BioMetals **17:** 317–323, 2004.

Methylation and expression of the lactoferrin gene in human tissues and cancer cells

Christina Teng*, Wesley Gladwell, Ibrahim Raphiou & Eric Liu
Gene Regulation Section, Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709, USA
**Author for corresepondence (Tel: 919-541-0344; Fax: 919-541-0696; E-mail: Teng@niehs.nih.gov)*

Key words: bisulfite genomic sequencing, cancer cells, lactoferrin, methylation

Abstract

The lactoferrin gene promoter contains GC-rich regions that harbor consensus sequences for a variety of transcription factors. Previous work in our laboratory has demonstrated a link between methylation at the CpG sites of the mouse lactoferrin gene promoter and the level of its expression. The current work investigates the methylation profile in three regions of the human lactoferrin gene by bisulfite genomic sequencing. In addition, the methylation profiles of normal leukocyte DNA, and leukemia cell line and patient DNA were compared. The three regions are located at the −504/−190, which includes the estrogen response element, the −282/+271 which contains the lactoferrin promoter and the +1087/+1476, a region within the first intron that has the alternative delta lactoferrin promoter. Differential methylations were found within all the regions. Increased methylation at the CpG sites and the presence of non-CpG methylation of the lactoferrin promoters were found in the cancer samples.

Introduction

Lactoferrin, an iron-binding glycoprotein, is a major constituent of the secondary granules in neutrophils. It is expressed in mucosa membrane and is present in the milk and biological fluids (Masson & Heremans 1971, Masson *et al.* 1969, Teng 2002). Although lactoferrin is abundant in many tissues, it is absent or decreased in expression in the tumor counterpart and tumor-derived cell lines (Panella *et al.* 1991, Siebert & Huang 1997). Recently, an alternative form of lactoferrin called delta lactoferrin (ΔLF), which is identical to the lactoferrin except for exon 1, was found missing from all tumor cell lines examined (Siebert & Huang 1997). Our laboratory has previously found that the methylation pattern of lactoferrin gene in leukemic and breast cancer cells was altered (Panella *et al.* 1991) and an inverse relationship of methylation state of the lactoferrin gene promoter and the expression in various mouse tissues existed (Grant *et al.* 1999).

DNA methylation at CpG dinucleotides has been shown to have a considerable effect on gene expression (Razin *et al.* 1991). The presence of methyl groups at position five of cytosine residues within CpG dinucleotides in gene promoter region is often associated with the inactivation of the corresponding gene (Lorincz *et al.* 2001). The current investigation examines the methylation profiles from DNA of normal human tissues and cancer cells (from leukemia patients and cancer cell lines) at three regions of the lactoferrin gene promoters. Within these regions, a series of transcription factors response elements has been characterized (Teng 2002). These regulatory elements play a critical role in steroid hormone response (Teng 1999), hematopoesis and leukemogenesis (Khanna-Gupta *et al.* 2000), and tissue specific expression of the ΔLF (Liu *et al.* 2003).

Materials and methods

Preparation of genomic DNA

Cancer samples from patients were obtained from Dr. Panella (University of Tennessee at Knoxville) after consent of the informed patients. The normal human tissue specimens were obtained from the Cooperative Human Tissue Network of the University of Alabama at Birmingham. To use the existing human tissue samples for research was approved by the Office of Human Subjects Research (OHSR). Human breast cell lines, MCF-7 (ATCC #HTB-22) and MCF-10a (ATCC #CRL-10317) were obtained commercially. DNA from the human samples was isolated as previously described (Panella *et al.* 1991).

PCR primer sequences

Primer sets designed to amplify the upper strand of lactoferrin gene sequence after bisulfite modification are as follows: the ERE region (−504 to −190), forward primer, 5′-TTAGTTGTTTTTAGGTTGTTGTTG-3′ and reverse primer, 5′-CCTATCCTACTAATTCTAC CTAACTAC-3′; the lactoferrin promoter region (−282 to +271), forward primer, 5′-TGGGATAGGGT TTAGGGGGTTGTG-3′ and reverse primer, 5′-ACTCCTATTCCTCCCCATATA-3′; the delta lactoferrin promoter (ΔLF) region (+1087 to +1476), forward primer, 5′-TAAGATAGATTGGGATTGGT-3′ and reverse primer, 5′-CCAACTAACCCCACTCACC-3′.

