Decreased expression of the gut-enriched Krüppel-like factor gene in intestinal adenomas of multiple intestinal neoplasia mice and in colonic adenomas of familial adenomatous polyposis patients

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Abstract Gut-enriched Krüppel-like factor (GKLF) is a zinc finger-containing transcription factor, the expression of which is associated with growth arrest. We compared *Gklf* expression in intestinal and colonic adenomas to normal mucosa in multiple intestinal neoplasia (*Min*) mice and familial adenomatous polyposis (FAP) patients, respectively, using semi-quantitative RT-PCR. In *Min* mice, the level of *Gklf* transcript is highest in normal-appearing intestinal tissues and decreases as the size of the adenoma increases. In FAP patients, the level of *GKLF* transcript is lower in adenomas compared to paired normal-appearing mucosa from the same patient or normal colonic mucosa from control individuals without FAP. The possibility of DNA methylation as a cause for the decreased expression of *Gklf* in adenomas of *Min* mice was investigated by methylation-specific PCR. Results indicate that the *Gklf* gene is not methylated in either normal or tumorous tissues. The findings of our study are therefore consistent with the potential role of GKLF as a negative growth regulator of gut epithelial cells.

Key words: Gut-enriched Krüppel-like factor; Tumorigenesis; *Min* mouse; Familial adenomatous polyposis; Reverse transcription-polymerase chain reaction; DNA methylation

1. Introduction

The mammalian intestinal epithelium is a rapidly proliferating system that is highly susceptible to neoplastic transformations. Approximately 50% of individuals in the Western populations will develop a colorectal neoplasm by age 70, and colon cancer is the second leading cause of cancer deaths in the USA [1,2]. As delineated by Fearon and Vogelstein [3], colonic tumors arise in a stepwise fashion, evolving from sequential mutations in key genes that control growth of epithelial cells. The adenomatous polyposis coli (*APC*) gene is an important example, which when mutated predisposes to the development of colorectal tumors [4–8]. APC functions as a gatekeeper of colonic epithelial cell proliferation by regulating the activity of β-catenin and Tcf-4 [9,10]. Mutations in *APC* can often be identified in early neoplastic changes such as aberrant crypt foci and adenomas [11]. Subsequent mutations in other genes cause tumor progression. This paradigm is evident in patients with the autosomal dominant syndrome familial adenomatous polyposis (FAP), who carry a single germline mutant allele of the *APC* gene. These patients develop adenomas when they acquire a somatic mutation of their wild-type *APC* allele, per Knudson's 'two-hit' hypothesis [8,12]. Consequently, FAP patients develop hundreds to thousands of colonic adenomas starting in teenage years with inevitable progression to colorectal cancer. A murine model for FAP, the multiple intestinal neoplasia (*Min*) mouse, was generated by *N*-ethyl-*N*-nitrosourea mutagenesis [13]. These mice carry a germline mutation in murine *Apc* [14]. On a C57BL/6J genetic background, *Min* mice develop an average of 50 adenomas per animal throughout their intestinal tract by the time of their death, usually at 150 days of age [13].

The gut-enriched Krüppel-like factor (GKLF; also called Krüppel-like factor 4 or KLF4) is a recently identified Krüppel-type transcription factor with three C_2H_2 zinc fingers [15,16]. *Gklf* is highly expressed in the epithelial cells of the gastrointestinal tract [16–18] and the skin [18,19], as well as in vascular endothelial cells [20]. Several lines of evidence suggest that GKLF is important in regulating proliferation and differentiation of epithelial cells. During fetal development in mice, the level of *Gklf* transcript is low initially, starts to rise on embryonic day 13 and peaks at embryonic day 17, which corresponds to a critical period of gut epithelium morphogenesis [21]. In vivo, *Gklf* is primarily expressed in the post-mitotic, terminally differentiated epithelial cells of the intestine [16] and the skin [18,19]. In cultured cells, expression of *Gklf* is temporally associated with conditions that promote growth arrest due to serum deprivation or contact inhibition, and constitutive production of GKLF results in the inhibition of DNA synthesis [16]. Lastly, in mice deficient in *Gklf*, late-stage differentiation of the epidermis is disturbed and they die shortly after birth due to inability to maintain a skin barrier [19].

