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Evaluation of *MYB* Promoter Methylation in Salivary Adenoid Cystic Carcinoma

Chunbo Shao[a], **Weiliang Bai**[b], **Jacqueline C. Junn**[c], **Mamoru Uemura**[a], **Patrick T. Hennessey**[a], **David Zaboli**[a], **David Sidransky**[a], **Joseph A. Califano**[a,d], and **Patrick K. Ha**[a,d]

[a] Department of Otolaryngology-Head and Neck Surgery, Johns Hopkins Medical Institutions, Baltimore, Maryland, United States of America

[b] Department of Otorhinolaryngology, Shengjing Hospital, China Medical University, Shenyang, China

[c] New York Medical College, Valhalla, New York, United States of America

[d] Milton J. Dance, Jr. Head and Neck Center, Greater Baltimore Medical Center, Baltimore, Maryland, United States of America

Summary

The transcription factor *MYB* was recently proposed to be a promising oncogene candidate in salivary gland adenoid cystic carcinoma (ACC). However, the up-regulation of *MYB* in ACC could not be explained solely by deletion of its 3′ end. It is widely accepted that the promoter methylation status can regulate the transcription of genes, especially in human cancers. Therefore, it is important to know whether *MYB* promoter demethylation could explain the over-expression of *MYB* in ACC. By using the Methprimer program, we identified nine CpG islands in the promoter of *MYB*. All of these CpG islands were located within the −864 to +2,082 nt region relative to the transcription start site of *MYB*. We then used bisulfite genomic sequencing to evaluate the methylation levels of the CpG islands of *MYB* in 18 primary ACC tumors, 13 normal salivary gland tissues and nine cancer cell lines. Using cell lines, we also determined the relative *MYB* expression levels and correlated these with the methylation levels. With bisulfite genomic sequencing, we found no detectable methylation in the CpG islands of *MYB* in either ACC or normal salivary gland tissues. There was a variable degree of *MYB* expression in the cell lines tested, but none of these cell lines demonstrated promoter methylation. Promoter hypomethylation does not appear to explain the differential expression of *MYB* in ACC. An alternative mechanism needs to be proposed for the transcriptional control of *MYB* in ACC.

Keywords

adenoid cystic carcinoma; *MYB*; DNA methylation

Corresponding Author: Patrick K. Ha, Johns Hopkins Department of Otolaryngology-Head and Neck Surgery, Johns Hopkins Head and Neck Surgery at GBMC, 1550 Orleans St, Rm 5M06, David H Koch Cancer Research Building, Baltimore, MD 21231, Phone – 410-502-5144, Fax – 410-614-1411, pha1@jhmi.edu; cshao1@jhmi.edu.

Conflict of Interests Statement
None declared.

Introduction

Salivary gland adenoid cystic carcinoma (ACC) is an aggressive, pernicious malignancy known for its persistent slow growth, proclivity for perineural invasion, high rate of recurrence, and formation of distant metastasis. While the five-year survival is favorable at approximately 75%, the long-term survival is poor, with only 39.6% of patients alive after 15 years [1]. Histopathologically, ACC is characterized by three patterns: solid, cribriform, and tubular. All three patterns may be found within the same tumor. The solid pattern is rare but typically has a worse clinical course 1. Surgical therapy with adjuvant radiation remains the main treatment for ACC and typically provides excellent locoregional control. Chemotherapy has been considered as a treatment for ACC due to its frequent distant metastasis, though only limited response has been found thus far. The basis for these disappointing results is a lack of understanding of the basic biologic mechanisms involved in ACC carcinogenesis 2.

Several oncogene candidates have been proposed for ACC. Among them, the transcription factor *MYB* seems to be a very promising one. *MYB* was recently shown to be overexpressed in 100% of ACC samples tested by a novel mechanism of forming a t(6;9)(q22–23;p23–24) translocation, which fuses the *MYB* and *NFIB* genes [3]. In an independent study with a larger primary ACC cohort, the overexpression of *MYB* was confirmed in the majority of salivary ACC cases. However, the formation of the fusion *MYB-NFIB* product was found in only 28% of primary and 35% of metastatic ACCs [4]. Therefore, fusion *MYB-NFIB* formation must not be the only mechanism to explain the overexpression of *MYB* in ACC.

