

# A possible tumor suppressor role of the *KLF5* transcription factor in human breast cancer

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The 13q21 tumor suppressor locus, as defined by chromosomal deletion, harbors the *KLF5* transcription factor which may have tumor suppressor function. To investigate whether *KLF5* plays a role in breast cancer, we evaluated all genes and/or expressed sequence tags (ESTs) within a 3.3 Mb common region of deletion at 13q21. Of these, only *KLF5* mRNA was expressed at high levels in non-neoplastic breast epithelial cells and in normal human mammary tissue, but at lower levels in various breast cancer cell lines. Using the real time TaqMan PCR assay, hemizygous deletion at *KLF5* was detected in 13 out of 30, or 43% of breast cancer cell lines tested, and various degrees of loss of expression were detected in 21 out of 30, or 70% of these cell lines. Each of the cases with hemizygous deletion also exhibited loss of *KLF5* expression, suggesting that loss of expression can result from chromosomal deletion, and that *KLF5* may undergo haploinsufficiency during carcinogenesis. Only one of the 30 breast cancer cell lines tested exhibited a mutation in *KLF5*, and neither promoter methylation nor homozygous deletion was detected in any of the cell lines. In contrast, loss of heterozygosity (LOH) was frequently detected at *KLF5*. Re-expression of wild-type *KLF5* in T-47D breast cancer cells significantly inhibited colony formation in these cells. Of the *KLF5*-transfected clones that did form colonies, none were found to express *KLF5* mRNA. These findings suggest that loss of function by deletion and/or loss of expression frequently occurs at *KLF5*, and *KLF5* suppresses tumor cell growth in breast cancer.

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## Introduction

It has been well established that breast cancer can arise from specific genetic alterations, including the inactivation

of tumor suppressor genes. Chromosomal deletion is a typical characteristic of tumor suppressor gene inactivation. Thus, the identification of regions of chromosomal deletions has been widely used to localize such genes in cancer. The q21 band of chromosome 13 (13q21) has been identified in various comparative genomic hybridization (CGH)<sup>3</sup> analyses as a region that is frequently deleted in breast cancer; 13q21 has been reported to be deleted in as many as 37% of breast tumors analysed (Kainu *et al.*, 2000; Knuutila *et al.*, 1998; Roylance *et al.*, 1999; Tirkkonen *et al.*, 1997). In addition to sporadic tumors, somatic deletion at 13q21 also often occurs in hereditary breast cancer (Kainu *et al.*, 2000; Tirkkonen *et al.*, 1997). Furthermore, higher grade breast cancers more frequently exhibit deletions at 13q21 than do lower grade tumors (Roylance *et al.*, 1999). These studies suggest that one or more tumor suppressor genes are localized at 13q21 and may play an important role in breast cancer.

Deletion at 13q21 also occurs in other types of cancer. About 200 CGH studies of over 70 types of human malignancies demonstrated that 13q21 was the second most frequently deleted region, occurring in 47% of all tumors analysed (Knuutila *et al.*, 1998). In our previous analyses, deletion at 13q21 was detected in carcinomas of the breast, cervix, endometrium, ovary, and prostate. Among these tumor samples, a common region of hemizygous deletion was defined within a 3.3 Mb segment at 13q21, and this deletion occurred most frequently in breast cancer (12 out of 18, or 67%) among the various cancers analysed (Chen *et al.*, 2001; Dong *et al.*, 2000; Hyytinen *et al.*, 1999).

In this study, we analysed 30 breast cancer cell lines to identify the target gene deleted at 13q21. It has been previously demonstrated that 13q21 harbors *KLF5*, a member of the Kruppel-like family of transcription factors (KLFs). Some of the KLFs, i.e., *KLF4* and *KLF6*, have been shown to function as tumor suppressor genes. By evaluating the individual genes and ESTs located within the common region of deletion at 13q21, we found that *KLF5* is the only gene that frequently exhibited hemizygous deletion and loss of expression. Gene mutation, promoter methylation, and homozygous deletion were rarely detected in any of these samples. Functionally, *KLF5* inhibited cell growth *in vitro*. These data suggest that functional inactivation of *KLF5* frequently occurs in breast cancer.

