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Genetic analysis identifies putative tumor suppressor sites at 2q35–q36.1 and 2q36.3–q37.1 involved in cervical cancer progression

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We performed comparative genomic hybridization (CGH) and high-resolution deletion mapping of the long arm of chromosome 2 (2q) in invasive cervical carcinoma (CC). The CGH analyses on 52 CCs identified genetic losses at 2q33–q36, gain of 3q26–q29, and frequent chromosomal amplifications. Characterization of 2q deletions by loss of heterozygosity (LOH) in 60 primary tumors identified two sites of minimal deleted regions at 2q35–q36.1 and 2q36.3–q37.1. To delineate the stage at which these genetic alterations occur in CC progression, we analysed 33 cervical intraepithelial neoplasia (CIN) for LOH. We found that 89% of high-grade (CINII and CINIII) and 40% of low-grade (CINI) CINs exhibited LOH at 2q. To identify the target tumor suppressor gene (TSG), we performed an extensive genetic and epigenetic analyses of a number of candidate genes mapped to the deleted regions. We did not find inactivating mutations in *CASP10*, *BARD1*, *XRCC5*, or *PPP1R7* genes mapped to the deleted regions. However, we did find evidence of downregulated gene expression in *CFLAR*, *CASP10* and *PPP1R7* in CC cell lines. We also found reactivated gene expression in CC cell lines *in vitro* after exposure to demethylating and histone deacetylase (HDAC) inhibiting agents. Thus, these data identify frequent chromosomal amplifications in CC, and sites of TSGs at 2q35–q36.1 and 2q36.3–q37.1 that are critical in CC development.
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Introduction

Cervical carcinoma (CC) is the second most common malignancy among women in both incidence and mortality (NIH Consensus Statement, 1996). Although much is known about the etiology and treatment of CC, the role of genetic alterations in the multistep pathway of cervical tumorigenesis is largely unknown. Converging points of evidence implicate infection by high-risk human papilloma virus (HPV) types as a critical etiologic factor (Kubbutat and Vousden, 1996). Epidemiological and experimental data, however, show that only a small fraction of HPV-infected cervical intraepithelial neoplastic (CIN) lesions progress to invasive CC (Murthy *et al*., 1990; Ostor, 1993; zur Hausen and Rosl, 1994). These findings, therefore, suggest that other somatic genetic mutations play a major role in the initiation and progression of CC. Delineation of these genetic changes is critical in understanding the molecular basis of CC.

The development of CC is preceded by distinct morphological changes from normal epithelium to carcinoma through low-grade and high-grade squamous intraepithelial lesions (SILs), which represent mild to severe dysplasias. The genetic basis of this progression is poorly understood. Molecular studies on early and invasive CC has identified several structural and functional alterations in oncogenes and candidate tumor suppressor gene (TSG) sites (Lazo, 1999). Despite this molecular characterization of cervical precancerous and cancerous lesions, no critical genes or chromosomal regions that play role in the development of CC have been identified so far. Studies on comparative genomic hybridization (CGH) have identified chromosomal changes involving loss of 2q, 3p, 4p, 4q, 5q, 6q, 11q, 13q, and 18q regions and gain of 1q, 3q, 5p, and 8q regions at various stages of CC (Heselmeyer *et al*., 1996, 1997; Dellas *et al*., 1999; Kirchhoff *et al*., 1999; Hidalgo *et al*., 2000; Umayahara *et al*., 2002). Although a limited number of CGH studies (Heselmeyer *et al*., 1997; Hidalgo *et al*., 2000) and low-resolution loss of

heterozygosity (LOH) mapping (Rader *et al*., 1998; Kersemaekers *et al*., 1999) have provided evidence of recurrent deletions on the 2q in invasive CC. The precise site of deletions that harbor putative TSG is unknown because of the lack of systematic and detailed deletion mapping studies.

In our attempts to discover critical genetic changes in CC progression, in the present study we first identified 2q33–q36 deletions, 3q gains, and chromosomal amplifications as the characteristic genetic changes by CGH. Followed by this, we identified two common minimal deleted regions of allelic loss at 2q35–q36.1 and 2q36.3–q37.1 by high-resolution LOH mapping. The 2q genetic alterations have also been found at early stages of progression in high- and low-grade CINs, suggesting that these changes affect genes critical in tumor progression. Furthermore, we have excluded several known candidate genes mapped to these regions as targets of mutational inactivation suggesting either the presence of so far unidentified gene(s) or alternative mechanisms of loss of tumor suppression resulting from 2q genetic loss. Gene expression analysis identified lack of or downregulated expression of a number of genes mapped in the proximity of the common regions of LOH. We have also observed reactivation of gene expression upon exposure of cells to demethylating and histone deacetylase (HDAC) inhibiting agents without evidence for hypermethylation of promoter sequences. These results, therefore, imply that the 2q genetic loss occurs very early in progression and the gene(s) affected may play an important role in cervical tumorigenesis.

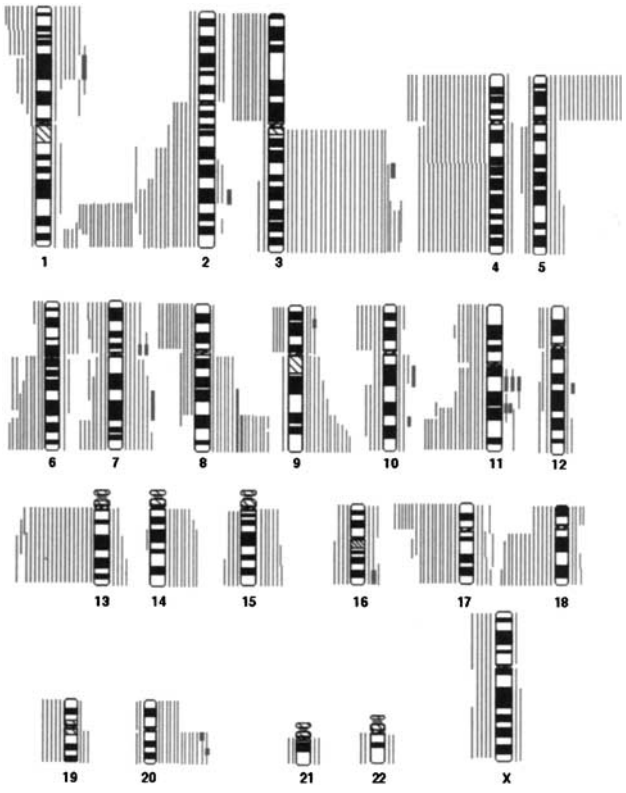

Figure 1 Ideogram showing DNA copy-number changes identified by CGH in 44 primary tumors and eight cell lines derived from CC. Thin vertical lines on either side of the ideogram indicate losses (left) and gains (right) of chromosomal region. The chromosomal regions of the high-level amplification were shown in thick lines (right)

