Analysis of Clonality by X Chromosome Inactivation in Uterine Cervix Cancer

The determination of a unicellular or a multicellular origin of a tumor is an important clue for understanding its etiology. To investigate this issue, we performed clonality assay of cervix cancer using polymerase chain reaction based on highly polymorphic locus of the androgen receptor gene, in which methylation of DNA correlates with inactivation of X chromosome. DNA samples were obtained from formalin-fixed, paraffin-embedded tissue of 20 invasive epidermoid carcinomas and 10 carcinoma in situ. Seven of ten carcinoma in situ, heterozygous for the androgen receptor locus, were monoclonal pattern. Among twenty invasive epidermoid carcinomas, eighteen of which showed heterozygous, twelve were monoclonal pattern and six were polyclonal pattern. We conclude that carcinoma in situ arises from a single cell. In invasive epidermoid carcinoma, most cases were monoclonal, although some cases were polyclonal. These suggest that invasive carcinoma of the cervix does not always arise from a single cell, but may arise from several cells with different mechanisms. ***(JKMS 1997; 12: 322~6)***

Key Words : *Androgen receptor gene, X chromosome inactivation, Uterine cervix cancer, Clonality*

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INTRODUCTION

Although the etiology and mechanism of the development of human cancer are largely unknown, all hypotheses must incorporate observations concerning the clonality of neoplastic cell populations. Mutational theories of carcinogenesis predict that neoplasms will have a monoclonal composition (1). Alternative mechanisms for tumor formation, such as those involving aberrant differentiation processes or field effects, could lead to neoplasms with a polyclonal composition (2). It has been demonstrated that the vast majority of human and experimental tumors are monoclonal (3~5). The implication of this concept is that tumors emerge from a collection of relative rare events (somatic mutation or loss of chromosomal material) occurring in only one cell. But the finding of clonal dominance does not necessarily imply a clonal origin because the demonstration of monoclonality at the relative late stage of tumorigenicity does not mean necessarily that early genetic and epigenetic events contributing to tumor progression are confined to only one original cell. If multiclonality existed at earlier stages of tumor progression, this would suggest that initiating events were more common than previously had been believed and that the events leading to clonal dominance were later events in tumor progression.

To investigate the clonality in the early and late stage of tumor progression, we performed a polymerase chain reaction (PCR) clonality assay for human androgen receptor gene (HUMARA) with archival paraffin-embedded tissues and applied it to the uterine cervical neoplasms from carcinoma in situ to invasive carcinomas.

MATERIALS AND METHODS

Case selection and sample preparation

All tissues for these studies came from the Department of Pathology, Chonnam University Hospital. Tissue specimens were fixed in 10% buffered neutral formalin overnight and embedded in paraffin.

Carcinoma in situ

Unstained 10 μm-thick paraffin sections adhered to standard glass microscope slides were utilized. After referring to a hematoxylin and eosin-stained serial section, unstained wax sections were visualized with dissecting microscope (Olympus SZ-PT) under darkfield optics. The tumor cells were scraped off with a razor blade and subjected to DNA isolation as described below.

Invasive epidermoid carcinoma

Hematoxylin-eosin stained tissue sections from the formalin-fixed, paraffin-embedded tissue blocks were examined to identify areas where the carcinoma cells composed greater than 90% of the total cell population. To obtain only tumor DNA, areas of normal tissue together with excess paraffin of paraffin block were cut off. Ten duplicate 10 μm sections of the remaining tissue were collected into a 1.5 mL tube. For benign control tissues from the same cases, only excess paraffin was scraped away and sampled by the same method. When available, control tissues were selected from the lymph node. If it was not available, then appendix, benign endometrium or myometrium were selected.

DNA Extraction

DNA extraction was performed using a procedure adapted from a combination of several previously described methods (6~9). Paraffin was removed by suspending the tissue section in 1 ml of xylene for 30 min. After centrifugation in a microcentrifuge (14,000 rpm), the xylene was decanted and the tissue was resuspended in ethanol. The tissue was centrifuged again and the excess ethanol was decanted. The tissues were allowed to vacuum dry for 10 min at room temperature. The tissues were then resuspended in 400 μl of proteinase K lysis buffer (10 mM Tris, pH 8.0, 400 mM NaCl, 2 mM EDTA, pH 8.2, Proteinase K 20 mg/ml), at 54℃ for 24 h. The tissues were then extracted once with phenol once with phenol-chloroform-isoamyl alcohol and once with chloroform. A 33% volume of 10 M ammonium acetate and 2.5 volumes of ethanol were added; the DNA pellet was recovered by centrifugation. After washing with 70% ethanol, the DNA was resuspended in 50 μl distilled water.

