained at least 2 cm distant from the tumour in which tumour cell infiltration was ruled out by histologic assessment. All specimens were snap frozen and stored at –80 °C. All patients gave informed written consent for obtaining the specimens, and the study was approved by the Clinical Research Ethics Committee of the Chinese University of Hong Kong. Genomic DNA from frozen gastric tissues was extracted by using the High Pure PCR Template Preparation kit (Roche, Germany). Microdissection was performed on both tumour and surrounding normal tissue. The diagnosis was validated by pathology review. Additional clinical characteristics for the cohort analysed include average age 64 ± 13 years; 70% of the patients analysed were male; the ethnicity of all the patients analysed was Chinese and metastasis was present in 30% of the patient samples analysed.

2.2. LOH and DNA mutation analysis

Fluorescent LOH analysis using genomic DNA from matched normal/tumour gastric tissue and markers had been previously described.[4] Briefly, fluorescent LOH analysis using genomic DNA microdissected from matched normal/tumour gastric tissue was performed as previously described (see above references). Fluorescently labelled microsatellite markers flanking *KLF6* and ordered according to the Marshfield map (http://research.marshfieldclinic.org/genetics/) were generated. PCR was performed according to manufacturers' suggestions (Perkin Elmer). The exponential range of the PCR was determined for each marker and each sample, and was between 30 and 38 cycles. The data were analysed by the ABI Genescan and Genotyper software packages (Perkin Elmer), and an allelic loss was scored by two independent observers. In our system, a relative allele ratio of less than 0.7, which correlates with an allelic loss of approximately 40%, was defined as the loss of heterozygosity (LOH). The XLOH was confirmed at least twice for each marker.

In order to finely map the region of chromosome 10 p loss in gastric cancer to the *KLF6* gene locus, we used three microsatellite markers, KLF6M1, KLF6M2 and KLF6M4, that were designed specifically to tightly flank the *KLF6* gene by 40 kb to the D10S591 and D10S594 markers that flank the gene by approximately 1 Mb. The heterozygosity scores for these markers in the normal population were calculated by amplifying genomic DNA isolated from over 100 healthy Caucasian individuals. Samples that had either loss of at least one of the *KLF6* specific markers flanking the *KLF6* gene, M1, M2 and M4, or a flanking marker when the contiguous *KLF6* specific marker was non-informative, were regarded as having LOH. All sample marker combinations were analysed at least twice.

PCR products were directly sequenced, following purification (Qiagen, QIAquick PCR purification kit), on an ABI Prism 3700 automated DNA Analyser, and data were analysed using the program Sequencher (Gene Codes Corporation). The following sets of intronic primers (sense and antisense, respectively) were used to amplify the coding region and intron/exon boundaries of *KLF6* exon 2: Exon 2 Fwd: 5′-CGG GCA GCA ATG TTA TCT GTC CTT C-3′ and Exon 2 Rev: 5′-CCC TCC AGG GCT GGT GCA -3′. PCR cycling conditions were 94 °C (10 min) for 1 cycle, 94 °C (30 s), 55 °C (30 s), 72 °C (1 min) for 45 cycles and a final extension of 72 °C (5 min).

2.3. Real-time PCR analysis

For quantitating target gene expression, RNA isolation from cultured cells and tumour xenografts was performed using RNeasy Mini kits (Qiagen). All RNA was treated with DNAse (Qiagen). One ug of RNA was reverse transcribed for each reaction using first strand cDNA synthesis with random primers (Biorad). mRNA levels were quantified by quantitative real-time polymerase chain reaction (qRT-PCR) using the following PCR primers on an ABI PRISM 7900HT (Applied Biosystems): *KLF6* Forward: 5′-CGG ACG CAC ACA GGA GAA AA-3′ and Reverse: 5′-CGG TGT GCT TTC GGA AGT G-3′; *GAPDH* Forward: 5′-CAA TGA CCC CTT CAT TGA CC-3′ and Reverse: 5'-GAT CTC GCT CCT GGA AGA TG-3′ and *c-myc* Forward: 5′-CAG CTG CTT AGA CGC TGG ATT T and Reverse: 5′-ACC GAG TCG TAG TCG AGG TCA T. Quantitative real-time PCR primers to *p21* were described previously.[23] All the values were calculated by normalising the levels of each target for each cDNA to *GAPDH* and *β-actin* and then by using this normalised value to calculate the fold change compared to control. All the experiments were done in triplicate and repeated three independent times. Statistical significance was determined by one-way ANOVA using a Bonferroni correction.

2.4. Cell culture and transfection

AGS and HS746T gastric cancer cell lines were purchased from the American Tissue Culture Collection (ATCC). Stable cell lines were generated by retroviral infection of pBABE, pBABE-R71Q or pBABE-S180L and selected with 2 μg/ml of puromycin, as previously described.[23] Polyclonal pools of each infected cell line were collected, and *KLF6* expression was determined by qRT-PCR and Western blotting. Genomic DNA was isolated from each cell line as previously described,[4] and sequence analysis to confirm each point mutation was performed using the following sets of primers and PCR conditions Exon 2 Fwd: 5′-CGG GCA GCA ATG TTA TCT GTC CTT C-3′ and Exon 2 Rev: 5′-CCC TCC AGG GCT GGT GCA-3′. PCR cycling conditions were 94 °C (10 min) for 1 cycle, 94 °C (30 s), 55 °C (30 s), 72 °C (1 min) for 45 cycles and a final extension of 72 °C (5 min). Transient transfection and cotransfection of the pciNEO, pciNEO-KLF6, R71Q, and S180L, and site-directed mutagenesis were performed as previously described.[4]

2.5. Western blot analysis and densitometric analysis

Cell extracts were harvested in RIPA buffer (Santa Cruz Biotechnology, standard protocol). Tumour tissue extracts were harvested and prepared in the T-PER reagent (Pierce). Equal amounts of protein (50 μg; determined by the BioRad DC Protein quantification assay (BioRad) were loaded and separated by polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Actin (SC-1616), *KLF6* (SC-7158) and p21 (H164) antibodies were obtained from Santa Cruz Biotechnology, C-myc (Calbiochem anti-cmyc (ab-1) mouse mab (9e10). Enhanced chemiluminescent immunoblot images were analysed by scanning densitometry and quantified with a BIOQUANT NOVA imaging system. Values were expressed as the fold change relative to control and normalised to actin as a loading control.

