CDK10 Is Not a Target for Aberrant DNA Methylation in Breast Cancer

GERWIN HELLER[1], BARBARA ZIEGLER[1], ANITA BRANDSTETTER[1], SABINE NOVAK[1], MARGARETHA RUDAS[2], GUIDO HENNIG[3], MATHIAS GEHRMANN[3], TORSTEN ACHT[3], SABINE ZÖCHBAUER-MÜLLER[1] and MARTIN FILIPITS[1]

Departments of [1]Medicine I and [2]Pathology, Medical University of Vienna, A-1090 Vienna, Austria; [3]Siemens Healthcare Diagnostics Products GmbH, Molecular Research Germany, D-50829 Cologne, Germany

Abstract. *Background: Loss of cyclin-dependent kinase (CDK) 10 expression may be an important mechanism of tamoxifen resistance and the 5' CpG island associated with the CDK10 gene has been suggested to be a target for aberrant methylation in breast cancer. Patients and Methods: The methylation status of CDK10, RASSF1A (Ras association domain family 1A) and DAL-1 (differentially expressed in adenocarcinoma of the lung) was determined by means of methylation-specific PCR (MSP) in the formalin-fixed, paraffin-embedded (FFPE) surgical specimens of 96 breast carcinoma patients. Reverse transcription kinetic PCR (RT-kPCR) was used for assessment of the expression of CDK10. Results: The unmethylated form of CDK10, RASSF1A and DAL-1 was detected in all the samples analyzed. Methylation of the CDK10 5' region was not found in any of the 96 breast cancer samples. RASSF1A methylation was detected in 75 out of 96 (78%) and DAL-1 in 9 out of 15 (60%) breast cancer samples, respectively. Consistent with the methylation results, the expression of CDK10 was detected in all 96 samples. Conclusion: CDK10 is not a target for aberrant DNA methylation in breast cancer.*

Endocrine therapy with the anti-estrogen tamoxifen is an effective treatment for women with hormone receptor-positive breast cancer, but *de novo* and acquired resistance remains a major problem (1). A considerable fraction of patients do not respond to tamoxifen despite having estrogen receptor-positive tumors. These patients may need other therapeutic interventions. Therefore, the ability to predict the outcome of tamoxifen treatment should significantly improve the management of early-stage breast cancer.

Cyclins, their associated cyclin-dependent kinases (CDK) and CDK inhibitory proteins play a central role in cell cycle progression and may also affect the response to tamoxifen (2, 3). Besides the central role in cell cycle regulation, cyclin D1 directly affects the estrogen receptor and may be involved in the response to estrogens and anti-estrogens (4, 5). *In vitro* studies have linked tamoxifen resistance to the expression of cyclin D1 in cell lines and several clinical studies have demonstrated early relapse and shorter survival in women with cyclin D1-positive breast cancer who received tamoxifen treatment (6-13). Recently, it has been shown that in patients with estrogen receptor-positive breast cancer, low levels of CDK10 may be an important determinant of tamoxifen resistance (14). Furthermore, low levels of CDK10 have been shown to be associated with methylation of the *CDK10* promoter (14). Aberrant methylation (referred to as methylation) of normally unmethylated CpG-rich areas, also known as CpG islands, which are located in or near the promoter region of many genes, has been associated with transcriptional inactivation of genes in human cancer (15). These findings led us to investigate the expression and the methylation pattern of *CDK10* in primary breast carcinomas.

Patients and Methods

Patients and tumor specimens. Ninety six breast cancer patients surgically treated at the Department of Surgery, Medical University of Vienna, from May 2006 to June 2008, were included in the study. A representative formalin-fixed, paraffin-embedded (FFPE) tumor block from each patient was collected after obtaining appropriate Institutional Review Board permission and written informed consent from the patients. All the tumor specimens had been obtained at the time of surgery before adjuvant therapy and the paraffin blocks had been stored at room temperature at the Department of Pathology,

Correspondence to: Martin Filipits, Ph.D., Associate Professor, Institute of Cancer Research, Department of Medicine I, Medical University of Vienna, Borschkegasse 8a, A-1090 Vienna, Austria. Tel: +43 1427765237, Fax: +43 1427765196, e-mail: martin.filipits@meduniwien.ac.at

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Medical University of Vienna. From each tumor block sections were cut at 4 to 10 μm. One 4 μm section was stained by hematoxylin and eosin to confirm the presence of invasive carcinoma histologically and further sections were used as described.

