RESEARCH COMMUNICATION

Chromosome 3p Alterations in Northeastern Thai Women with Cervical Carcinoma

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Abstract

　　The purpose of this study was to determine the incidence of the loss of heterozygosity (LOH) among normal cervixes, cervical intraepithelial neoplasias (CINs) and invasive cervical cancers (ICCs). DNA samples (136) were obtained from 31 normal cervixes, 49 CINs and 56 ICCs. Four polymorphic microsatellite markers (D3S1300, D3S1351, D3S1478 and D3S4103) covering the chromosome 3p arm, were employed. LOH at one or more loci were identified in: 9/31 (8.1%) normal cervixes, 17/49 (14.6%) CINs and 26/56 (22.1%) invasive cancers. The incidence of the LOH at 3p varied for each locus and ranged from 5.6% for D3S1351 to the highest rate of 16.6% for D3S1300. We thus found that LOH of chromosome 3p can occur in normal cervixes and that incidences increase in CINs and ICCs. Deletion in the 3p14.2 (D3S1300) and 3p21.2 (D3S1478) regions might be an early event and, in fact, necessary for cervical cancer progression. The loss of function of tumor suppressor genes (TSGs) located in these regions may have a sequential effect in cervical cancer carcinogenesis.

Key Words: Chromosome 3p - cervical carcinoma - loss of heterozygosity

Introduction

　　Cervical cancer (CCA) is one of the most common malignancies in women in developing countries. In Northeast Thailand, CCA is the most common female cancer (Cancer Unit, Khon Kaen University, 2003) and causes the highest mortality among females (Vatanasapt et al., 1998). Most CCAs are squamous cell carcinomas (90%) and likely originate from a series of pre-malignant lesions (cervical intraepithelial lesions, CINs) (Kumar et al., 2005). CIN I has a low rate of progression before becoming an invasive cervical cancer (ICC). By contrast, CINs II and III have a much greater potential for progression and are high-risk precursors for development of ICCs.

　　The loss of heterozygosity (LOH) is a common genetic finding in many human neoplasms. LOH studies of primary CCAs have shown allelic loss on several chromosomal arms such as 2p, 3p, 6p, 9p, 11q, 13q and 18q (Krul et al., 1999; Miyai et al., 2004; Mullokandov et al., 1996). The frequency of the LOH in 3p has been variously reported as 35, 39 and 45 percent in ICCs (Krul et al., 1999; Mullokandov et al., 1996) and 21 and 16 percent in the CIN III and II/IIIs regions, respectively (Nishimura et al., 2000; Wistuba et al., 1997).

　　Deletions of chromosome 3p are a frequent event in ICCs and likely play an important role in the transition of CINs to ICCs (Chung et al., 2000; Dasgupta et al., 2003; Guo et al., 2000; Guo et al., 2001; Nishimura et al., 2000).

　　Our aim was to detect the incidence of the LOH at chromosomal regions 3p14.2, 3p21.3-3p21.2 and 3p24.2 in normal cervixes, CINs and ICCs from women in Northeast Thailand.

Materials and Methods

Patients and Samples

　　Tissue samples were collected from 136 patients with normal cervixes from leiomyoma, CINs from LEEP conization and ICCs from biopsy at the Department of Obstetrics and Gynecology, Faculty of Medicine, Khon Kaen University, Thailand. Peripheral blood samples were collected from these patients before the procedure. Histological diagnosis was confirmed by a pathologist. All the collected samples were formalin-fixed and paraffin-embedded. Histologically, the 136 samples comprised: 31 normal cervixes, 49 CINs, and 56 ICCs (including 54 squamous cell carcinomas and 2 adenocarcinomas).

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Table 1. Microsatellite Markers and Their PCR Product Sizes

Band location	Locus	Forward Primer (5'→3')	Reverse Primer (5'→3')	PCR product (bp)
3p14.2	D3S1300	HEX-AGCTCACATTCTAGTCAGCCT	GCCAATTCCCCAGATG	217-241
3p14.2	D3S4103	HEX-TTCTACTGCAATCCAGCCTGG	GCCTTGGGTAGATTTATACCT	165-185
3p21.3-21.2	D3S1478	HEX-GATGAAACTGTGATAGCACC	CTGCCAGTAATGTAAATCTCC	109-152
3p22-24.2	D3S1351	HEX-AGCCTCCATAACTGCATGAA	ACAGAACCAGAACCAATAGG	112-124

Histologic diagnosis and grade were determined according to WHO criteria. This study was approved by the Ethics Committee of Faculty of Medicine, Khon Kaen University.

DNA Extraction from Tissue Microdissection

Serial sections of cells from normal cervixes, CINs and ICCs were cut, deparaffinized, stained with Mayer's haematoxylin, visualized under an inverted microscope, and an area of epithelium was microdissected using a needle. The microdissected tissues were digested overnight at 50°C in 50 μl buffer containing 50 mM Tris-HCl, 4 mM EDTA (pH 8.0), and 20 μg/ml proteinase K. The lysate was heated at 99°C for 8 min and stored at -20°C until subjected to PCR reaction.