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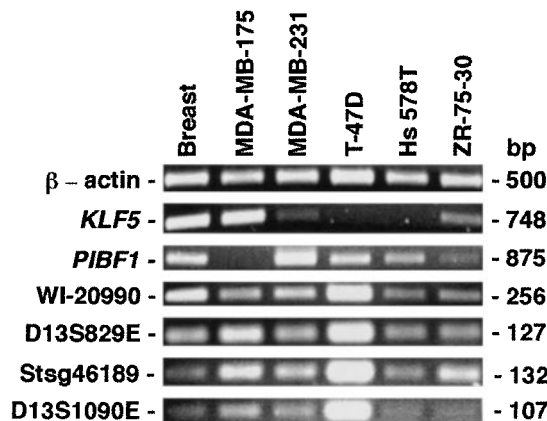
**Results**

In previous studies, we identified a 3.3 Mb common region of deletion at 13q21 (Chen *et al.*, 2001; Dong *et al.*, 2000). We first analysed all the possible genes and/or ESTs in this region for expression in non-neoplastic and neoplastic breast epithelial cells. RT-PCR analyses of a normal mammary gland showed that, among 22 genes and/or ESTs tested, four (*KLF5*, *PIBF1*, WI-20990, and D13S829E) were highly expressed, two (stSG46189 and D13S1090E) were expressed at intermediate levels, four (stSG50450, stSG15188, 361B5-FE, and AA213647) had very low but detectable levels of expression, and 12 were not detectable (Figure 1). In breast cancer cell lines, most of the genes and/or ESTs we evaluated were either unchanged or expressed at increased levels compared to the normal sample. Only *KLF5* was expressed at lower levels in the majority of the cell lines analysed (four out of five, or 80%) (Figure 1). Loss of *KLF5* expression was confirmed by Northern blot analysis, which showed lower levels of expression for *KLF5* in seven out of eight breast cancer cell lines (Figure 2).

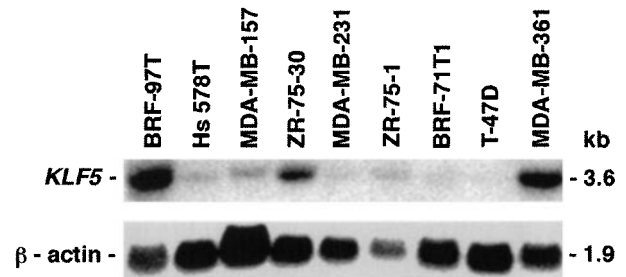
Because genomic deletion at 13q21 frequently occurs in breast cancer, we proceeded to examine 30 breast cancer cell lines and controls by duplex PCR analysis to determine whether *KLF5* exhibited hemizygous or homozygous deletion. Although this procedure is less sensitive than real time PCR, we were still able to detect clear hemizygous deletion in eight out of 30, or 27% of the cancer cell lines we tested. Representative results from these experiments are shown in Figure 3, and the results obtained from all 30 cell lines are summarized in Figure 4. As expected, neither normal human placenta nor the immortalized non-neoplastic breast epithelial cell line (BRF-97T) showed a deletion (Figure 3). The deletion occurred not only at *KLF5*, but also at its surrounding markers, indicating that the

deletion encompasses a region that is larger than the *KLF5* gene itself. We tested additional STS markers from the 3.3 Mb region of deletion, with the intention of defining a smaller region of deletion. Unfortunately, in all samples with *KLF5* deletion, the STS markers also showed a deletion (data not shown). Furthermore, no homozygous deletions were detected in any of the cell lines.

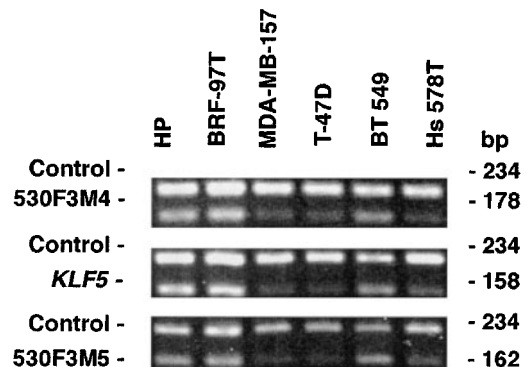
To more accurately determine the extent and frequency of deletion and loss of expression for *KLF5* in breast cancer, we performed real time TaqMan PCR using a pair of primers from exon 2 of the gene. As shown in Figure 4, 13 out of 30, or 43% of the breast cancer cell lines we tested showed deletions at *KLF5* and 21 out of 30, or 70%, showed varying degrees of loss of expression. Compared to the results from duplex PCR assays, deletion was detected in five additional cases using this more sensitive analysis. Whereas the *KLF5* to  $\beta$ -actin ratio for expression was as low as 1% in some samples relative to that in control BRF-97T cells, the lowest ratio of *KLF5* to *KAI1* for hemizygous deletion was 32% of control, indicating that the deletion was most likely to be hemizygous (Figure 4).



