FEBS 24578 FEBS Letters 490 (2001) 75–78

Analyses of the genomic methylation status of the human cyclin A1 promoter by a novel real-time PCR-based methodology

Carsten Müller-Tidow*, Christina Bornemann, Sven Diederichs, Annette Westermann, Silvia Klümpen, Peijun Zuo, Wenbing Wang, Wolfgang E. Berdel, Hubert Serve

Department of Medicine, Hematology and Oncology, University of Münster, Domagkstr. 3, 48129 Münster, Germany

Received 19 September 2000; revised 6 January 2001; accepted 9 January 2001

First published online 19 January 2001

Edited by Takashi Gojobori

Abstract The role of CpG methylation in the regulation of tissue-specific gene expression is highly controversial. Cyclin A1 is a tissue-specifically expressed gene that is strongly methylated in non-expressing tumor cell lines. We have established a novel real-time PCR method to quantitate genomic CpG methylation of the cyclin A1 promoter. Genomic DNA samples from different human organs were treated with bisulfite and amplified with methylation-specific primers and with primers amplifying methylated as well as non-methylated DNA. PCR product quantitation was obtained by using a fluorogenic probe labeled with FAM and TAMRA. These analyses demonstrated that the human cyclin A1 promoter was methylated in kidney, colon, spleen, testis, and small intestine, but not in brain, liver, pancreas, or heart. Expression of cyclin A1 was predominantly found in testis. Low level expression of cyclin A1 was present in spleen, prostate, leukocytes, colon, and thymus. Taken together, our data provide evidence that CpG methylation patterns of the human cyclin A1 promoter in human organs do not generally correlate with cyclin A1 gene expression in vivo.

Key words: Real-time quantitative polymerase chain reaction; CpG methylation; Tissue-specific gene expression; Transcriptional regulation; Cyclin A1

1. Introduction

The tight control of gene expression is of fundamental importance for the development of higher organisms. Several layers of regulation are known that coordinate the correct spatial and temporal regulation of gene expression [1]. Methylation of CpG dinucleotides in genomic DNA is a phenomenon that has long been known in mammals and may provide a layer of epigenetic information [2].

In general, most of the human genome is methylated at CpG dinucleotides, but transcriptionally active promoters in CpG islands are usually non-methylated. Consequently, close correlations between methylation and repressed transcription on one hand and between non-methylation and active transcription on the other hand exist for several genes [3,4]. These findings and others have prompted investigators to speculate that CpG methylation itself is actively involved in transcriptional regulation [2]. While the association between methylation and transcriptional repression appears to be strong in tumor cells [5], the role of CpG methylation in normal development and tissue-specific gene expression is highly controversial [6]. To date, most data deal with cell culture models, but data on CpG methylation patterns in different human organs exist so far only for few genes.

Recently, we have shown that the promoter of the human cyclin A1 gene is heavily methylated in non-expressing solid tumor cell lines [7]. No methylation of the cyclin A1 promoter was found in cyclin A1-expressing cell lines derived from acute myeloid leukemia. In addition, we were able to demonstrate that a transgenic cyclin A1 promoter in mice directed GFP expression in the testis independent of the methylation status [7].

In the current study we developed a novel method to quantitate CpG methylation and used it for the analyses of the endogenous cyclin A1 promoter in different human organs. A significant degree of cyclin A1 promoter CpG methylation was found in kidney, colon, spleen, testis, and small intestine, whereas CpG methylation was absent in most other organs. The methylation status of the cyclin A1 promoter in human organs did not correlate with cyclin A1 mRNA expression as shown by quantitative real-time RT-PCR. These data provide evidence that CpG methylation is unlikely to be involved in directing tissue-specific expression of cyclin A1.

2. Materials and methods

2.1. Materials

HeLa cells and U937 monocytic leukemia cells were cultured in Dulbecco's modified Eagle's medium and RPMI medium, respectively. All media were supplemented with 10% fetal calf serum and streptomycin and penicillin. Genomic DNA was extracted from organs obtained in the pathology department at the time of autopsy. Human cDNA derived from the different organs was purchased from Clontech. These cDNAs represent RNA derived independently from several persons.