2.6. Analysis of proliferation

Proliferation was determined by performing tritiated thymidine incorporation assays as previously described.[23] The stable cell lines containing pBABE, R71Q and S180L were plated at a density of 100,000 cells/well in 12 well dishes.

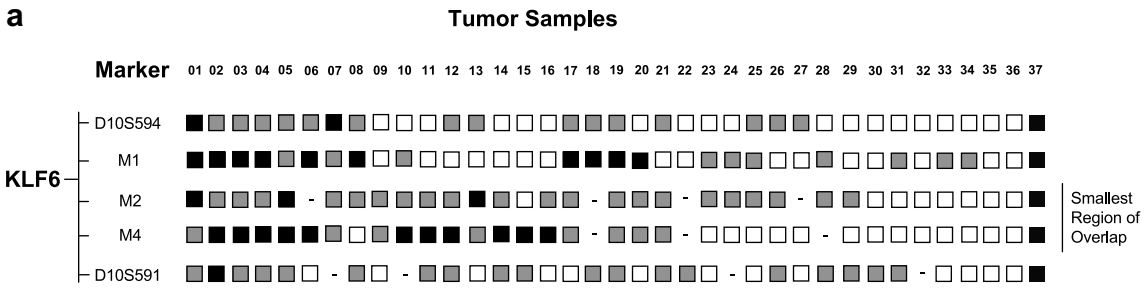
2.7. Tumourigenicity assays

Stable HS746T cells (1×10^7) were injected into the left flank of female 6–8-week old BALB/c *nu/nu* mice as previously described.[23] Tumour volume was assessed weekly and determined using the formula (length × width × width × 0.4). After 6 weeks, mice were sacrificed, and tumours were removed for RNA and protein analysis. All animal studies were approved by the MSSM IACUC.

3. Results

3.1. Functional Inactivation and decreased expression of *KLF6* tumour suppressor gene in gastric cancer

A total of 37 patient samples with detailed clinical information were collected under Institution Review Board approval. Briefly, 65% were of the diffuse type, and 16% of the intestinal type of gastric cancer with a complete range of stages represented. The clinical-pathologic profile of these samples is representative of the varied clinical spectrum of gastric cancer. We determined the frequency of *KLF6* loss using five microsatellite markers: KLF6M1, KLF6M2 and KLF6M4 which tightly flank the *KLF6* gene locus,[4] and D10S594 and D10S591 which are more distal to the gene. This marker set rendered 34 of the 37 analysed samples informative for at least one marker at the *KLF6* locus (Fig. 1a). Fluorescent LOH analysis using genomic DNA from matched normal/tumour tissue and these specific microsatellite markers had been previously described.[4] The exponential range of the PCR was determined for each microsatellite marker, and a relative allele ratio of less than 0.7, correlating with an allelic loss of approximately 40%, was defined as LOH.[4] Samples that had either loss of at least one of the *KLF6* specific markers flanking the *KLF6* gene, M1, M2 and M4, or a flanking marker when the contiguous *KLF6* specific marker was non-informative, were regarded as having LOH. All sample marker combinations were analysed at least twice. In addition, patients (18 and 22) with failed PCRs for both the M2 and M4 microsatellite markers that represent the smallest region of overlap for *KLF6* loss in gastric cancer and patients with microsatellite instability (MSI) (patient 37) were removed from the analysis. Clinical information on the cohort of samples analysed is provided in Fig. 1b. Overall, 53% (18/34) of the gastric cancer samples demonstrated LOH of the *KLF6* locus. LOH was present in both the diffuse and intestinal types of gastric cancer at the similar frequencies (data not shown). Interestingly, an allelic loss of the *KLF6* locus was significantly more frequent ($p < 0.001$) in advanced stage diseases (III and IV) than when compared to stage I and II cancers (Fig. 1c). The frequency of *KLF6* loss in the present study is consistent with the previous report by Cho et al. who reported the allelic loss of the *KLF6* gene in 43.2% of the informative cases.



b

Histological Type

Diffuse	24 (65%)
Intestinal	6 (16%)
Mixed	2 (5%)
No data	5 (13%)

Stage

I	7 (18%)
II	5 (13%)
III	9 (24%)
IV	13 (35%)
No data	3 (8%)

c

Stage	Allelic loss of 10p15
I	1 (14%)
II	1 (20%)
III	5 (55%)
IV	10(76%)
No data	2 (13%)

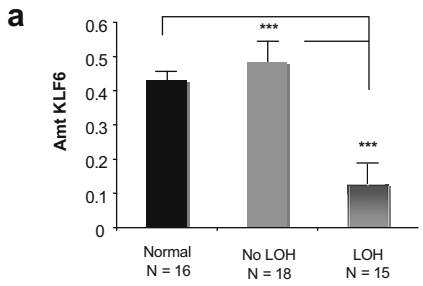
*Allelic loss of KLF6 was significantly more frequently found in advanced stage tumors (III and IV) ($p < 0.001$)

Fig. 1 – (a) LOH status of *KLF6*. LOH of the *KLF6* locus was analysed using microsatellite markers (vertical axis) from the 10p15 region and *KLF6*-specific markers KLF6M1, M2 and M4, which have been previously described.[4] Cases are arranged on the horizontal axis. Black filled square – LOH; grey – non-informative (NI); white square – no evidence of loss; '-' – represents a PCR that failed three or more times. Tumours were defined as having LOH if one or all of the markers flanking the *KLF6* locus were lost. (b) Clinical profile of gastric cancer patient samples. A summary of the relevant clinical information for the 37 gastric cancer patient samples analysed is presented. A complete range of stages and histological types are represented. (c) Summary of *KLF6* allelic loss by stage of disease. Statistical analysis was performed using a Student *t*-test with two-tailed distribution and two samples equal variance ($p < 0.001$). Allelic loss of the *KLF6* locus was significantly more frequent in advanced disease ($p < 0.001$).