**Figure 1** Loss of expression for *KLF5*, but not for other genes from the common region of deletion at 13q21 in various breast cancer cells. Expression levels were determined by RT-PCR analysis, followed by electrophoresis and ethidium staining on agarose gels. Samples are listed at the top, gene/EST names at the left, and molecular sizes of PCR products are shown at the right. Breast, a normal human mammary tissue



**Figure 2** *KLF5* mRNA expression in various breast cancer cell lines. Upper panel: Samples containing 15  $\mu$ g of total RNA from various cell lines were subjected to *KLF5* Northern blot analysis, as indicated. Lower panel: The same blot was stripped and rehybridized with a cDNA probe specific for  $\beta$ -actin. BRF-97T is an immortalized nonneoplastic breast epithelial cell line



**Figure 3** Detection of hemizygous deletion of *KLF5* and surrounding markers by duplex PCR analysis in various breast cancer cell lines. The control marker was derived from exon 5 of the *KAI1* gene. Samples are listed at the top, marker names are shown at the left, and molecular sizes of PCR products are shown at the right. HP, normal human placenta

Samples	Expression	Deletion	LOH	D13S1291	D13S1318	D13S131	D13S258	D13S152	D13S745	D13S791	D13S1326	D13S166	D13S269
<b>Nonneoplastic</b>													
Breast 89	+	+											
BRF-97T 100	+	+	●	●	○	○	●	●	○	●	●		
<b>Neoplastic</b>													
HCC1806 135	+	+	○	●	○		●	●	○	●	○	●	
BT-20 125	+	-	●	●	●	●	●	●	●	●	●	●	●
HCC70 120	+	-	●	●	●	●	●	●	●	●	●	●	○
MDA-MB-175 118	+	+	●	○	○	○	○	○	○	○	○	○	○
BT-549 87	+	-	●	●	●	●	●	●	●	●	●	●	●
HCC1599 73	+	-	●	●	●	●	●	●	●	●	●	●	●
MDA-MB-361 65	+	-	●	●	●	●	●	●	●	●	●	●	●
MDA-MB-134 61	+	+	○	●	○	○	○	○	○	○	○	○	○
HCC1937 61	+	-	●	●	●	●	●	●	●	●	●	●	●
ZR-75-30 29	-	+	○	○	○	○	○	○	○	○	○	○	○
HCC1143 28	-	-	●	●	●	●	●	●	●	●	●	●	●
MDA-MB-415 24	-	+	●	○	○	○	○	○	○	○	○	○	○
HCC1500 21	-	-	●	●	●	●	●	●	●	●	●	●	●
HCC1395 18	+	-	●	●	●	●	●	●	○	●	●	○	○
BRF-71T1 16	-	+	○	○	○	○	○	○	○	○	○	○	○
BT-474 16	-	-	●	●	●	●	●	●	●	●	●	●	●
UACC-893 14	-	-	●	●	●	●	●	●	●	●	●	●	●
HCC2218 13	+	+	●	●	●	●	○	○	○	○	○	○	○
ZR-75-1 8	+	-	●	●	●	○	○	○	○	○	○	○	○
MCF7 7	+	+	●	●	●	●	●	●	●	●	●	●	●
MDA-MB-231 6	-	-	●	●	●	●	●	●	●	●	●	●	●
HCC38 6	-	-	●	●	●	●	●	●	●	●	●	●	●
DU4475 5	+	+	○	○	○	○	○	○	○	○	○	○	○
MDA-MB-453 4	+	-	●	●	●	●	●	●	●	●	●	●	●
BT-483 3	+	+	○	○	○	○	○	○	○	○	○	○	○
CAMA-1 2	+	+	●	●	○	○	○	○	○	○	○	○	○
HCC202 2	-	+	●	●	●	●	○	○	○	○	○	○	○
MDA-MB-157 2	-	-	●	●	●	●	●	●	●	●	●	●	●
Hs 578T 1	-	-	●	●	●	●	●	●	●	●	●	●	●
T-47D 1	-	-	●	●	●	●	●	●	●	●	●	●	●

**Figure 4** Summary of expression level, hemizygous deletion, and LOH at *KLF5* in various nonneoplastic and neoplastic breast specimens. Expression is indicated by the ratio of *KLF5* to  $\beta$ -actin which was normalized to that of BRF-97T control (100). Hemizygous deletion was defined by TaqMan analysis. LOH was determined by the HOMOD analysis. ‘-’ indicates the occurrence of deletion or LOH, where ‘+’ indicates the absence of deletion or LOH. Microsatellite markers and their allele status in each of the samples are also shown, with empty circles and filled circles indicating two alleles and one allele, respectively, for a marker in a sample. Consecutive markers indicating an LOH are underlined

