Mapping the Sites of Putative Tumor Suppressor Genes at 6p25 and 6p21.3 in Cervical Carcinoma: Occurrence of Allelic Deletions in Precancerous Lesions[1]

Anupam Chatterjee,[2, 3] Hugo Arias Pulido,[2] Sanjay Koul, Nestor Beleño, Ana Perilla, Hector Posso, Mahesh Manusukhani, and Vundavalli V. V. S. Murty[4]

Department of Pathology, College of Physicians & Surgeons of Columbia University, New York, New York 10032 [A. C., H. A. P., S. K., M. M., V. V. V. S. M.], and Departments of Genetics, Pathology, Gynecology, and Epidemiology, Instituto Nacional de Cancerología, Santa Fe de Bogotá, Colombia [H. A. P., N. B., A. P., H. P.]

ABSTRACT

Allelic deletions on the short arm of chromosome 6 (6p) are one of the common, possibly early, genetic changes that occur in the pathogenesis of cervical carcinoma (CC). Previous loss of heterozygosity (LOH) studies in CC identified a number of critical regions of deletions on 6p. However, the precise location of minimally deleted regions and their role in precancerous lesions have not been well characterized. To address these questions, we first performed a detailed LOH analysis on 6p in 59 cases of invasive CC. The pattern of LOH identified two minimal regions of deletions, one spanning a 5 cM genetic distance at 6p25 and a second site of 10.3 cM deletion mapping to 6p21.3. The 6p21.3 minimal deletion spans HLA class I genes. To understand the role of 6p genetic alterations in the development of CC, we also investigated 12 high-grade and 4 low-grade cases of cervical intraepithelial neoplasia (CIN) for LOH after laser microdissection. The high-grade CINs exhibited 91.7% LOH, and low-grade CINs had 50% LOH. These findings implicate the presence of at least two tumor suppressor genes on 6p relevant to CC and suggest that these genetic alterations occur very early in CC development. This study should therefore facilitate the identification of tumor suppressor genes on 6p and may identify which CINs are at high risk of progressing to invasive CC.

INTRODUCTION

The short arm of chromosome 6 (6p) is frequently affected by LOH[5] in a high proportion of CCs (1–5), suggesting the presence of one or more TSGs on this chromosomal arm. Previous allelotype studies have indicated potential sites of TSGs at 6p23 (6), and at 6p21.3 (5, 7, 8) in CC. The latter region of deletion spans the HLA class I antigen genes, thus suggesting a potential role for HLA genes in the development of CC. These studies have also indicated for a third candidate TSG site of deletion at subtelomeric loci on 6p, but the boundaries of the deletion have not been identified (7, 8). Although these data provide strong evidence for the presence of TSGs on 6p that may be relevant to CC, the critical regions of loss on 6p have not been identified precisely.

To identify the critical regions of LOH, we performed a detailed deletion mapping on 6p in invasive CC and identified two regions of minimal deletions at 6p25 and 6p21.3 spanning a 5 cM and a 10.3 cM genetic distance, respectively. We have also found that these genetic alterations occur in CIN, suggesting that they represent genetic changes that occur early in the process of cervical tumorigenesis.

MATERIALS AND METHODS

Tumor/Normal Tissues. A total of 75 tumor biopsies representing 59 frozen, previously untreated, primary invasive CCs with corresponding peripheral blood samples and 16 formalin-fixed, paraffin-embedded specimens from low-grade (CINI) and high-grade (CINII, CINIII/carcinoma *in situ*) CINs comprised the material. The tissues were obtained from patients treated at the Instituto Nacional de Cancerologia (Santa Fe de Bogotá, Colombia) after appropriate informed consent and approval of the protocol by the institutional review board. Clinically, the tumors were classified as follows: (*a*) FIGO stage 1B, 4 tumors; (*b*) FIGO stage IIB, 15 tumors; (*c*) FIGO stage IIIB, 36 tumors; and (*d*) FIGO stage IV, 4 tumors. Histologically, 56 tumors were classified as squamous cell carcinomas, and 3 tumors were classified as adenocarcinomas. The samples from CINs included 12 high-grade and 4 low-grade lesions.