2.2. Detection of CpG methylation

Genomic DNA was extracted using DNAzol (Gibco Life Technology). Subsequently, the DNA was treated with bisulfite as described [7]. Methylation-specific primers (5′ to 3′: forward: CGT ACG TTT GTC GCG GTC G, reverse: GAC TAA ACT ACC CAC CCG CG) were designed that were specific for methylated cyclin A1 promoter sequences (MSP). A second set of primers was used that amplified bisulfite-treated non-methylated cyclin A1 promoter DNA (5′ to 3′: forward: TGG AGA GTG TAT GTT TGT TGT GGT TG, reverse: AAA CTT AAC AAT ACA ACT AAA CTA CCC ACC C). To amplify DNA sequences irrespective of their methylation status, equal amounts of both sets of primers were mixed (All). In addition to the

*Corresponding author. Fax: (49)-251-835 2673.
E-mail: muellerc@uni-muenster.de

PII: S0014-5793(01)02128-7

primers, a probe was designed that could bind to the bisulfite-treated DNA irrespective of the methylation status (5′ to 3′: ACR CCC CCR AAC CTA ACR AAA ACR TTT CCA). This probe was labeled with FAM at the 5′ end and with the quencher TAMRA at the 3′ end. The quencher molecule inhibited 5′ FAM fluorescence when the probe was intact. During the amplification of the PCR products, the probe annealed to the antisense strand and the 5′ exonuclease activity of the Taq polymerase freed the fluorescent molecule. The resulting fluorescence was analyzed during each cycle and the cycle in which a significant increase of the fluorescence could be noted was determined as the threshold cycle. Because of the exponential nature of PCR, the degree of CpG methylation was calculated according to the formula:

fraction of methylated molecules =

2 (threshold non − specific primers minus threshold methylation−

specific primers)

All PCR reactions were performed at least three times. The means of the threshold values were used for the final calculation.

2.3. Quantitation of human cyclin A1 expression

Cyclin A1 expression was determined by quantitative real-time RT-PCR. The cDNA was amplified in a SDS7700 sequencing detector (PE Biosystems) using cyclin A1-specific primers and a Taqman probe (for primer sequence information see [7]). The probe was FAM-labeled at the 5′ end and labeled with TAMRA at the 3′ end. The 5′ exonuclease activity of the Taq polymerase released the FAM molecule and led to an increase in fluorescence which was analyzed by the SDS7700 during each PCR cycle. A standard curve derived from serial dilutions of U937 cell line cDNA was used to obtain relative quantitation data. These data were standardized using expression data of GAPDH mRNA which was also analyzed by the 5′ nuclease assay [7]. The GAPDH probe was VIC-labeled at the 5′ terminus. Relative GAPDH expression was also quantitated using a standard curve which was run on every plate that was analyzed. Cyclin A1 expression data were then calculated by dividing the relative concentrations of cyclin A1 by the GAPDH values.

3. Results

To quantitate methylation of the human cyclin A1 promoter in healthy organs, we developed a new method, which is based on real-time PCR amplification of bisulfite-treated DNA. Bisulfite exposure of genomic DNA led to a change of cytidine to uracil if the cytosine was not methylated. Methylated cytosine remained unchanged. Primers were designed that either specifically amplified methylated cyclin A1 promoter sequences (MSP) or were able to amplify methylated as well as non-methylated sequences (All) (Fig. 1). All primers were specific for bisulfite-modified DNA and did not amplify primary genomic DNA. In between the primer pairs, we designed a fluorogenic probe that could bind to methylated as well as to non-methylated sequences. Consequently, amplification by either primer pair led to the generation of fluorescence that was analyzed by the laser detector. Comparisons of the threshold cycle (the PCR cycle when a significant increase of fluorescence was observed for the first time) allowed the calculation of the fraction of methylated DNA sequences (for details see Section 2).

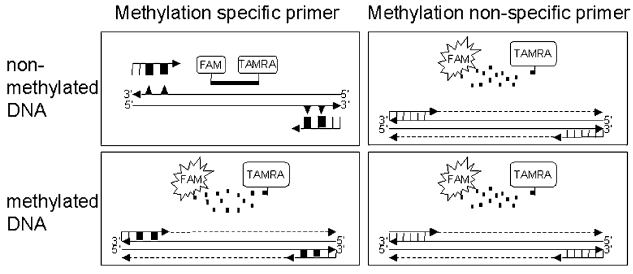

Fig. 1. Novel real-time PCR-based assay for rapid analysis of CpG methylation. DNA was isolated and bisulfite-treated (for details see Section 2). In the real-time quantitative PCR reaction, methylation-specific primers only bound to methylated DNA and did not anneal to non-methylated DNA. Consequently, Taq polymerase only amplified methylated DNA and cleaved the annealed probe through its exonuclease activity, thereby setting free the fluorogenic probe FAM. With non-methylated DNA, the probe stayed intact and TAMRA quenched the fluorescence of FAM. The methylation non-specific primers amplified both kinds of DNA, resulting in a signal indicating the total amount of cyclin A1 promoter sequences in the sample.