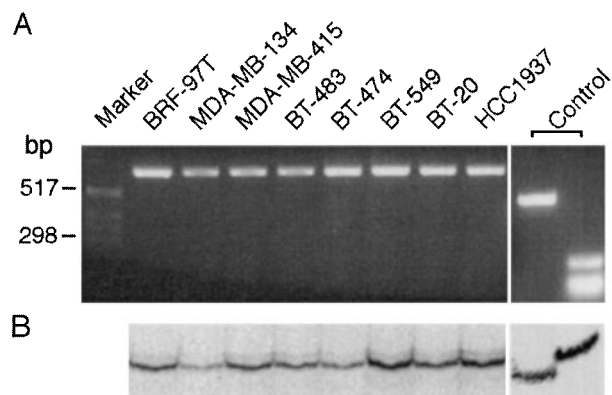
Using the HOMOD analysis, we then examined the allelic status of *KLF5* with 10 polymorphic microsatellite markers surrounding the gene. As shown in Figure 4, loss of heterozygosity (LOH) at *KLF5* was detected in 19 out of 30, or 63% of the breast cancer cell lines tested, which appears to be more frequent than hemizygous deletion. To test whether *KLF5* was mutated in breast cancers, we analysed each of its four exons by SSCP followed by DNA sequencing. Only one cell line, MDA-MB-231, exhibited a point mutation of A>G, which changed codon 294 from methionine to valine. This cell line also underwent deletion and loss of expression at *KLF5* (Figures 1, 2 and 4). These data indicate that, whereas deletion at *KLF5* occurs frequently in breast cancer, mutation of this gene is a relatively rare event.

Epigenetic promoter methylation is a well documented mechanism for the loss of expression for many tumor suppressor genes. Thus, we next examined the 632 bp GC-rich region of the *KLF5* promoter and exon 1, to determine whether the promoter of this gene was methylated in breast cancer. No methylation was detected in any of the samples by methylation-specific PCR followed by either restriction enzyme digestion or by SSCP analysis (Figure 5).

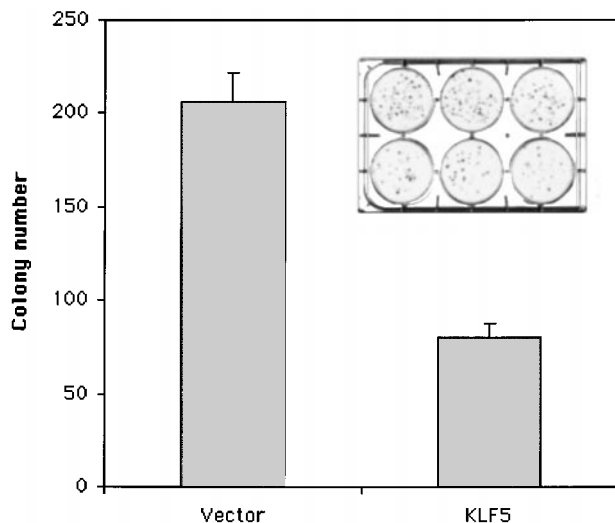
To test the effect of *KLF5* expression on cell growth, we transfected T-47D breast cancer cells with pcDNA-KLF5. T-47D cells express very low endogenous levels of *KLF5* (Figure 2). As shown in Figure 6, over-expression of *KLF5* significantly reduced the colony forming efficiency in T-47D cells. Among the colonies that did form after transfection of *KLF5*, most lacked a functional *KLF5*, as *KLF5* mRNA was undetectable in any of the 50 clones isolated from the transfection, as determined by Northern blot hybridization. In contrast, *KLF5* expression was detected in clones isolated from a bladder cancer cell line transfected with pc DNA-KLF5 (data not shown). We also transfected pcDNA-KLF5 into MDA-MB-231 cells, with similar results as were obtained in T-47D cells.

## Discussion

Each of the cell lines with hemizygous deletion at *KLF5* exhibited loss of expression. On the other hand, there were six breast cancer cell lines that exhibited LOH, but not hemizygous deletion or loss of expression at *KLF5*. These results suggest that quantitative hemizygous deletion induces loss of expression and that, in the absence of quantitative hemizygous deletion, LOH is insufficient to induce loss of expression. Thus, *KLF5* appears to undergo haploinsufficiency during breast carcinogenesis. In eight out of 30, or 27% of breast cancer cell lines that



**Figure 5** Lack of promoter methylation at *KLF5* in breast cancer cell lines. (a) *Bst* UI digested PCR products from samples. The control shows the PCR products without (on the left) or with (on the right) *Bst* UI digestion. See Materials and methods for details. Molecular weight sizes of markers are indicated at the left. (b) SSCP analysis of the same PCR products as in (a). The control is exon 2b of *KLF5* with a known T>G point mutation (on the left) or without a mutation (on the right)