Laser Microdissection, DNA Isolation, and LOH Analysis. High molecular weight DNA from frozen tumor and peripheral blood specimens was isolated as described previously (9). Tumor cells from paraffin-embedded CIN tissue specimens were isolated by laser capture microdissection (Arcturus, Mountain View, CA) after methyl green staining, and DNA was extracted by digestion with proteinase K for 72 h. Twenty-three STRP (20 dinucleotide and 3 tetranucleotide) markers were chosen on the basis of their map position and heterozygosity (Table 1; Gene Map 99).[6] A standard PCR reaction containing [γ-^{32}P]dATP end-labeled forward primer, analysis of PCR products on denaturing polyacrylamide sequencing gels, and scoring of LOH on autoradiograms were performed as described previously (9). The criteria used for scoring LOH were described previously (10). All autoradiograms were independently scored visually by three investigators (A. C., H. A. P., and V. V. V. S. M.). The definition of the minimal region of deletion was based on LOH of the loci that span common deletions in several tumors and retention of heterozygosity of adjoining markers at both the boundaries in at least two tumors. The LOH analysis was performed at least twice on all tumors with the corresponding markers that define minimal deletion.

RESULTS

Analysis of LOH in Invasive CC Identifies Two Common Regions of Deletions at 6p25 and 6p21.3. Evaluation of LOH on a panel of 59 invasive CCs with 23 STRP markers mapped to chromosome 6p revealed deletions in at least one marker in 66.1% (39 cases) of the tumors. Of the 39 tumors that had LOH on 6p, 8 (21%) showed LOH at all of the informative markers, suggesting 6p monosomy. The patterns of LOH in the remaining 31 tumors that exhibited regional losses on 6p were used to identify minimal regions of deletion (Table 1; Fig. 1). This LOH pattern in invasive CCs revealed two common regions of minimal deletions at 6p25 and 6p21.3 (Fig. 1).

The 6p25 minimal region of deletion derived from 20 tumors spanned the marker AFMB034Ya5 flanked by marker D6S344 proximally and D6S1617 distally. The deletion boundaries were defined

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[3] Present address: Department of Zoology, North-Eastern Hill University, Shillong, 793022 India.

[4] To whom requests for reprints should be addressed, at Department of Pathology, College of Physicians & Surgeons of Columbia University, 630 West 168th Street, New York, New York 10032. Phone: (212) 305-7914; Fax: (212) 305-5498; E-mail: vvm2@columbia.edu.

[5] The abbreviations used are: LOH, loss of heterozygosity; CC, cervical carcinoma; TSG, tumor suppressor gene; CIN, cervical intraepithelial neoplasia; FIGO, International Federation of Gynecologists and Obstetricians; STRP, sequence-tagged repeat polymorphic; ZNF, zinc finger.

[6] http://www.ncbi.nlm.nih.gov/genemap.

Table 1 *Frequency of LOH on chromosome 6p in cervical carcinoma*

Chromosome band	Locus	Genetic map position	No. studied/ informative	LOH (%)
6p25	*D6S1600*	0.0	56/47	20 (42.6)
	D6S344	1.0	57/48	26 (54.2)
	AFMbO34ya5	1.4	57/27	19 (70.4)
	D6S1617	6.4	59/47	26 (55.3)
	D6S1640	11.4	59/43	20 (46.5)
6p24	*CHLC.GATA23E10*	14.0	57/37	9 (24.3)
6p22–23	*D6S1267*	25.9	59/44	22 (50.0)
	D6S260	29.6	58/51	26 (51.0)
	D6S469	29.6	58/39	19 (48.7)
	D6S1584	32.4	53/41	19 (46.3)
	CHLC.GATA87C11	34.0	58/40	21 (52.5)
6p22.1	*D6S422*	35.7	59/41	22 (53.7)
6p21.3	*D6S1691*	42.7	56/42	24 (57.1)
	D6S265	44.3	59/43	22 (51.2)
6p21.1–21.3	*MOG* $(CA)_n$	44.5	57/49	24 (49.0)
	D6S273	44.9	59/42	23 (54.8)
	D6S276	44.9	59/41	19 (46.3)
	TAP1 $(CA)_n$	45.4	59/44	19 (43.2)
	D6S291	49.6	58/37	17 (45.9)
6p11.2–12	*D6S1549*	62.3	59/41	15 (36.6)
	D6S1650	68.7	57/47	18 (38.3)
6p11.1	*D6S272*	74.1	58/49	15 (30.6)
6p10	*D6S1681*	85.0	57/46	10 (21.7)