Sodium bisulfite gemonic DNA sequencing

The sodium bisulfite treatment of DNA was performed according to Frommer (Frommer *et al.* 1992) with minor modifications. The DNA was digested with NcoI restriction endonuclease to generate a 1 kb (+550 to −542) fragment with the ERE and promoter regions present before the chemical modification. We also used the commercial bisulfite kit (CpGenome DNA modification kit, Intergen) to modify DNA from the normal human tissue samples and the MCF-7 and MCF-10a cells. The three regions of lactoferrin gene were PCR amplified with the specific sets of primers with a thermocycler (Perkin Elmer PCR System 9600). PCR products were run on 1.8% agarose gels. Correctly identified bands were excised, purified with a gel purification kit (Qiagen) and cloned into either pT-Adv (Clontech) or TOPO (Invitrogen) vectors. At least 10 positive clones were selected randomly. The plasmids were prepared by the automatic DNA isolation system AutoGene α 850 (AutoGen) and the insert sequenced with automated sequencing by the sequencing core laboratory of NIEHS. A Statistical Analysis System (SAS) protocol was used to analyze the sequencing data.

Northern blot analysis

Human tissue blots I and II (MTN^{TM}, Clontech) containing poly A RNA (2 μg/lane) from various adult tissues were hybridized with the [^{32}P]dCTP-labeled human lactoferrin cDNA probe (p1212) (Panella *et al.* 1991). The filters were exposed to X-ray film with intensify screen at −70 °C.

Results and discussion

Sodium bisulfite chemical conversion of the human genomic DNA

We have previously analyzed the methylation status of human lactoferrin gene in cancer cells (Panella *et al.* 1991) and mouse lactoferrin gene in different tissues (Grant *et al.* 1999) with methylation sensitive restriction enzymes. The method is limited to a few CpG sites that happen to be located at the restriction enzyme cutting site. Bisulfite chemical conversion method, on the other hand, allows the detection of methylated cytosines within a specific region of interest on a single stranded DNA, whether it is in a CpG or non-CpG dinucleotide form (Frommer *et al.* 1992). Three regions of the lactoferrin gene that includes the ERE, promoter and ΔLF promoter regions (Figure 1) were analyzed. The specific primer set that produces the 314 bp ERE region (−504/−190) contains four CpG sites with the −345 CpG (site 2) present in the middle of ERE element. The 553 bp region (−282/+271) includes the 5′ flanking region, lactoferrin gene promoter, first exon and a partial intron sequence that consist of 33 CpG sites (Figure 1A). The five CpG sites within the ΔLF promoter and the alternative first exon (exon 1β) of the lactoferrin gene were amplified by the primer set that produces a 389 bp PCR product (Figure 1B). By agarose gel electrophoresis, we demonstrated that the three regions were successfully amplified and produced correct sizes of PCR products with the bisulfite modified DNA as template and the specific primer sets designed for the modified DNA (Figure 1, gel picture under the arrows). The

