

Loss of Heterozygosity and PCR Artifacts in a Microsatellite Analysis of Psoriasis and Colorectal Cancer

Although a loss of heterozygosity (LOH) is commonly observed using microsatellite markers in a cell-proliferating malignant disorder, controversial findings of psoriasis, a keratinocyte-outgrowth disease, remain to be explained. It was hypothesized that unstable natures of the microsatellite markers for the polymerase chain reaction (PCR) might give a rise to either a false-positive or -negative LOH. Twenty-one frozen skin tissues and 33 formalin-fixed paraffin-embedded archives were obtained from patients with psoriatic plaques and colorectal cancers, respectively. In the frozen psoriatic skin, two of the 17 microsatellite markers selected from 11 chromosomal arms were associated with artifact LOHs that were not reproduced in repeated PCRs. The remaining 15 stable microsatellite markers with few PCR artifacts demonstrated a borderline-level LOH in cases with an ambiguous heterozygosity such as a juxtaposed allelic band. Infrequent LOHs (3 out of 242 heterozygous markers, 1.2%) were detected in psoriatic cases with two separate alleles. In colorectal cancers, a set of the 15 stable microsatellite markers identified a minimal borderline-level LOH at the cut-off point that was same with that of psoriasis. These results indicate that the selection of reproducible microsatellite sequences and the cautious criteria for informative heterozygosity are required to obtain the reliable LOH results from variable genomic DNAs, and that psoriatic lesions harbor few LOH.

Key Words : Psoriasis; Loss of Heterozygosity; Microsatellite Repeats

Jeong Sun Hyun, Bo-Kyong Jo*,
Chul Jong Park, Jong Yuk Yi,
Jun Young Lee, Mun-Gan Rhyu*

Department of Dermatology and Microbiology*,
College of Medicine, The Catholic University of
Korea, Seoul, Korea

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Address for correspondence

Mun-Gan Rhyu, M.D.
Department of Microbiology, College of Medicine,
The Catholic University of Korea, 505 Banpo-dong,
Sochu-gu, Seoul 137-701, Korea
Tel : +82.2-590-1215, Fax : +82.2-596-8969
E-mail : rhyumung@catholic.ac.kr

INTRODUCTION

Psoriasis is a persistent relapsing papulosquamous disease of the skin, and is characterized by an aberrant differentiation and hyperproliferation of keratinocytes. Genetic susceptibility and environmental factors such as physical trauma, infection, psychological stress, or drugs contribute to the development of psoriasis (1). In relation to a hereditary tendency, family studies have shown that the incidence of psoriasis is higher among relatives and offspring of the affected proband and a concordant occurrence of psoriasis is observed among monozygotic twins (1-4). Linkage disequilibrium studies have reported psoriasis-associated genetic loci within the major histocompatibility complex (MHC) (5) and chromosome 4q (6) and 17q (7).

In addition to a hereditary tendency, genetic alterations such as a chromosomal loss in a somatic cell have been observed frequently in the previous study of psoriatic lesions (8). Since studies of colorectal cancer reported multiple chromosomal losses on the basis of the loss of heterozygosity (LOH) detected by the polymorphic markers (9, 10), malignant outgrowth disorders of the skin including basal cell carcinoma (11), squamous carcinoma (12), and malignant melanoma (13) were

also reported to harbor a LOH on multiple chromosomes. PCR-based microsatellite analysis, which requires only a small sample of DNA for a number of genetic markers (14), has indicated the presence of LOH in a variety of cell proliferating disorders, supporting the hypothesis that the inactivation of the regulatory genes caused by a LOH results in hyperproliferation and abnormal differentiation of keratinocytes (8). However, chromosomal losses are unlikely to be responsible for the symmetric psoriatic plaques of the common type. This is because such acquired genetic alterations conferring a selective growth advantage are more likely to contribute to a segmental plaque manifestation resulting from the clonal expansion of a disordered single cell (15). Therefore, there is some debate as to whether or not multiple LOH events truly exist in psoriasis.

Although PCR-based LOH analysis has been widely used over the past decade to investigate chromosomal losses, there appear to be contrasting results among the individual studies on the incidence and pattern of chromosomal loss occurring in a given cell proliferation disorder (16). Sampling of genomic DNA and the unstable natures of the microsatellite markers are known to be the two major causes of PCR artifacts giving a rise to false-positive and -negative results in

a LOH diagnosis (16, 17). Genomic DNA extracted from formalin-fixed, paraffin-embedded tissue is highly fragmented and cross-linked, and are sometimes prone to artificial PCR amplification (16, 17). A set of PCR primers encompassing microsatellite repetitive sequences are superimposed to the erroneous products because a slippage in the repeat units commonly results in a stutter or satellite extra-ladder bands (18).