by tumors T-86, T-118, and T-55 proximally and tumors T-6 and T-55 distally (Fig. 1). The 6p21.3 minimal deletion was deduced from the pattern of LOH in 24 tumors as shown in Fig. 1 and Fig. 3. This deletion was spanned by the STRP markers D6S422, D6S1691, and D6S265 flanked by markers CHLC.GATA87C11 proximally and $MOG(CA)_n$ distally. The 6p21.3 deletion boundaries were defined by tumors T-2 and T-13 proximally, and tumors T-13 and T-55, distally.

All but 1 of the 39 tumors with 6p deletions fell within the two defined regions of minimal deletions. Twenty-two tumors exhibited LOH at both regions, whereas 6 tumors showed deletions only at 6p25, and 10 others had deletions only at 6p21.3. Thus, we identified two discrete sites of minimal deletions in CC at 6p25, which was restricted to a 5 cM genetic distance, and 6p21.3, which spans a 10.3 cM genetic distance.

Identification of LOH at 6p25 and 6p21.3 in Precancerous and Early Cancerous Lesions. To evaluate LOH in early cervical cancerous lesions, we studied 16 microdissected CIN specimens using 7 STRP markers spanning 6p25 and 6p21.3, the regions that exhibited a high frequency of LOH in invasive CCs. The pattern of LOH in high-grade and low-grade CINs is shown in Fig. 2.

Eleven of 12 (91.7%) high-grade CINs had LOH at one or more informative markers. Five of these lesions had LOH at all markers studied on 6p, and four others showed deletions encompassing both the 6p25 and 6p21.3 regions but retained heterozygosity of proximal markers. The remaining two high-grade CINs exhibited LOH only at the 6p23–25 region and retained constitutional heterozygosity at all proximal markers including the 6p21.3 region.

Of the four low-grade CINs studied, two (50%) exhibited LOH, one each at the 6p23–25 and 6p21.3 regions (Fig. 2 and Fig. 3). Thus, deletions at 6p25 and 6p21.3 were found in both high- and low-grade CINs, suggesting that these genetic alterations represent very early change in the development of CC.

DISCUSSION

The high frequency of LOH on 6p in CC suggests the possible existence of critical genes in tumor suppression (4, 5, 7, 8). However, the locations of the exact regions of LOH are not known due to lack of systematic LOH mapping studies, except for a single report by Rader *et al.* (6) identifying a minimal deletion at the 6p23 region. In the present study, we performed high-density LOH mapping on 6p and identified two minimal regions of deletions at 6p25 and 6p21.3. The 6p25 deletion exhibited LOH in 74% (28 of 39 tumors) of the cases, and the 6p21.3 minimal deletion exhibited LOH in 87% (32 of 39 tumors) of the cases among the specimens that had 6p deletions.

The 6p25 minimal deletion that spans a 5 cM genetic distance is a novel site that has not been described previously. Although the 6p25 region has been shown to have a high frequency of LOH in several previous studies (4, 5, 7, 8), the exact location of the minimal deletion is not known. Rader *et al.* (6) restricted the deletion at 6p23 to a 1 cM distance between 26–27 cM genetic distance. In contrast, our analysis identified the minimal deletion between 1.0 and 6.4 cM genetic distance, (Fig. 1), which is 20 cM distal to the one reported by Rader *et al.* (6). In the present study, despite the fact that we found a high frequency of LOH at markers spanning the 6p23 region, the patterns of LOH identified the minimal deletion at 6p25. Consistent with this observation, marker AFMb34ya5 mapping to the 6p25 region exhibited the highest frequency (70.4%) of LOH among all of the tested loci (Table 1). The discrepancy between our study and that of Rader *et al.* (6) may be due to differences in the number and density of markers used in the region or may represent two separate targets of deletions.

