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Mapping common deleted regions on 5p15 in cervical carcinoma and their occurrence in precancerous lesions

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Abstract

Background: Previous studies have shown that the short arm of chromosome 5 (5p) exhibit frequent genetic changes in invasive cervical carcinoma (CC), and that these changes arise early during the carcinogenesis, in precancerous lesions. These data therefore suggest that loss of candidate tumor suppressor genes located on 5p is associated with the development of CC. However, the precise location of 5p deletions is not known.

Results: We performed a detailed deletion mapping of 5p in 60 cases of invasive CC. We found that 60% of the tumors exhibit a 5p loss of heterozygosity (LOH). The patterns of LOH allowed us to identify two minimal regions of deletions, one at 5p15.3 spanning a 5.5 cM genetic distance and a second site of 7 cM at 5p15.2-15.3. In addition, we also identified 5p deletions in 16% lesions of high-grade cervical intraepithelial neoplasia (CIN). 5p LOH was found in 63% of HPV 16 positive tumors, while only 33% tumors with other HPV-types had 5p LOH. The differences in frequency of 5p LOH between tumors harboring HPV16 in combination with other HPV types and tumors harboring HPV16 DNA alone were significantly higher, suggesting a synergistic effect of high-risk types in causing genomic instability.

Conclusion: These findings implicate the presence of tumor suppressor gene(s) on 5p relevant to CC tumorigenesis.

Background

Converging points of evidence implicate infection by high-risk human papilloma virus (HPV) types as a critical etiologic factor in cervical tumorigenesis [1]. Epidemiological and experimental data, however, show that only a small fraction of HPV-infected cervical intraepithelial neoplastic (CIN) lesions progress to invasive cervical carcinoma (CC) [2]. These findings suggest somatic genetic