DNA methylation in the promoter region is a key mechanism in regulating gene transcription. Many tumor suppressor genes are found to be silenced by methylation, and oncogenes re-activated by demethylation in various human cancers [5]. In fact, the methylation status of some genes can be used as biomarkers for tumorigenesis [6]. In salivary gland ACC, promoter methylation has been reported in several candidate tumor suppressor genes [7–13]. Furthermore, promoter methylation of these candidates was correlated with clinical and pathologic parameters, such as tumor histological grade or subtype [10,11,13]. However, DNA methylation status in the *MYB* promoter in ACC has remained unexplored. In our current study, we investigated the methylation status of the *MYB* promoter region in ACC in hopes of elucidating an alternative mechanism to explain *MYB* overexpression in ACC.

Materials and methods

Clinical samples

Eighteen primary ACC tissues were obtained via the Johns Hopkins Pathology Department under the Johns Hopkins Institutional Review Board approved protocol IRB#92-07-21-01. Written informed consent was obtained from all those patients. Normal salivary gland samples were collected through a Johns Hopkins IRB-approved waste tissue protocol through the Department of Pathology that waived patient consent, and no patient identifiers were maintained. Tumor tissues from fresh surgical specimens were snap frozen in liquid nitrogen immediately after surgical resection and stored at −196°C until use. Tumor blocks with at least 80% tumor cells were used for DNA isolation. A cohort of normal parotid tissues obtained from separate patients with benign or inflammatory disease were also freshly frozen and utilized for this study as controls. The normal tissue used from benign tumors was selected to be distant from the tumor and confirmed histologically that no tumor or inflammation was found within the sample block before proceeding to DNA isolation.

DNA and RNA extraction

Samples were digested in 1% SDS and 50 μg/mL proteinase K (Boehringer Mannheim) at 48°C overnight, for removal of proteins bound to DNA. DNA was then purified by phenol-chloroform extraction and ethanol precipitation as described previously [14]. The DNA was subsequently resuspended in 500 μL of LoTE (EDTA 2.5 mmol/L and Tris-HCl 10 mmol/L) and stored at −80°C until use.

Total cellular RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Cell strain and cell lines

The normal salivary gland epithelial cell strain HPAF1 was obtained from ATCC (with the kind permission of Dr. Dharam Chopra) and grown according to ATCC instructions. Cells were grown in collagen IV coated flasks (BD Bioscience, San Jose, CA). The medium used was Keratinocyte Basal Medium (Lonza, Walkersville, MD) supplemented with KGM Single Quots (Lonza).

The ACC83 cell line was a kind gift from Dr. Osamu Tetsu (personal communication), who performed the genotyping of the available ACC cell lines. While ACC83 is thought to be of epithelial origin, its genotype could not necessarily be proven to be of ACC origin, though it did not overlap with other known cell lines. ACC83 was cultured in RPMI1640 medium with 10% FBS and 1% penicillin/streptomycin in a 5% CO_2, 37°C incubator.

Human head and neck squamous cell carcinoma (HNSCC) cell lines JHU-O22, and JHU-O28 were from the Johns Hopkins University Department of Otolaryngology-Head and Neck Surgery [15]. UM22A and UM22B cell lines were provided by Ajay Verma (Merck & Co., North Wales, PA). FaDu cells originated from the American Type Culture Collection.

Three non-small cell lung cancer (NSCLC) cell lines, H1703, H1299 and H1437 were maintained in RPMI 1640 medium with 10% fetal bovine serum [16].