The 6p25 minimal deletion interval contains at least 9 genes, 17 UniGenes, and 5 expressed sequence tags.[7] The genes include *PECI*, *PRP4*, *TUBB*, *PI6*, *ELANH2*, *NMOR2*, *BPHL*, *FKHL6*, and *FKHL7*. The biological functions of the*TUBB*, *PI6*, *ELANH2*, *FKHL6*, and *FKHL7* genes suggest a putative TSG role for these genes. The *TUBB* β-tubulin gene is a member of tubulin multigene family that forms microtubules, which is a constituent of the eukaryotic cytoskeleton and mitotic apparatus. The β-tubulin gene modulates drug responses in human cancer cells by binding to the β-tubulin component of α-/β-tubulin heterodimers that facilitate blocking cells at G_2-M phase and lead to cell death. Mutations and differential expression of isotypes in the β-tubulin gene confer acquired resistance to anticancer drug taxanes in cancer (11, 12). We have performed mutation analysis of the coding region of the *TUBB* gene by single-strand conformational polymorphism in 30 invasive CCs that exhibited 6p LOH, followed by direct sequencing of PCR products of tumors suspected of conformational variations by single-strand conformational polymorphism (data not shown). No pathogenic mutations were identified by this analysis, suggesting that *TUBB* is not a target TSG of the deletion at 6p25 in CCs.

The *PI6* and *ELANH2* genes belong to a superfamily of serine protease inhibitors that play a role in many cellular processes including matrix remodeling and apoptosis. A member of this family, the *PI5* (maspin) gene, has been shown previously to have a tumor suppressor role in breast carcinoma (13). The *FKHL6* and *FKHL7* genes belong to a large family of forkhead (*Drosophila*) genes that regulate transcription. Mutations in forkhead genes cause developmental anomalies (14). The presence of these genes in the 6p25 deletion interval suggests that they may be the targets of deletion in CC and may function as cervical cancer TSGs.

The second site of minimal deletion at 6p21.3 identified in the present study has also been reported to have frequent LOH in invasive CC (4, 7, 8). Although the precise location of the minimal deletion was unclear, the HLA class I genes have been shown to be part of the 6p21.3 deletion interval. In the present study, we identified a 10.3-cM minimal deletion between 34.0 and 44.3 cM genetic distance (Fig. 1). This 6p21.3 deletion interval is highly gene rich because the region harbors at least 64 genes, 67 UniGene clusters, and 30 expressed sequence tags.[7] The genes in the 6p21.3 deletion interval include clusters of 12 histone family genes, 5 butyrophilin family genes, 8

[7] http://www.ncbi.nlm.nih.gov/genemap/map.cgi?CHR=6.