Results

CGH identified a common site of deletion at 2q33–q36, gain of 3q26–q29, and frequent chromosomal amplifications in invasive CC

We have performed CGH analysis of 52 DNAs from invasive CCs (44 primary tumors and eight cell lines), and the results are summarized in Figure 1. Underrepresentation of DNA copy number in $\geqslant 25\%$ tumors was noted at chromosomal regions 2q (65%), 13q (36.5%), 4p (34.6%), 17p (32.7%), 11q (32.7%), 4q (30.8%), and 18q (25%). Chromosomal gains in $>15\%$ of tumors were identified at 3q (52%), 5p (32.7%), 8q (26.9%), 20q (25%), 9q (23.0%), 14q (15.4%), and 1p (15.4%). Although the pattern of chromosomal changes observed in both primary tumors and cell lines was similar, the DNA copy number losses predominated in primary tumors (65% losses versus 35% gains). However, the cell lines exhibited almost identical frequencies of DNA losses and gains (47 and 53%).

The most striking observation of the CGH analysis was the identification of loss of chromosome 2 regions in 34 (65%) tumors. All eight cell lines studied exhibited 2q losses, while 26 (59%) of the primary tumors had losses. Mapping CGH losses identified an overlapping region between 2q33 and q36 in all tumors that showed deletions on 2q (Figure 1). Thus, the chromosome 2q33–q36 region was identified as a common site of deletion in a high proportion of CCs. These data, therefore, suggest that the 2q33–q36 region contain one or more genes that are critical for CC tumorigenesis.

Of the DNA copy number gains seen in CC, the 3q was most frequently affected. We found 3q copy number gains in 24 (54.5%) of the primary tumors and in three (37.5%) of the cell lines studied. The common region of gain identified in the present study was 3q26–q29.

We identified 20 high-level chromosomal amplifications at 15 different chromosomal sites in 15 of 52 (28.8%) tumors studied. The amplifications were noted at 1p31, 2q32, 3q21, 7p11.2–p12, 7q22–q31, 8q23–q24, 9p22, 10q21, 10q24, 11q13, 11q21, 12q15, 16q23–q24, 20q11.2, and 20q13.1 (Figure 1). Recurrent sites of amplification were noted in four chromosomal sites: 7p11 (Ca Ski and MS751), 8q23–q24 (CC102 and Ca Ski), 11q13 (CC52, MS751, and HT-3) and 11q21 (CC98 and CC116). Nonrecurring amplifications were found at 1p31 (CC24), 2q32 (CC968), 3q21 (SiHa), 7q22–q31 (CC892), 9p22 (CC86), 10q21 (ME-180), 10q24 (CC968), 12q15 (CC52), 16q23–q24 (SiHa), 20q11.2 (SW756), and 20q13.1 (C-33A). These data, therefore, suggest that gene amplification is a common genetic alteration in this tumor type. Our results suggest that the 2q33–q36 losses, the 3q26–q29 gains, and chromosomal

amplifications are characteristic genetic changes in invasive CC.

High-resolution deletion mapping in invasive CC identifies two minimal deleted regions of LOH at 2q35–q36.1 and 2q36.3–q37.1

To further characterize the 2q losses identified by CGH analysis, we evaluated LOH in 60 primary CCs, using 31 STRP markers mapped to this chromosomal arm (Figure 2). A total of 41 of these tumors were also studied by CGH. We found LOH of at least one marker in 37 (61.7%) of the tumors. Two of these tumors showed LOH at all the informative markers, while the remaining 35 tumors exhibited partial deletions on 2q. The patterns of LOH from the 33 tumors with partial losses identified two common sites of minimal deletions at the 2q36.3–q37.1 and 2q35–q36.1 regions (Figure 2).

The 2q36.3–q37.1 minimal deletion derived from 32 tumors span six markers (D2S1392, D2S427, D2S2176, D2S206, D2S331, and D2S2348). The deletion boundaries were identified based on the patterns of LOH in at least five tumors (T-29, T-869, T-939, T-968, and T-1068) (Figure 2). The 2q35–q36.1 minimal deletion spans five STRP loci (D2S163, D2S120, D2S377, D2S339, and D2S126) and was identified based on the patterns of LOH in 26 tumors. The 2q35–q36.1 deletion boundaries were identified from the pattern of LOH in at least six tumors (T-93, T-63, T-98, T-58, T-70, T-28, and T-21) (Figure 2). A total of 21 tumors commonly exhibited LOH at both sites of minimal deletions, whereas nine tumors had deletions only at 2q36.3–q37.1 and three had deletions only at the 2q35–q36.1 region (Figure 2). The 2q36.3–q37.1 deletion spans a 10.6-cM distance between 242.4 and 253 cM on the genetic map, and the 2q35–q36.1 minimal deletion maps to a 6.8-cM distance between 223.2 to 230 cM on the genetic map. Thus, our deletion analysis identified two distinct but closely mapped sites of common regions of deletions on 2q in CC.

2q deletions occur in cervical precancerous lesions

To evaluate whether the 2q genetic losses occur early in the progression of CC, we studied LOH in 33 cervical intraepithelial neoplasia (CIN) specimens (28 high-grade and five low-grade lesions) using six STRP markers mapped to 2q23–2q37.2 regions. In total, 25 of 28 (89.3%) high-grade CINs exhibited LOH in at least one informative locus. Of the five low-grade CINs, two (40%) had LOH (Figure 3). Consistent with the localization of common deleted regions to the distal 2q in invasive CC, the CINs also had a higher incidence of LOH with the markers D2S1392, D2S2176, and D2S2348 mapped to 2q36.3–q37.1 regions compared to the proximal marker D2S321, which maps to the 2q23 region and the distal marker D2S338 that maps to 2q37.2 (Figure 3). The finding of 2q deletions in both low-grade and high-grade CINs suggests that these genetic alterations occur at very early stages in the development of CC.