**Figure 6** Reduced colony forming efficiency by *KLF5* in T-47D breast cancer cells. The graph shows the numbers of colonies formed in clones of T-47D cells stably transfected with pcDNA-KLF5, as described in Materials and methods. Data are expressed as average  $\pm$  s.e.m. Inset: Representative samples of T-47D cells transfected with either vector alone (pcDNA3.1, three wells in the upper row) or pcDNA-KLF5 (three wells in the lower row).  $P=0.000$  (*t*-test)

exhibited loss of expression, neither hemizygous deletion nor promoter methylation was detected. These findings suggest that other mechanisms may also be involved in regulating *KLF5* expression in certain breast cancers. Furthermore, only one of the 30 cancer cell lines tested exhibited a mutation within the *KLF5* gene, indicating that *KLF5* mutation occurs relatively rarely in breast cancer. Consistent with our mutation data, no germline mutations of *KLF5* were found in 19 breast cancer families (Rozenblum *et al.*, 2002). Because promoter methylation is not responsible for the loss of expression in the cancer cell lines, these findings suggest that hemizygous deletion and associated loss of expression, or haploinsufficiency, is likely to be a major mechanism by which *KLF5* function becomes insufficient during breast carcinogenesis.

Knudson's two-hit theory of tumor suppressor inactivation, in which one allele is mutated while the other is often deleted, has led to the identification of many tumor suppressor genes. As chromosomal deletion can confer one hit, it has been widely used to define tumor suppressor loci. To date, a large number of chromosomal regions with deletions have been identified in various common human cancers. In contrast, the number of genes found with mutations in human cancers is relatively small. One possible explanation for this discrepancy is haploinsufficiency, where the loss of one chromosome at a locus reduces gene expression levels. Our current findings support the hypothesis that haploinsufficiency plays a role in breast cancer. Role of tumor suppressor haploinsufficiency in carcinogenesis has been demonstrated for several genes, including *PTEN* and *p27/Kip* in knockout mouse models (Di Cristofano *et al.*, 1998; Fero *et al.*, 1998).

We are currently analysing the effect of haploinsufficiency at *KLF5* on cell growth and differentiation in a knockout mouse model.

Our data suggest that LOH occurs more frequently than quantitative hemizygous deletion, as 19 out of 30, or 63% of cases exhibited LOH *versus* 13 out of 30, or 43% that exhibited hemizygous deletion. Nine of the 13 (69%) cases with hemizygous deletion also exhibited LOH. However, the other four cases showed quantitative deletion at *KLF5*, but LOH was not detected by the HOMOD method. By definition, a case with hemizygous deletion should also show LOH. One possible explanation for these four cases with hemizygous deletion in the absence of LOH is that LOH occurred in a smaller region around *KLF5* that was not detectable by the HOMOD method.

*KLF5* belongs to the Kruppel-like family of transcription factors, which is characterized by three zinc-finger domains. These genes are believed to play important roles in regulating a diverse range of biological processes, including cellular proliferation and differentiation (Dang *et al.*, 2000). This family of transcription factors is involved in several aspects of tumorigenesis, including growth control, apoptosis, and angiogenesis (Black *et al.*, 2001). It has been demonstrated that *KLF5* can bind to CACCC motifs and thereby regulate the expression of other genes (Conkright *et al.*, 1999; Shi *et al.*, 1999). Another member of this family, *KLF4*, is significantly down-regulated during intestinal tumorigenesis (Ton-That *et al.*, 1997). More recently, *KLF6* was reported to act as a tumor suppressor gene in prostate cancer (Narla *et al.*, 2001). Thus, it is likely that down-regulation of *KLF5* alters the expression levels of a series of genes that control cellular proliferation and/or differentiation. Such alterations may represent an important step in the development and progression of cancer.

In a recent study, loss of 13q was predicted to be one of the earliest genetic events in hereditary breast cancers (Kainu *et al.*, 2000). The minimal region of deletion was identified between markers *D13S1317* (51.02 cM) and *D13S166* (55.3 cM) at 13q21-22 by both CGH and linkage analysis (Kainu *et al.*, 2000). *KLF5* is located in this region, approximately 1.1 Mb centromeric to *D13S166* (Chen *et al.*, 2001). This finding suggests that *KLF5* may also play a role in hereditary breast cancer.

In previous studies, we have shown that deletions in the same region of 13q21 are also frequent in carcinomas of the prostate, ovary, endometrium, and cervix (Chen *et al.*, 2001). CGH analysis has also detected quantitative deletion of 13q21 in many other cancers (Knuutila *et al.*, 1999). Thus, it is likely that inactivation of *KLF5* by hemizygous deletion-induced loss of expression plays a role in the development of many types of neoplasm.

In summary, *KLF5* is the only gene from a common region of deletion at 13q21 that frequently undergoes hemizygous deletion and loss of expression in breast cancer. In the absence of quantitative hemizygous deletion, LOH was insufficient to induce loss of *KLF5*

expression. Neither mutation nor promoter methylation was a common occurrence at *KLF5* in breast cancer. The functional restoration of *KLF5* by exogenous overexpression inhibited cell proliferation. These findings suggest that *KLF5* is a candidate tumor suppressor gene that is inactivated in breast cancer primarily through hemizygous deletion-induced loss of expression. This study provides the basis for further analysis of *KLF5* in cancer development.