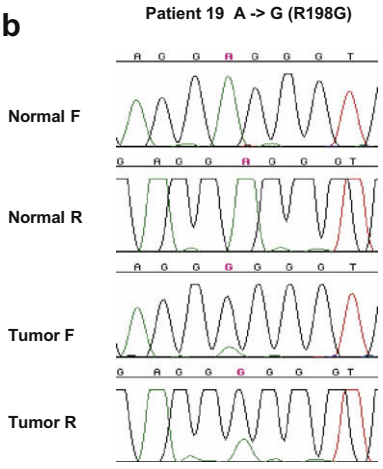
We next explored whether allelic loss was associated with decreased *KLF6* mRNA expression. RNA was available from a total of 16 normal and 33 of the tumour samples from gastric tissue specimens. Quantitative real-time PCR (qRT-PCR) using wild-type KLF6 (wtKLF6) specific primers[30] was used to determine *KLF6* expression in all 33 RNA samples. One microgram of RNA was reverse transcribed for each reaction using first strand cDNA synthesis with random primers, and levels of wtKLF6 were determined as previously described.[30] Both β-actin and GADPH were used as housekeeping genes for all analysis. On average, patients with *KLF6* loss had a 80% reduction in wtKLF6 expression when compared to tumours without LOH ($p < 0.001$) or when compared to normal gastric tissue ($p < 0.001$) (Fig. 2a). Interestingly, *KLF6* expression levels in tumours without LOH were identical to normal gastric tissue (Fig. 2a). There was a significant correlation ($p < 0.01$) between *KLF6* loss and decreased *KLF6* expression as demonstrated in Fig. 2a ($p < 0.01$). *KLF6* mutation status was examined by direct sequencing of exon 2 in all patient samples as previously described.[4] This exon encodes three quarters of the wild-type protein and contains the majority of mutations previously identified in other human cancers.[4–19] Consistent with its role as a tumour suppressor gene, mutations were identified in 4/37 cases (10%) (Fig. 2b and c). All four of the identified mutations were somatic and resulted in non-conservative amino acid changes in the *KLF6* protein (Fig. 2c). Interestingly, three of the four mutations, S180L, R71Q and T179I, had been previously identified in prostate, colorectal and hepatocellular carcinoma patient samples.[4,6,7,14] Combined, these findings demonstrate that an inactivation of the *KLF6* tumour suppressor gene occurs through either LOH and/or somatic mutation with decreased expression in wtKLF6 expression occurring in a significant percentage of gastric cancer patients. Furthermore, loss of the *KLF6* locus is associated with later stage disease suggesting a potential role for the *KLF6* gene in gastric cancer progression.

3.2. *Targeted reduction of KLF6 results in increased proliferation in gastric cancer cell lines*

Having identified both genetic alterations in the *KLF6* gene and correlations between *KLF6* loss and clinical-pathological parameters in patient samples, we next directly explored the biological role of *KLF6* in gastric cancer by using siRNA-mediated gene silencing to specifically downregulate its expression in gastric cancer cells. Both the AGS and Hs746T cell lines had no somatic mutations in the *KLF6* gene and express approximately 50% less *KLF6* than pooled normal gastric tissue (data not shown). Specific siRNA to wtKLF6 has been generated and validated for target specificity as



	LOH	No LOH
Low Expression	12	4
High Expression	3	14



c

Patient Number	LOH	Mutation
Patient 1	Yes	T179I
Patient 9	NI	R198G
Patient 13	Yes	R71Q
Patient 19	Yes	S180L

Fig. 2 – (a) *KLF6* expression in gastric cancer. Quantitative real-time PCR using wtKLF6 specific primers as previously described[23] demonstrated a 80% reduction in wtKLF6 expression compared to both patient samples without LOH of the *KLF6* locus and to normal gastric tissue ($p < 0.0001$). As the table demonstrates, there was a significant correlation between *KLF6* loss and decreased wtKLF6 expression. (b) *KLF6* mutations in gastric cancer. Microdissected tumour DNA was amplified using the following sets of intronic primers (sense and antisense, respectively) *KLF6* Exon 2 Fwd: 5′-CGG GCA GCA ATG TTA TCT GTC CTT C-3′ and Exon 2 Rev: 5′-CCC TCC AGG GCT GGT GCA-3′. PCR cycling conditions were 94 °C (10 min) for 1 cycle, 94 °C (30 s), 55 °C (30 s), 72 °C (1 min) for 35 cycles and a final extension of 72 °C (5 min). PCR products were directly sequenced, following purification (Qiagen, QIAquick PCR purification kit), on an ABI Prism 3700 automated DNA Analyser, and data were analysed using the program Sequencher (Gene Codes Corporation). All mutations were confirmed by an indendepent PCR and all sequencing reactions were done in both the forward and reverse directions as previously described.[4] (c) Summary of *KLF6* mutation and LOH status in gastric cancer patient samples.

previously described.[19,30,31] Transient transfection of a siRNA specific to wtKLF6 in the AGS gastric cancer cell line resulted in a 50% decrease in wtKLF6 expression at both the mRNA and protein levels as measured by qRT-PCR analysis and Western blotting when compared to a si-NTC non-targeting siRNA control (Fig. 3a). No differences in *KLF6* expression were seen at time point 0, and no effect on *KLF6* expression was seen in control cells that had not been transfected compared to cells that were transfected with either siRNAs (data not shown). Tritiated thymidine incorporation assays of transiently transfected cells demonstrated that the targeted reduction of wtKLF6 resulted in a nearly 2-fold increase in cellular proliferation when compared to the si-NTC transfected control cells (Fig. 3b). Similar results were obtained with 2 additional siRNAs specifically targeting wtKLF6 (data not shown). This increase in cellular proliferation was associated with the changes in the expression of two well-characterised transcriptional targets of *KLF6*, the proto-oncogene c-myc and the cyclin-dependent kinase inhibitor p21.[4] Targeted reduction of *KLF6* resulted in an increased expression of c-myc with a concomitant decrease in p21 expression at both the mRNA and protein levels (Fig. 3c). Similar results were obtained in the HS746T gastric cancer cell line (data not shown). In addition, expression of *KLF6* at the mRNA and protein levels was not significantly different between the scrambled oligo control and si-KLF6 transfected cells at time zero, and furthermore there were no major differences in KLF6 expression between transfected and untransfected cells. The incorporation of tritiated thymidine at time 0 was equal between the control and si-KLF6 transfected cells, which is expected given that there was no difference in *KLF6* expression at this time point (data not shown).