Analysis of candidate genes at the 2q deleted regions

Lack of inactivating mutations in PPP1R7, BARD1, XRCC5, and CASP10 genes Despite the well-defined morphologic changes at various stages in the development, the genetic alterations associated with the initiation and progression of CC are unclear. To find inactivated genes at the 2q36.3–q37.1 and 2q35–q36.1 minimal deleted regions, we identified genes with potential tumor suppressor function mapped to these regions by searching the human genome resources at the NCBI web site (http://www.ncbi.nlm.nih.gov/genome/guide/human/). We identified three candidate genes for tumor suppressor function, *NCL*, *NEDD5* and *PPP1R7*, in the vicinity of the 2q36.3–q37.1 deletion. The *PPP1R7* gene encodes a protein phosphatase 1, regulatory subunit 7 that is required for the completion of mitosis in *Saccharomyces pombe* (Renouf *et al*., 1995). The *NEDD5* gene plays an essential role in cytokinesis (Kinoshita *et al*., 1997). The *NCL*, nucleolin, gene is a major nucleolar protein in growing eucaryotic cells; it induces chromatin decondensation by binding to histone H1. Although *NCL* is upregulated in proliferating cells, its multiple roles in the cell cycle implicate this gene in the regulation of cell growth (Srivastava and Pollard, 1999). We analysed the *PPP1R7* gene for mutations in 30 primary tumors with a 2q36–q37 region of LOH and did not find any inactivating genetic alterations.

The vicinity of 2q35–q36.1 deleted region contains five potential TSGs. These are *BARD1*, *XRCC5*, *CFLAR*, *CASP10*, and *CASP8*. The *BARD1* gene, which encodes brca1-associated ring domain protein, is implicated in brca1-mediated tumor suppression, cell growth regulation, and the cellular response to DNA damage *in vitro* (Wu *et al*., 1996). The *BARD1* gene has earlier been shown to exhibit infrequent mutations in sporadic and hereditary breast, ovarian, and endometerial tumors (Thai *et al*., 1998). In view of the high frequency of 2q35–q36.1 deletions in cervical precancerous and cancerous lesions, and the chromosomal localization of the *BARD1* gene at this region, we analysed 73 DNA samples from invasive CC for mutations. We found a very high frequency of conformational variations in CC. Follow-up studies on DNA from the corresponding blood specimens, together with sequencing analysis confirmed these changes as polymorphic variants (Table 1). We found 12 different single-nucleotide alterations in the *BARD1* gene. All of these were within the coding region except two that were in the 5′ noncoding sequence (Table 1). One of these two was a splice donor variant of exon 3. In addition, we found a two base pair AT deletion in intron 2. Both these changes were also found in the corresponding normal DNA. Thus, our extensive mutation analysis excluded *BARD1* as the target of mutational inactivation at 2q35–q36.1 deletions in CC.

The *XRCC5* gene mapped to 2q35 encodes the 80-kDa subunit of the Ku autoantigen. The *Ku80* is essential for maintaining genomic integrity through its ability to bind DNA double-strand breaks and to facilitate repair by the nonhomologous end-joining