PCR assay for clonality

For each DNA sample, two reactions were prepared; in one tube, 2 μg of DNA was digested with 20 U *Hpa*II (Boehringer Mannheim); in the other, 2 μg of DNA was incubated with the enzyme digestion buffer with no enzyme. All reactions were 20 μl in total volume, and all incubations were for 12 h at 37℃. After digestion, the reactions were terminated by incubating the mixture at 95℃ for 10min from this reaction, 3 μl was added to 30 μl total volume PCR reaction containing both oligonucleotide primers at a concentration of 1 μM, 250 μM dNTPs (Promega), 1.5 U *Taq* polymerase/μl (DyNA Zyme), 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.8), and 0.1% Triton X-100. The sequences of the HUMARA primers were obtained from sequences reported elsewhere (10): forward primer 1, 5'-GCTGTGAAGGTTGCTGTTCCTCAT-3'; and reverse primer 2, 5'-TCCAGAATCTGTTCCAGAGCGTGC-3'. Samples were amplified using a Perkin-Elmer thermocycler 2400 for 31 cycles (each comprising 1 min at 94℃, 1 min at 63℃ and 1 min at 72℃) with an initial denaturation at 94℃ for 2 min. Three microliters of the PCR product was mixed with 3 μl of STR 2X loading solution (10 mM NaOH, 95% formamide, 0.05% xylene cyanol FF, and 0.05% bromophenol blue). This mixture was loaded on a denaturing 6% acrylamide gel (7 M urea and 0.5x TBE), and electrophoresis was performed at 650~700V for 4 h. The gel was silver stained using a modified procedure from previously described methods (11, 12). The silver staining procedure used is outlined in Table 1.

Table 1. Procedure for silver staining of nucleic acids

Step	Solution	Time
1. Fix	10% acetic acid	20 min
2. Rinse	deionized H_2O	2 min / 3 times
3. Impregnation	0.5 g $AgNO_3$	
	750 μl 37% HCOH	30 min
	500 ml deionized H_2O	
4. Rinse	deionized H_2O	10 sec
5. Develop	30 g Na_2CO_3	
	1.5 ml 37% HCOH	2-5 min
	200 μl $Na_2S_2O_3.5H_2O$	
	1000 ml deionized H_2O	
6. Stop	10% acetic acid	5 min
7. Rinse	deionized H_2O	2 min

RESULTS

A 280-bp PCR amplification unit was developed for the human androgen-receptor locus. We interpreted as skewed all DNA samples which generated two fold or greater shift in allele band relative intensity. If there was no skewing in normal control tissues, then visible skewing in the lesional tissue was viewed as evidence for monoclonal pattern. Cases in which control polyclonal tissues had apparent skewing in the same pattern as lesional tissues were interpreted as undetermined.

Clonality assay of invasive epidermoid carcinoma

Eighteen invasive epidermoid carcinomas were heterozygous at the HUMARA locus (Table 2). Two cases were excluded from clonality determination. One was homozygous for the HUMARA locus and the other showed X inactivation skewing of *Hpa*II-digested normal control tissue. Twelve were monoclonal and the remaining 6 had a polyclonal pattern (Fig. 1A, 1B). To assess the possibility that normal tissues mixed with the tumor specimens, we repeated the clonality analysis on