3.3. *Overexpression of KLF6 results in cell cycle arrest in gastric cancer cell lines*

To further define the role of the wild-type *KLF6* tumour suppressor gene in gastric cancer cell lines, we overexpressed wtKLF6 in the AGS gastric cancer cell line. Transient transfection of wtKLF6 resulted in a greater than 40-fold increase in *KLF6* expression at both the mRNA and protein levels (Fig. 4a). This was associated with a significant decrease in

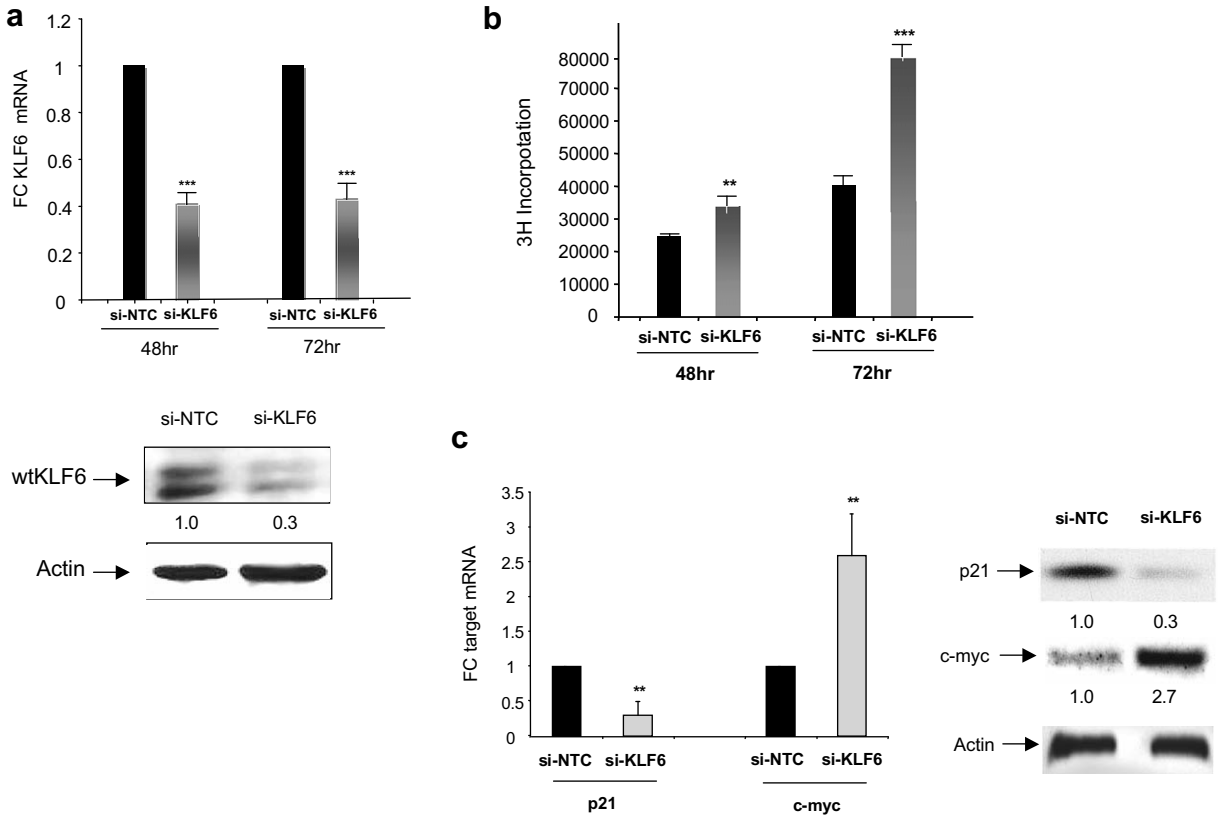


Fig. 3 – Targeted reduction of wtKLF6 in gastric cancer cell lines. (a) Targeted reduction of wtKLF6 in gastric cancer cell lines results in decreased *KLF6* expression at both the mRNA and protein levels as measured by wtKLF6 specific quantitative real-time PCR and Western blotting respectively. AGS cells transfected with siRNA specific to wtKLF6 and a non- targeting scrambled siRNA control were harvested 72 h after transfection. The experiment was repeated three independent times (*$p < 0.0001$). (b) Targeted reduction of wtKLF6 results in increased cellular proliferation. Tritiated thymydine incorporation was measured at 48 and 72 h after siRNA transfection of AGS cells. At 72 h, there is a nearly 2-fold increase in cellular proliferation (***$p < 0.0001$). The experiment was repeated three independent times. (c) Targeted reduction of wtKLF6 results in dysregulated *c-myc* and *p21* expressions. Quantitative real-time PCR and Western blotting using *c-myc* and *p21* specific primers, and antibodies demonstrated a 70% reduction in *p21* expression with a concomitant 2-fold increase in *c-myc* expression in gastric cancer cells transfected with *KLF6* siRNAs (**$p < 0.001$).**

c-myc expression with a concomitant increase in *p21* expression at both the mRNA and protein levels (Fig. 4a). Furthermore, overexpression of wtKLF6 resulted in a 80% decrease in cellular proliferation as measured by tritiated thymidine incorporation (Fig. 4b). This decrease in cellular proliferation was associated with a G1/S arrest as demonstrated by FACS analysis (Fig. 4c). Combined, these data demonstrate that wild-type KLF6 is growth suppressive in gastric cancer cell lines through the transcriptional regulation of p21 and c-myc.