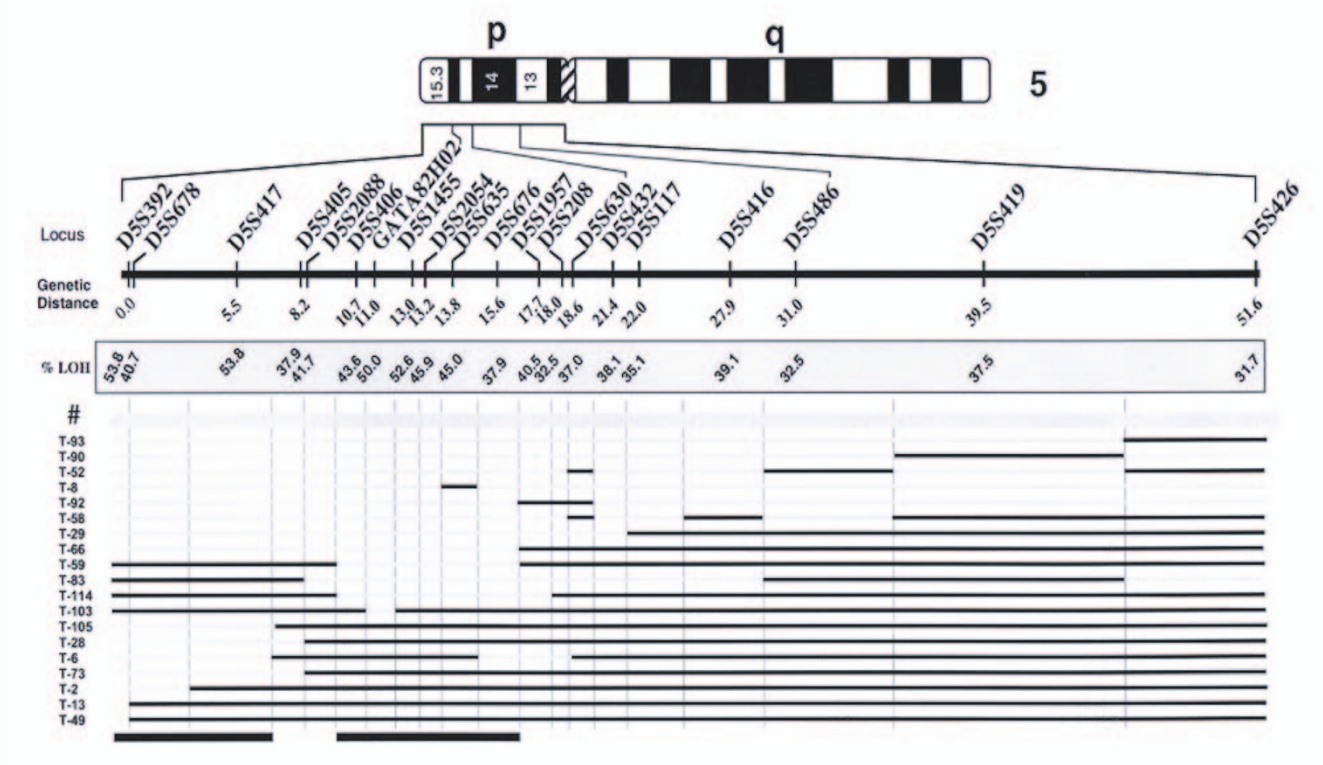

Figure 1
Identification of minimal deleted regions by high-resolution LOH mapping on 5p in invasive CC. Patterns of LOH. Thirty-six tumors with 5p LOH are shown. A G-banded ideogram of chromosome 5 is shown on *top*. Thick horizontal line shown *below* the ideogram represents the corresponding chromosomal region (scaled only approximately). Small vertical lines show STRP markers on the *thick horizontal* line 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. 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 *left* of *horizontal lines*. The *thick light shaded line* denoted on left with # represents 17 tumors that showed LOH of the entire 5p. *Thick black rectangles* at the bottom indicate region of minimal deletion.

mutations play a critical role in the initiation and progression of CC. Delineation of these genetic changes is crucial to understanding the molecular basis of CC. A number of genetic changes have been identified so far in invasive CC. However, the precise mechanism of each of these genetic alterations in the multi-step pathway of cervical tumor development is largely unknown. 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). The genetic basis of this progression is also poorly understood.

Cytogenetic and molecular genetic analyses have identified the short arm of chromosome 5, 5p, as frequently affected by duplications and deletions in a high proportion of CCs [3–6]. These findings suggest the presence of positively regulated amplified oncogenes and negatively regulated tumor suppressor genes (TSGs) on this chromosomal arm. Comparative genomic hybridization (CGH), a molecular cytogenetic method that identifies chromosomal gains and losses, and loss of heterozygosity (LOH) studies have also demonstrated recurrent deletions on the 5p in precancerous lesions [7–10]. Although, these studies have identified 5p as a commonly affected region, the precise site of deletion that harbor putative TSG is unknown because of the lack of systematic and detailed deletion mapping studies.

To further characterize the 5p region in CC, we performed a high-resolution deletion mapping in invasive cancer and found that 60% of patients exhibit LOH. We identified two sites of minimal deletions at 5p15.3 and 5p15.2-15.3 spanning 5.5 cM and 7 cM genetic distance, respectively. We have also shown that the 5p15 region deletions occur in 16% of high-grade CIN lesions. Here, we have identified two sites of candidate tumor suppressor genes and confirmed the previous reports that the 5p deletions occur in a small proportion of precancerous lesions.