## Materials and methods

### Non-neoplastic and neoplastic human breast epithelial cells

A sample of total RNA derived from normal human mammary gland was purchased from Clontech (Palo Alto, CA, USA). The immortalized non-neoplastic breast epithelial cell line BRF-97T and the breast cancer cell line BRF-71T1 were purchased from Biological Research Faculty & Facility (BRFF, Ijamsville, MD, USA). All other breast cancer cell lines, listed in Figure 4, were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and were propagated according to the conditions recommended by ATCC. Genomic DNA samples were isolated using the Blood & Cell Culture DNA Midi Kit from Qiagen (Valencia, CA, USA), and total RNA was isolated from cytoplasm of cells using Qiagen's RNeasy Maxi Kit.

### Reverse transcription–polymerase chain reaction (RT–PCR)

RNA samples were treated with DNase I to remove contaminating genomic DNA. Using the Superscript cDNA synthesis system (Invitrogen, Carlsbad, CA, USA), first strand cDNA was synthesized from 2  $\mu$ g of total RNA with oligo(dT) primers. Ribonuclease H was then added to the reaction mixture, which was heated to 37°C for 15 min. The resulting cDNA was purified using the Qiaquick PCR Purification Kit (Qiagen) and resuspended in 100  $\mu$ l of water. Two  $\mu$ l of this purified cDNA were included in each PCR. Primer sequences for the genes/ESTs tested have been either described previously (Chen *et al.*, 2001) or are as follows: 5'-ATCAAGACAGAACCTGTTGCC-3'/5'-CAGCCTTCCCA-GGTACACTTG-3' (*KLF5*), 5'-GCAAGAAATCTCACACAGTG-3'/5'-ATGGTCTACAGGTGCTTCCC-3' (*PIBF1*), 5'-GAAATCGTGCCTGACATTAAG-3'/5'-CTAGAAGCA-TTTGCGGTGGACGATGGAGGGGCC-3' ( $\beta$ -actin). The Platinum Taq Polymerase (Invitrogen) was used for RT–PCR. In a volume of 20  $\mu$ l, 28 cycles were used to amplify  $\beta$ -actin, whereas 35 cycles were used to amplify the various other genes and/or ESTs.

### Duplex PCR

Duplex PCR was performed as previously described (Dong *et al.*, 2000). Primer sequences for markers in Figure 3 were: 5'-GATTTGATCCATTTAGGGGC-3'/5'-CACTGTGGACTT-TGATACTTGG-3' (530F3M4), 5'-GACTGAGGCACTGT-CTCGTC-3'/5'-GTTTCGTTCTTCCCAGATGG-3' (*KLF5*), and 5'-AGACTAAATCTGTGTTGCC-3'/5'-AATTAGT-GGCCTGGCAGTGG-3' (530F3M5).

### Real time PCR assay

Real time TaqMan PCR was performed using the ABI PRISM 7700 Sequence Detection System (PE Applied

Biosystems, Foster City, CA, USA). All reagents, including synthesized primers and probes, were purchased from PE Applied Biosystems. DNA sequences and final concentrations for primers and TaqMan probes were: 5'-CTTCCACAA-CAGGCCACTTACTT-3' (*KLF5* forward, 60 nM), 5'-AGA-AGCAATTGTAGCAGCATAGGA-3' (*KLF5* reverse, 120 nM), 5'-VIC-TCACCACCAAGCTCAGAGCCTGGA-TAMRA-3' (*KLF5* probe, 100 nM), 5'-CAGGTGGGCAC-GGGTTT-3' (*KAI1* forward, 30 nM), 5'-TCCTCGCG-ACTGCTGTTGTA-3' (*KAI1* reverse, 60 nM), and 5'-FAM-AGGAAATCTGACCCTGACCTTTGTCCTCC-TAMRA-3' (*KAI1* probe, 100 nM). Primers and TaqMan probe for  $\beta$ -actin were purchased from PE Applied Biosystems. For detection of deletions, samples containing 20 ng of genomic DNA were subjected to amplification by PCR in a volume of 50  $\mu$ l with one pair of primers derived from exon 2 of *KLF5* and one pair derived from the *KAI1* gene, which served as an internal control (non-deleted) sequence. For expression analysis, the same pair of *KLF5* primers were used with  $\beta$ -actin as an internal control. All reactions were performed in triplicate with 40 cycles using standard program, and DNA or RNA levels were determined by the standard curve method. For deletion analysis, the average ratio of *KLF5* to the internal control *KAI1* in non-neoplastic samples, which included the BRF-97T non-neoplastic cell line and 15 normal DNA samples derived from the blood of healthy individuals, was defined as 100, and the ratio for each sample was normalized accordingly. For expression analysis, the ratio of *KLF5* to internal control  $\beta$ -actin in the non-neoplastic cell line BRF-97T was defined as 100, and the ratio for each sample was normalized accordingly. A sample was designated as having a deletion or loss of expression if the ratio of *KLF5* to *KAI1* or *KLF5* to  $\beta$ -actin, respectively, was  $50 \pm 5$  or less.