5-Aza dC/TSA treatment

In order to evaluate whether *MYB* could be under the control of promoter methylation, we treated the ACC83 cell line in triplicate with 5-aza-deoxycytidine (5-Aza dC) and trichostatin (TSA) as described previously [17]. Briefly, cells were split to low density (5×10^5/100 mm dish) 24 hours before treatment. Stock solutions of 5-Aza dC (Sigma, St. Louis, MO) and TSA (Sigma, St. Louis, MO) were dissolved in 50% acetic acid and 100% ethanol, respectively. Cells were treated with 5 uM 5-Aza dC for 96 hours and 300 nmol/L TSA for the last 24 hours. Baseline expression was established by mock-treated cells with the same volume of acetic acid or ethanol in triplicate. Total cellular RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Bisulfite treatment and bisulfite genomic sequencing

The EpiTect Bisulfite kit (Qiagen, Valencia, CA) was used to convert unmethylated cytosines to uracils in DNA according to the manufacturer's instructions. Converted DNA was stored at −80°C. Subsequently, bisulfite-treated DNA was amplified using primers designed by MethPrimer to span areas of CpG islands in the promoter of *MYB*. Primer sequences were designed to contain no CG dinucleotides and are listed in Table 1. Touch-down PCR was performed as follows: 95°C for 5 min, followed by cycles consisting of a 30 second denaturation step at 95°C, holding at an annealing temperature for 30 sec, initial extension at 72°C for 1 min, with a final extension at 72°C for 5 min. The annealing temperature was gradually decreased, e.g. two cycles at 64°C, 62°C, 60°C, 58°C and then 35

cycles at 56°C. The PCR products were purified using the QIAquick 96 PCR Purification Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Purified PCR products were then subjected to direct sequencing (Genewiz Inc, Germantown, MD).

By using Methprimer [18], the criteria for CpG islands are: 1) an Observed/Expected CpG ratio over 0.6, 2) the percentage of G plus C over 50%, and 3) a window size of at least 100 bp. By these criteria, there are nine clustered CpG islands within −864 to +2,082 nt region relative to the transcription start site of *MYB*. We designed bisulfite sequencing primers in each of the CpG islands (Table 2). All islands, except for island number 8, were amplified successfully.

Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was measured and adjusted to the same amount for each cell line, and then cDNA synthesis was performed using the iScript™cDNA Synthesis Kit (Bio-Rad, Hercules, CA). The final cDNA products were used as the templates for subsequent qRT-PCR with SYBR green and primers designed spanning Exon 5 and Exon 6 of *MYB*. The *MYB* RT primers are: forward. 5′-GCT GGC TTT TGA AGA CTC CT -3′ and reverse, 5′-AAA TGA CTG TTC TTC TGG AAG -3′. PCR conditions were 95°C for 5min, 45 cycles of 95°C for 15 sec, and 64°C for 30 sec. β-actin was examined to ensure accurate relative quantitation in qRT-PCR. The β-actin RT primers are: forward, 5′-AGT CCT GTG GCA TCC ACG AAA CTA-3′ and reverse, 5′-ACT GTG TTG GCG TAC AGG TCT TTG-3′. PCR conditions were the same as *MYB* RT primers. All reactions were performed in triplicate with water controls, and relative quantity was calculated after normalizing to β-actin expression.

Results

Clinical samples

The demographic characteristics of the patients are summarized in Table 1. Patients with ACC ranged in age from 26 to 68 years, whereas the controls ranged in age from 29 to 81 years. The ACC groups had similar male and female patients, while the normal groups had more males than females: 76.9% vs 23.1%. The smoking status of patients with ACC and subject with controls were similar.

DNA methylation in CpG islands of MYB in primary ACCs, normal salivary glands, and cancer cell lines

In the UCSC human genome browser (http://genome.ucsc.edu/), Feb. 2009 (GRCh37/hg19) assembly, there are 33 splice variants for *MYB*. Twenty-nine of these transcripts spanned a ~38 kb region within chromosome 6 (chr6:135,502,453–135,540,313). We first used Methprimer [18] to screen for CpG islands in the *MYB* region plus a 5 kb region upstream of *MYB*. The default parameters for defining CpG islands are, 1) an Observed/Expected CpG ratio over 0.6, 2) the percentage of G plus C over 50%, and 3) a window size of at least 100 bp. Methprimer located a total of 11 CpG islands, the sizes of which ranged from 112 bp to 654 bp. Nine of total 11 CpG islands were clustered in the −864 ~ +2,082 nt region relative to the *MYB* transcription start site (TSS) (Figure 1). We then used Methprimer to design primers that did not contain CpG dinucleotide sequences (Figure 1, Table 2). The other two CpG islands were short (<150 bp) and located in the introns of *MYB* at +21,096 nt and +24,613 nt relative to TSS. Their short length and long distance from the TSS suggest that they might have little effect on the transcriptional control of *MYB*. Therefore, we did not analyze the methylation status of these two CpG islands.