Table 1: Frequency of LOH on 5p in cervical carcinoma

Chromosome Band	Locus	Genetic map position (in cM)	No. studied/ Informative	LOH (%)
5p15.3	D5S392	0.0	60/39	21 (53.8)
	D5S678	0.0	59/27	11 (40.7)
	D5S417	5.5	59/28	15 (53.6)
	D5S405	8.2	58/29	11 (37.9)
	D5S2088	8.2	59/48	20 (41.7)
	D5S406	10.7	60/39	17 (43.6)
	GATA82H02	11.0	60/42	21 (50.0)
5p15.2	D5S1455	13.0	60/38	20 (52.6)
	D5S2054	13.2	60/37	17 (45.9)
	D5S635	13.8	60/40	18 (45.0)
	D5S676	15.6	57/29	11 (37.9)
	D5S1957	17.7	59/42	17 (40.5)
	D5S208	18.0	60/40	13 (32.5)
	D5S630	18.6	60/46	17 (37.0)
5p14	D5S432	21.4	58/42	16 (38.1)
	D5S117	22.0	58/37	13 (35.1)
	D5S416	27.9	59/46	18 (39.1)
	D5S486	31.0	57/40	13 (32.5)
5p13.2	D5S419	39.5	60/48	18 (37.5)
5p11-12	D5S426	51.6	60/41	13 (31.7)

Results

Identification of two regions of minimal deletions at 5p15.2-p15.3

Analysis of LOH in 60 invasive CCs using 20 STRP markers mapped to 5p showed deletions in 36 of 60 (60%) tumors in at least one marker (Table 1). Of the 36 tumors with LOH, 17 (47.2%) had LOH at all the informative markers suggesting genetic monosomy of 5p (Fig. 1). The patterns of LOH in the remaining 19 tumors that exhibited regional losses on 5p were used to map the common deleted regions (Fig. 1). This has identified two minimal deletions at 5p15.3 and 5p15.2-15.3 (Fig. 1 & Fig. 2). All the tumors that defined the minimal deletions showed more than 60% reduction in signal intensity in the deleted alleles.

The distal 5p15.3 deletion spans the telomeric region encompassing D5S392, D5S678, and D5S417 markers in 32 tumors. Although two tumors (T-13 and T-49) showed deletions of terminal marker D5S392 alone at 0 cM genetic distance, which is the distal most marker on 5p, we considered the deletion boundaries defined by the proximal marker D5S417 in tumors T-6 and T-105 (Fig. 1 & Fig. 2). The second and proximal region of deletion spans 6 markers (D5S406, GATA82H02, D5S1455, D5S2054, D5S635 and D5S676), and was deleted in 28 tumors. The deletion boundaries were defined proximally by the tumors T-59 and T-66, while the distal boundary was identified by the tumors T-59 and T-114 (Fig. 1 & Fig. 2). All tumors that showed 5p deletions fell within the two defined regions of deletions, while 25 tumors exhibited deletions at both regions. Thus, this analysis identified two discrete and closely mapped sites of minimal deletions at 5p15.3 and 5p15.2-15.3, which were restricted to 5.5 cM and 7 cM genetic distance, respectively.

Gene expression analysis of candidate tumor suppressor genes in CC cell lines

In an effort to identify the genes inactivated in the recurrent 5p deletions in CC, we identified candidate genes with tumor suppressor function. Three genes were identified at the terminal 5p15.3 deletion. These include, *PDCD6*, a protein that plays a role in T cell receptor-, FAS-, and glucocorticoid-induced cell death [11]; *TERT*, a gene that encodes a reverse transcriptase component of telomerase, dysfunction of which promotes chromosomal instability [12]; and *TRIP13*, a thyroid hormone receptor interacting protein that binds with the human papillomavirus type 16 (HPV16) E1 protein [13]. We have identified *POLS* gene that encodes a DNA polymerase sigma, and plays a role in DNA replication and sister chromatid cohesion at the proximal minimal deleted region [14]. We performed a semi-quantitative RT-PCR analysis of all four genes (*PDCD6*, *TERT*, *TRIP13*, and *POLS*) in eight CC cell lines and did not find evidence for lack of or down regulated expression compared to normal cervix (Fig. 3).