3.4. *Tumour-derived KLF6 mutations fail to suppress cell growth*

Having identified both genetic alterations in the *KLF6* gene, correlations between *KLF6* loss and clinical-pathological parameters in patient samples, and functionally characterised the biological function of wtKLF6 in gastric cancer cell lines, we next directly explored the biological role of *KLF6* tumour-derived mutants in the gastric cancer cell lines, AGS and HS746T. Stable cell lines for each of these *KLF6* mutants were generated as previously described[14,19] in each of the gastric cancer cell lines. We were unable to generate stable cell lines overexpressing the R198G and T179I tumour-derived mutants for technical reasons. Previous reports, however, have demonstrated that the T179I mutant fails to transactivate p21 and suppress cellular proliferation in colorectal cancer cell lines.[6] In addition, the R198G mutation disrupts the predicted nuclear localisation signal of the *KLF6* tumour suppressor gene, and would therefore most likely result in cytoplasmic accumulation of the mutant protein. Because of its growth suppressive effects on gastric cancer cell lines, we were unable to successfully generate stable cell lines overexpressing wtKLF6 (data not shown). Sequence and quantitative real-time PCR analysis of the R71Q and S180L retrovirally derived cell lines demonstrated the overexpression of each *KLF6* mutant in their respective cell lines compared to the control pBABE cell line. Overexpression of both the R71Q and S180L mutants resulted

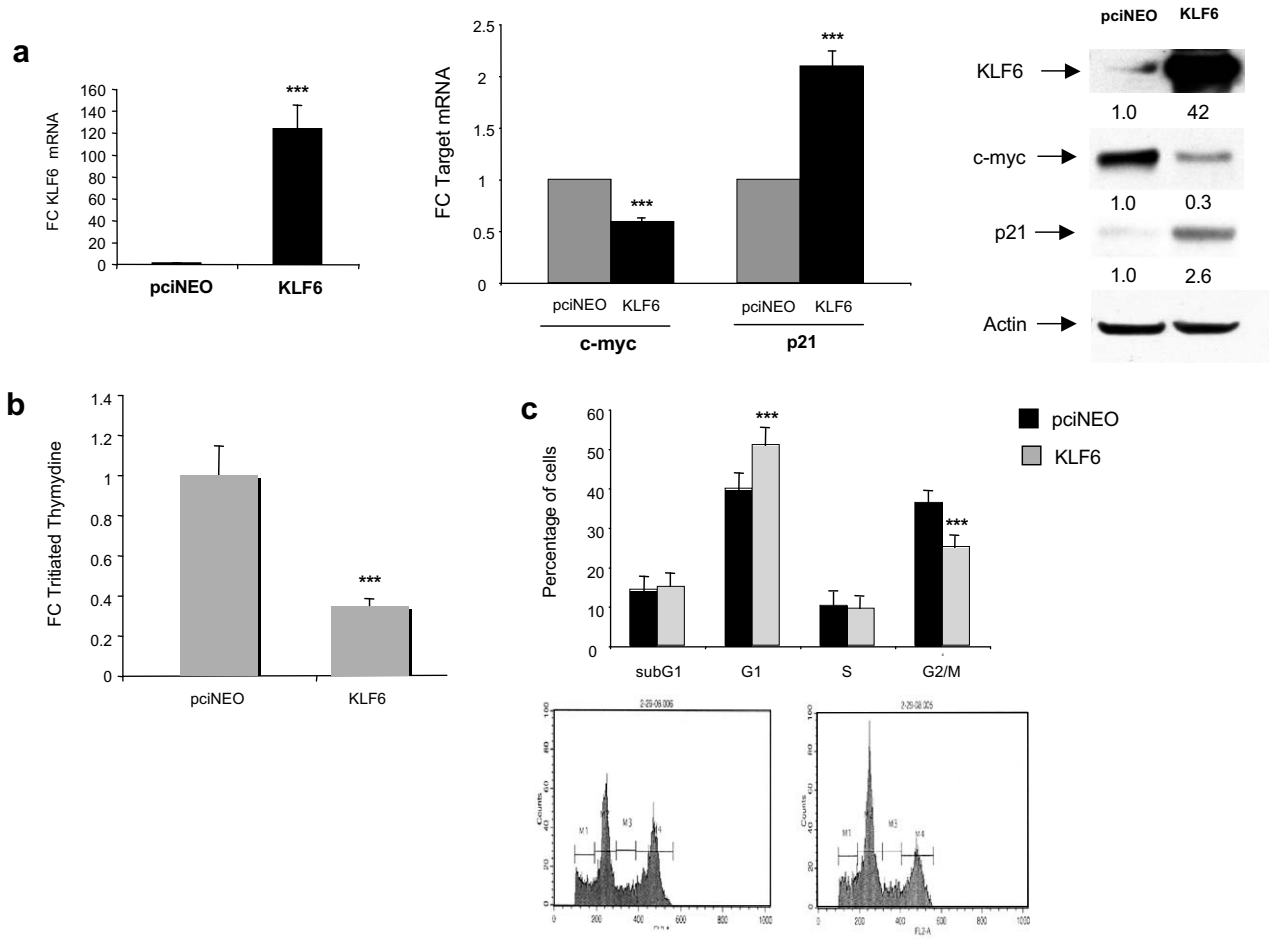


Fig. 4 – Functional role of wtKLF6 in gastric cancer cell lines. (a) Overexpression of wtKLF6 results in increased *KLF6* expression as well as decreased c-myc expression and increased *p21* expression at mRNA level and protein level as measured by wtKLF6 specific quantitative real time PCR and Western blotting respectively. AGS cells transfected with pciNEO-KLF6 and an empty pciNEO vector control were harvested 72 h after transfection. The experiment was repeated three independent times (*$p < 0.0001$). (b) Overexpression of wtKLF6 results in decreased cellular proliferation. Triatiated thymydine incorporation was measured at 72 h after transfection of AGS cells. At 72 h there is a nearly 5 fold decrease in cellular proliferation (***$p < 0.0001$). The experiment was repeated three independent times. (c) Overexpression of wtKLF6 results in increased amount of cells in G1 and decreased amount of cells in G2/M. FACS analysis was performed after transfection of AGS cells (**$p < 0.001$).**

in marked changes in the expression of both c-myc and p21 at both the mRNA and protein levels (Fig. 5a and b). These changes in c-myc and p21 expressions were associated with the changes in cellular morphology in the HS746T cell line and increased cellular proliferation when compared to control as shown in Fig. 5c. Analysis of cellular proliferation using an assay of the R71Q and S180L cell lines demonstrated a greater than 3-fold increase in growth when compared to control cells (Fig. 5c). These results were confirmed in the AGS cell line (data not shown).