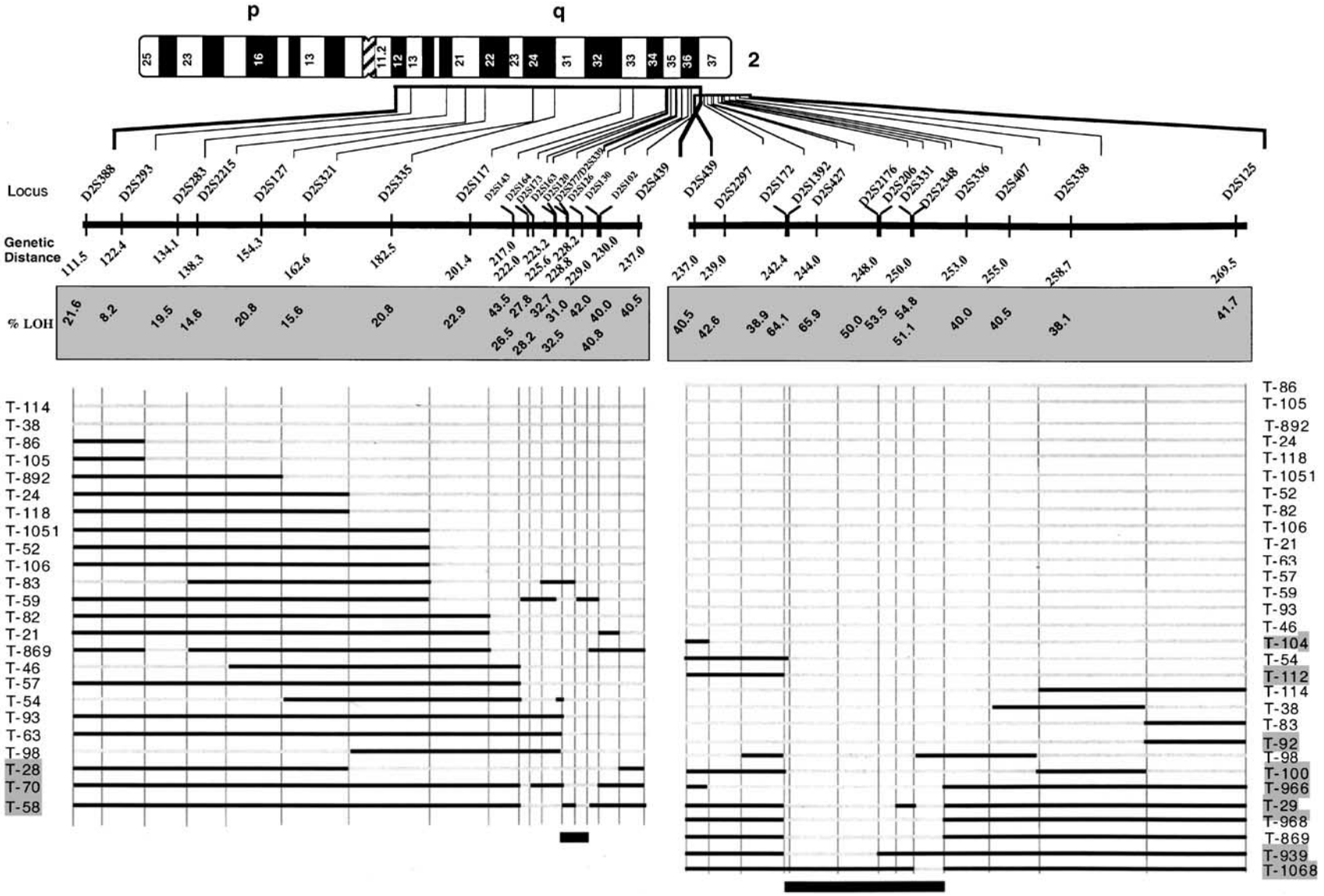

Figure 2 Identification of minimal regions of deletions by high-resolution LOH mapping on 2q in invasive CC. Patterns of LOH. A total of 33 tumors with partial deletions are represented. A G-banded ideogram of chromosome 2 is shown on *top*. Thick horizontal lines shown *below* the 2q ideogram represents the corresponding chromosomal regions. Small vertical lines show STRP markers on the *thick horizontal* lines and their corresponding genetic map distances. The percentage of LOH is shown in horizontal box *below* each STRP marker. The patterns of LOH are shown below. For the clarity of presentation, derivation of two minimal deleted region at 2q35–q36.1 (*left* panel) and 2q36.3–q37.1 (*right* panel) are separated. A horizontal line represents each tumor; marker region with LOH is shown by *light shaded* lines, and *dark shaded* lines show the retention of heterozygosity. Tumor numbers are shown on the *right* or the *left* of *horizontal lines*. Shaded tumor numbers indicate LOH specific to that panel. *Thick black rectangles* at the bottom indicate region of minimal deletion

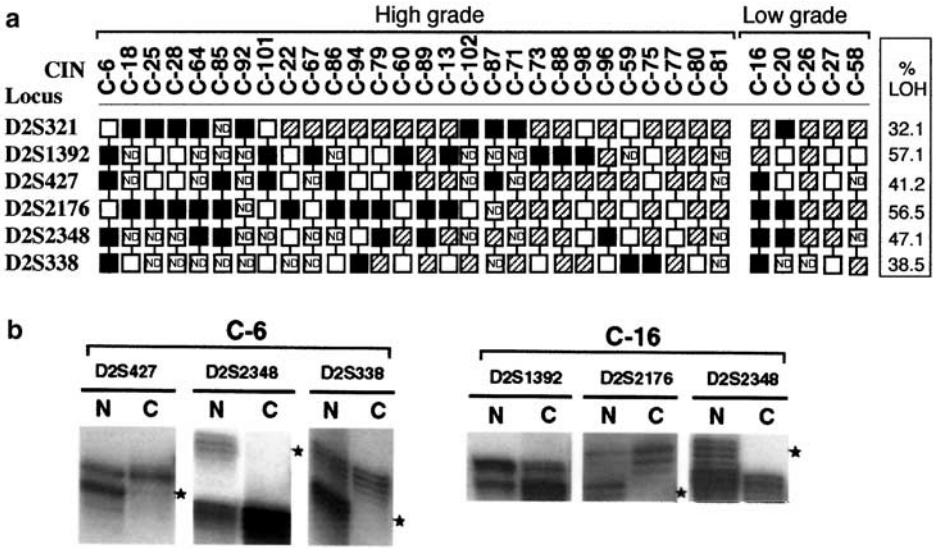

Figure 3 Identification of 2q LOH in high-grade and low-grade CINs. (**a**) Pattern and frequency of LOH in CINs. In total, 14 high-grade and five low-grade CINs studied are shown on *top*. The STRP markers studied and their patterns of LOH are shown *below*. Filled square, LOH; hatched square, retention of heterozygosity; empty square, homozygous and uninformative; ND, not done. Percentage of LOH of each marker is shown on *right* in the box. (**b**) Illustration of LOH in CINs. C-16 is a low-grade CIN and C-6 represents a high-grade CIN. The STRP markers and the CIN numbers are shown above each panel. N, normal; C, CIN; Asterisks indicate LOH

Table 1 Nucleotide variations in genes mapped to the common regions of deletions at 2q35–q36.1 and 2q36–q37 in CC

Gene	*Nucleotide variation*	*Amino-acid change*	*No of cases*[a]
BARD1	A26G	Noncoding region	7
	C44G	Noncoding region	7
	C143T	Pro24Ser	12
	AT deletion	Intron 2	4
	A373C	Gly100His	11
	A378C	Asp102Ala	15
	G385C	Met104Ile	11
	Exon 3 splice donor GT→CT	—	2
	C1126G	Thr351Thr	6
	G1207C	Arg378Ser	6
	C1591T	His506His	2
	G1592A	Val507Met	4
	G1743C	Cys558Ser	2
XRCC5	T1218C	Tyr395Tyr	1
	A58416G (intron 12)[b]	—	2
	C1590T	Pro519Leu	1
	G2361C	Noncoding region	5
	T2263A	Noncoding region	1
CASP10	C402G	Leu70Val	1
	C1293T	Val367Ile	2

[a]The frequency is based on combined SSCP and sequencing data. [b]The nucleotide number is based on the clone AC009521

pathway in mammalian cells (Taccioli *et al*., 1994). *XRCC5* functions as a caretaker gene, acting synergistically with the loss of p53 in mice. *XRCC5*$^{-/-}$/*p53*$^{-/-}$ mice exhibit hypersensitivity to gamma radiation and develop a spectrum of cancers, including pro B-cell lymphomas with specific chromosome translocations and gene amplifications (Difilippantonio *et al*., 2000; Lim *et al*., 2000). As expression of the high-risk HPV E6 gene results in functional inactivation of p53 through binding and degradation (Munger *et al*., 1992), the *XRCC5* could be a strong candidate TSG at the 2q35–36.1 deletion. However, our mutation analysis of this gene in 30 cases of CC provided no evidence of the presence of inactivating mutations (Table 1).

The *CFLAR*, *CASP10*, and *CASP8* genes, which encode functionally related cysteine proteases and play a central role in apoptosis, have been mapped within a 150-kb genomic region proximal to the *BARD1* gene at the 2q35–q36.1 minimal deletion (Fernandes-Alnemri *et al*., 1996; Shu *et al*., 1997). These caspases have been implicated in various diseases, including cancer. For example, germline mutations in the *CASP10* gene result in autoimmune lymphoproliferative syndrome, type II; ALPS2 (Wang *et al*., 1999). Caspase 8 has been shown to function as a tumor suppressor resulting from 2q deletions in neuroblastoma (Teitz *et al*., 2000, 2001; Takita *et al*., 2001). To assess the role of the *CASP10* gene in cervical tumorigenesis, we performed mutation analysis in 22 primary tumors and eight cell lines. We found no pathogenic mutations in the *CASP10* gene, except two single-nucleotide changes that were present in both the tumors and the corresponding normal DNAs (Table 1). Thus, we conclude that the four candidate genes we tested so far are not targets of mutational inactivation of 2q deletions in CC.