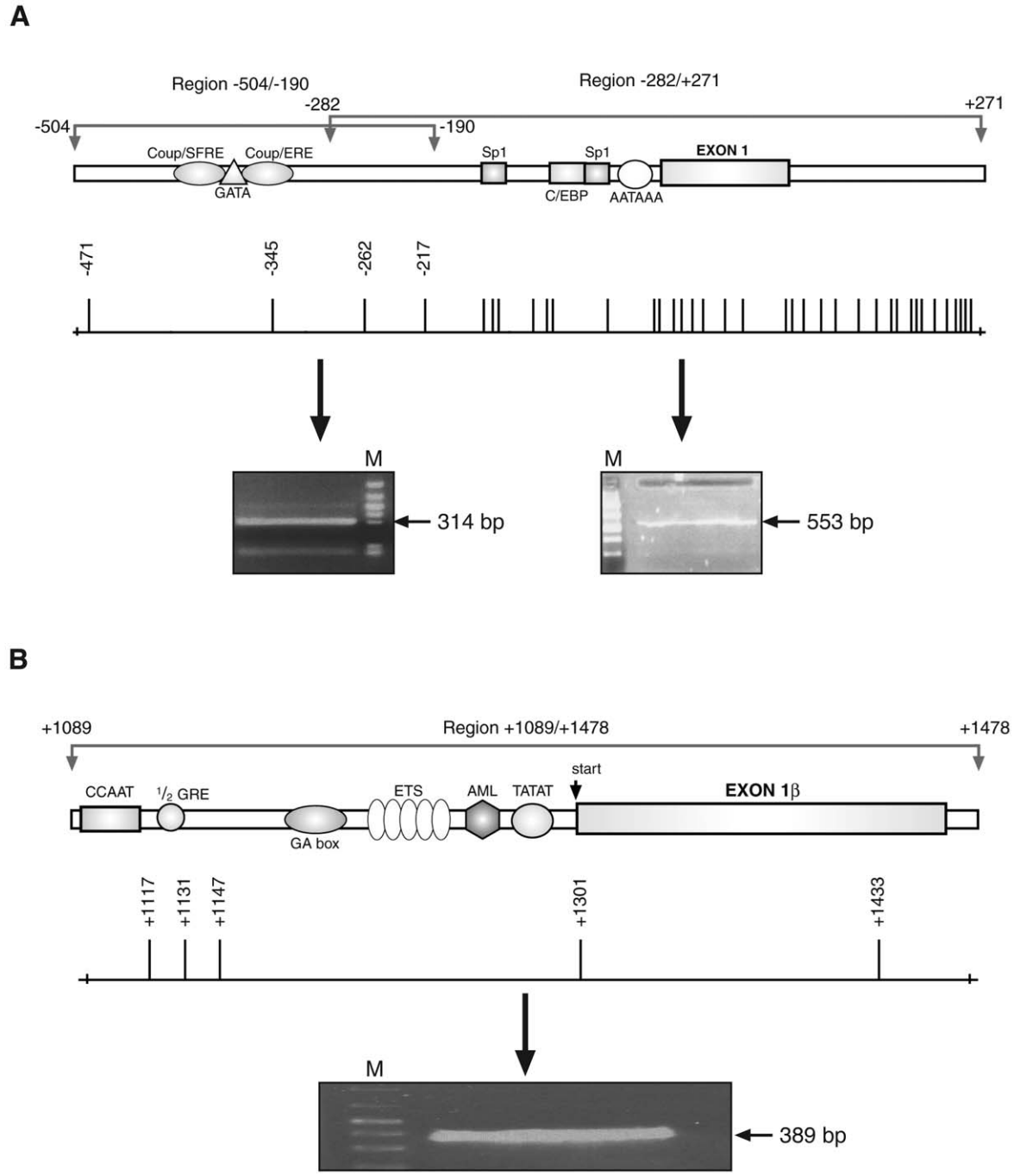

Fig. 1. The three regions of lactoferrin gene that are under investigation were amplified by the bisulfite modified DNA template and specific primer set designed to the modified DNA. A. Schematic presentation of the ERE and the promoter regions of lactoferrin gene (upper). Amplification of the 314 bp ERE and 553 bp promoter regions by PCR were verified by agarose gel electrophoresis (under the arrows). B. Schematic presentation of the region around the ΔLF promoter (upper). The consensus transcription factor binding elements are indicated. The 389 bp PCR product of this region was verified by agarose gel electrophoresis (under the arrow). Locations of the CpG site are marked by vertical bars.

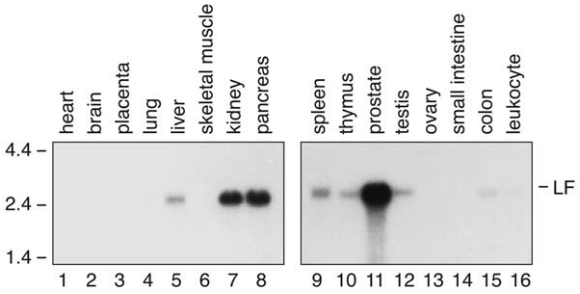

Fig. 2. Northern blot analysis of lactoferrin mRNA from various human tissues. Two μg of poly A RNA from each tissue were gel separated and blotted onto a nitrocellular membrane (Clontech, human tissue blot I and II). The tissue blots were hybridized with radiolabeled lactoferrin cDNA (p1212) (Panella *et al.* 1991) and the signal was detected by X-ray film. Molecular markers are indicated on the left and position of the lactoferrin mRNA is marked on the right.