The reliability of PCR-based LOH analysis was examined using stable microsatellite markers that were expected to confer the unequivocal microsatellite amplification in the genomic DNA from both the formalin-fixed paraffin-embedded colorectal cancer tissue (poor quality DNA) and frozen psoriatic skin tissue (intact DNA). The microsatellite markers were selected from 11 chromosomal arms, 3p, 4p, 4q, 5q, 7p, 8p, 9p, 11p, 13q, 17p and 18q, which are known to suffer from frequent LOH in colorectal cancer and/or psoriasis (8, 19). A set of stable microsatellite markers showing a few PCR artifacts proved that the loss of a total or extensive chromosomal arm, rather than an interstitial region, was a major event in malignant lesions, but not in psoriatic lesions.

MATERIALS AND METHODS

Biological material and DNA extraction

Twenty-one psoriatic patients were selected by reviewing the medical records from the Uijongbu St. Mary's Hospital of the Catholic University of Korea and were enrolled according to the amount of available skin sample. Psoriatic epidermal samples were obtained from 3-mm punch biopsies that were digested using dispase (Boehringer Mannheim Co., Indianapolis, IN, U.S.A.). Matched blood samples were used as the negative controls. The genomic DNA was extracted from the blood and tissue of psoriasis patients with a QIAamp DNA

Minikit according to the manufacturer's instructions (Qiagen Inc., Crawshaw, CA, U.S.A.).

Formalin-fixed, paraffin-embedded colorectal carcinoma tissue was obtained from surgical resections conducted from 1995 to 1999. Seven-micrometer sections were stained with hematoxylin-eosin, and the tumor-rich areas representative of the histological features were then chosen by a microscopic examination. Normal tissues in the tumor portions were manually removed from the slides by a scalpel under stereomicroscopic guidance (Fig. 1). For each case, approximately 100 microdissected cells were digested with 1 μ L of Tween 20-Proteinase K lysis buffer. The lysate mixtures were used as the genomic DNA for PCR analysis.

Microsatellite analysis

For the PCR-based LOH analysis, 27 primer pairs of the microsatellite loci were obtained from Research Genetics (Huntsville, AL, U.S.A.) on chromosome 3p, 4p, 4q, 5q, 7p, 8p, 9p, 11p, 13q, 17p, and 18q (Table 1). The allelic frequency and PCR product size were from the Genome Database (<http://www.gdb.org/>). Each PCR was initiated at the so-called hot-start condition by heating the template-primer mixtures containing 1 μ L of the tissue lysate or the extracted DNA template and 5 μ M of the sense and antisense primers for 5 min at 95°C. A 5- μ L solution of the reaction mixture including 0.1 μ L dNTP (1.25 mM mixture of dATP, dCTP, dGTP, and dTTP, Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A.), 1 μ L of a 10 \times reaction buffer, 0.6 μ L of 25 mM MgCl₂, 1 U *Taq* DNA polymerase (Promega, Madison, WI, U.S.A.), and 0.5 μ Ci [α -³²P]dCTP (3,000 Ci/mmol, Amersham Pharmacia Biotech, Sweden) were added to the template-primer mixture at 95°C. PCR was carried out using an MJR gene cycler (MJ Research Co., Waltham, MA, U.S.A.) in 32 amplification cycles; 94°C for 50 sec, an annealing temperature 55-62°C for 50 sec, and 72°C for 1