Nucleic acid isolation and bisulfite treatment of genomic DNA. The genomic DNA was isolated from the FFPE breast cancer samples using a QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Four 5 μm sections from each tumor block were used for the DNA preparation. In addition, genomic DNA from MCF-7 breast cancer cells and from human B lymphocytes was isolated by digestion with proteinase K followed by standard phenol-chloroform extraction and ethanol precipitation as reported previously (16). Afterwards, 2 μg of genomic DNA were modified by treatment with sodium bisulfite using an EpiTect 96 Bisulfite Kit (Qiagen).

The total RNA was extracted with a Siemens, silica bead-based and fully automated isolation method for RNA from one 10 μm whole FFPE tissue section on a Hamilton MICROLAB STARlet liquid handling robot (17). The robot, buffers and chemicals (unless indicated differently) were part of a Siemens VERSANT® kPCR Molecular System (Siemens Healthcare Diagnostics, Tarrytown, NY; not commercially available in the USA). Briefly, 150 μl FFPE buffer (Buffer FFPE, research reagent, Siemens Healthcare Diagnostics, not commercially available) were added to each section and incubated for 30 minutes at 80˚C with shaking to melt the paraffin. After cooling down, proteinase K was added and incubated for 30 minutes at 65˚C. After lysis, residual tissue debris was removed from the lysis fluid by a 15 minutes incubation step at 65˚C with 40 μl silica-coated iron oxide beads. The beads with surface-bound tissue debris were separated with a magnet and the lysates were transferred to a standard 2 ml deep well-plate (96 wells). There, the total RNA and DNA was bound to 40 μl unused beads and incubated at room temperature. Chaotropic conditions were produced by the addition of 600 μl lysis buffer. Then, the beads were magnetically separated and the supernatants were discarded. Afterwards, the surface-bound nucleic acids were washed three times followed by magnetization, aspiration and disposal of supernatants. Afterwards, the nucleic acids were eluted by incubation of the beads with 100 μl elution buffer for 10 minutes at 70˚C with shaking. Finally, the beads were separated and the supernatant incubated with 12 μl DNase I Mix (2 μL DNase I (RNase free); 10 μl 10x DNase I buffer; Ambion/Applied Biosystems, Darmstadt, Germany) to remove contaminating DNA. After incubation for 30 minutes at 37˚C, the DNA-free total RNA solution was aliquoted and stored at –80˚C.

mRNA expression analysis by reverse transcription kinetic PCR (RT-kPCR). All the samples were analyzed with one-step RT-kPCR for the gene expression of one reference gene, *RPL37A* (ribosomal protein L37a) and three target genes *CDK10, CCND1* (cyclin D1) and *ESR1* (estrogen receptor 1) in an ABI PRISM® 7900HT (Applied Biosystems, Darmstadt, Germany). The SuperScript® III Platinum® One-Step Quantitative RT-PCR System with ROX (6-carboxy-X-rhodamine) (Invitrogen, Karlsruhe, Germany) was used according to the manufacturer's instructions. The PCR conditions were as follows: 30 minutes at 50˚C, 2 minutes at 95˚C followed by 40 cycles of 15 seconds at 95˚C and 30 seconds at 60˚C. All the PCR assays were performed in triplicate. As surrogate marker for RNA yield, the housekeeper gene, *RPL37A* cycle threshold (Ct) value was used as described elsewhere (17). The relative gene expression levels of the target genes were calculated by the ΔCt method using the formula: $20 - (Ct_{target} - Ct_{RPL37A})$. The sequences of the primers and probes were as follows: *RPL37A*: forward: 5'-TGTGGTTCCTGCATGAAGACA-3', reverse: 5'-GTGACAGCG GAAGTGGTATTGTAC-3', probe: 5'-TGGCTGGCGGTGCCT GGA-3'; *CDK10*: forward: 5'-GCACGCC CAGTGA GAACAT-3', reverse: 5'-CAGGTTGTTGTAGGGCTG CTT-3', probe: 5'-CCGGGCTTTTCCAAGCTGCCA-3'; *CCND1*: forward: 5'-CACGCGCAGACCTTCGTT-3', reverse: 5'-CCGCTGCC ACCATGGA-3', probe: 5'-TGTGCCACAGATGTGAAGTTCATT TCC-3' and *ESR1*: forward: 5'-GCCAAATTGTGTTTGATGG ATTAA-3', reverse: 5'-GACAAAACCGAGTCACATCAGTAATAG-3', probe: 5'-ATGCCCTTTTGCCGATGCA-3'. All the RT-kPCR assays were performed using 1 μl of eluate in a 10 μl reaction volume and run in triplicate. The mean of the triplicates is reported.