PCR products were cloned into plasmids, and between 10–30 individual clones selected. The conversion or no conversion of the CpG sites in the above mentioned regions (the vertical bars) were analyzed.

DNA methylation profiles of lactoferrin gene in various human tissues

The human tissues samples were from different individuals. The methylation profiles presented in Table 1 thus reflects the pattern for an individual and his/her specific tissue. The percentage of methylation at the CpG sites in the ERE, promoter and the exon 1 are presented in Table 1, and the first intron and ΔLF promoter regions are presented in Table 1-continued. Variations on the specific CpG sites existed among different human tissues, nonetheless, a general pattern of methylation appears among them. For example, colon, lung, testis and prostate shared an overall similar methylation profile with some sites highly methylated and some sites not methylated. The spleen, kidney and leukocyte have an overall low methylation profile. In particular, the CpG sites around the TATA area were under methylated (Table 1, boxed region in spleen, kidney and leukocyte). This finding is consistent with our previous results that the mouse lactoferrin gene of spleen and kidney are under methylated (Grant *et al.* 1999). In addition, abundant lactoferrin mRNA was found in kidney and spleen by Northern analysis of the human tissue blots (Figure 2, lanes 7 and 9, respectively). The CpG site 2, which is located in the middle of the ERE of lactoferrin gene, is under methylated in liver, an estrogen target tissue, while this site is highly methylated in the majority of tissues examined. There are also discrepancies between the degree of methylation and the expression. The discrepancies could result from the difference in age and physiological conditions of the human tissues used in the methylation study and in the mRNA detection. The ΔLF promoter region was highly methylated in most tissues examined except the normal leukocytes (Table 1-continued, boxed).

DNA methylation profiles of cancer cells

To examine the methylation profile of lactoferrin gene between normal leukocytes and leukemic cells, we analyzed leukocyte DNA from seven volunteers (NLD) and 10 DNA samples from leukemic cells of leukemia patients and the established leukemia cell lines (LKD). We found great variations in the percentage of methylation at each CpG site among the cancer samples. The leukemia sample presented in Table 1 falls into the heavily methylated group. In general, the LKD and breast cancer cell infiltrated lymph node samples were methylated at a higher level than the corresponding NLD and normal lymph node. In addition, we found non-CpG and unusual methylation of the LKD samples (data not shown). A unique feature of the NLD methylation profile lies in the ΔLF promoter region. The five CpG sites in the ΔLF promoter region were under methylated in the NLD samples as compared to other human tissues (Table 1-continue). These CpG sites in the LKD samples showed significant increase of methylation. Statistical analysis of the methylation status within the ΔLF promoter indicates significant differences at three CpG sites located at position +1147, +1301 and +1433 (Figure 3). In conclusion, the level of methylation at the regulatory regions of the lactoferrin gene exhibits inverse relationship of its expression.

Acknowledgements

We thank Drs Shuanfang Li and Theodora Devereux for critically reading the manuscripts. The excellent technical assistant of the undergraduate student, Clara Beard and Adele Hodges is acknowledged. Sequencing provided by the NIEHS sequencing core laboratory is appreciated. L. Moore edited the manuscript.