5p deletions in relation to HPV

5p deletions did not show any significant differences with age at diagnosis and stage of the tumor (data not shown). Fifty-eight (96.7%) of the 60 tumors studied were positive for HPV DNA. The remaining two tumors were HPV-neg-

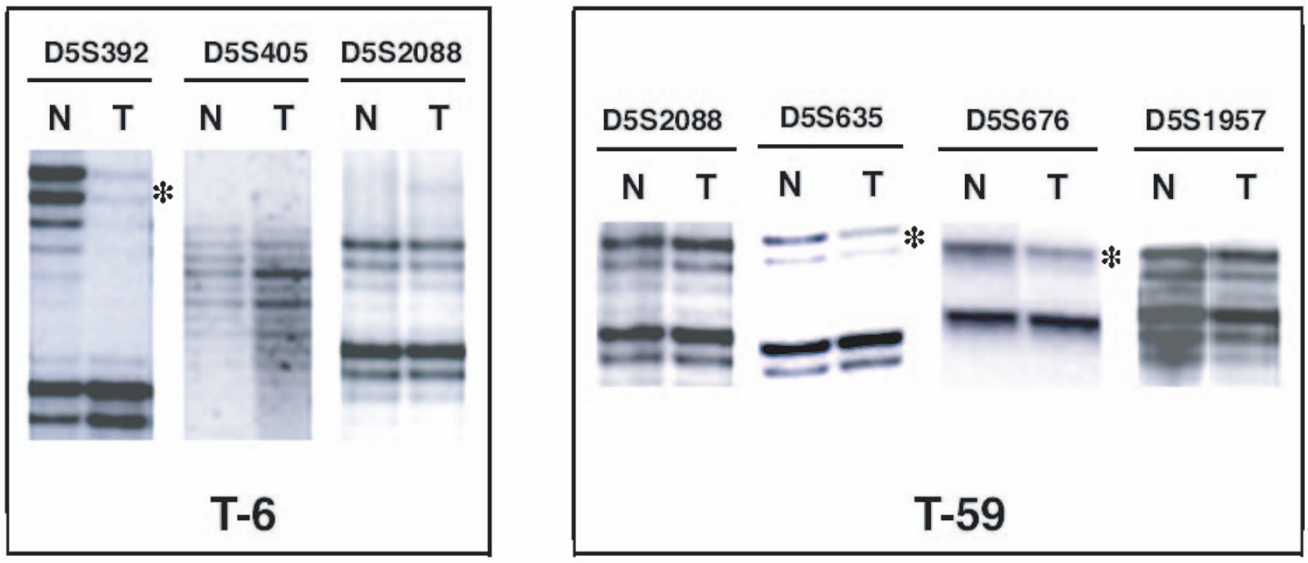

Figure 2
Illustration of LOH of tumors representing minimal deletions at 5p15.3 (T-6) (left panel) and 5p15.3-15.2 (T-59) (right panel). The STRP markers are shown above and the tumor numbers below of each panel. N, normal; T, tumor; Asterisks indicate LOH. Reduction of signal intensity by 50% or more of one of the alleles in tumor DNA compared with the intensity of constitutional alleles was considered LOH.

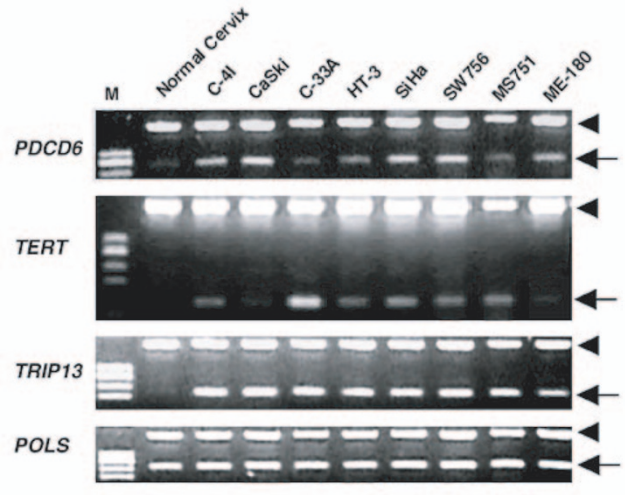

Figure 3
Multiplex RT-PCR analysis of expression of 5p candidate genes in CC cell lines. Genes are shown on *left*; cell lines are indicated on *top*; arrowhead indicates the PCR product of actin gene used as control and arrow represents the indicated gene. Note the complete lack of expression of *TERT* and *TRIP13* genes in normal cervix.