Gene expression and methylation analysis in CC cell lines In a further effort to identify the mechanisms of gene inactivation involved in the recurrent 2q deletions in CC, we performed a semiquantitative RT–PCR analysis on eight genes (*CFLAR*, *CASP8*, *CASP10*, *BARD1*, *XRCC5*, *NCL*, *NEDD5*, and *PPP1R7*) in eight CC cell lines (Figure 4a). Five of these genes (*CASP8*, *BARD1*, *XRCC5*, *NCL*, and *NEDD5*) showed no evidence of downregulated expression relative to their

levels in the normal cervix. The remaining three genes (*CFLAR*, *CASP10*, and *PPP1R7*) exhibited relative decrease in expression in some cell lines. The most striking observation of this analysis was the complete lack of expression, using two different sets of RT–PCR primers, of *CASP10* in the C-33A cell line (Figure 4a). In addition, expression of the *CASP10* gene was also reduced in one other cell line (Ca Ski). Thus, two (25%) of the CC cell lines studied showed evidence of downregulated gene expression. Expression of *CFLAR*, another caspase gene in this cluster, was also showed decreased expression in the same two cell lines (Ca Ski and C-33A). Expression of the *PPP1R7* gene was downregulated in three (37.5%) of the cell lines (C-33A, SiHa, and MS751) (Figure 4a). These results, therefore, suggest that several genes mapped to the 2q deletions have downregulated steady-state levels of gene expression in CC cell lines. Since we found no pathogenic mutations in CC cell lines and primary tumors in *CASP10* and *PPP1R7* genes, these results also suggest that another genetic or epigenetic gene inactivation mechanism may be involved.

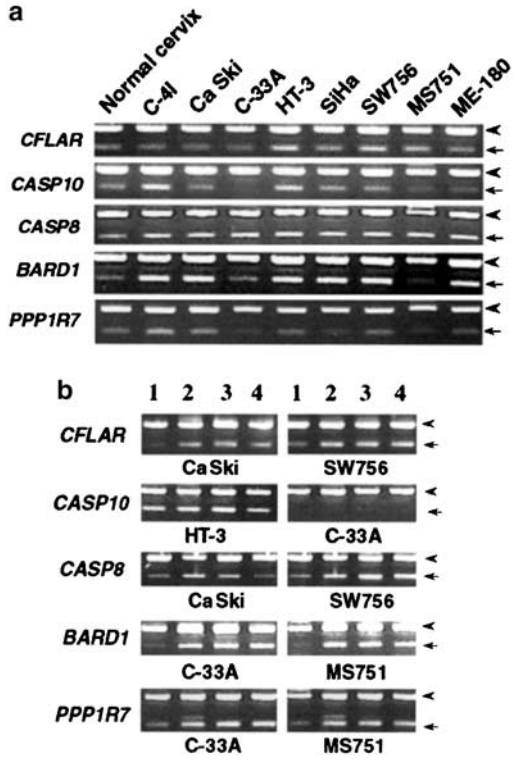

Figure 4 Analysis of gene expression in CC cell lines. (**a**) Semiquantitative multiplex RT–PCR analysis of 2q candidate genes. The C-33A cell line showed complete lack of expression in *CASP10* gene. Reduced expression was found in *CFLAR* (Ca Ski and C-33A), *CASP10* (Ca Ski), and *PPP1R7* (C-33A, SiHa and MS751). (**b**) Effect of demethylation and acetylation on gene expression. Note the lack of reactivation of gene expression in C-33A in *CASP10*. Lane 1, untreated; lane 2, butyrate-treated; lane 3, 5-Aza-2′ deoxycytidine + butyrate treated, and lane 4, 5-Aza-2′ deoxycytidine-treated. Genes are shown on *left*; cell lines are indicated on *top* (panel "a") or bottom (panel "b"); arrowhead indicates the PCR product of actin gene used as control and arrow represents the indicated gene

To identify the possible epigenetic mechanism, we treated CC cell lines with a demethylating drug 5-Aza-2′ deoxycytidine, and an inhibitor of HDAC, *n*-butyrate. Demethylation of promoter regions and inhibition of histone deacetylation has been shown to reactivate gene expression by 5-Aza-2′ deoxycytidine and *n*-butyrate, respectively (Tycko, 2000). Some of the cell lines treated with these drugs either separately or in combination have shown reactivation of gene expression as compared to untreated cells (Figure 4b). However, one of the caspase genes, *CASP10*, did not show any evidence of reactivation of mRNA after treatment including the C-33A cell line that showed the complete lack of expression (Figure 4b). The *CFLAR* gene was reactivated either after the treatment to 5-Aza-2′ deoxycytidine or combined with *n*-butyrate in two cell lines (CaSki and C-33A) that showed downregulated expression. In addition, another cell line (SW756) that showed no relative decrease in expression in untreated cells also showed reactivation to these drugs (Figure 4b). The *CASP8* gene that did not show evidence of downregulated expression showed reactivation in all eight cell lines studied. Similarly, the *BARD1* that did not show downregulated expression showed reactivation in three of eight cell lines (Figure 4b). The *PPP1R7* gene was reactivated in two of three cell lines that showed downregulated expression and another cell line (CaSki) that did not show reduced expression also showed reactivation (Figure 4). Thus, these data suggest that both the demethylating agent and the deacetylase inhibitor have the potential ability to reactivate gene expression over the basal levels.

We then tested to see if this gene reactivation was because of promoter hypermethylation. We searched the 5′ end of genomic sequences of the genes included in this study for CpG islands using Grail Experimental Gene Discovery software (http://grail.lsd.ornl.gov/grailexp/). This analysis has identified that the *BARD1* and *PPP1R7* genes each contain an approximately 2 kb CpG island spanning exon 1 and intron 1 at the 5′ end of the gene. We also found two CpG islands spanning 600 and 360 bp of DNA located, respectively, 14 and 15 kb upstream of *CFLAR*, in the caspase gene cluster. Methylation-specific PCR of the CpG islands followed by sequencing of at least 200 bp of each CpG yielded no evidence of CpG hypermethylation in these genes (data not shown). Thus, our data suggest that extensive hypermethylation does not occur in these genes in CC, but that the reactivation of gene expression occurs in response to demethylating or HDAC-inhibiting agents.