From bisulfite genomic sequencing, our results showed that none of the analyzed CpG islands demonstrated detectable methylation in the 18 primary salivary adenoid cystic

carcinomas or in the 13 normal salivary gland tissues. The representative chromatography sequencing results are shown in Figure 2. The methylation status of all CpG sites were clearly unmethylated as demonstrated by singly predominant T peaks seen on sequencing chromatographs.

We further analyzed one normal salivary gland epithelial cell strain, HPAF1, several head and neck squamous cell carcinoma (HNSCC), and non-small cell lung carcinoma (NSCLC) cell lines by bisulfite genomic sequencing. Similar to the primary tissue samples, we did not find detectable methylation in any of the CpG islands.

Expression levels of MYB

Since the all cell strain/lines were similarly unmethylated in most CpG islands in the *MYB* promoter, we then wanted to know the expression status of *MYB*. We quantified the exon 5/6 junction of *MYB* by qRT-PCR in those cell lines. The expression level of *MYB* in the normal cell strain HPAF1 was assigned as 1. The *MYB* levels in cell lines were quite variable despite having no detectable promoter methylation. The NSCLC cell lines overall showed higher expression (H1703 was 247.0, H1299 was 50.0, H1437 was 62.9) than the HNSCC cell lines (Fadu was 14.6, UM22A was 3.8, UM22B was 2.2, JHU-O28 was 1.5, and JHU-O22 was 0.6). SACC83 also had relatively high expression level of 231.0 (Figure 3). The methylation levels of the *MYB* promoter region were low in all examined cells lines: none of the cytosines were methylated in a total of 121 interpretable CpG sites (Figure 3). Our results confirm that the variable expression levels of *MYB* were not correlated with promoter methylation status.

Validation of the effects of 5-Aza dC/TSA treatment in MYB re-expression

5-Aza dC/TSA treatment can induce global demethylation in the genome, which may reactivate the transcription of genes that were silenced via promoter methylation. A cell line with methylated *MYB* would be very helpful to investigate whether *MYB* were subjected to methylation control. However, since none of the screened cancer cell lines, including SACC83, showed detectable methylation, we did a tentative 5-Aza dC/TSA treatment in the only available ACC cell line, SACC83. After 5-Aza dC/TSA treatment, the expression levels of *MYB* were actually decreased from 231.0 to 127.7. Therefore, there was no significant reactivation of *MYB* expression (data not shown).

Discussion

The transcription factor, *MYB*, plays a critical role in regulating stem and progenitor cells in the bone marrow, colonic crypts and neurogenic tissues [19]. *MYB* has been implicated in various human malignancies including colon cancer, breast cancer, melanoma, pancreatic cancer, esophageal cancer, and more recently, salivary ACC [19,20]. *MYB* target genes were summarized into three categories by Ramsay, et al, including housekeeping genes (*MAT2A, GSTM1*), differentiation genes (*ELA2*, *MIM1*, *CD4* and *PTCA*), and genes with oncogenic potential (*MYC, KIT, BCL2, GATA3*) [20]. *MYB* has been studied as a therapeutic target in colon cancer and breast cancer [21,22]. For example, a DNA vaccine against *MYB* has been shown to suppress tumor growth in colon cancer [21].

The mechanism of *MYB* overexpression in salivary ACC is postulated to be related to creation of a fusion gene product formed by a chromosomal translocation, t(6;9)(q22–23;p23–24), which leads to a loss of the 3′ untranslated region (UTR) harboring miR-15a/16 and miR-150 binding sites [3]. In a separate study with a larger cohort of salivary ACC's, the overexpression of *MYB* was found in, but not limited to, the primary ACC with such chromosomal translocations [4]. This discrepancy suggested that the overexpression of *MYB*

in salivary ACC has an alternative regulatory mechanism in addition to miRNA. The mechanism of regulating *MYB* expression has been proposed to be through external signals, such as mitogens, signal pathway through extracellular signal-regulated kinases, and Wnt [20]. Promoter methylation control is an important epigenetic mechanism for tumor suppressor genes or oncogenes; this has been frequently demonstrated in human cancers. For the *MYB* gene, its TSS is surrounded by nine clustered CpG islands. Therefore, we hypothesized that *MYB* transcription might be under the control of promoter methylation.