### Northern blot analysis

The probe used in Northern blot analysis was amplified from the *KLF5* cDNA by PCR using primers 5'-CCCTACCCAG-CAGGGCCACC-3' and 5'-GGAACGGGTACACGGGC-AG-3'. After purification with the Qiaquick PCR Purification Kit (Qiagen), the DNA probe was labeled by random priming with  $\alpha$ -<sup>32</sup>P-dCTP (Invitrogen). Blots were hybridized at 68°C for 1 h in QuikHyb solution (Stratagene, La Jolla, CA, USA) and washed according to a standard protocol. After *KLF5* blots were exposed to X-ray film, the same membrane was stripped and re-hybridized with a  $\beta$ -actin probe.

### PCR-single strand conformational polymorphism (SSCP) and direct DNA sequencing

SSCP analyses and direct DNA sequencing were performed as previously described (Chen *et al.*, 2001). PCR primers flanking each of the *KLF5* exons were used and the sequences were: 5'-AGCTGCGCCCCGAGTG-3'/5'-CCCAGCCTG-GAGCGGCCCTGT-3' (exon 1), 5'-GTTTCGTTCTTCC-CGAGATGG-3'/5'-AATGGCAACAGGTTCTGTCTTG-3' (exon 2a), 5'-ATCAAGACAGAACCTGTTGCC-3'/5'-TCC-ATTGCTGCTGTCTGATTTGTAG-3' (exon 2b), 5'-CCC-TACCCAGCAGGGCCACC-3'/5'-AGGCTCTGAGCTTG-GTGGTG-3' (exon 2c), 5'-CTTCCACAACAGGCCACT-TACTT-3'/5'-TTCAACCAGGTAAGAGCACATAC-3' (exon 2d); 5'-GATCTCCAAAATGACATGCTG-3'/5'-TAAAAG-GATGAATACCACTTACAC-3' (exon 3), and 5'-GCAG-GCCGCTTACCTCCTTTG-3'/5'-GGAACGGGTACAC-GGGCAG-3' (exon 4).

### Detection of DNA methylation

For methylation detection, genomic DNA was treated with bisulfite, as described previously (Herman *et al.*, 1996). Samples were then subjected to amplification by PCR using primers 5'-GGAGTTGGGTGAAATAGAGG-3' and 5'-CC-TCTTACCTCTCCCAAACC-3', which spanned 632 bp of GC-rich DNA in the promoter region and exon 1 of *KLF5*. Purified PCR products were then subjected to two independent procedures to detect methylation. First, the PCR products were digested with *Bst* UI, which cleaves the wild-type fragment at 13 sites, and analysed by agarose gel electrophoresis. A change in the pattern of fragmentation indicated the presence of methylation. Second, the <sup>33</sup>P-labeled PCR products were subjected to SSCP analyses, to search for shifted bands, which would indicate the presence of methylation. In *Bst* UI digestion experiments, the positive control was a 392 bp PCR product amplified from untreated BR-97T DNA using primers 5'-CCGCTGTCTGAG-GAGTCCACCC-3' and 5'-ACCAGGTCCTCTGGAGGCA-GCCG-3'. There are 10 *Bst* UI sites in the positive DNA fragments. The positive control in SSCP analysis was a PCR fragment amplified from IMAGE cDNA clone 2256867 which has a T>G point mutation at 635 from ATG start codon, with primers for exon 2b of *KLF5* as described in the mutation screening section.

### Homozygosity-mapping-of-deletion (HOMOD) analysis

HOMOD analyses were performed as described in our previous study (Chen *et al.*, 2001). Ten polymorphic markers

### References

Black AR, Black JD and Azizkhan-Clifford J. (2001). *J. Cell. Physiol.*, **188**, 143–160.

Chen C, Brabham WW, Stultz BG, Frierson HFJ, Barrett JC, Sawyers CL, Isaacs JT and Dong JT. (2001). *Genes Chromosomes Cancer*, **31**, 333–344.

Conkright MD, Wani MA, Anderson KP and Lingrel JB. (1999). *Nucleic Acids Res.*, **27**, 1263–1270.

Dang DT, Pevsner J and Wang VW. (2000). *Int. J. Biochem. Cell Biol.*, **32**, 1103–1121.

Di Cristofano A, Pesce B, Cordon-Cardo C and Pandolfi PP. (1998). *Nat. Genet.*, **19**, 348–355.