Discussion

Our CGH analysis identified a consensus region of deletion at 2q33–q36 regions in all 34 cervical tumors that showed 2q losses. Previous CGH studies have shown a low frequency of genetic losses at 2q in precancerous lesions, CIS, and invasive CC (Heselmeyer *et al*., 1996; Dellas *et al*., 1999). Thus, the identification of a high incidence of 2q33–q36 losses in CC strongly

suggests that this region harbor one or more tumor suppressor genes. Of the DNA copy-number gains seen in CC, the 3q was most frequently affected. The common region of gain identified in the present study was 3q26–q29, which is identical to that in most previously reported studies (Heselmeyer *et al*., 1996, 1997). The gain of 3q genetic material has been shown in a diverse types of tumors, with highest prevalence of gain occurring in carcinomas of mucosal origin, including those arising from the head and neck, cervix, lung, and esophagus (http://www.nhgri.nih.gov/DIR/LCG/CGH/index.html). In CC, it has been demonstrated that copy-number gains in the 3q region are associated with progression from high-grade cervical intraepithelial neoplasia to invasive cancer (Heselmeyer *et al*., 1996). A number of candidate genes have been identified on 3q26–q27 regions implicated as candidate oncogenes in carcinomas of the cervix, ovary, and head and neck (Shayesteh *et al*., 1999; Ma *et al*., 2000; Singh *et al*., 2001).

Increase in gene dosage by DNA amplification is a common mechanism to achieve overexpression of genes in tumors. It has been shown that gene amplification commonly is associated with tumor progression, resistance to chemotherapy, and poor clinical outcome in a variety of tumor types (Schwab, 1999). However, the scope of gene and chromosomal amplifications has been limited in CC (Mitra *et al*., 1994). In the present study, we identified a high frequency of amplified chromosomal sites in CC. These data, therefore, comprise the first distinct evidence of chromosomal amplification in CC. Several of the amplified sites in the present study are of particular interest, such as 8q24 region, which harbors the *MYC* gene. A recently identified protein tyrosine phosphatase (*PRL3*) gene, which also maps to 8q24, is expressed at high levels in metastatic colon cancer (Saha *et al*., 2001). It has been shown that the amplified *CCND1*/*PRAD1* gene on 11q13 is overexpressed in CC cell lines (Kurzrock *et al*., 1995). A newly identified *A1B1* gene on 20q12 is frequently amplified and overexpressed in breast tumors, although its exact role is not yet known (Anzick *et al*., 1997). Thus, the identification of many chromosomal regions of amplification in the present study suggests that gene amplification is a common genetic alteration in invasive CC. Further molecular characterization of the amplified regions should facilitate identification of the target amplified genes in CC. Thus, in the present study, we identified a novel site of deletion at the 2q33–q36, confirmed the previously reported gains in the 3q26–q29 region and identified frequent occurrence of gene amplifications. Our results, therefore, suggest that these are the characteristic genetic changes in invasive CC.

To identify the critical regions of deletion on 2q, we performed high-density LOH mapping and identified two common regions of deletions at cytogenetic bands 2q36.3–q37.1 and 2q35–q36.1. The major site of deletion at 2q36.3–q37.1 spans a 4.7-Mb physical distance exhibiting LOH in 32 of 37 (86.5%) tumors that showed 2q losses. Consistently, the marker D2S427 mapped to this region exhibited the highest frequency (65.9%) of LOH (Figure 2). The second region at 2q35–q36.1, spanning a 4.8-Mb physical distance, exhibited deletion in 26 of 37 (70%) tumors that showed 2q LOH. Among the 41 primary invasive CCs that we studied for both CGH and LOH, the results were compatible in 35 tumors by both methods. In the other six tumors, which showed copy-number losses by CGH, did not exhibit LOH. It is likely that the LOH analysis was not sensitive enough to detect losses in specimens containing less than 50% tumor cells, while the CGH might be more sensitive in detecting these changes. However, our extensive deletion mapping allowed us to identify two distinct sites of minimal deletions, providing evidence of the existence of critical TSGs at these regions.

The 2q33–37 region has been shown to affect a number of other tumor types, including neuroblastoma (Takita *et al*., 2001), as well as carcinomas of lung (Otsuka *et al*., 1996), head and neck (Ransom *et al*., 1998), esophagus (Hu *et al*., 2000), and follicular thyroid (Tung *et al*., 1997). It has also been shown that the 2q LOH has associated with advanced stage cancer and predicts a poor prognosis for patients with head and neck cancer and follicular thyroid carcinoma (Tung *et al*., 1997; Ransom *et al*., 1998). 2q37 microcell hybrids effectively restore cellular senescence in the CC cell line SiHa, identifying a putative senescence gene to this region (Uejima *et al*., 1998). Thus, one or more genes that control cell growth and senescence are likely to be located on 2q35–37 and may play a common role in different tumor types.

To identify target genes at these deletions, we performed semiquantitative gene expression in CC cell lines. This analysis identified *CFLAR* and *CASP10* (mapped to 2q34–q35), and *PPP1R7* (mapped to 2q36.3–37.1) genes as having downregulated steady-state levels of gene expression in a number of cell lines. As downregulated gene expression is not a sole criterion for identifying TSGs, we then performed mutation analysis of the entire coding regions of the *CASP10*, *BARD1*, *XRCC5*, and *PPP1R7* genes in primary tumors that showed LOH on 2q and in cell lines. Direct sequencing of all SSCP variants suggested that none of these genes exhibit inactivating mutations in CC. Surprisingly, the *BARD1* genomic region is highly polymorphic showing at least 87 variants in the 73 tumor DNAs studied (Table 1). The majority of the variants that we found have also been reported in the single-nucleotide polymorphism (SNP) database (http://www.ncbi.nlm.nih.gov/SNP/) and by a previous study (Thai *et al*., 1998). In addition, we found several variants that have not been reported earlier, including a splice donor variant in exon 3. We found five changes in *XRCC5* gene, two in the coding region and three in either the noncoding region or in intronic sequence (Table 1). All changes except Pro519Leu were confirmed as polymorphisms by their presence in the corresponding constitutional DNA. We have also found two SNPs in the coding region of the *CASP10* gene that are not in the SNP database (Table 1). Germline mutations in the *CASP10* gene have been shown to cause defective lymphocyte and dendritic cell apoptosis in ALPS type II

(Wang *et al*., 1999). The V367I polymorphism that we found in two tumors has previously been reported in ALPS patients as an inherited mutation that causes decreased apoptotic activity. Whether or not these polymorphisms have any effect on the efficiency of apoptotic mechanisms in tumors remains to be investigated.