Methylation-specific PCR (MSP). The methylation status of *CDK10, RASSF1A* (Ras association domain family 1A) and *DAL-1* (differentially expressed in adenocarcinoma of the lung) was determined by MSP (18). Bisulfite-treated DNA was subjected to PCR amplification using primers designed to anneal specifically to the methylated or unmethylated bisulfite-modified DNA sequence within the gene. *CDK10* MSP was performed with two different primer sets. Primer set A amplified a 328 bp fragment and was used as reported previously (14). In addition, a search for CpG islands in the 5' region of *CDK10* (ENSG00000185324, www.ensembl.org, release 52) was performed using the CpGplot tool (http:// www.ebi.ac.uk/Tools/ emboss/cpgplot/index.html) which found a CpG island located at -225 bp to +632 bp relative to the transcription start site of *CDK10*. The sequences of *CDK10* MSP primer set B yielding a 126 bp fragment were designed using MethPrimer (19). The primers were designed to amplify a region within a predicted promoter region at -138 bp to -12 bp relative to the transcription start site of *CDK10* (Figure 1). The forward and reverse primer sequences of *CDK10* MSP primer set B were: 5'-AGGTTCGAATTG TAGTAGTCGGAG-3' and 5'-GACGCAAACGCGAAAACTCC TTCC-3'. The PCR conditions were as follows: initial denaturation for 12 minutes at 95˚C followed by 40 cycles of denaturation for 30 seconds at 95˚C, annealing for 40 seconds at 60˚C and extension for 30 seconds at 72˚C with a final extension for 7 minutes at 72˚C. The MSP primer sequences and PCR conditions for *RASSF1A* and *DAL-1* were used as reported previously (20-22). The MSP products were separated in 2% agarose gels stained with GelRed™ (Biotium, Hayward, CA, USA) and visualized under UV spectrophotometry. The DNA extracted from the normal B-lymphocytes and the breast cancer cell line MCF-7 was treated with Sss1 methylase (New England Biolabs, Beverly, MA, USA) and used as a positive control for the methylated alleles. Furthermore, DNA from FFPE breast tissue was treated with Sss1 methylase (New England Biolabs) and used as a positive control for the methylated alleles to ensure the efficiency of MSP on fragmented DNA. Water blanks were used as the negative controls.

Statistical analyses. Comparison of *CDK10, CCND1* and *ESR1* mRNA expression with the clinico-pathological parameters was performed using the Mann-Whitney *U*-test or the Kruskal-Wallis test. Correlations among *CDK10, CCND1* and *ESR1* mRNA levels were assessed using the Spearman's Correlation Coefficient Method. All the *p*-values are the result of two-sided tests. A *p*-value equal to or less than 5% was considered statistically significant. The SPSS 15.0 software (SPSS Inc., Chicago, IL, USA) was used for the calculations.