3.5. *Dominant negative function of KLF6 tumour-derived mutants*

We next sought to determine if the R71Q and S180L had a dominant negative effect on wtKLF6 function. Transient cotransfection of either the R71Q or S180L mutant expression vectors with wtKLF6 resulted in equal levels of *KLF6* overexpression at both the mRNA and protein levels (Fig. 6a and data not shown). Consistent with the previous results, wtKLF6 overexpression resulted in a 40% reduction in cellular proliferation in the HS746T cell line, which was completely abrogated by cotransfection with either the R71Q or S180L *KLF6* expression vectors. This dominant negative effect of the R71Q and S180L proteins was associated with the suppression of p21 induction and failure to repress c-myc expression at both the mRNA and protein levels (Fig. 6c and data not shown). Combined, these data suggest that the R71Q and S180L *KLF6* mutants function as dominant negative regulators of wild-type KLF6 function through their ability to effect p21 and c-myc transcription.

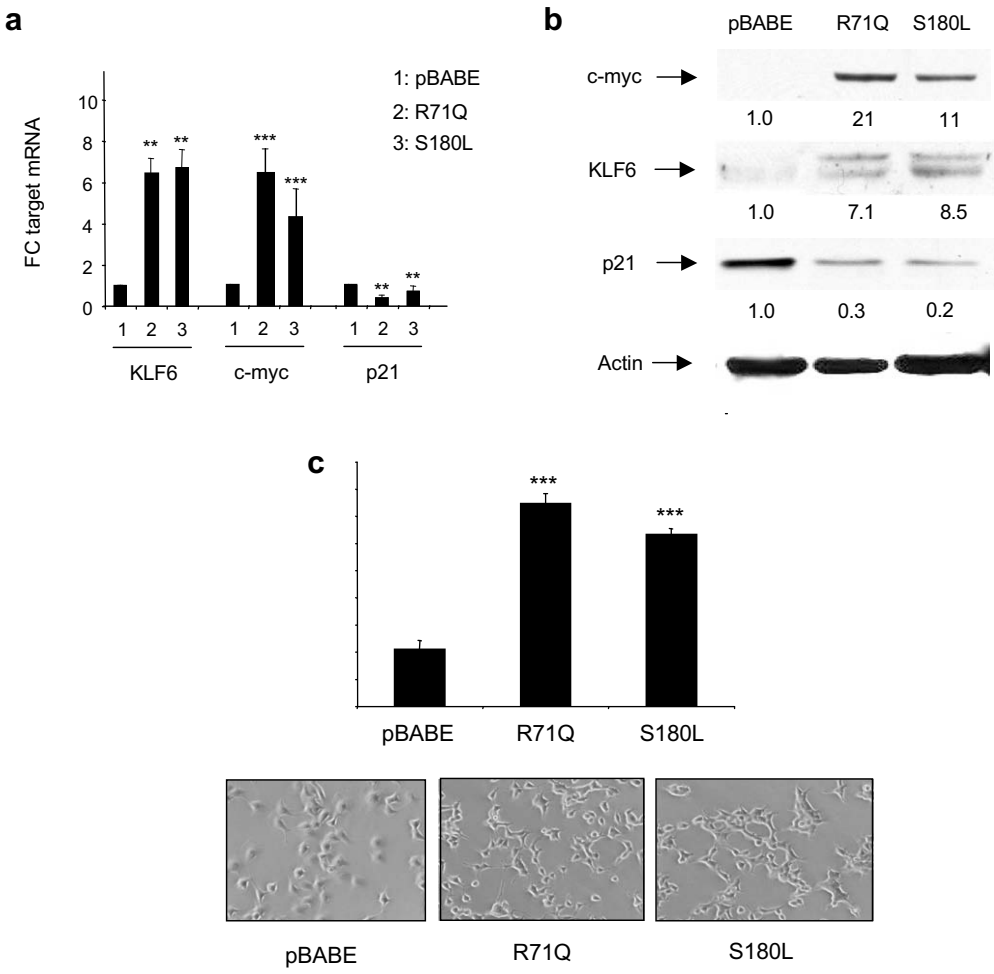


Fig. 5 – Functional characterisation of the R71Q and S180L patient-derived mutations. (a) Target gene expression was analysed by qRT-PCR and correlated to the control pBABE cell line. For quantitating target gene expression, one microgram of RNA was reverse transcribed for each reaction using first strand cDNA synthesis with random primers. mRNA levels for each target were quantified by qRT-PCR, and all the values were calculated by normalising the levels of each target for each cDNA to both GAPDH and β-actin to determine the relative amount of expression compared to control. All the experiments were done in triplicate and repeated three independent times. *KLF6* was overexpressed in the R71Q and S180L cell lines approximately 8-fold ($p < 0.001$). This was associated with a 7-fold increase in *c-myc* expression, respectively, with a concomitant reduction in expression of *p21* in these cell lines compared to control. (b) Western blot of the pBABE, R71Q and S180L cells was performed using the following antibodies, *c-myc*, *KLF6*, *p21*, and *actin* as a control for protein loading. Consistent with qRT-PCR data, the R71Q and S180L mutants expressed increased levels of *c-myc* and decreased levels of *p21* compared to control. (c) The R71Q and S180L mutants resulted in increased cellular proliferation (***$p < 0.001$). Photographs of retrovirally generated stable cell lines in HS746T gastric cancer cell line. The R71Q and S180L-derived cells exhibit markedly different cellular morphologies than the control pBABE cell line.**

3.6. *KLF6 tumour-derived mutants increase in vivo tumourigenicity*

To extend these findings to an *in vivo* tumourigenicity model, we injected the pBABE, R71Q and S180L HS746T subcutaneously in nude mice. Consistent with our cell culture findings, tumours derived from the R71Q and S180L cell lines were significantly larger than the pBABE control-derived tumours (Fig. 7a). H and E staining of tumours revealed a marked difference in cellular differentiation between the R71Q, S180L and control tumours (Fig. 7b). Specifically, tumours derived from the R71Q and S180L appeared less differentiated. Quantitative real-time PCR (qRT-PCR) analysis of these tumours demonstrated a marked upregulation in c-myc expression and decreased *p21* expression at both the mRNA and protein levels (Fig. 7c) consistent with our previous findings in cell culture. Combined, these studies suggest that tumour-derived somatic mutations in the *KLF6* gene result in an increased *in vivo* tumourigenicity through the regulation of *c-myc* and *p21* expressions.