DNA Extraction from Blood

The buffy coat of each patient sample was separated from the EDTA-blood sample and the red blood cells were lysed by adding lysis buffer. Hemolysate was removed and the leucocytes were washed twice in distilled water. The leucocytes were re-suspended in 1 ml of extraction buffer (10 mM Tris-HCl, 400 mM NaCl and 2 mM EDTA pH 8.2) containing 0.5% SDS and 20 μg/ml proteinase K. The reaction was incubated at 56°C for 30 min with rotating. Proteins were precipitated by salting out with saturated NaCl solution. The DNA was precipitated with cold absolute ethanol. Finally, the DNA was re-suspended in 1 ml TE buffer and stored at -20°C until used.

LOH Analysis

PCR amplification of microsatellite markers was performed using the 4 primers distributed along chromosome 3p (Table 1). The sense strand of each primer was labeled at 5' end with fluorescein dye, HEX. All locus-specific reactions were done with 20 μl of a mixture containing 50 ng of DNA template, 3.0 mM MgCl for tissue DNA (1.5 mM MgCl for leucocytes DNA), 50 mM KCl, 5 pmol each of primer, 1 U of Taq DNA polymerase (Promega), and 200 μM of each dNTP. The PCR mixtures were denatured for 5 min at 94°C and passed through 35 cycles for tissue DNA (30 cycles for leucocytes DNA) of amplification consisting of 30 sec denaturation step at 94°C, 30 sec annealing step at 55°C, 30 sec elongation step at 72°C, and followed by a final cycle with an extension of 7 min at 72°C. The PCR products were mixed with an equal volume of loading buffer heated for 5 min at 95°C, and rapidly chilled on ice. Five microlitres of the denatured DNA were loaded on a denaturing 6% polyacrylamide gel containing 7 M urea and electrophoresed using 0.6X TBE as a running buffer. The

LOH of each sample was analyzed by DNA fragment autoanalyzer 2000 (Corbett Research, Australia). For informative cases, LOH or allelic loss was scored if the signal of one allele was at least 50% reduced in the tumor DNA, compared with the corresponding normal allele. The frequency of each marker showing the LOH was used for data analysis.

Results

Table 2 shows the LOH incidence at 3p varied for each locus and ranged from 5.6% on D3S1351 to the highest rate of 16.6% on D3S1300. The LOH at one or more loci of chromosome 3p were identified in: 1) 9/31 (8.1%) of normal cervixes; 2) 17/49 (14.6%) of CINs; and, 3) 26/56 (21.1%) of ICCs (Table 3).

The highest incidence of the LOH (9.3%, 9.2%) in ICCs were at loci D3S1478 (3p21) and D3S1300 (3p14.2), respectively. Six cases of one normal, one human papilloma virus infection, one CIN I, one CIN III, and 2 ICC showed the LOH in two consecutive loci. Only one case of ICC had the LOH in three consecutive loci of D3S1300, D3S1351, and D3S1478. Figure 1 is representative of negative LOH at the D3S1351 locus in women with ICC. Representative images scored as the LOH at four loci of chromosome 3p in women with CIN and ICC are shown in Figure 2. One allele of CIN and ICC DNA was lost compared with DNA from the leucocytes.

Discussion

The involvement of chromosome 3p deletions in the development of ICCs has been confirmed by studying the LOH using chromosome specific microsatellite markers. Several discrepancies between reports defining the commonly deleted regions and frequencies of the LOH have been reported. Mullokandov et al. (1996) found an overall frequency of 39% the LOH at the 3p21.3, 3p22.1-24.1 and 3p25.1-25.3 regions, whereas Krul et al. (1998) found a high frequency of allelic imbalance on chromosome 3p at

Table 2. LOH Incidence at Each Locus of Chromosome 3p

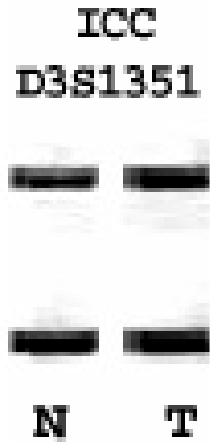| Band Location	Locus	LOH/informative cases (%)
3p14.2	D3S1300	18/108 (16.6)
3p14.2	D3S4103	10/116 (8.6)
3p21.3-21.2	D3S1478	18/128 (14)
3p22-24.2	D3S1351	6/106 (5.6)

Figure 1. Example of LOH Negative at the D3S1351 Locus: Two Alleles are Found in all DNAs, i.e., N = normal DNA, T = Tumor DNA from ICC Cells.

D3S2456 with the LOH rates of 47% and 41% in specimens from Surinam and the Netherlands.