In this study, we investigated the methylation levels in almost all CpG islands overlapping the promoter region of *MYB* in primary ACC and normal salivary gland tissues. No detectable methylation was observed in either primary ACC or normal salivary gland tissue. While we would expect that promoter hypomethylation would lead to overexpression, this was not an ACC tumor specific event, but occurred in the normal samples as well. We further examined several HNSCC and NSCLC cell lines, none of which demonstrated detectable methylation in the *MYB* promoter. These cell lines also demonstrated variable expression levels, further suggesting that *MYB* is not under the influence of promoter methylation. Our final conclusion is that the variable expression levels of *MYB* could not be explained by differential methylation in the promoter.

There are very few studies investigating the relationship of *MYB* methylation and its transcription. One study in 1998 suggested *MYB* promoter methylation might be linked to the transformation in MNNG-exposed Bloom Syndrome (BS) B-lymphoblastoid cell populations, which have been reported to be highly susceptible to malignant transformation after carcinogen treatment [23]. The authors showed unexpected hypermethylation of the c-myb locus in MNNG-BS cell populations compared to control cell lines. The DNA methylation analysis was performed by using methylation sensitive enzymes, *Hpa*II and *Msp*I followed by Southern blotting. However, only a limited number of CpG sites were studied, and *MYB* transcription levels were not determined. In our study, we were unable to identify a cell line with a methylated *MYB* promoter, nor were there an existing, reliable ACC cell line model; therefore, there was no way to investigate whether *MYB* could be re-expressed after inducing promoter demethylation with 5-Aza dC/TSA. This study could be carried out when appropriate cells lines are available.

Additionally, the *MYB* promoter harbors many GGA sequences which might potentially form G-quadruplex structures [24]. A G-quadruplex is a four-stranded DNA or RNA structure stabilized by G-quartets, in which four guanines interact via Hoogsteen hydrogen bonds to form a planar ring [25,26]. The biological implications of these sequences in promoter region are suggestive of their involvement in the regulation of *MYB* gene expression [27].

In summary, we found no methylation in the promoter regions of *MYB* in ACC or normal controls, and therefore, promoter demethylation does not appear to explain the over-expression of *MYB* in ACC tumors. An alternative mechanism needs to be proposed for the transcriptional control of *MYB* in ACC, as growing evidence shows that this may be an important event in ACC carcinogenesis.

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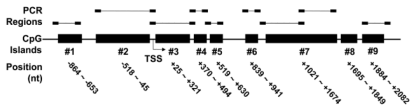

Figure 1. CpG islands of *MYB* and regions analyzed by bisulfite genomic sequencing

This cartoon shows the location of the 9 CpG islands of *MYB* (indicated by solid black boxes), which are numbered #1 to #9. Subregions analyzed by bisulfite genomic sequencing (not to scale) are indicated above each CpG island. Two subregions in CpG island #7 were analyzed. The transcription start site (TSS), was assigned as nt 0 in this cartoon. All positions of the CpG islands are relative to the TSS: the positions upstream are assigned negative values, and the positions downstream are positive. CpG islands were clustered in a region from −864~+2,082 nt region. The relative position of each CpG island is indicated below each CpG island.

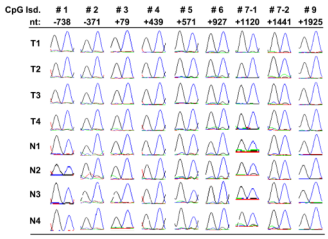

Figure 2. Representative sequencing results in ACC and normal salivary gland tissues

We used bisulfite genomic sequencing to evaluate the DNA methylation levels of *MYB* CpG islands. Here we are showing the sequencing results (in chromatograph form) from randomly selected CpG sites within *MYB* CpG islands in four salivary ACC and four normal salivary gland tissues. Numbered CpG islands (CpG Isd) from #1 to #9 correspond to those shown in Figure 1. Nt indicates the position of selected CpG dinucleotides relative to the TSS of *MYB*. The blue peak stands for G; black, T; green, C; red, A. All CpG dinucleotides deomstrated dominant peaks of TG, which indicate unmethylated CpG. There was no discernible difference in methylation between ACC and normal salivary tissue.