ative. HPV 16 alone or in combination with other HPV types was found in 46 tumors, while 12 tumors had other HPV types including HPV 18. 5p LOH was found in 29 of 46 (63%) HPV 16 positive tumors, while only 4 of 12 (33%) tumors with other HPV-types had 5p LOH. The two tumors that did not harbor HPV infection also had LOH at 5p. These differences were found to be statistically not significant (Chi square 4.93; p = 0.10). However, when the analysis was restricted to HPV16 harboring tumors, it was found that the patients harboring HPV16 DNA alone had 5p LOH in 20 of 36 (55.6%) tumors, while the patients infected with HPV16 in combination with other HPV types exhibited 9 of 10 (90%) LOH. This difference was found to be statistically significant (Chi square, 8.75; p = 0.05).

5p deletions occur at low frequency in precancerous lesions

To understand the role of 5p deletions in cervical cancer progression, we evaluated LOH in 26 microdissected CIN specimens (25 high-grade CINs and one low-grade CIN) using four STRP markers (Table 2). Four of the 25 informative tumors had LOH at one or more markers. One low-grade CIN did not show LOH. Thus, deletions at 5p identified in high-grade CINs suggest that these genetic alterations occur prior to development of invasive carcinoma.

Discussion

Extensive genetic studies have documented frequent alterations affecting various chromosomal regions in CC. LOH and CGH analyses have consistently shown frequent loss of genomic material in a number of chromosomal regions

suggesting the presence of potential tumor suppressor genes at these sites [4,6,15,16]. Of these, chromosome 5p deletions have been noted to be very frequent in invasive CC [5–7]. However, the locations of exact regions of LOH are not known because of a lack of systematic and high-resolution deletion mapping of the 5p region in CC. We have previously identified that markers mapped to 5p15 regions are more frequently deleted [4,7]. To further characterize the 5p deletions in CC, we performed a high-density LOH mapping in the present study. We found high frequency of LOH and identified two minimal regions of deletions at 5p15.3 and 5p15.2-15.3.

The 5p15.3 minimal deletion spans between 0–5.5 cM genetic distance. Consistent with this pattern, the markers D5S392 and D5S417 exhibited the highest frequency (53.8% and 53.6%, respectively) of LOH among all the tested loci. The genomic size of the 5p15.3 deletion interval is approximately 3.2 Mb. A database search has identified three candidate genes, *PDCD6*, *TERT*, and *TRIP13*, with suggestive tumor suppressor gene function. The programmed cell death gene, *PDCD6*, is a member of the family of intracellular $Ca2^{+}$-binding proteins and a part of the apoptotic machinery controlled by T-cell receptor (TCR), Fas, and glucocorticoid signals [11,17]. *TERT* encodes a reverse transcriptase required for the replication of chromosome termini and plays a role in telomere elongation. Although telomerase expression is a hallmark of cancer, the mice lacking the RNA component of telomerase (mTERC) exhibit progressive telomere shortening and chromosomal instability associated with epithelial tumors [12]. Consistent with the established role of this enzyme in cell immortalization and cancer pathogenesis [18], we did not find expression in normal cervix while it was abundantly present in all eight cell lines studied (Fig. 3). Recently, it has been shown that the hTERT amplification and over expression is common in CC [19]. *TRIP13*, thyroid receptor interacting protein 13, is a transcription factor that regulates expression of a variety of specific target genes including the human papillomavirus type 16 (HPV16) E1 protein [13]. Similar to *TERT*, the *TRIP13* also showed very low expression in normal cervix and highly elevated levels of expression in CC cell lines (Fig. 3). Presence of these genes at the 5p15.3 interval suggests that they may be target TSGs of these deletions in CC. However, expression analysis of these genes did not provide evidence of down-regulation in CC cell lines.