Since we obtained evidence of downregulated expression of several genes in the absence of inactivating mutations, we tested to see if this downregulation was a consequence of epigenetic mechanisms. We found detectable upregulation in expression of four of the five genes tested upon exposure to 5-Aza-2′ deoxycytidine, *n*-butyrate, or combination of the two. To determine whether or not promoter hypermethylation plays a role in reactivation, we performed methylation-specific PCR, and then sequenced a part of the CpG islands of *BARD1* and *PPP1R7*. We also tested two CpG islands upstream of the caspase gene cluster. We did not find any evidence of methylation. Thus, we ruled out extensive promoter hypermethylation as the mechanism of inactivation of these genes. The absence of CpG islands in the proximal promoter regions of caspase genes and their reactivation by methylation and HDAC inhibitors suggests an indirect mechanism of epigenetic gene downregulation affecting the transactivating regulators or transcription factors by methylation (Soengas *et al*., 2001; Zhu *et al*., 2001). However, the cell line C-33A, which showed complete lack of expression of *CASP10*, did not reactivate in response to 5-Aza-2′ deoxycytidine or *n*-butyrate (Figure 4b). Complete absence of the expression may also result from lack of primer binding because of polymorphism. However, this possibility was ruled out since we have tested two different sets of primers to amplify the *CASP10* gene. The fact that SSCP analysis of the C-33A cell line did not show mutations in the coding region of *CASP10* excludes homozygous deletion as a mechanism for the loss of gene expression. Thus, gene inactivation in this cell line may be the result of other mechanisms such as a promoter mutation.

The high frequency of 2q loss of genetic material and the identification of regions of critical deletions strongly indicate that these regions harbor TSGs. Thus, it is possible that there may be other genes that are targets of mutational inactivation at 2q35–q36.1 and 2q36.3–q37.1 in CC. The partial downregulation of expression and the reactivation of genes mapped to the minimal deletions is, however, intriguing. It is possible that several genes are influenced by genetic alterations of the region and that their downregulation below physiologic levels is associated with disease progression. Our data on restoration of expression to physiologic levels following exposure to demethylating agents, in the absence of demonstrable promoter hypermethylation, suggest that the downregulated gene expression may be because of defects in transcription factors that normally activate promoters or partial methylation. Such defects may involve methylation and deacetylation of those transcription factors.

In summary, we have identified two sites of common regions of frequent deletions at 2q35–q36.1 and 2q36.3–q37.1 in CC that harbor critical TSGs. We have excluded *CASP10*, *BARD1*, *XRCC5*, and *PPP1R7* as targets of mutational inactivation of these deletions in CC. Further, we have identified an indirect epigenetic effect on a number of genes linked to methylation or deacetylation mechanisms, or both. These results should further aid in identifying the TSGs at the 2q-deleted region in cervical carcinogenesis. In the present study, we also have detected LOH at 2q35–q37 regions in 85% of precancerous lesions, both high grade and low grade. Our data, therefore, support the hypothesis that 2q deletions occur very early in cervical carcinogenesis and that the TSG affected by these deletions may play a role in the progression of precancerous lesions to invasive cancer. Thus, the 2q genetic deletions identified in invasive CC and in precursor lesions may have biologic and clinical implications. Biologically, the identification of target TSG at 2q deletions will provide a better understanding of the genetic basis of cervical carcinogenesis. Since only a fraction of precancerous lesions have the potential to progress to invasive cancer (zur Hausen and Rosl, 1994), the 2q-specific gene alterations may facilitate the clinical identification of dysplasias at high risk for progression. The chromosomal amplifications reported here have the potential to uncover amplified genes. Molecular characterization of these chromosomal changes utilizing the new genomic technologies should provide important advances in our understanding of the genetic mechanisms of initiation and progression. The identification of amplified genes will provide new insights into the clinical behavior and management of CC.

Materials and methods

Tumor-normal tissues and cell lines

We analysed 98 tumor biopsies consisting of 65 frozen tumors from previously untreated primary invasive CCs with the corresponding peripheral blood samples, and 33 formalin-fixed, paraffin-embedded specimens from CINs. The tissues were obtained from patients treated at the Instituto Nacional de Cancerologia, Santa Fe de Bogota, Colombia and from the Department of the Obstetrics and Gynecology of Friedrich Schiller University, Jena, Germany after appropriate informed consent and approval of the protocols by the institutional review boards. The invasive tumors were clinically classified as FIGO stage IB (eight tumors), IIB (16 tumors), IIIB (37 tumors), and IV (four tumors). Histologically, 60 tumors were classified as squamous cell carcinomas and five as adenocarcinomas. A total of 28 CIN specimens were classified as high-grade and five as low-grade lesions. Eight CC cell lines SiHa, SW756, C-4I, Ca Ski, C-33A, HT-3, MS751, and ME-180 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and were grown in tissue culture according to the supplier's recommendations.

Microdissection, DNA and RNA isolation, and LOH analysis

High-molecular weight DNA from frozen tumor and peripheral blood specimens, and frozen cell pellets from cell lines was isolated as previously described (Pulido *et al*., 2000).

Tumor cells from paraffin-embedded CIN tissue specimens were isolated by laser capture microdissection (Arcturus, Mountain View, CA, USA) after methyl green staining and the extraction of DNA as described earlier (Chatterjee *et al*., 2001). Total RNA was isolated from semiconfluent cell cultures using TRIzol reagent (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's protocol. A panel of 31 sequence-tagged repeat polymorphic (STRP) (27 di- and four tetra-nucleotide) markers was chosen on the basis of their map position and heterozygosity (Figure 2) (Gene Map 99-http://www.ncbi.nlm.nih.gov/genemap). A standard polymerase chain reaction (PCR) containing gamma [^{32}P]ATP end-labeled forward primer, analysis of PCR products on denaturing polyacrylamide sequencing gels, and scoring of LOH on autoradiograms were performed as previously described (Mitra *et al*., 1995; Pulido *et al*., 2000). All autoradiograms were visually analysed. The definition of minimal region of deletion was defined earlier (Chatterjee *et al*., 2001). The LOH analysis was performed at least twice on all tumors with the corresponding markers that define the minimal deletion.

Comparative genomic hybridization

CGH analysis was performed as previously described (Rao *et al*., 1998). Briefly, labeled tumor (fluorescein-12-dUTP; NEN-DuPont, Boston, MA, USA) and normal (Texas Red-5-dUTP; NEN-DuPont, Boston, MA, USA) DNAs were mixed in equal amounts in the presence of human Cot-1 DNA (GIBCO/BRL, Gaithersburg, MD, USA), and were hybridized to normal human metaphase chromosomes. Seven to 10 metaphases from DAPI counter-stained slides were captured for each tumor using a cooled charge-coupled device camera attached to a Nikon Eclipse 800 microscope and processed using the Quantitative Image Processing System (Applied Imaging, Santa Clara, CA, USA). Red, green, and blue fluorescence intensities were analysed for all metaphases, normalized to a standard length, and statistically combined to show the red : green signal ratio and 95% confidence intervals for the entire chromosome. Copy-number changes were detected based on the variance of the red : green ratio profile from the standard of 1.0. Ratio values of 1.20 and 0.80 were used as upper and lower thresholds to define gains and losses, respectively. High-level amplification was defined as the occurrence of fluorescein intensity values in excess of 2.0 along with a strong localized fluorescein isothiocynate (FITC) signal at the chromosomal site.