Table 1. Methylation profile of lactoferrin gene in various human tissues. Total of 42 CpG sites were examined by the bisulfite DNA modification method. The three regions of lactoferrin gene are indicated. Frozen samples were as follows: colon (75 yr. white male, surgery); lung (73 yr. white female, surgery); testis (81 yr. white male, surgery); prostate (BPH and low grade PIN, 62 yr. Asian male, surgery); liver (40 yr. male, surgery); muscle (54 yr. black male, surgery); heart (30 yr. black female, post mortem); ovary (56 yr. white female); pancreas (43 yr. white male); spleen (48 yr. white female); kidney (57 yr. white female); leukocyte (from blood, normal volunteer), lymph, N (lymph node from cancer patient with no cancer), leukemia (leukemic cells from leukemia patient), lymph, C (lymph node from cancer patient with cancer cells); MCF-10a (immortal breast cell line); MCF-7 (breast cancer cell line). Boxed regions highlighted the under methylation CpG sites. % Me, % methylation based on number clones with methylation/number clones sequenced (10–25 for each sample).

CpG / % Me / Tissue	ERE									TATA			Exon 1						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
colon	97	100	0	93	0	100	93	93	0	100	93	64	86	100	100	100	100	0	7
lung	9	14	0	100	0	93	100	100	0	86	93	93	93	100	100	100	100	0	0
testis	4	35	0	100	0	100	100	100	0	100	67	87	93	100	100	93	100	0	0
prostate	16	48	4	4	8	100	100	100	0	92	100	100	100	100	100	100	100	0	8
liver	6	7	0	0	94	0	93	93	0	92	92	92	92	92	92	92	92	0	0
muscle	12	54	4	71	86	100	100	100	14	43	93	71	86	57	100	71	86	36	43
heart	6	33	0	42	23	69	54	69	15	15	23	23	23	62	62	85	46	15	30
ovary	24	78	0	0	17	92	75	75	0	58	50	50	67	83	67	100	58	0	0
pancreas	30	88	0	50	17	92	75	67	0	38	50	50	67	83	67	100	85	0	0
spleen	14	79	0	0	20	64	54	68	50	12	0	17	20	16	33	41	41	13	6
kidney	14	14	0	0	0	50	13	75	10	0	0	10	40	0	40	20	0	0	0
leukocyte	41	90	6	23	46	77	77	51	0	14	14	14	43	29	57	57	14	0	7
lymph, N				74	0	76	90	82	0	75	61	57	64	64	75	79	75	0	0
leukemia	43	98	4	100	22	100	89	100	11	89	89	89	89	89	89	89	84	11	11
lymph, C				90	8	90	90	95	5	67	74	66	74	90	90	92	84	0	3
MCF-10a	12	51	0	83	0	83	75	67	0	50	58	58	58	58	58	58	67	0	0
MCF-7	74	95	12	90	10	90	100	100	10	80	80	90	90	90	90	90	80	0	10

Table 2. Continued

% Me / CpG / Tissue	Intron 1																		Δ LF/Exon 1β				
	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
colon	0	100	100	100	100	100	0	0	93	100	0	0	100	93	93	0	86	93	0	100	100	100	100
lung	0	100	100	100	86	93	0	0	93	93	0	0	100	100	93	0	93	93	0	100	100	100	100
testis	0	100	93	93	93	87	0	0	87	100	0	0	100	87	87	0	100	93					
prostate	8	100	100	100	100	100	8	8	100	100	0	0	100	100	100	0	100	100					
liver	0	96	96	96	96	96	0	0	25	96	95	0	0	95	100	94	0	94					
muscle	79	86	93	93	100	86	36	57	57	100	14	64	79	35	92	43	100	71	0	100	100	100	100
heart	0	46	46	23	46	46	21	14	57	57	0	14	51	29	29	14	36	29	0	100	100	100	100
ovary	8	58	58	50	50	58	0	0	67	75	0	0	75	58	58	0	67	58					
pancreas	17	50	50	58	50	50	0	0	50	67	0	0	68	50	67	0	67	50					
spleen	25	25	31	19	31	25	38	12	25	63	13	38	19	13	38	31	31	19	100		100	100	92
kidney	20	20	20	30	50	10	10	40	0	70	0	0	10	0	30	10	30	30	100		89	89	67
leukocyte	27	13	13	13	13	13	0	0	13	40	0	0	40	20	40	7	47	13	15	23	26	29	27
lymph, N	4	75	75	75	71	75	0	0	68	79	0	0	79	75	79	4	61	68					
leukemia	22	89	100	100	100	90	20	10	90	100	10	10	80	90	100	20	100	90	33	70	56	59	54
lymph, C	8	74	79	79	84	82	8	3	79	90	0	5	90	71	76	5	82	68					
MCF-10a	0	58	58	58	58	58	0	0	58	92	0	8	83	67	50	0	68	68					
MCF-7	10	90	90	80	90	90	20	10	90	100	10	10	90	90	90	20	90	90	50		38	100	0