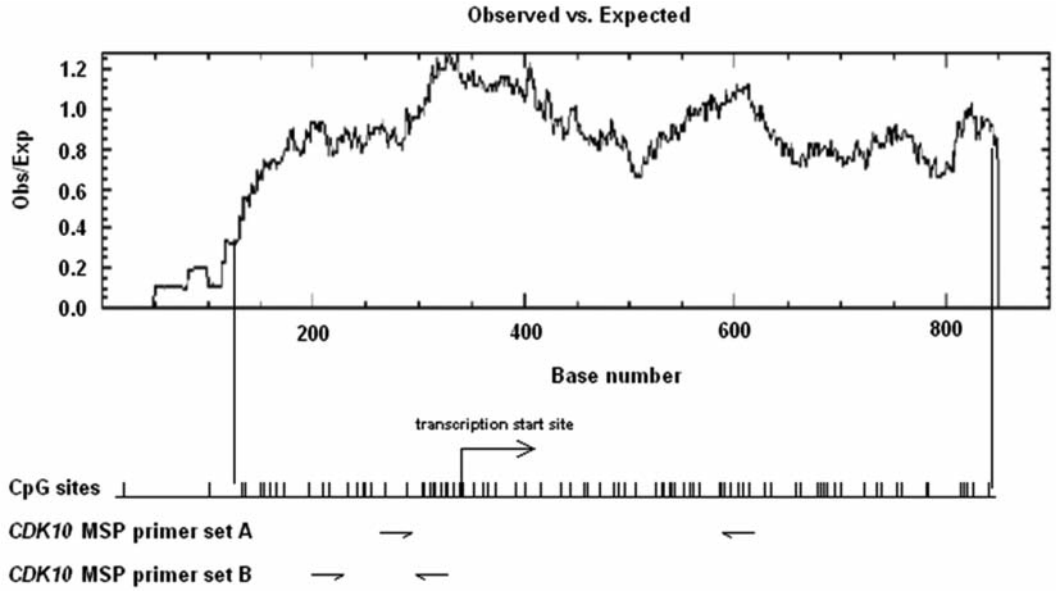

Figure 1. *CpG island search and location of MSP primer sets A and B in the CDK10 5' region. A 1000 bp fragment which included the transcription start site of CDK10 was analyzed for the presence of a CpG island using the EMBOSS CpGplot program. CpG sites are shown as vertical bars. MSP primer binding sites of primer set A and B are shown as arrows.*

Results

Expression of CDK10, CCND1 and ESR1 in breast carcinoma specimens. The isolation of mRNA was successful in all 96 tumor blocks. The Ct values for *RPL37A* ranged from 17.7 to 30.5 (median 19.8) and, therefore, sufficient RNA for the expression analyses was available. Figure 2 shows the distribution of *CDK10, CCND1* and *ESR1* mRNA levels in the studied population. The median value of mRNA expression normalized to the expression value of *RPL37A* (20 - ΔCt) for *CDK10* was 7.43 (range, 4.44-14.78), for *CCND1* 15.69 (range, 12.40-19.54) and for *ESR1* 15.49 (range, 8.84-18.73), respectively (Figure 2).

The relationship between *CDK10, CCND1* and *ESR1* mRNA status and the standard clinical and pathological factors is listed in Table I. No significant association between the *CDK10* mRNA expression and age, tumor size, lymph node status, tumor grade, histology, estrogen receptor or progesterone receptor status, HER2, p53 or Ki-67 expression was observed. The *CCND1* mRNA expression was significantly correlated with lymph node status (p=0.03) and estrogen receptor status (p=0.002). The *ESR1* mRNA expression was inversely correlated with tumor grade (p=0.001), HER2 (p=0.01) and p53 expression (p=0.002), whereas a positive association between the *ESR1* mRNA expression and age (p=0.007), estrogen receptor (p<0.001) as well as progesterone receptor (p<0.001) protein expression determined by means of immunohistochemistry was observed.

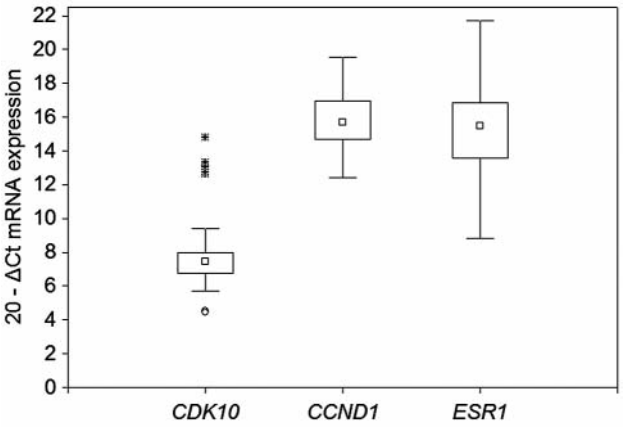

Figure 2. *Distribution of CDK10, CCND1 and ESR1 mRNA levels in the studied population.*

The *CDK10* mRNA status was significantly correlated with both the *CCND1* (Spearman rank correlation r=0.35, p=0.001) and *ESR1* mRNA status (Spearman rank correlation r=0.23, p=0.02). Moreover, the *CCND1* and *ESR1* mRNA values were positively correlated to each other (Spearman rank correlation r=0.57, p<0.001).

DNA methylation analysis. To further define the role of *CDK10* and its epigenetic silencing in breast cancer, we analyzed the methylation status of *CDK10* in 96 primary

Table I. *Characteristics of patients*.