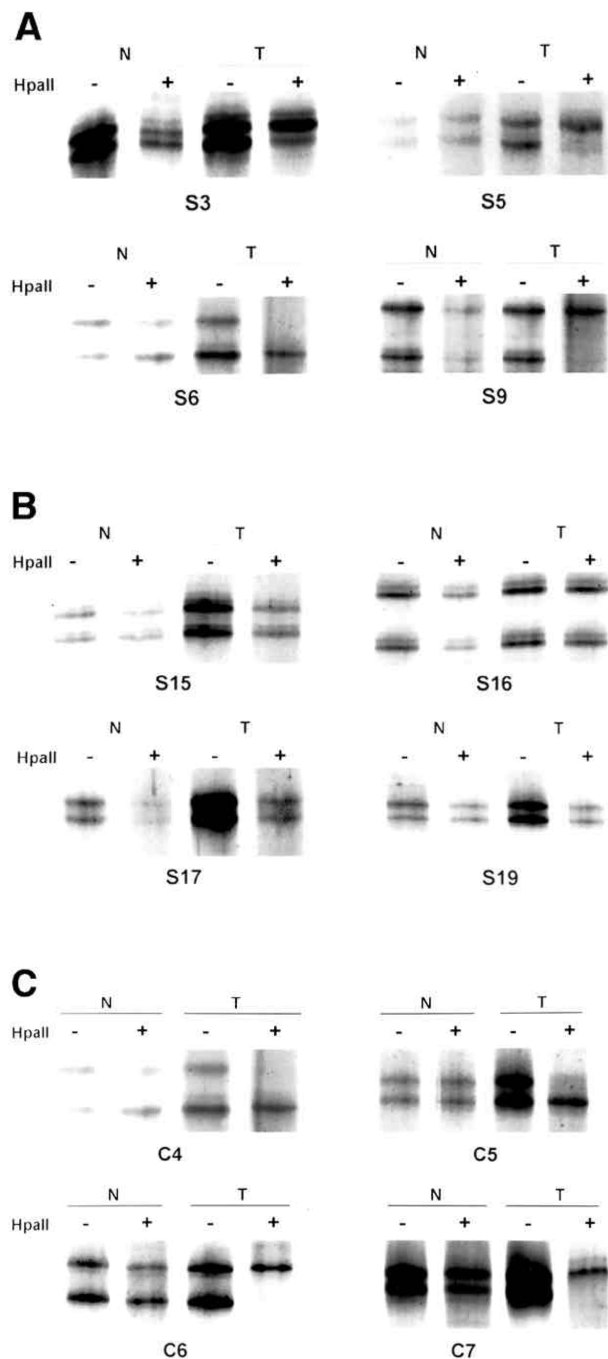

Fig. 1. Examples of PCR results of the HUMARA locus in uterine cervix cancer. PCR products were around 280 bp. DNAs for normal(N) and tumor(T) were digested with(+) or without(−) HpaII restriction enzyme.

(A) Monoclonal pattern in invasive epidermoid carcinoma showed skewing of PCR signals towards one of the two alleles in *Hpa*II-digested lane of the tumor.

(B) Polyclonal pattern in invasive epidermoid carcinoma showed two different allele approximately evenly distributed in *Hpa*II-digested lane of the tumor.

(C) Monoclonal pattern in carcinoma in situ

Table 2. Results of clonality analysis of uterine cervical cancer

Diagnosis	Tumor Sample	Single band	Double band	Comment
Invasive squamous cell carcinoma				
S1	1	(-)	(-)	Homozygous
S2	1	(-)	(-)	skewed, undetermined
S3	1	1	0	
S4	1	1	0	
S5	1	1	0	
S6	1	1	0	
S7	1	1	0	
S8	1	1	0	
S9	2	2	0	
S10	2	2	0	
S11	2	2	0	
S12	3	3	0	
S13	4	4	0	Lymph node metastasis
S14	5	5	0	Lymph node metastasis
S15	3	0	3	
S16	3	0	3	
S17	3	0	3	
S18	3	0	3	
S19	3	0	3	
S20	4	0	4	Lymph node metastasis
Carcinoma in situ				
C1	1	(-)	(-)	Homozygous
C2	1	(-)	(-)	skewed, undetermined
C3	1	(-)	(-)	skewed, undetermined
C4	1	1	0	
C5	1	1	0	
C6	1	1	0	
C7	1	1	0	
C8	1	1	0	
C9	1	1	0	
C10	1	1	0	

DNAs re-extracted from minimized lesional sample (5×5 mm) and another multiple sites. But the results were still polyclonal in origin. In addition, each experiment was repeated at least twice, and the results were the same.

Clonality assay of carcinoma in situ

Of the 10 cases of carcinoma in situ, 7 informative cases were monoclonal (Fig. 1C). One case was homozygous for the HUMARA locus. Two cases showed X inactivation skewing of normal tissue.

DISCUSSION

Analysis of clonality by X chromosome inactivation has proven a useful study of neoplastic and preneoplastic tissue (4, 13) as well as in the study of female heterozygotes of X-linked diseases (14, 15). Early studies of

tissue clonality base on X inactivation analyzed glucose-6-phosphate dehydrogenase (G-6-PD) isoenzymes (4) ; however this technique suffered from the major limitation that only a small fraction of women are heterozygous for isoenzymes. Later, analysis using differences in methylation of DNA in the active and inactive X chromosomes was developed (5). This analysis required at least two restriction enzymes and then analysis by Southern blot hybridization. This technique requires moderate amounts of relatively intact DNA (about 1~10 μg/analysis). Such amounts of DNA are readily obtainable from fresh or frozen specimens but are rarely available in formalin fixed and paraffin embedded tissues. The PCR approach has been possible in DNA extracted from formalin-fixed and paraffin embedded tissues and concentrated usually on the PGK or HPRT gene.