4. Discussion

Our findings in patient samples and biological model systems define a high frequency of *KLF6* allelic loss and/ or somatic

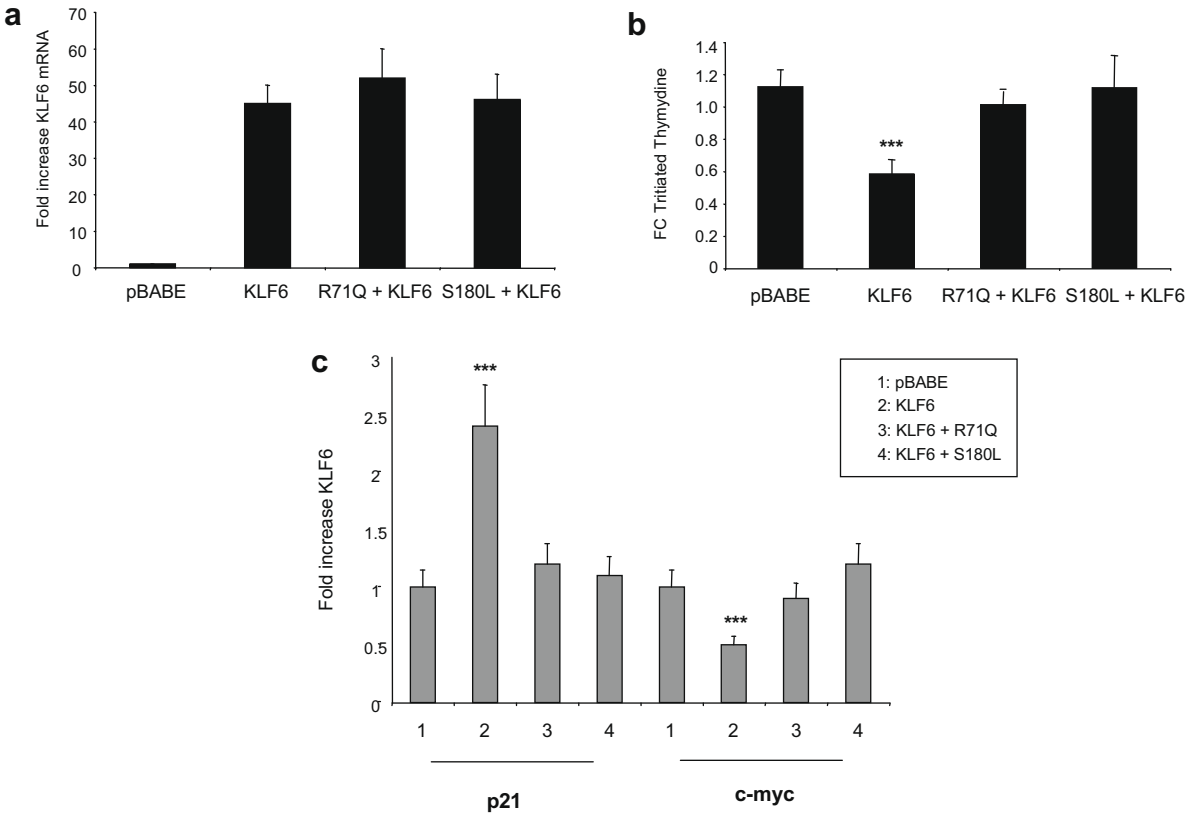


Fig. 6 – Dominant negative function of the *KLF6* tumour-derived mutants. (a) *KLF6* gene expression was analysed by qRT-PCR and compared to the control pBABE cell line. mRNA levels for wtKLF6 were quantified by qRT-PCR, and all the values were calculated by normalising the levels of each target for each cDNA to both GAPDH and β-actin to determine the relative amount of expression compared to control. All the experiments were done in triplicate and repeated three independent times. *KLF6* was overexpressed in all three cotransfected AGS cell lines to equal amounts. (b) Tritiated thymydine incorporation was measured at 72 h after transfection of AGS cells. WTKLF6 overexpression resulted in a 40% reduction in cellular proliferation in the AGS cell line, which was completely abrogated by cotransfection with either the R71Q or S180L *KLF6* expression vectors. (c) *KLF6* overexpression resulted in a 2-fold increase in p21 expression and a 50% reduction in C-myc expression as measured by qRT-PCR (*$p < 0.0001$). These changes in target gene expression were completely abrogated by cotransfection with either the R71Q or S180L *KLF6* tumour-derived mutant constructs.**

mutation suggesting that dysregulation of *KLF6* may be an important event in the development and progression of gastric cancer. Mounting evidence in other tumours has highlighted a variety of *KLF6* inactivating mechanisms relevant to tumour growth and spread. These mechanisms include (1) LOH and somatic mutation in prostate,[4,5] colorectal,[6,7] malignant glioma,[11,12] nasopharyngeal carcinomas,[13] HCC[14–17] and gastric cancer,[10] (2) transcriptional silencing through promoter hypermethylation in oesophageal cancer cell lines[32] and (3) dysregulated alternative splicing in prostate cancer and ovarian cancer.[19,30,31] The current findings are consistent with a previous report in gastric cancer reporting a LOH frequency of 43.2% with somatic mutations occurring in approximately 10% of the 80 gastric cancer patient samples analysed. The results between these two studies are remarkably consistent, and demonstrate that *KLF6* loss is a frequent event in gastric cancer and that functional inactivation of the gene through somatic mutations occurs in a minority of cases.