Characteristic	No. of patients	%
Total	96	100
Age		
<60 years	46	48
≥60 years	50	52
Tumor size		
T1	43	45
T2	39	41
T3	12	12
T4	2	2
Lymph node status		
Negative	54	56
Positive	42	44
Tumor grade		
G1	10	10
G2	43	45
G3	43	45
Histology		
Ductal	64	67
Lobular	23	24
Other	9	9
Estrogen receptor		
Negative	29	30
Positive	67	70
Progesterone receptor		
Negative	42	44
Positive	54	56
HER2		
Negative	82	85
Positive	14	15
p53		
Negative	70	73
Positive	26	27
Ki-67		
Negative	23	24
Positive	73	76

breast cancer samples by MSP. We first used primers (MSP primer set A) specific for methylated or unmethylated DNA sequences of the *CDK10* gene as described previously by Iorns *et al*. (14). As shown in Figure 3A, the MSP primer set A worked well on the non-fragmented, SssI CpG methylase treated DNA isolated from both normal human B-lymphocytes and MCF-7 cells. However, no amplification product could be detected using methylated primer set A on fragmented DNA from the FFPE breast tumor samples. In addition, PCR amplification using the primer sequences for unmethylated *CDK10* was found in only one of the breast tumor samples. As seen in Figure 3A, *CDK10* methylation was detected in all the positive controls including the SssI methylase treated fragmented DNA with the MSP primer set B. Interestingly, using primer set B, methylation of the *CDK10* 5' region was not found in any of the 96 primary breast cancer samples. In contrast, using primer set B the unmethylated form of *CDK10* was detected in all the samples analyzed indicating that both the extraction of genomic DNA from the FFPE breast tissue samples and the treatment of this DNA with sodium bisulfite worked well in this sample cohort.

To ensure that the MSP assay was suitable for methylation analysis of DNA extracted from paraffin embedded tissues the methylation status of the *RASSF1A* and *DAL-1* genes in the breast carcinoma samples was also determined using previously reported primer sequences (20-22). The unmethylated form of *RASSF1A* and *DAL-1* was detected in all the FFPE breast tissue samples analyzed. *RASSF1A* methylation was detected in 75 out of the 96 (78%) breast cancer samples and *DAL-1* in 9 out of 15 (60%) samples analyzed, respectively. Examples of MSP results are shown in Figure 3B.

Discussion

Tamoxifen has been the standard of care for women with hormone receptor-positive breast cancer. Although aromatase inhibitors are a slightly more effective endocrine strategy against hormone-dependent breast cancer, tamoxifen still plays an important role in adjuvant endocrine treatment. Whereas some patients are unsuitable for aromatase inhibitor therapy due to side-effects others may be unsuitable due to pre-existing bone problems which many consider an aromatase inhibitor contraindication. For those women who relapse or develop metastases after aromatase inhibitor therapy, tamoxifen may still be a reasonable treatment choice. Moreover, in many parts of the world, tamoxifen remains the only economically affordable treatment option for women with endocrine-responsive breast cancer. Therefore, tamoxifen remains in the adjuvant treatment armamentarium and the ability to predict the outcome of tamoxifen treatment is still of importance in the management of hormone-dependent breast cancer.

Several biomarkers have been identified which may predict tamoxifen response (1, 23). Recently, Iorns *et al*. using an RNA interference (RNAi) screen identified CDK10 as an important factor of resistance to tamoxifen and fulvestrant in estrogen receptor-positive MCF-7 breast cancer cells (14). Using quantitative PCR in estrogen receptor-positive breast tumors from patients treated with adjuvant tamoxifen they found that low *CDK10* expression was not associated with well-established prognostic factors such as age, tumor size, lymph node status, tumor grade, estrogen receptor, progesterone receptor, HER2, p53 and Ki-67, which was in line with the present results. Furthermore, low *CDK10* expression was associated with a shorter time to distant relapse and shorter overall survival in two independent data sets. Moreover, Iorns *et al*. using MSP