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Abbreviations: *APC*, adenomatous polyposis coli gene; FAP, familial adenomatous polyposis; GKLF, gut-enriched Krüppel-like factor; *Min*, multiple intestinal neoplasia; MSP, methylation-specific PCR; RT-PCR, reverse transcription-polymerase chain reaction; UTR, untranslated region

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Our group has been investigating the expression of *Gklf* during tumorigenesis. We previously reported that *Gklf* expression is decreased in the whole small intestines of *Min* mice when compared to controls, starting at 12 weeks of age [21]. While the level of *Gklf* transcript in the adenomas of *Min* mice was not directly evaluated, this previous study did suggest that a decreased expression of *Gklf* might be related to tumor development. Our current study measures the levels of *Gklf* transcripts in the intestinal tumors of *Min* mice and in the colonic adenomas of FAP patients compared to normal-appearing mucosa. The results indicate that levels of *Gklf* transcript are similarly decreased in tumors from *Min* mice and FAP patients comparing to the normal mucosa. These studies provide further evidence for the potential involvement of GKLF in regulating proliferation of gut epithelial cells.

2. Materials and methods

2.1. Animals

Wild-type (*Apc*+/+) and *Min* (*Apc*+/−) C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The *Min* pedigree was maintained by crossing male *Min* mice with female wild-type C57BL/6J mice. The *Min* mice were identified by an allele-specific polymerase chain reaction (PCR) as previously described [22]. All animals were killed at age 5 months by CO_2 asphyxiation in accordance with the animal protocol of the Johns Hopkins University. From *Min* mice, small intestine and colon were dissected and adenomas carefully removed under a dissecting microscope and snap-frozen. The adenomas were pooled according to the following sizes: <3 mm, 3–6 mm, and >6 mm. For controls, segments of intestine without tumors from *Min* mice and age-matched wild-type littermates were also retrieved and snap-frozen in liquid nitrogen.

2.2. Human tissues

Tissue samples from FAP patients were obtained from the Johns Hopkins Hospital tissue bank. Two sets of paired samples from three different patients with FAP were obtained. Each pair consisted of two standard biopsy specimens of normal-appearing mucosa and two standard biopsy specimens of an adenoma from each patient obtained during colonoscopy. Samples had previously been histologically confirmed as consisting of either normal mucosa or adenomas. Biopsy samples of the normal colon were also obtained from two individuals without FAP who underwent diagnostic colonoscopy for other medically indicated reasons.

2.3. Preparation of RNA and genomic DNA

Total RNA and genomic DNA were extracted from tissues using the TRIzol Reagent protocol from Life Technologies, Inc. (LTI, Gaithersburg, MD). Due to a paucity of materials, tumors and non-tumor-bearing sections of the intestines were pooled from three different *Min* mice prior to RNA extraction. Similarly, tissues from wild-type mice were pooled from three different animals. After determining the concentration of the RNA samples by optical density measurement, their integrity and quantity were verified with denaturing agarose gel electrophoresis.

2.4. Reverse transcription (RT) PCR

Semi-quantitative PCR following reverse transcription was used to assess transcript levels of the various genes in question. 5 μg of total RNA was treated with amplification-grade DNase I per protocol from LTI (Gaithersburg, MD). 1 μg of the DNase I-treated RNA was reverse transcribed in a 80 μl reaction containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, 3 mM $MgCl_2$, 0.5 mM each dGTP, dATP, dTTP, and dCTP, 80 U RNase inhibitor, and 100 pmol random primer, $pd(N)_6$. The reaction was heated to 90°C for 5 min, cooled slowly to 67°C, and followed by the addition of 200 U MMLV reverse transcriptase (LTI, Gaithersburg, MD). The reaction was then incubated at 42°C for 1 h followed by 95°C for 5 min.