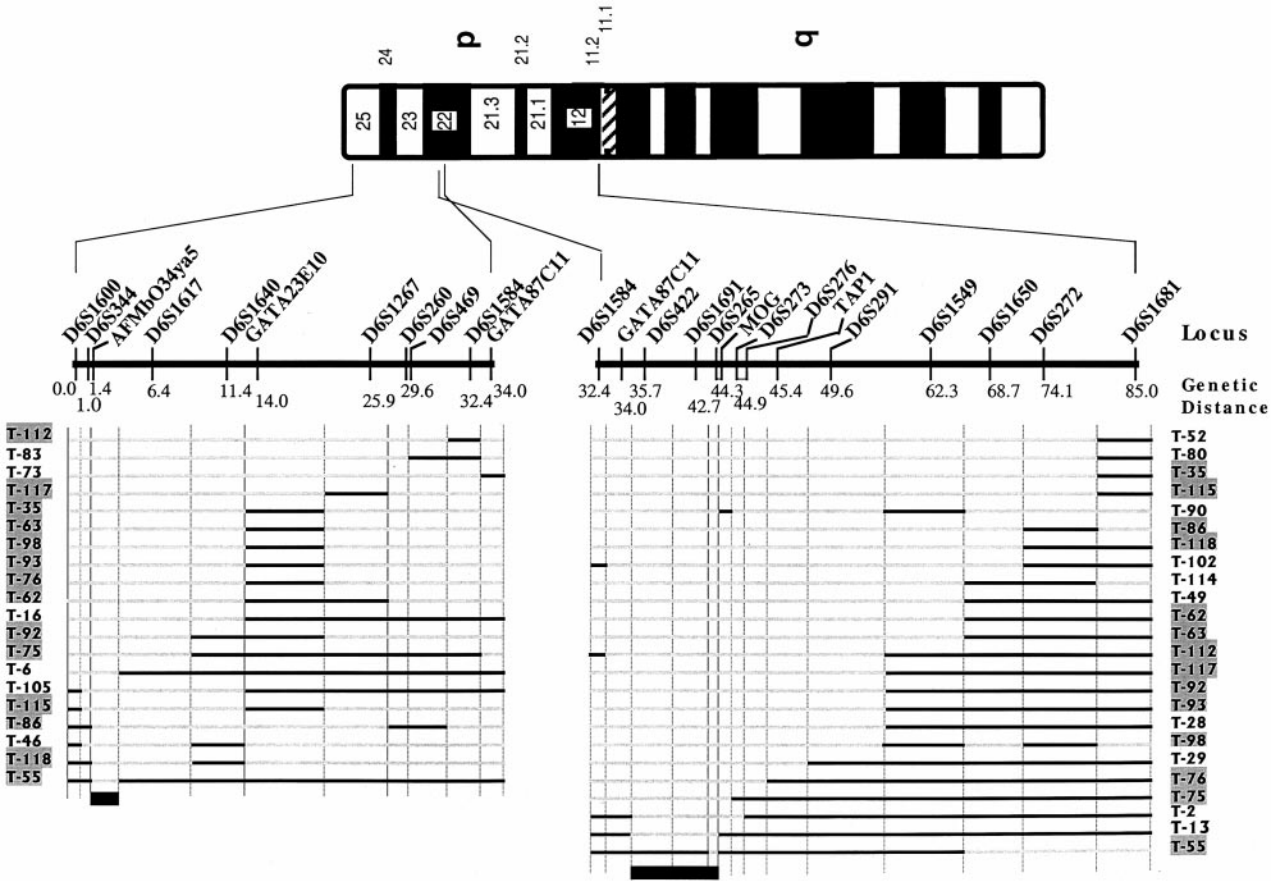

Fig. 1. Patterns of LOH on 6p in invasive CC. Thirty tumors that exhibited partial deletions are represented here. A G-banded ideogram of chromosome 6 is shown on *top*. *Thick horizontal lines* shown below the G-banded ideogram represent 6p chromosomal regions. STRP markers are shown by *small vertical lines* on the *thick horizontal lines*. Genetic map positions are shown *below* the *thick horizontal line*. Each tumor is represented by a *horizontal line*; marker/region of LOH is shown by *light shading*, and retention of heterozygosity is shown by *dark shading*. Tumor numbers are shown on the *right* or *left* of the *horizontal lines*. *Shaded tumor numbers* indicate a common deletion at both the 6p25 and 6p21.3 regions. For convenience of presentation, 6p25 and 6p21.3 regions of deletions are presented separately. *Thick black rectangles* at the *bottom* indicate regions of minimal deletion.