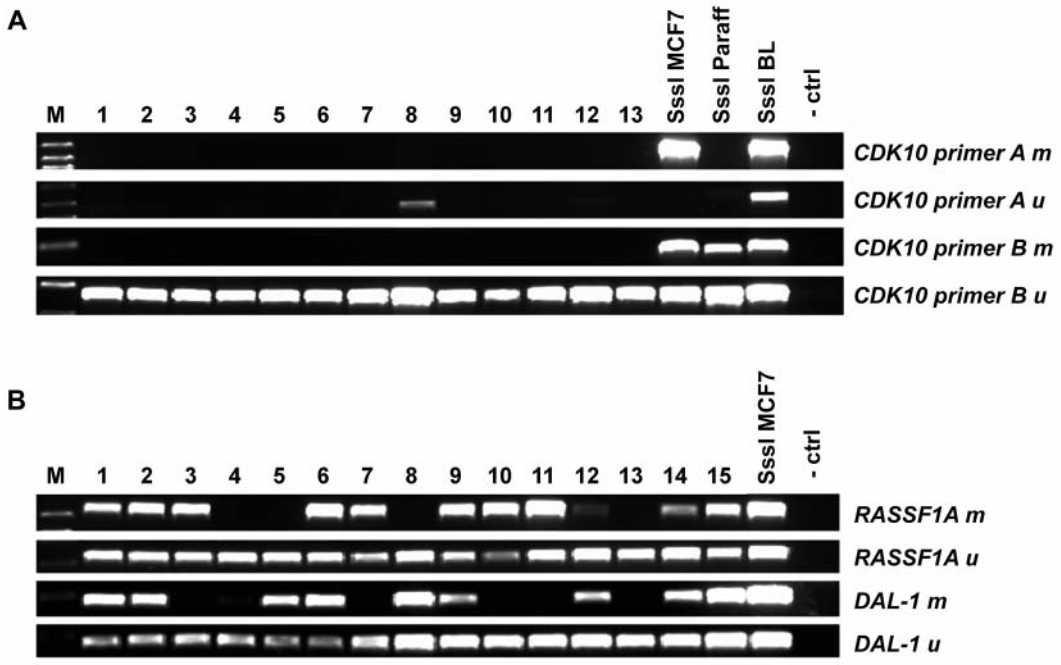

Figure 3. *(A) MSP analysis of CDK10 in DNA of FFPE breast tumor samples. M, 100 bp ladder; 1-13 primary breast tumor samples; SssI MCF7, SssI CpG methylase treated DNA from MCF7 cells used as positive control; SssI Paraff, SssI CpG methylase treated DNA from a FFPE breast tumor sample; SssI BL, SssI CpG methylase treated DNA from mononuclear cells. -ctrl. (B) MSP analysis of RASSF1A and DAL-1 in DNA of FFPE breast tumor samples. M, 100 bp ladder; 1-15 primary breast tumor samples; SssI MCF7, SssI CpG methylase treated DNA from MCF7 cells used as positive control; -ctrl, water blanks; m, results with primers specific for methylated sequences; u, results with primers specific for unmethylated sequences.*

found that 7 out of 38 (18%) FFPE breast tumors had methylation of the CpG island in the *CDK10* promoter, which correlated with low *CDK10* expression and was also significantly associated with a shorter time to disease progression ($p<0.001$) and shorter overall survival ($p<0.001$) (14). The authors concluded that methylation may be a mechanism by which tumors develop low *CDK10* expression levels resulting in tamoxifen resistance.

The present observations that using primer set A no amplification was detected in SssI CpG methylase treated DNA isolated from FFPE breast tissue and that the unmethylated form of *CDK10* was only seen in one sample suggested that the product amplified by MSP primer set A may have been too long for the detection of *CDK10* methylation in the present sample cohort. In general, using fragmented DNA obtained from FFPE tissue for MSP assays, MSP products should not exceed 150 bp in length (24). Thus, to improve MSP efficiency, additional MSP primer sequences for the 5' region of *CDK10* yielding a 126 bp fragment were designed in this study (Figure 1). Importantly, these primers were designed to anneal in the same region as the primer sequences used by Iorns *et al*. (14).

Surprisingly, even using the two different primer sets, no methylation of the *CDK10* promoter region was observed in the present 96 breast cancer samples. The data suggested that the lack of methylation of the *CDK10* promoter region in these tumor samples was not due to technical problems for the following reasons. Firstly, several enzymatically methylated positive controls were used to test primer sets A and B for their efficiency in identifying *CDK10* methylation in different sample types including FFPE breast tumors. All these controls were positive in the case of primer set B (Figure 2) suggesting that the MSP assay works well also on DNA extracted from FFPE breast cancer samples. Secondly, the unmethylated form of *CDK10* was detected in all the breast cancer samples analyzed using the unmethylated primer set B which indicated that after bisulfite treatment the DNA of the present samples was in good condition for subsequent MSP analysis. Thirdly, the methylation frequencies of *RASSF1A* and *DAL-1* were determined and found to be in accordance with other reports suggesting that the MSP assays were suitable for detecting methylation of DNA. Finally, it is noteworthy that *CDK10* mRNA expression was detected in all the samples analyzed.

In conclusion, the results strongly suggest that *CDK10* is not a target for aberrant DNA methylation in breast cancer which may be important information for future investigations of the clinical relevance of CDK10 in tamoxifen resistance.

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