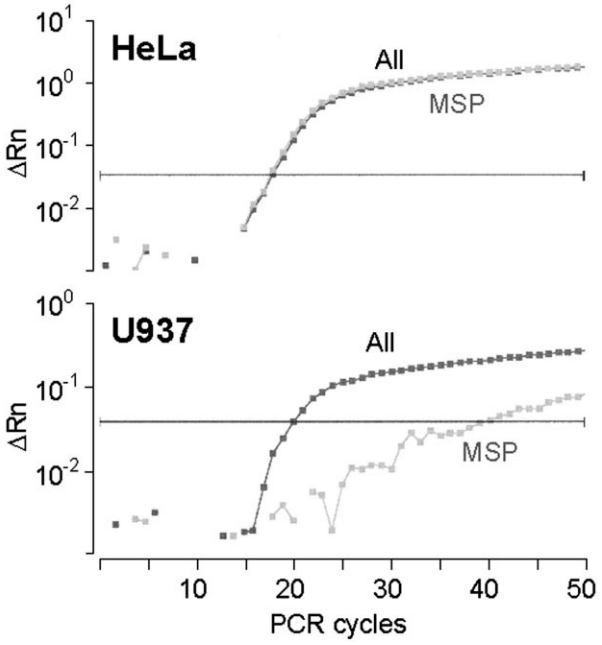

Fig. 2. Analyses of cyclin A1 promoter methylation status in cell lines. Quantitative analyses of the methylation status of the cyclin A1 promoter in the cell lines HeLa and U937 are shown. Bisulfite-treated DNA was amplified with primers specific for methylation (MSP) and primers amplifying the sequences irrespective of their methylation status (All). On the *x*-axis the number of PCR cycles is shown, on the *y*-axis the difference between measured fluorescence and the baseline (ΔRn). The threshold line is indicated as well.

We initially tested this method on two cell lines with well-known methylation patterns of the cyclin A1 promoter (Fig. 2). In HeLa cells, most of the sequences were methylated. Consequently, no difference in the threshold cycle was observed when bisulfite-exposed DNA was amplified by either methylation-specific primers (MSP) or primers amplifying all sequences (All). In contrast, almost no methylation of the human cyclin A1 promoter existed in strongly cyclin A1-expressing U937 leukemic cells. While the methylation non-specific primers amplified bisulfite-treated U937 cell DNA similar to HeLa cell DNA, the methylation-specific primers did not amplify the DNA derived from U937 cells, indicating the absence of genomic methylation.

Next, we focused on methylation of the cyclin A1 promoter in different human organs. About 40% of the cyclin A1 promoter sequences in the kidney were found to be methylated. A

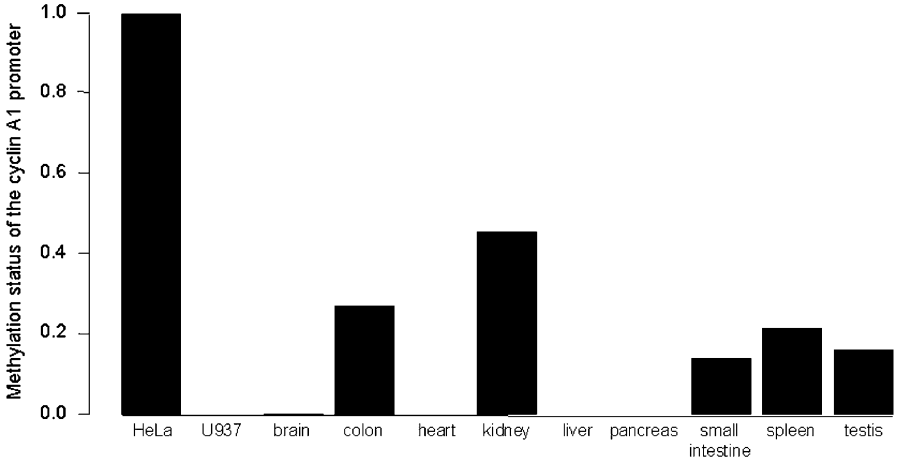

Fig. 3. Analyses of cyclin A1 promoter methylation status in human organs. The methylation status of the cyclin A1 promoter was analyzed in samples of different human tissues. DNA was isolated, bisulfite-treated, and amplified by real-time PCR techniques as described in Section 2. The calculated fraction of methylated sequences in cyclin A1 promoter sequences is indicated. Each sample was independently analyzed at least three times.