ZNF proteins, 5 HLA class I genes, and 4 KIAA gene products. Histones are the basic nuclear proteins responsible for the nucleosome structure of the chromosomal fiber in eukaryotes involved in the formation of higher order structures of chromatin (15). They contribute to virtually all chromosomal processes, such as gene regulation, chromosome condensation, recombination, and replication. Butyrophilin genes belong to the immunoglobulin superfamily that plays a role in the development of mammary epithelial cells during lactation

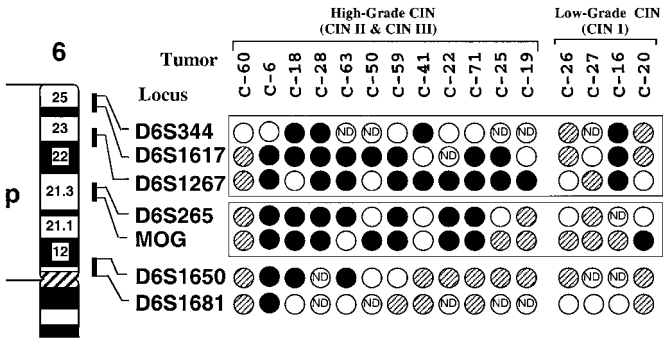

Fig. 2. LOH on 6p in high- and low-grade cervical CINs. Twelve high-grade CINs and four low-grade CINs are represented. A G-banded ideogram is shown on the *left*, and the corresponding polymorphic loci are shown to the *right* of the ideogram. Patterns of deletions are indicated by *circles below* the CIN numbers corresponding to each marker. ●, LOH; *hatched circle*, retention of heterozygosity; ○, homozygous and uninformative; *circle containing ND*, not done. *Two large rectangles* represent regions corresponding to 6p23–25 and 6p21.3.

(16). A large number of the ZNF family proteins exist in human genome. The ZNF genes perform many key functions, the most important of which is regulation of transcription. The ZNF domains are also thought to be involved in both normal and abnormal cellular proliferation and differentiation (see OMIM number 603971). HLA class I molecules regulate immune response by ligand binding to the T-cell receptor on cytotoxic T cells that recognize and destroy tumor cells. Genetic changes at the 6p21.3 region containing HLA class I molecules may affect the expression of these genes and therefore may allow tumor cells to evade the immune response. Among the other genes mapped to the 6p21.3 deletion interval, the human immediate early response 3 (*IER3*) gene contains binding sites for transcription factor *p53*, *NF-κB*, *CEBP*, and *SP1* genes and thus may have a role in cell growth regulation (17, 18). Mutation analysis of the coding region of the *IER3* gene in 30 cases of invasive CC that exhibited 6p LOH did not reveal any pathogenic alterations (data not shown).

Although the role of these gene(s) or gene clusters in cervical carcinogenesis remains unknown, the HLA class I genes have been proposed as targets of 6p21.3 deletions in CC. Loss of HLA class I antigen gene expression is a common phenomenon in most CCs (19), and the loss of HLA-A2 and B7 expression is associated with a poor prognosis in CC (20). The underlying mechanism for the loss of HLA class I gene expression in CC has recently been shown to be genetic alterations including LOH at 6p21.3 and mutations in the HLA-A and/or HLA-B alleles (21). Koopman *et al.* (21) found that 70% of CCs with loss of HLA class I expression harbor multiple genetic