Each PCR reaction of 50 μl consisted of cDNA made from 125 ng of total RNA, 1×REDTaq PCR buffer (10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1 mM $MgCl_2$, and 0.1% gelatin), 2.5 U REDTaq DNA polymerase (Sigma, St. Louis, MO), 200 μM each dGTP, dATP, dTTP, dCTP and 40 pmol each of the forward and reverse primer. The primers used in the PCR reaction were designed from the cDNA sequences of mouse *Gklf* and human *GKLF* (GenBank accession numbers U20344 and AF105036, respectively). For mouse *Gklf*, the forward primer is 5′-AGGTCGGACCACCTTGCCTTACACATG-3′, the reverse primer is 5′-CCTCACCCCAAGCTCACTGATTTAG-3′, and the expected PCR product is 439 bp. For human *GKLF*, the forward primer is 5′-AGGTCGGACCACCTCGCCTTACACATG-3′, the reverse primer is 5′-AAGGTAAAGAGAATACAAGGTGA-TCTTTTATGC-3′, and the expected PCR product is 345 bp. Water blank and RNA samples not reverse transcribed were used as negative controls.

To ensure equal loading of cDNA samples, concurrent PCR reactions for β-actin were performed. The primers for β-actin were designed from cDNA sequences of mouse and human β-actin (GenBank accession numbers M12481 and X00351, respectively). For mouse β-actin, the forward primer is 5′-TATGCCAACACAGTGTT-GTCTGG-3′, the reverse primer is 5′-TACTCCTGCTTGCTGATC-CACAT-3′, and the expected PCR product is 206 bp. For human β-actin, the forward primer is 5′-TACGCCAACACAGTGCTG-TCTGG-3′, the reverse primer is 5′-TACTCCTGCTTGCTGATC-CACAT-3′, and the expected PCR product is 205 bp. In addition, the levels of transcripts for cytokeratin 19 (CK19) in the collected mouse specimens were measured by RT-PCR as a means to provide an estimate of the amount of epithelial cells in the tissues. CK19 is uniformly distributed in the epithelial cells of rodent small intestine [23]. The primer sequences used to amplify mouse CK19 cDNA were 5′-GGGTTCAGTACGCATTGGG-3′ for the forward primer and 5′-CTCCACGCTCAGACGCAAG-3′ for the reverse primer, and the expected PCR product is 471 bp [24].

The number of amplification cycles in the semi-quantitative PCR was determined from the linear portion of the PCR cycle when amplification was performed with increasing numbers of cycles for *Gklf*, β-actin and CK19. Fig. 1 shows the standard curves for *Gklf* and β-actin, using cDNA from the distal small intestine of wild-type mice. For humans, cDNA product from normal-appearing colonic mucosa was used to generate a standard curve (data not shown). Each PCR reaction was started at 94°C for 5 min followed by the predetermined number of cycles, and terminated after a final 72°C incubation for 10 min. Each cycle included denaturation at 94°C for 45 s, primer annealing at 55°C (mouse *Gklf*, β-actin, and CK19) or 45°C (human *GKLF* and β-actin) for 1 min, and extension at 72°C for 1.5 min. All PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide.