Our findings demonstrate the importance and prevalence of *KLF6* inactivation in gastric cancer. Consistent with the previous reports, *KLF6* LOH and/or somatic mutation occurs in a significant number of gastric cancer patient samples. In addition, we define the functional significance of commonly identified *KLF6* mutations in multiple gastric cancer cell lines both in culture and *in vivo*. Specifically, we identify the regulation of the cyclin-dependent kinase inhibitor *p21* and the proto-oncogene *c-myc* by *KLF6* tumour-derived mutants resulting in increased tumourigenicity and decreased cellular differentiation. Combined, these studies highlight an important role for the *KLF6* tumour suppressor gene in gastric cancer pathogenesis, and demonstrate the diversity of mechanisms of functional inactivation and cellular pathways regulated by this gene in human cancer. Future studies of larger cohorts of patient samples will better define the exact prevalence and clinico-pathological associations with *KLF6* loss and mutation. In addition, further studies of the *KLF6* heterozygous mice, which display significantly enlarged livers and reduced *p21* expression,[31] are warranted to identify if haploinsufficiency of the *KLF6* gene plays a role in gastric cancer development and progression *in vivo*.

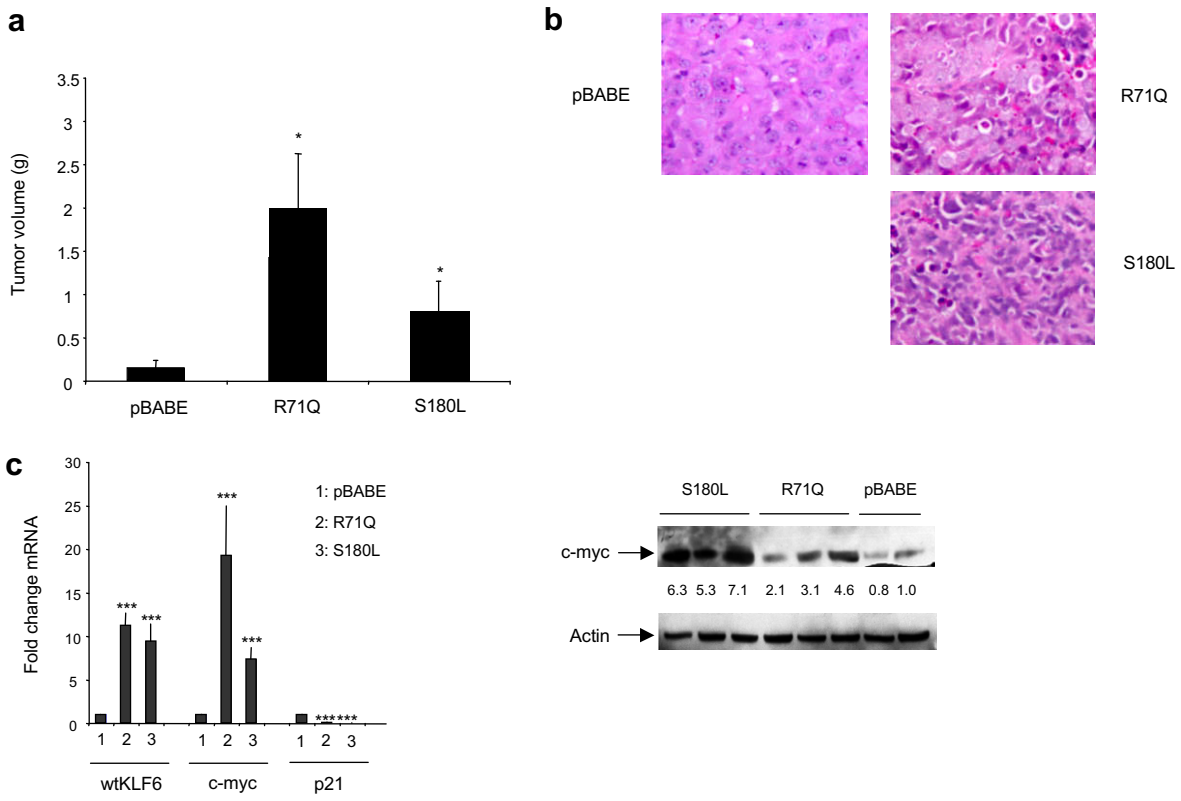


Fig. 7 – Tumour-derived *KLF6* mutations significantly increased in vivo tumourigenicity. Stable cell lines expressing the R71Q, S180L or the control pBABE HS746T cell line were injected into nude mice and their volumes were assessed as described in Materials and Methods. (a) Tumours derived from the R71Q and S180L cells were significantly larger (*$p < 0.01$) than control cell line-derived tumours. For each group, five tumours were analysed. (b) The R71Q and S180L-derived tumours were markedly less differentiated as shown by H and E staining. (c) mRNA levels for each target were quantified by qRT-PCR, and all the values were calculated by normalising the levels of each target for each cDNA to both GAPDH and β-actin to determine the relative amount of expression compared to control. qRT-PCR of cDNA derived from each tumour revealed a marked increase in c-myc and decrease in *p21* expression (*$p < 0.001$). Western blotting confirmed increased *c-myc* expression in tumours expressing either the R71Q or S180L patient-derived mutations. All the experiments were repeated in triplicate, and five tumours were analysed from each experimental group.**

The association between loss of the *KLF6* locus, decreased *KLF6* expression and advanced stage disease is consistent with other previous reports of decreased *KLF6* expression in advanced stages of disease in hepatocellular carcinoma and prostate cancer.[31,33] These findings suggest not only the potential use of *KLF6* as a prognostic marker in cancer, but also that additional epigenetic mechanisms of gene silencing such as promoter methylation may be responsible for the further decrease in *KLF6* expression in advanced stage cancers.

In summary, these findings demonstrate that the tumour suppressor gene *KLF6* plays an important role in gastric cancer development and progression. Ultimately, we believe these findings have relevance to both the identification of pathogenic associations between *KLF6* loss, expression and mutation and clinically relevant parameters such as tumour stage, grade and histological type.

Conflict of interest statement

None declared.

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