significant fraction of methylated sequences was also found in spleen, testis, colon, and small intestine. No significant degree of methylation was observed in brain, heart, liver, or pancreas (Fig. 3). To correlate these findings with cyclin A1 gene expression, we performed quantitative real-time RT-PCR on human cDNA samples derived from different human organs (Fig. 4). These analyses confirmed that high levels of cyclin A1 expression were restricted to the testis. Low level expression was detected in leukocytes, colon, and spleen. No significant cyclin A1 expression was found in kidney, brain, small intestine, heart, liver, pancreas, and muscle. Our findings provide strong evidence that methylation of the cyclin A1 promoter is not involved in directing tissue-specific cyclin A1 expression in humans. Cyclin A1 expression was found to be repressed in several organs where no significant degree of cyclin A1 promoter methylation was present, e.g. in brain or heart. Nevertheless, methylation of the cyclin A1 promoter was found in some organs, e.g. kidney, where cyclin A1 expression was notably absent.

4. Discussion

The role of genomic CpG methylation in the regulation of gene expression in mammals is highly controversial. In the current study, we established a new methodology that allows rapid analyses of the methylation status of a single gene in many samples. In addition, we show evidence for cyclin A1 promoter methylation in some human organs in vivo. Finally, methylation of the cyclin A1 promoter did not correlate with cyclin A1 gene expression in vivo as determined by quantitative real-time RT-PCR.

Recently, several methods have been introduced that allow analyses of the methylation status of small amounts of DNA. These methods are based on the conversion of cytidine to uracil following bisulfite exposure and subsequent treatment with sodium hydroxide [8]. Methylation of CpG dinucleotides inhibits this conversion and the different techniques aim to visualize this difference between methylated and non-methylated sequences.

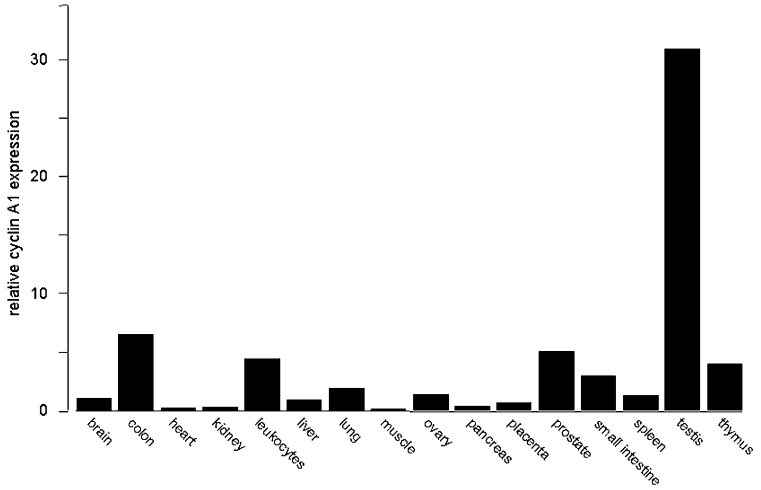

Fig. 4. Cyclin A1 expression in human organs. Expression levels of cyclin A1 were analyzed in a panel of different human cDNAs by real-time quantitative PCR. Expression levels were standardized using expression of the housekeeping gene GAPDH.

Methods that have been employed include PCR systems with methylation-specific primers [9]. An important drawback of this method is the lack of any quantitative information. Even when 99% of the sequences are non-methylated, methylation-specific primers might produce a positive band. The biological relevance of such a finding appears to be doubtful. More quantitative detection methods include single nucleotide primer extension methods [10], or DNA sequencing [8]. All of these methods are time-consuming, involve radioactivity, or do not allow accurate estimation of the fraction of methylated sequences.