The second site of minimal deletion at 5p15.2-15.3 between 10.7 and 17.7 cM genetic distance spans approximately 2.5 Mb genomic region. A database search has identified *POLS*, a topoisomerase-related function protein-4-1, gene that encodes polymerase (DNA directed) sigma within the deleted region. The yeast homolog TRF4 plays a critical role in chromosome segregation by coordinating between DNA replication and sister chromatid cohesion [14]. Analysis of expression of this gene in CC cell lines did not show evidence of down regulation. Although the genes tested so far showed no deregulated expression, it remains to be seen whether any of these genes or the others mapped to the minimal deleted regions are targets of mutations in CC. However, the present study identified two discrete sites of deletions at 5p15.3 and 5p15.2-15.3 that harbor putative TSGs important in CC development providing a basis for their identification.

5p deletions also have been reported in precursor CIN lesions with the frequencies between 14.7 and 42% [7–10]. We have previously shown, in a different geographical population, that 21% of CINs had 5p deletions. Based on the follow-up studies, we suggested that 5p genetic deletions might identify high-risk lesions for progression to invasive cancer [7]. In a more recent study it was also shown that the 5p deletions were significantly more frequent in dysplasias that were recurrent compared to non-recurrent dysplasias [9]. These data therefore suggest that 5p genetic alterations occur early in cervical carcinogenesis and they may be critical to the development of invasive cancer. Supporting previous reports, in the present study, we detected LOH at 5p15 in 16% of the high-grade CINs (Table 2).

Table 2: 5p LOH in cervical precancerous lesions

Locus	No. of CINs studied	No. Informative	LOH (%)
D5S2088	25	22	1 (4.5%)
GATA82H02	24	17	3 (17.6%)
D5S1455	18	9	1 (11.1%)
D5S635	23	17	2 (11.8%)

The role and mechanisms involved in high-risk HPV induced genomic instability has been of a considerable speculation [reviewed in ref. [20]]. High-risk HPV E6 and E7 gene expression can independently immortalize various human cell types *in vitro*. The E6 gene interacts with p53 and an apoptotic protein *BAK*, followed by its degradation, which results in chromosomal instability and resistance to apoptosis [21,22]. The E7 protein interacts with pRB followed by its degradation [23]. The p53 and pRB proteins have been shown to play multiple roles in cell growth and in genomic integrity. There have been contradictory reports on targeted 5p deletions in relation to HPV infection [5,24]. Ku and co-workers have shown that 5p deletions occur in significantly high proportion of CC infected with HPV 16 compared to low-risk HPVtypes [5]. Contradictory to this, another study has shown that 5p deletions are independent of HPV16 and HPV18 infec-

tion [24]. In the present study, we did not find any significant differences in 5p LOH between patients harboring HPV16 and other HPV types. However, we found that patients harboring HPV16 in combination with other HPV types showed significant increase in 5p loss compared to patients harboring HPV16 alone. These data, therefore, suggest that multiple high-risk HPV infections may have synergistic effect in causing genomic instability. Thus, currently it is unclear the role of high-risk HPV types in causing 5p genomic instability. Further studies are needed to address this question.

Conclusions

In summary, the present study confirms our previous observations that genetic loss of 5p is a common feature of invasive CC. Here we identified two common sites of deletions that harbor candidate tumor suppressor genes on this chromosomal arm and showed that multiple infection of high-risk HPV types may cause targeted genomic instability at 5p. In addition, we have shown that the genetic deletions on 5p occur early during the development of CC in high-grade CIN lesions. Our identification of minimal deletions on 5p and LOH in CINs has several potential implications. First, it should facilitate the identification of the target genes of importance in cervical tumorigenesis. Second, it may provide critical genetic markers for identifying cervical intraepithelial neoplasias at high-risk to progression.