2.5. Methylation-specific PCR (MSP)

MSP was performed on genomic DNA obtained from mouse tissues using 1 μg of DNA modified with bisulfite as previously described [25]. For unmethylated *Gklf*, the forward primer is 5′-AGTTTTT-TATTTAGTAATTTGTTTGTGATTTGT-3′ and the reverse primer is 5′-AACACAAAAACTATAATCCAAACAATAAACA-3′, which were designed to amplify the 172 bp sequence between nucleotides +107 and +279 of the 5′-untranslated region (UTR) of the mouse *Gklf* gene [26]. For methylated *Gklf*, the forward primer is 5′-TTATT-TAGTAATTCGTTCGTGATTCGC-3′ and the reverse primer is 5′-CGAAAACTATAATCCGAACGATAAACG-3′, which were designed to amplify the 162 bp sequence between nucleotides +113 and +275 of the 5′ UTR of the mouse *Gklf* gene [26]. The negative control was a water blank. The positive control for methylation-specific reaction was in vitro methylated DNA (IVD) from wild-type mouse, generated by treatment with Sss1 methylase per protocol from New England Biolabs (Beverly, MA). This treatment renders all cytosine residues in a CpG dinucleotide methylated. Each PCR reaction of 50 μl consisted of 40 ng DNA, 1×buffer as described [25], 1.25 mM each dGTP, dATP, dTTP, dCTP and 200 ng each forward and reverse primer. Each PCR reaction was hot-started at 94°C for 5 min followed by the addition of 2.5 U REDTaq DNA polymerase (Sigma, St. Louis, MO), and then denatured at 95°C for 30 s, annealed at 57°C for 30 s, and extended at 72°C for 30 s, for a total of 35 cycles. PCR products were visualized on a 6% non-denaturing polyacrylamide gel stained with ethidium bromide.

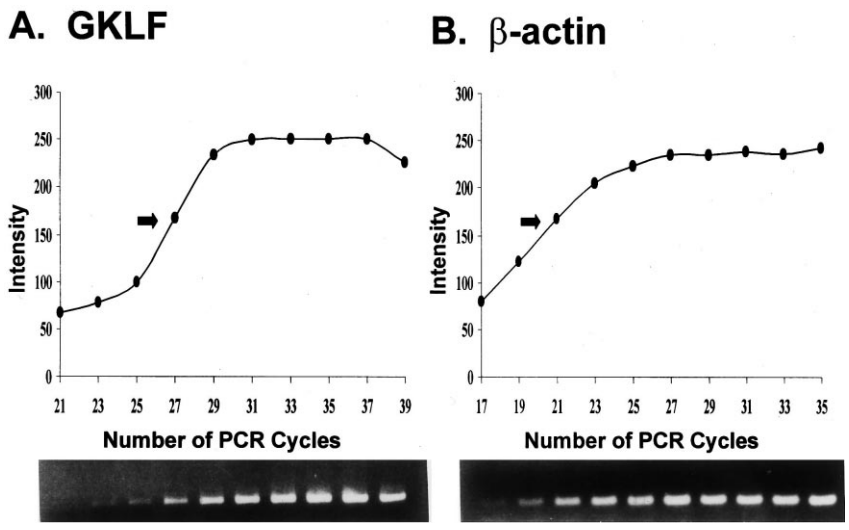

Fig. 1. Optimization of RT-PCR reactions for *Gklf* and β-actin. cDNA derived from 125 ng total RNA from wild-type mouse intestine was amplified for 21–39 cycles using primers for *Gklf* (A) and 17–35 cycles using primers for β-actin (B). The intensity of the RT-PCR bands, as measured by quantitative densitometric tracings, as a function of the number of cycles is plotted. The arrows indicate the numbers of cycles selected for subsequent experiments for *Gklf* and β-actin, respectively.

3. Results

Fig. 1 shows the optimization of PCR cycle numbers for the mouse *Gklf* and β-actin cDNA (panels A and B, respectively), reversed transcribed from RNA isolated from the small intestine of wild-type mice. The optimal numbers of cycles for the two genes were selected from the linear region of the curves (Fig. 1, arrows). Similar optimization was performed on human *GKLF* and β-actin, and mouse CK19 genes (results not shown).

Using the optimized cycle numbers, RT-PCR on RNA obtained from either the normal-appearing portion of the small intestine or size-selected, pooled intestinal adenomas from *Min* mice was performed and compared to RNA from the intestines of age-matched control littermates. As seen in Fig. 2, the level of *Gklf* transcript in the normal-appearing portion of the *Min* mouse intestine (Fig. 2, lane 2) is slightly lower than that of the wild-type mice (Fig. 2, lane 1). Importantly, Fig. 2 shows that the level of *Gklf* transcript is progressively decreased in RNA isolated from the adenomas of the *Min* mouse intestine and that this decrease is size-dependent (lanes 3–5). The transcript is barely detectable in the group of adenomas that are greater than 6 mm (lane 5). A similar finding was obtained with a second set of independently isolated tissues (data not shown). The loading of the samples was reasonably equal as judged by the similar transcript amounts for β-actin (Fig. 2). Moreover, the amounts of epithelial cells in the various tissues studied were also relatively equal as judged by the levels of CK19 transcript (Fig. 2).