Recently, a real-time PCR assay was published that used three fluorogenic probes for quantitative analyses of CpG methylation [11]. The method presented in our study uses only one probe and is thus much more cost-effective. In addition, the quality of probe labeling varies among different probes and this phenomenon complicates the quantitation when more than one probe is used. In our assay, the use of the same probe for two different primer pairs circumvents these problems.

Analyses of cyclin A1 promoter methylation in human organs revealed a significant degree of methylation in some organs. But methylation of all genomic cyclin A1 sequences as detected in HeLa cells was not observed. This finding probably hints at the mixed background of cells obtained from human organs compared to cell lines. In addition, significant fractions of methylated cyclin A1 sequences were found in only five out of nine organs analyzed.

Most of the organs where cyclin A1 promoter methylation was found did not express cyclin A1 as shown by real-time RT-PCR analyses. Interestingly, a small percentage of methylated cyclin A1 sequences was found in the testis, an organ with very high levels of cyclin A1 expression in pachytene spermatocytes. Since it is unknown which cell types were methylated in vivo, we cannot draw conclusions on an association between methylation and non-expression in the testis. Previously, we have demonstrated that methylation of a transgenic cyclin A1 promoter in mice did not prevent the promoter from being active during spermatogenesis [7]. Another interesting phenomenon concerns the high degree of methylation in kidney and spleen, two organs that also express no or only low levels of cyclin A1. In the transgenic mouse model aberrant activity of the cyclin A1 promoter was detected in collecting duct and glomerulus cells from the kidney as well as in some splenic B cells [7]. In contrast, no cyclin A1 promoter activity was detected in the kidney or in the spleen when the transgenic promoter was methylated, a situation recapitulated in the human tissue studies in this paper.

Taken together, these findings indicate that CpG methylation might be necessary for appropriate transcriptional control in some organs and cell types. So far, physiological methylation patterns in different organs are known for only very few genes. α-Actin is one gene that has been studied previously [12]. The authors described an association between CpG methylation and transcriptional repression in some organs. Appropriate repression in other organs occurred in the absence of CpG methylation. These findings are very similar to the findings concerning the cyclin A1 promoter presented in our study. Our data show evidence that the physiological expression pattern of cyclin A1 can be obtained in most organs in the absence of CpG methylation.

In conclusion, we established a novel, rapid, high-throughput assay for quantitative analyses of genomic CpG methylation patterns. Our analyses of cyclin A1 promoter methylation patterns demonstrate that a significant fraction of CpG dinucleotides was methylated in some of the human organs. Absence of methylation was not usually associated with transcriptional repression as shown by quantitative real-time RT-PCR analyses.

Acknowledgements: We are grateful to Dr. Dockhorn-Dworniczak for providing tissue samples. This work is part of the M.D. thesis work of C.B. This work is supported by grants from the Deutsche Krebshilfe (10-1539-Mü1), the Deutsche Forschungsgemeinschaft (Mu 1328/2-1) and the IMF Program (Mü429926, Mü529905, SE119908) at the University of Münster.

References

[1] Burley, S.K. and Roeder, R.G. (1996) Annu. Rev. Biochem. 65, 769–799.
[2] Singal, R. and Ginder, G.D. (1999) Blood 93, 4059–4070.
[3] De Smet, C., Lurquin, C., Lethe, B., Martelange, V. and Boon, T. (1999) Mol. Cell. Biol. 19, 7327–7335.
[4] Kudo, S. and Fukuda, M. (1995) J. Biol. Chem. 270, 13298–13302.
[5] Cameron, E.E., Bachman, K.E., Myohanen, S., Herman, J.G. and Baylin, S.B. (1999) Nature Genet. 21, 103–107.
[6] Walsh, C.P. and Bestor, T.H. (1999) Genes Dev. 13, 26–34.
[7] Müller, C. et al. (2000) Mol. Cell. Biol. 20, 3316–3329.
[8] Clark, S.J., Harrison, J., Paul, C.L. and Frommer, M. (1994) Nucleic Acids Res. 22, 2990–2997.
[9] Kawano, S. et al. (1999) Blood 94, 1113–1120.
[10] Gonzalgo, M.L. and Jones, P.A. (1997) Nucleic Acids Res. 25, 2529–2531.
[11] Lo, Y.M., Wong, I.H., Zhang, J., Tein, M.S., Ng, M.H. and Hjelm, N.M. (1999) Cancer Res. 59, 3899–3903.
[12] Warnecke, P.M. and Clark, S.J. (1999) Mol. Cell. Biol. 19, 164–172.