Methods

Tumor and normal tissues

We analyzed 60 tumor biopsies consisting of previously untreated primary invasive CCs with the corresponding peripheral blood samples, and 26 formalin-fixed and paraffin-embedded CIN specimens. The tissues were obtained from patients treated at the Instituto Nacional de Cancerologia, Colombia after appropriate informed consent and approval of the protocols by the institutional review board. The invasive tumors were clinically classified as FIGO stage IB (5 tumors), IIB (14 tumors), IIIB (37 tumors), and IV (4 tumors). Histologically, 57 tumors were classified as squamous cell carcinomas and 3 as adenocarcinomas. Twenty-five CIN specimens were classified as high-grade and one 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), and were grown in culture according to the supplier's recommendations.

Microdissection, DNA and RNA isolation and LOH analysis

High-molecular weight DNA from frozen tumor and the corresponding peripheral blood specimens, and frozen cells from cell lines was isolated as previously described [25]. Tumor cells from paraffin-embedded CIN tissue specimens were isolated by laser capture microdissection (Arcturus, Mountain View, CA) after methyl green-staining and the extraction of DNA as described earlier [26]. Total RNA was isolated from semi-confluent cell cultures using the TRIzol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's protocol. A panel of 20 sequence tagged repeat polymorphic (STRP) (18 di- and two tetra-nucleotide) markers was chosen on the basis of their map position and heterozygosity (Fig. 1) (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 was performed as previously described [25,26]. All autoradiograms were visually analyzed. Reduction of signal intensity by 50% or more of one allele over the other allele in tumor DNA compared with the intensity of constitutional alleles was considered LOH. The definition of minimal region of deletion was defined earlier [25]. The LOH analysis was performed at least twice on all tumors with the corresponding markers that define the minimal deletions.

Detection of HPV types

The PGMY09/11 L1 consensus HPV L1 primers and reverse line blot hybridization system was used to detect 38 genital HPV types as described earlier [27,28]. Appropriate control experiments were set up using bulk master mix components to eliminate potential contamination. Individual assay sensitivity was analyzed by the use of serial dilutions of SiHa cell line crude cell digest, targeting 10^4, 10^3, 10^2, 10^1, and 10^0 input copies of HPV-16. A no template negative control was set up in all experiments.

Analysis of 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). Semi-quantitative analysis of gene expression was performed by 26 to 28 cycles of multiplex RT-PCR with β-actin as control and gene specific primers spanning at least 2 exons. The gene primers used and their positions in cDNA were:

PDCD6-F 5'-GCTTCCTGTGGAACGTTTTC-3' (108–127 bp)

PDCD6-R 5'-TCTTATCGATCATCCCGGAG-3' (349–368 bp)

TERT-F 5'-GCGTTTGGTGGATGATTTCT-3' (2647–2666 bp)

TERT-R 5'-CAGGGCCTCGTCTTCTACAG-3' (2778–2797 bp)

TRIP13-F 5'-CAGCAGCACTGCAAAGAAAG-3' (132–151 bp)

TRIP13-R 5'-TGTGAAGTGCAACAGTGCAT-3' (321–340 bp)

POLS-F 5'-CCCTTGTCCTGAAGAAGCAG-3' (43–62 bp)

POLS-R 5'-AGGACTTTGATGGAACACGG-3' (263–282 bp)

The PCR products were run on 1.5-% agarose gels, visualized by ethidium bromide staining and quantitated using the Kodak Digital Image Analysis System (Kodak, New Haven, CT).

Authors' contributions

Author 1 (HAP) carried out the molecular genetic analysis of LOH on invasive cancer and performed HPV typing. Author 2 (GN) performed LOH analysis on CIN lesions and gene expression analysis. Author 3 (HV) participated in the collection of tumor material and clinical information. Author 4 (MM) participated in microdissection of CIN lesions. Author 5 (VVVSM) has conceived and coordinated the study. All authors read and approved the final manuscript.

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