Compared to the PGK gene, the human androgen receptor gene has a number of features making it a better marker for clonal analysis (16). The HUMARA locus spans >90 kb of DNA and located in Xcen-q13 (17). The DNA contains a highly variable region of CAG trinucleotide repeats encoding 11~31 glycine residues in the first exon. Approximately 90% of females are heterozygous and 20 alleles associated with repeat have been identified, corresponding to 11~31 CAG repeat units (10). Since the CAG repeats result in PCR products of different sizes from the two alleles, a second restriction enzyme digestion is unnecessary for the analysis and the reaction products are analyzed directly by electrophoresis through a denaturing polyacrylamide gel.

Thirty specimens were collected, of which 28 were heterozygous at the HUMARA locus (93.5%). Three cases (10%) were excluded from clonality determination due to severe skewing towards one allele in HpaII-digested normal control tissue. The frequency of skewness is similar to observation in other clonality studies (18,19).

Seven carcinoma in situ had monoclonal patterns. Smith et al. studied five cervical dysplasia and one carcinoma in situ using G-6-PD isoenzyme; single-enzyme phenotype were found in seven separate samples of the carcinoma in situ and eight dysplasia samples, which is consistent with our results (20). The results strongly suggest that early stage of uterine cervical carcinoma originates in a single cell.

Among 18 informative invasive carcinoma, twelve exhibited a monoclonal pattern. Multiple tumors from the different sites (including metastatic lymph nodes) were all concordant for the markers in the same patients. These data strongly support the monoclonal origin. Although viruses infect many cells, the clonal origin of a neoplasm does not necessarily exclude viral cause. If the oncogenic changes induced by the virus were a relatively rare one such as a specific alteration in DNA, single cell origin would occur. No matter what the mechanism, the clonal origin of malignant viral disease (cervical carcinoma) contrasts with the multicellular origin of the benign viral growth (Condyloma acuminatum) (21). This difference suggests that virus-infected cells give rise to a malignant one only if one or more additional mutations occur.

Six of the eighteen invasive carcinomas were identified as having a polyclonal pattern. This is a striking finding and is unlikely to be explained by experimental evidence. Incomplete digestion of DNA was possible but unlikely, since complete digestion was observed in monoclonal samples, which shared the master mixture for *Hpa*II digestion with other polyclonal samples. In addition, the same results were obtained in at least two repetitive experiments and multiple sites (blocks). Significant contamination by normal tissue or non-tumor tissue intermixed with tumor mass was likely to occur. The tumor samples were usually taken from the center of the tumor masses and minimized to avoid contaminated normal tissues. Polyclonality in uterine cervix carcinoma was observed in other studies. Smith et al. found that 2 of 7 invasive carcinoma were polyclonal (20) and Park and Jones reported that 3 of 8 invasive carcinomas were polyclonal by the G-6-PD isoenzyme study (22). Based on our and previous data, it may be a question why all carcinoma in situ showed monoclonal but some invasive carcinoma showed polyclonal pattern. In one, the invasive carcinoma arising from carcinoma in situ can have monoclonal, however, a relatively large number of cells may be simultaneously affected by the tumorigenic process in de novo developing invasive carcinoma. Alternatively, although the oncogenic mechanism initially altered only a single cell (in carcinoma in situ), this influenced the pattern of growth in neighboring cells and then some invasive cacinoma may have polyclonal pattern. It means that the the tumor may be heterogenous in composition containing clonal tumor cells and another polyclonal component. This will be further investigated within a single neoplasm at the DNA level.

Before we conclude that some invasive cervical carcinoma are of polyclonal origin, however, the validity of X-chromosome inactivation method for clonal analysis must be reconsidered. There has been concern that differential methylation is not a reliable marker as to the state of the X chromosome or that variable methylation may occur in association with malignancy (23~27). This is well exemplified at the DXS255 locus analyzed with M27 β probe, which is hypermethylated in a significant proportion of acute lymphoblastic leukemia (19, 28). Therefore, although benign or preinvasive tumor can be analyzed by the methylation pattern of the HUMARA

locus, it may not always serve as an indicator of malignant tumor clonality.

In conclusion, our study of clonality demonstrated that even very small numbers of carcinoma in situ were monoclonal, suggesting that this process of selective outgrowth must begin during the early stage of neoplasm. In invasive epidermoid carcinoma, our results were more complex, most cases were monoclonal, although some cases were polyclonal. These suggest that invasive carcinoma of the cervix does not always arise from a single cell, but may arise from several cells with different mechanisms or further DNA mutation during progression of carcionoma in situ to invasive carcinoma. However, the possibility of the latter hypothesis requires further investigation using additional gene probes.

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