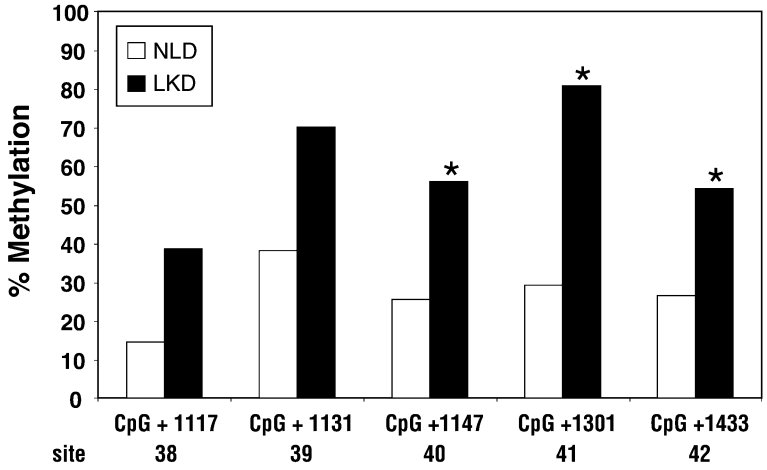

Fig. 3. Comparison of the methylation status from NLD and LKD samples at the ΔLF promoter region. Methylation profiles from seven NLD and 10 LKD samples were compared. The % of methylation from each sample was determined by sequencing at least 10–25 clones. Data was statistically analyzed. The statistically significant difference between the two samples is indicated by *.

References

Frommer M, McDonald LE, Millar DS *et al.* 1992 A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc Natl Acad Sci USA* **89**, 1827–1831.

Grant DJ, Shi H, Teng CT 1999 Tissue and site-specific methylation correlates with expression of the mouse lactoferrin gene. *J Mol Endocrinol* **23**, 45–55.

Khanna-Gupta A, Zibello T, Simkevich C *et al.* 2000 Sp1 and C/EBP are necessary to activate the lactoferrin gene promoter during myeloid differentiation. *Blood* **95**, 3734–3741.

Liu D, Wang X, Zhang Z, Teng CT. 2003 An intronic alternative promoter of the human lactoferrin gene is activated by Ets. *Biochem Biophys Res Commun* **301**, 472–479.

Lorincz MC, Groudine M, Bird AP *et al.* 2001 C(m)C(a/t)GG methylation: a new epigenetic mark in mammalian DNA? *Proc Natl Acad Sci USA* **98**, 10034-10036.

Masson PL, Heremans JF 1971 Lactoferrin in milk from different species. *Comp Biochem Physiol* **39B**, 119–129.

Masson PL, Heremans JF, Schonne E. 1969 Lactoferrin, an iron-binding protein in neutrophilic leukocytes. *J Exp Med* **130**, 643-658.

Panella TJ, Liu YH, Huang AT, Teng CT. 1991 Polymorphism and Altered Methylation of the Lactoferrin Gene in Normal Leukocytes, Leukemic-Cells, and Breast-Cancer. *Cancer Research* **51**, 3037–3043.

Razin A, Cedar H, Britigan BE *et al.* 1991 DNA methylation and gene expression. *Microbiol Rev* **55**, 451–458.

Siebert PD, Huang BCB. 1997 Identification of an alternative form of human lactoferrin mRNA that is expressed differentially in normal tissues and tumor-derived cell lines. *Proc Natl Acad Sci USA* **94**, 2198–2203.

Teng CT. 1999 Regulation of lactoferrin gene expression by estrogen and epidermal growth factor - Molecular mechanism. *Cell Biochem Biophys* **31**, 49–64.

Teng CT. 2002 Lactoferrin gene expression and regulation: an overview. *Biochem Cell Biol* **80**, 7–16.