Next, the level of *GKLF* transcript in colonic adenomas was measured and compared to the neighboring, normal-appearing colonic mucosa obtained from three FAP patients and to biopsied normal colonic specimens from two control individuals. As shown in Fig. 3, in each of the three pairs of speci-

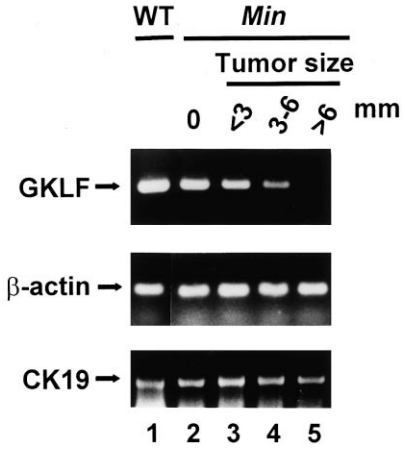

Fig. 2. Semi-quantitative RT-PCR of *Gklf*, β-actin and CK19 transcripts in *Min* and wild-type mouse intestines. RNA was extracted from the intestines of wild-type mice (WT) and the normal-appearing mucosa of *Min* mice (0), as well as the pooled adenomas of *Min* mouse intestines based on size: <3 mm, 3–6 mm, and >6 mm. cDNA produced from 125 ng total RNA was subject to PCR using primers for *Gklf*, β-actin and CK19. Products were resolved on a 1.5% agarose gel.

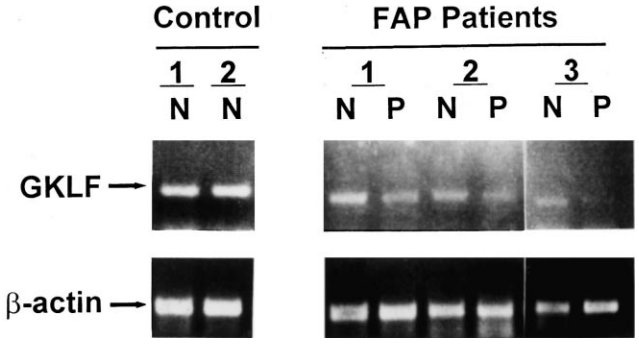

Fig. 3. Semi-quantitative RT-PCR of *GKLF* and β-actin transcripts in control individuals and FAP patients. RNA was extracted from biopsied specimens of normal colon from two control individuals (left panel) and normal-appearing colonic mucosa (N) and adenomatous polyps (P) from three FAP patients (right panel). cDNA template made from 125 ng total RNA was subject to PCR using primers for human *GKLF*. To ensure even loading, the same amount of template was subjected to PCR using primers for β-actin.

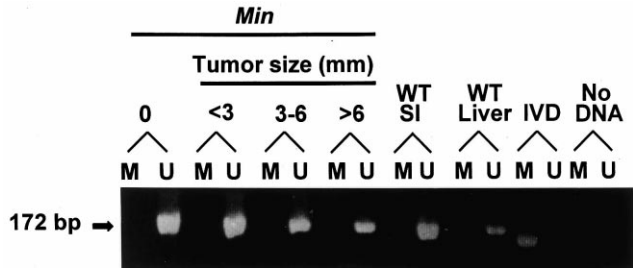

Fig. 4. MSP of genomic DNA from mouse tissues. Genomic DNA was extracted from normal-appearing intestinal mucosa and adenomas of different size of *Min* mice, and from the small intestine (SI) and liver of wild-type (WT) control littermates. Following treatment of the DNA with bisulfite, MSP was performed according to the protocol described in Section 2 using primers designed to amplify either methylated (M) or unmethylated (U) DNA present in the 5′-UTR of the mouse *Gklf* gene [26]. The expected product for the unmethylated DNA is 172 bp. As a control for methylated product, in vitro methylated mouse genomic DNA (IVD) was similarly analyzed by MSP and the methylated DNA product is 162 bp. Water was used as a negative control (No DNA).