Dong JT, Chen C, Stultz BG, Isaacs JT and Frierson Jr HF. (2000). *Cancer Res.*, **60**, 3880–3883.

Fero ML, Randel E, Gurley KE, Roberts JM and Kemp CJ. (1998). *Nature*, **396**, 177–180.

Herman JG, Graff JR, Myohanen S, Nelkin BD and Baylin SB. (1996). *Proc. Natl. Acad. Sci. USA*, **93**, 9821–9826.

Hyytinen ER, Frierson HF, Boyd JC, Chung LWK and Dong JT. (1999). *Genes Chromosomes Cancer*, **25**, 108–114.

Kainu T, Juo SH, Desper R, Schaffer AA, Gillanders E, Rozenblum E, Freas-Lutz D, Weaver D, Stephan D, Bailey-Wilson J, Kallioniemi OP, Tirkkonen M, Syrjakoski K, Kuukasjarvi T, Koivisto P, Karhu R, Holli K, Arason A, Johannesdottir G, Bergthorsson JT, Johannsdottir H, Egilsson V, Barkardottir RB, Johannsson O, Haraldsson K, Sandberg T, Holmberg E, Gronberg H, Olsson H, Borg A, Vehmanen P, Eerola H, Heikkila P, Pyrhonen S and Nevanlinna H. (2000). *Proc. Natl. Acad. Sci. USA*, **97**, 9603–9608.

(*D13S1291*, *D13S1318*, *D13S131*, *D13S258*, *D13S152*, *D13S745*, *D13S791*, *D13S1326*, *D13S166*, and *D13S269*) surrounding *KLF5* were tested to determine allelic status. Primer sequences for these markers are available from the Genome Database (<http://gdbwww.gdb.org>).

### Gene transfection and colony forming assay

The coding region of the *KLF5* cDNA was cloned into the *Eco*RI and *Xho*I sites of the mammalian expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA, USA). The resulting construct was designated as pcDNA-KLF5. Positive clones were identified by restriction enzyme digestion and verified by DNA sequencing. Six-well tissue culture clusters were seeded with 5000 T-47D cells per well. Purified plasmid DNA (0.5 µg) for either pcDNA-KLF5 or vector alone was transfected into cells using the LipofectAMINE reagent (Invitrogen). Twenty-four hours after transfection, the culture medium was replaced with medium including G418 (1 mg/ml, Invitrogen), and thereafter the selection medium was replaced every 3 days. After 2 weeks of selection in G418, cells were fixed with 2% formaldehyde and stained with 0.5% crystal violet. The number of colonies in each well was counted.

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Knuutila S, Aalto Y, Autio K, Bjorkqvist AM, El-Rifai W, Hemmer S, Huhta T, Kettunen E, Kiuru-Kuhlefelt S, Larramendy ML, Lushnikova T, Monni O, Pere H, Tapper J, Tarkkanen M, Varis A, Wasenius VM, Wolf M and Zhu Y. (1999). *Am. J. Pathol.*, **155**, 683–694.

Knuutila S, Bjorkqvist AM, Autio K, Tarkkanen M, Wolf M, Monni O, Szymanska J, Larramendy ML, Tapper J, Pere H, El-Rifai W, Hemmer S, Wasenius VM, Vidgren V and Zhu Y. (1998). *Am. J. Pathol.*, **152**, 1107–1123.

Narla G, Heath KE, Reeves HL, Li D, Giono LE, Kimmelman AC, Glucksman MJ, Narla J, Eng FJ, Chan AM, Ferrari AC, Martignetti JA and Friedman SL. (2001). *Science*, **294**, 2563–2566.

Roylance R, Gorman P, Harris W, Liebmann R, Barnes D, Hanby A and Sheer D. (1999). *Cancer Res.*, **59**, 1433–1436.

Rozenblum E, Vahteristo P, Sandberg T, Bergthorsson JT, Syrjakoski K, Weaver D, Haraldsson K, Johannsdottir HK, Vehmanen P, Nigam S, Golberger N, Robbins C, Pak E, Dutra A, Gillander E, Stephan DA, Bailey-Wilson J, Juo SH, Kainu T, Arason A, Barkardottir RB, Nevanlinna H, Borg A and Kallioniemi OP. (2002). *Hum. Genet.*, **110**, 111–121.

Shi H, Zhang Z, Wang X, Liu S and Teng CT. (1999). *Nucleic Acids Res.*, **27**, 4807–4815.

Tirkkonen M, Johannsson O, Agnarsson BA, Olsson H, Ingvarsson S, Karhu R, Tanner M, Isola J, Barkardottir RB, Borg A and Kallioniemi OP. (1997). *Cancer Res.*, **57**, 1222–1227.

Ton-That H, Kaestner KH, Shields JM, Mahatanankoon CS and Yang VW. (1997). *FEBS Lett.*, **419**, 239–243.