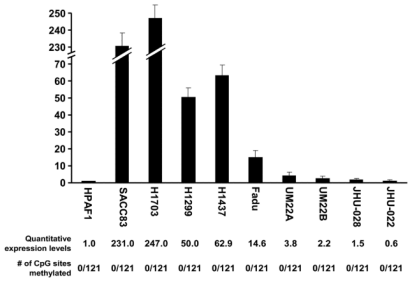

Figure 3. Variable *MYB* expression in cancer cell lines

We used qRT-PCR with SYBR green to investigate the expression levels of *MYB* in various head and neck squamous cell carcinoma cell lines (Fadu, UM22A, UM22B, JHU-O28 and JHU-O22), non-small cell lung cancer cell lines (H1703, H1299, and H1437) and SACC83. HPAF1, which is a normal salivary gland epithelial cell strain, was used as a control and assigned a relative value of "1" for its expression level of *MYB*. Other cell lines were then compared to HPAF1. The quantitative expression levels and the number of CpG sites methylated of *MYB* are shown below each indicated cell line. Error bars indicate the standard deviation. The methylation level of *MYB* was determined by counting the number of methylated CpG in total 121 interpretable CpG sites in all examined CpG islands.

Table 1

Clinical and pathologic characteristics of patient populations

Category	Subcategory	Normal	ACC
Patients, *n*		13	18
Age, yr, median (range)		54 (29–81)	47 (26–68)
Sex, *n* (%)	Male	10 (76.9%)	9 (50%)
	Female	3 (23.1%)	9 (50%)
Smoking status, n (%)	Never	6 (46.2%)	7 (38.9%)
	Former	4 (30.8%)	5 (27.8%)
	Current	3 (23.1%)	5 (27.8%)
	Unknown	0 (0%)	1 (5.6%)
Tumor location, *n* (%)	Parotid	-	4 (22.2%)
	Submandibular	-	5 (27.8%)
	Minor	-	9 (50%)
Stage, *n* (%)	I	-	6 (33.3%)
	II	-	3 (16.7%)
	III	-	2 (11.1%)
	IV	-	4 (22.2%)
	Unknown	-	3 (16.7%)
Perineural invasion, n (%)	Yes	-	6 (33.3%)
	No	-	6 (33.3%)
	Unknown	-	6 (33.3%)
Local recurrence, *n* (%)	Yes	-	6 (33.3%)
	No	-	11 (61.1%)
	Unknown	-	1 (5.6%)
Metastasis, *n* (%)	Yes		4 (22.2%)

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Table 2

Bisulfite genomic sequencing primers for *MYB*

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
Isd 1[a]	TTTGTAGGTGTGTAATTTAGGGTG	TCTCTAATTTAACTTATTAATAACAAAAAA
Isd 2	TAGAGGGTATAGTTGTAAATTTTGA	ACAAAAATACCATCAAACAAAAAACTT
Isd 3	GGGGAGTGTTTAAAGTTTTTTGTT	AAATCTAACAACCCCTCAAATAATC
Isd 4	GGATTATTTGAGGGGTTGTTAGATT	TCCCCATACAACTTACAAAATAAAC
Isd 5	TAAGTTGTATGGGGATATATTTTTTTT	ACCATATACTCTACACTTCCAAATCC
Isd 6	AAAGGGGTTTTTATTTTTGTAAATTGT	ATAAAAAAACTACACCCATCTTCCC
Isd 7-1	GGGTGTAGTTTTTTTATATTTTTGGT	AATTTACTTTACCTCCATAAACTTC
Isd 7-2	TTTATGGAGGTAAAGTAAATTTATT	AACTATCCTATAAAATCCTAAAAAC
Isd 9	TTTTGAGGTTTTAGGTAGTTTAGAGG	TAACCTAATTAATATAATCCCTACC

[a]Isd 1, CpG island #1, the PCR amplified regions are in Figure 1.