mens, the level of the *GKLF* transcript in the adenoma (P) was lower than that of the paired normal colon (N). Quantitative densitometric tracings of the band intensities for *GKLF*, after normalizing to the β-actin levels, revealed a 25–40% reduction in the *GKLF* transcript level in the adenomas compared to paired normal-appearing mucosa.

As an initial attempt to understand the mechanism responsible for the decreased expression of *Gklf* in the tumorous tissues, MSP on genomic DNA obtained from wild-type or *Min* mice was performed. The rationale to address this possibility first is that the promoter and 5′-UTR of the mouse *Gklf* gene contains a large CpG island [26]. Aberrant methylation of 5′ CpG islands has been correlated with the silencing of gene expression, especially during tumorigenesis [27]. However, as seen in Fig. 4, in contrast to the positive result obtained from the IVD, no evidence of methylation of the 5′-UTR of *Gklf* was found in either the tumorous or non-tumorous tissues, including the liver, an organ that does not express *Gklf*. This result suggests that methylation is not involved in the tissue-specific or tumor-related regulation of expression of *Gklf*.

4. Discussion

This study represents a continuing interest of our group in understanding the relationship between *Gklf* expression and intestinal tumorigenesis. Our prior work suggests that GKLF is a negative regulator of cell growth [16]. We previously showed that the level of *Gklf* transcript is decreased in the whole intestine of *Min* mice compared to wild-type controls during the period of tumor formation [21]. The current study extends this observation by demonstrating that expression of *Gklf* and *GKLF* is selectively decreased in the intestinal adenomas of *Min* mice and FAP patients, respectively. Whether decreased expression initiates tumorigenesis or is a result of tumor progression is unclear. However, the observation that *Gklf* transcript level is slightly decreased in normal-appearing intestine of *Min* mice compared to control mice suggests a potential role for GKLF in the early stage of tumorigenesis.

The mechanism for the decreased expression of *Gklf* in tumors is presently unknown. Our data show that aberrant methylation in the 5′-UTR of *Gklf* as defined by MSP is not responsible for the decreased expression of *Gklf* in tumor tissues from *Min* mice. Several recent studies suggest that there may be an alternative explanation. Adenomas of *Min* mice and FAP patients arise from the loss of the wild-type *Apc* in addition to the inherited germline mutated *Apc* [8,10]. APC functions by facilitating β-catenin degradation, thus down-regulating β-catenin translocation into the nucleus and subsequent binding to Tcf-4 [9,10]. When APC is mutated, expression of target genes of the β-catenin/Tcf-4 complex including those encoding c-Myc, cyclin D1, Tcf-1, and PPAR-δ are aberrantly increased, which leads to accelerated growth [28–31]. Recently, APC has been shown to induce expression of *Cdx-2*, a homeodomain-containing nuclear protein specifically expressed in the gut epithelium [32,33]. Similar to GKLF, Cdx-2 regulates proliferation and differentiation of the intestinal epithelium [34] and *Cdx-2* expression is decreased in human and rat adenomas, carcinomas, and colon cancer cell lines [35,36]. Moreover, 90% of heterozygous *Cdx-2*-deficient mice develop multiple intestinal adenomatous polyps by 3 months of age [37]. We recently reported that a Cdx-2 binding site is present in the *Gklf* promoter, and that Cdx-2 transactivates a reporter gene linked to 1 kb of the *Gklf* 5′-flanking region [26]. These results suggest that GKLF is potentially a downstream target gene of Cdx-2, and hence APC. Additional experiments are in progress to further delineate this relationship.

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