Analysis of mutations and gene expression

Single-strand conformational polymorphism (SSCP) analysis was performed on all coding exons using primers flanking intronic sequences of four candidate genes. These were protein phosphatase 1, regulatory subunit 7 (*PPP1R7*) (Renouf *et al*., 1995), caspase 10, apoptosis-related cysteine protease (*CASP10*) (Vincenz and Dixit, 1997), BRCA1-associated RING domain 1 (*BARD1*) (Wu *et al*., 1996), and X-ray repair complementing defective repair in Chinese hamster cells 5 (double-strand break rejoining; Ku autoantigen) *XRCC5* (Taccioli *et al*., 1994). Standard PCR was performed using 25 ng of DNA in the presence of 0.5 μCi of alpha [^{32}P]dCTP. The diluted PCR products were denatured in sequencing stop buffer and was run overnight at room temperature in 6% nondenaturing polyacrylamide gels containing 10% glycerol. Dried gels were autoradiographed and examined for conformational changes. Purified PCR products were sequenced for all exons that showed conformational variations. Primer sequence information and the PCR conditions of these genes are available from authors on request.

To assess gene expression, total RNA isolated from the cell lines was reverse transcribed using random primers and the Pro-STAR first strand RT–PCR kit (Stratagene, La Jolla, CA, USA). Semiquantitative analysis of gene expression was performed in replicate experiments by 26–28 cycles of multiplex RT–PCR with β-actin as control and gene-specific primers spanning at least two exons. The gene primers used and their positions in cDNA were:

CFLAR-F	5′-CTTGGCCAATTTGCCTGTAT	(535–554 bp)
CRLAR-R	5′-CTCGCTTCTGAGCCTTGAAT	(810–829 bp)
		(product size-295 bp)
CASP10-F	5′-ACCCGACAAAGGGTTTCTCT	(516–535 bp)
CASP10-R	5′-GATACGACTCGGCTTCCTTG	(800–819 bp)
		(product size-304 bp)
CASP10-F2	5′-AAGAGGAAGTGGAGCGACTG	(490–509 bp)
CASP10-R2	5′-GTCCTCCAGGCATGTCAGAT	(691–710 bp)
		(product size-221 bp)
CASP8-F	5′-GTCTGTACCTTTCTGGCGGA	(616–635 bp)
CASP8-R	5′-ACTCCTCCCCTTTGCTGAAT	(892–911 bp)
		(product size-296 bp)
BARD1-F	5′-CATGGGCACCTGAAGGTAGT	(1484–1503 bp)
BARD1-R	5′-AAGAGGTCCATCCCTACGCT	(1764–1783 bp)
		(product size-300 bp)
XRCC5-F	5′-ATCCCCATTTGAACAAGCAA	(117–136 bp)
XRCC5-R	5′-AATCACATCCATGCTCACGA	(368–387 bp)
		(product size-271 bp)
PPP1R7-F	5′-AGAATCTGGAGGCGCTAACA	(422–441 bp)
PPP1R7-R	5′-CCCCAAAAACAAACTCTCCA	(644–663 bp)
		(product size-242 bp)
NCL-F	5′-CAGCCAAAGCAGTTACCACA	(410–429 bp)
NCL-R	5′-GGTGCTTCTTTGACAGGCTC	(922–949 bp)
		(product size-532 bp)
NEDD5-F	5′-CCTGGCTATGGTGACGCTAT	(565–584 bp)
NEDD5-R	5′-TCTGGGTTCTCCACTTCCAC	(1045–1064 bp)
		(product size-500 bp).

The PCR products were run on 1.5% agarose gels, visualized by ethidium bromide staining and quantified using the Kodak Digital Image Analysis System (Kodak, New Haven, CT, USA). The effect of methylation on gene expression was similarly assessed on total RNA isolated from the cell lines treated with the demethylating agent 5-Aza-2′ deoxycytidine (Sigma) for 5 days at a concentration of 5 μM, the deacetylase inhibiting agent *n*-butyric acid (Sigma) for 24 h at a concentration of 5 μM or with both of these agents.

Analysis of promoter hypermethylation

Methylation-specific PCR (MSP) was performed with primers designed specific for methylated and unmethylated DNA as previously described (Herman *et al*., 1996). These primers were:

BARD1	U-F	5′-TATTAATGTGAGTAGTGTAGTA; U-R 5′-CACCATCCCAAACACAAAAAA
BARD1	M-F	5′-TATTAACGCGAGTAGCGTAG; M-R 5′-CGCCGTCCCAAACGCGAA
PPP1R7	U-F	5′-GTGTGGTTTTATGATGGAATTA; U-R 5′-ACAACTATTACTACCCCACAC
PPP1R7	M-F	5′-GCGCGGTTTTATGACGGAAT; M-R 5′-ACGACTATTACTACCCCGCG
CFLAR	U-F1	5′-TTGGTTTATTTATGTTTGGGTAG; U-R1 5′-CAATAAATAACAAAAATTAAACATAA
CFLAR	M-F1	5′-TCGGTTTATTTACGTTCGGGTA; M-R1 5′-CGATAAATAACGAAAATTAAACGTA
CFLAR	U-F2	5′-TTGTAAAGTTTGTGGTAAATAATTG; U-R2 5′- CACACTAACAACACCAAAACTAT
CFLAR	M-F2	5′-TCGTAAAGTTCGCGGTAAATAATC; M-R2 5′- GCGCTAACGACGCCGAAACTA

(underlined letters indicate converted nucleotides).

Briefly, tumor DNA was modified by sodium bisulphite treatment. We used placental DNA treated *in vitro* with *Sss*I methyltransferase (New England Biolabs, Beverly, MA, USA) and normal lymphocyte DNA treated similarly to tumor DNAs as positive controls for methylated and unmethylated templates, respectively. PCR products were run on 2% agarose gels and viewed after ethidium bromide staining. PCR products generated from bisulfite-converted tumor DNA were sequenced to identify partially methylated residues.

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