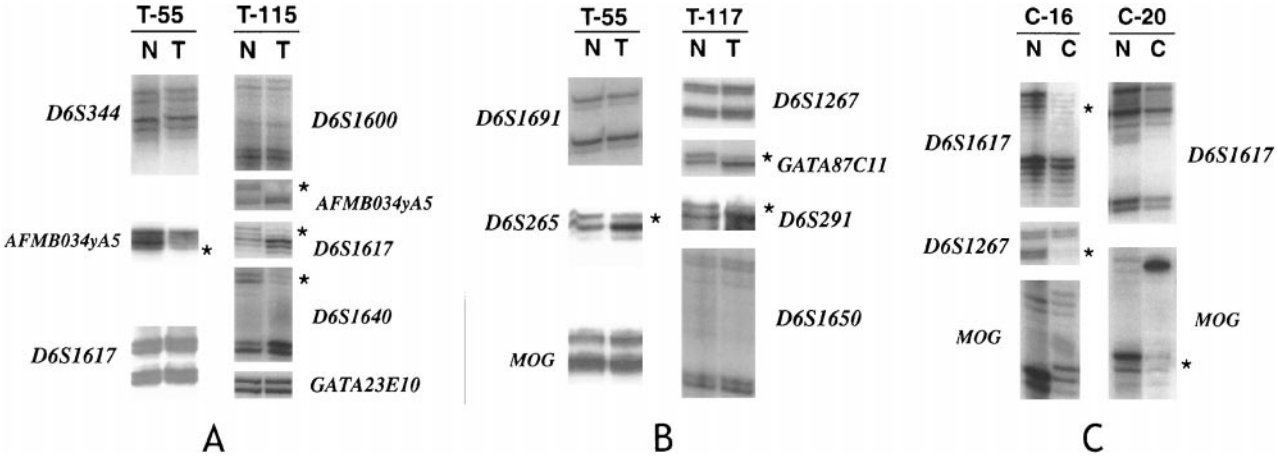

Fig. 3. Illustration of LOH at 6p25 and 6p21.3 common regions of deletions in invasive cervical cancer and precancerous lesions. *A*, the 6p25 region of deletions in invasive cancer is represented by two tumors, T-55 and T-115. Tumor T-55 shows LOH of 6p25 marker AFMB034yA5 but retains heterozygosity of both proximal and distal markers. T-115 shows LOH of all markers except the proximal GATA23E10 marker. *B*, the 6p21.3 region of deletions is represented by two tumors, T-55 and T-117. Tumor T-55 shows LOH of marker D6S265 but retains heterozygosity of both proximal and distal markers. T-117 shows LOH of markers GATA87C11 and D6S291 but retains heterozygosity of both proximal and distal markers. *C*, LOH in low-grade CINs. Case C-16 shows LOH at the 6p23–25 region (*D6S1617* and *D6S1267*), whereas the proximal marker (*MOG*) mapped to 6p21.3 is heterozygous. Case C-20 shows LOH at 6p21.3 (*MOG*) but retains heterozygosity at 6p25 (*D6S1617*). *N*, normal; *T*, tumor; *C*, CIN. *Asterisks* indicate LOH. Tumor numbers are shown at the *top* of each panel, and markers are indicated on the *sides* of the panels.

alterations, including 50% of the cases with LOH on 6p. Our present definition of 6p21.3 minimal deletion includes several HLA class I genes (*HLA-A*, *P5–1*, *HLA-C*, *HLA-Bw72*, *MICA*, and *HLA-E*) at the proximal boundary, supporting the observations made by Koopman *et al.* (21). Our data therefore suggest that the loss of expression of HLA class I genes seen in many CCs may be due to targeted deletions at 6p21.3. Furthermore, 6p21 LOH has been reported to be predictive of disease recurrence after radiotherapy in CC (22). It remains to be seen whether any TSGs exist at the 6p21.3 minimal deletion and whether there is any synergy between the TSG and MHC class I genes in CC development.

Our study identified two discrete sites of deletions at 6p25 and 6p23.1 that harbor candidate gene(s) important in CC development. This provides a basis for further investigations in finding TSGs in these deleted targets. The 6p genetic deletions have also been reported in CINs, which are precursor lesions for invasive CCs (5, 23). These data therefore suggest that the 6p genetic alterations occur early in cervical carcinogenesis and that they may be critical to the development of this tumor. In the present study, we detected LOH at 6p25 and 6p21.3 in 91.7% of high-grade CINs and 50% of low-grade CINs, respectively (Fig. 2), supporting previous reports that these alterations are very early events in cervical carcinogenesis (5, 23). The 6p genetic alterations documented here in low-grade CINs and high-grade CINs have two potential implications. First, our data should facilitate uncovering the target genes of importance, which may provide a better understanding of the genetic basis of cervical carcinogenesis. Second, because only a certain fraction of precancerous lesions has the potential to progress to invasive cancer (24), the markers and/or regions deleted in CIN lesions may facilitate the identification of high-risk dysplasias. The present study thus provides a basis for the identification of TSGs on 6p involved in CC and for the analysis of a large cohort of precancerous lesions to validate the usefulness of the deleted markers in identification of high-risk dysplasias.

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