Wistuba et al. (1997) searched for the presence of the LOH at four loci on chromosome 3p in CCA and accompanying CIN and found that the LOH at any 3p region was displayed in 70% of cancers. LOH was the most common at 3p14.2 (56%) and 3p21 (57%). Region 3p14.2 contained at least three tumor suppressor genes (TSGs), viz.: 1) fragile histidine triad (FHIT); 2) transforming growth factor-beta receptor II (T beta R-II); and, 3) Von Hippel-Lindon (Herzog et al., 2001). Nishimura et al. (2000) detected the LOH of chromosome 3p in 14.6% of ICCs and in 11.1% of CIN IIIs. Guo et al. (2001) detected the LOH at the FHIT gene region (i.e. 60% D3S1300 and 54.6% D3S4103), 3p21.3-21.2 (45.5% D3S1478) and 3p24.2-22 (50% D3S1283).

Dasgupta et al. (2003) concluded that the location of TSGs in regions 3p26.1, 3p22.3, 3p21.2 and 3p13 may have a cumulative effect on tumor progression. Guo et al. (2000) suggested that two regions, 3p22-21.3 and 3p21.1 are novel tumor-suppressor loci, which may play a role in early transition of CINs to ICCs.

Chung et al. (2000) suggested that genetic alterations on chromosome 3p are common in high grade CINs and probably early events in cervical carcinogenesis. Larson et al. (1997) detected an overall frequency of 70% the LOH in ICC, with the most frequent deletion being at the 3p14 region. From this, they hypothesized that chromosome 3p harbors several TSGs relevant to CCA.
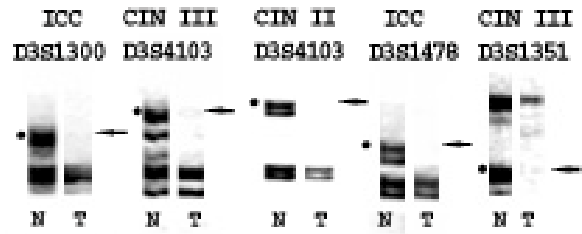

Figure 2. Examples of LOH at the Four Loci of Chromosome 3p in Women with CIN and ICC (Note: N = normal DNA, T = tumor DNA from CIN and ICC cells; dot = normal alleles, arrow = LOH).

In our study, the LOH incidence varied from 3p14.2 region (D3S1300, 16.6% D3S4103, 8.6%), 3p21.3-3p21.2 (D3S1478, 14%) to 3p22-24.2 (D3S1351, 5.6%) (Table 2). Like our results, many reports (Chung et al., 2000; Dasgupta et al., 2003; Guo et al., 2000; Guo et al., 2001; Larson et al., 1997; Wistuba et al., 1997) have detected a high frequency of the LOH located on the short arm of chromosome 3 at or near 3p14.2-13, 3p21.2-21.1, 3p24.2-22, and 3p26.1-25.1. The incidence of the LOH in ICCs (22.1%) was higher than that in CINs (14.6%) and normal cervixes (8.1%) (Table 3), suggesting that the LOH is associated with the development of CIN to ICC.

Deletion in the 3p14.2 (D3S1300) and 3p21.2 (D3S1478) regions increased gradually from CINs to ICCs and these regions showed the highest percentages (9.2%, 9.3%) of deletion in ICC of all four loci. The results of our study and previous reports (Connolly et al., 2000; Helland et al., 2000; Herzog et al., 2001; Lin et al., 2000; Wong et al., 1997) suggest that there are TSGs on the short arm of chromosome 3 that play a role in CCA pathogenesis and are likely within, or close to, 3p14-21.

Connolly et al. (2000) suggested that the loss of FHIT expression might be associated with tumor progression. Helland et al. (2000) confirmed that the reduced FHIT protein and FHIT gene alterations are important in cervical carcinogenesis. Lin et al. (2000) suggested that frequent microsatellite alterations in the recurrent group may create susceptibility to the LOH and increase the risk of recurrence or progression. Wong et al. (1997) indicated that the LOH at or near 3p13 appears to be a late event in tumor progression.

Many reports conclude that the LOH of the chromosome 3p is an early event in cervical carcinogenesis and a potential marker for risk of progressive disease in patients with pre-

Table 3. LOH Incidence of Chromosome 3p in Women with Normal Cervix, CIN and ICC

	D3S1300 LOH (%)	D3S4103 LOH (%)	D3S1478 LOH (%)	D3S1351 LOH (%)	Total LOH (%)
Normal (31)	5 (4.6)	3 (2.6)	0	1 (0.9)	9 (8.1)
CIN (49)	3 (2.8)	5 (4.3)	6 (4.7)	3 (2.8)	17 (14.6)
ICC (56)	10 (9.2)	2 (1.7)	12 (9.3)	2 (1.9)	26 (22.1)
Total (136)	18 (16.6)	10 (8.6)	18 (14)	6 (5.6)	52 (44.8)

malignant lesions. The difference in deletion frequency from each report may be due to differences in ethnicity.

In conclusion, our results and other reports agree that the LOH of chromosome 3p can occur in normal cervixes and the incidence increases in CINs and ICCs. The deletion in the 3p14.2 (D3S1300) and 3p21.2 (D3S1478) regions might be the earliest event and necessary for the development of CCA progression. The loss of function of TSGs located in these regions may have a sequential effect in cervical cancer carcinogenesis and progression.

Acknowledgments

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