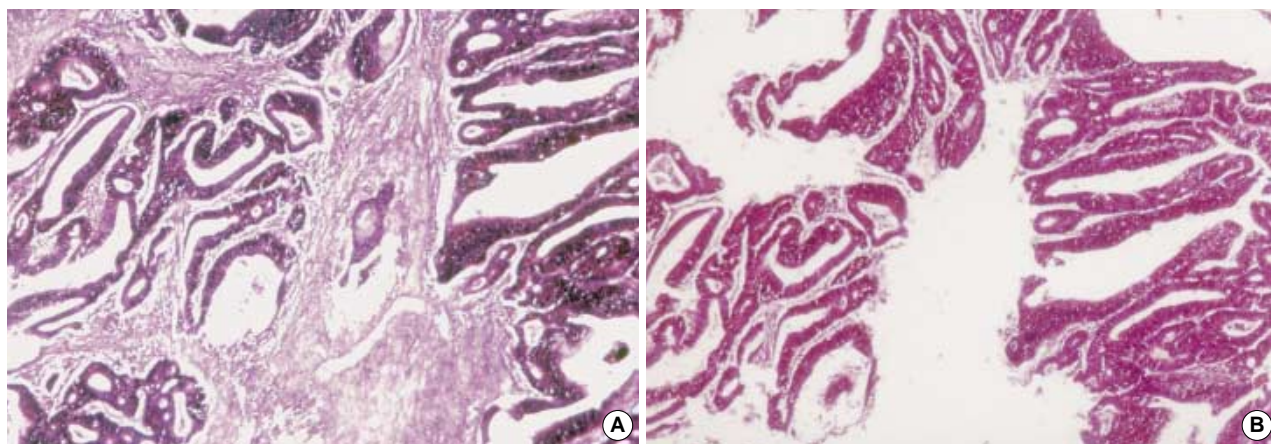


Fig. 1. Manual microdissection from formalin-fixed paraffin-embedded tissues on a microscopic slide (H&E, $\times 40$). Admixtures of normal stroma and tumor tissue (A) were manually scraping off the stromal tissue under the stereomicroscopic guidance (B). The tumor cell content was found to be >70 % to obtain pure tumor DNA, which represented the true allelic status of the tumor.

Table 1. Microsatellite markers used for LOH detection in psoriasis (N=21)

Chromosome	Marker	Size (bp)	Location	Heterozygosity (%)	LOH* (%)
3p	D3S1298	194-220	24.2-22	52.4	0
	D3S1478	109-152	21.3-21.2	60	8.3
	D3S1597	80-100	24.2	73.3	0
	D3S1611	277-288	24.2-22	40	0
4p	D4S391	165-185	15.1	53.3	0
4q	FGA	199	28	66.7	0
5q	D5S346	96-122	22-21	60	0
	D5S299	156-182	15-22	53.3	0
6p	D6S105	138-168	21.1-21.33	80	0
7p	D7S519	256-268	15-22	42.9	0
8p	ANK1	332	12-11.2	NI [†]	NI [†]
	D8S7	250-252	23	NI [†]	NI [†]
	D8S261	128-144	22	53	0
	D8S262	114-128	23	73	0
	D8S503	240	22	50	0
	D8S552	180	22	40	0
	D8S1734	100-120	22-21.3	53	0
	D9S157	133-149	23-22	93	14
9p	D9S199	144-164	23	67	20
	D9S200	107-127	21-12	73	0
	D9S270	97	21	73	0
	D9S288	120-140	pter-p22	60	0
11p	D11S861	154	15.2	87	0
13q	D13S263	149	14	60	0
17p	D17S1566	179-209	13.3	53	0
	D17S796	144-174	13.2	53	0
18q	D18S67	113-129	12.2-12.3	43	0

*LOH: Loss of heterozygosity; [†]NI: noninformative PCR artifacts.

min. No additional PCR cycles were performed in order to avoid over-amplification leading to PCR artifacts. Two microliters of the PCR products were diluted with 5 μ L of a formamide-dye loading buffer, heated at 80°C for 3 min, and electrophoresed on 6% polyacrylamide gel containing 7 M urea. The radioisotope-labeled microsatellite bands were visualized by an autoradiograph of denaturing gel electrophoresis. Repeated exposures of each autoradiograph were performed to obtain an optimal range of the allelic band intensities for densitometric analysis.

Analysis of microsatellite alleles

The highly heterogeneous patterns of the microsatellite sequences amplified by PCR were converted into one-dimensional peaks to standardize the LOH scores with the use of the "profile" option in TINA (raytest Isotopenmeßgeräte GmbH, Straubenhardt, Germany) image software (Fig. 2). In the heterozygous cases, the diverse peaks including both main and shadow bands were divided into two compartments for a single allelic intensity. In the cases with the two alleles in proximity, where excessive shadow bands co-migrate, the overlapping peaks were subtracted from the allelic intensity

measurement.

The difference in the allelic intensity between the normal and lesion DNAs was scored as the relative allelic ratio calculated by dividing the intensity ratio of the lesion by the normal allelic ratio. If gain of allele were found in the lesion DNA, they were interpreted as a microsatellite instability, which were not included in LOH analysis.

RESULTS

Microsatellite allelic signals in frozen psoriatic tissue

Of the 21 psoriasis patients examined, 10 (47%) and 11 (53%) were females and males, respectively. Seventeen patients (81%) were afflicted with early onset psoriasis before they were 40 yr-old, of which 8 (47%) had a family history. The remaining 4 patients suffered from a late onset of the disease without a family history. The psoriatic epidermal tissue separated from the dermis by a dispase digestion of the punch-biopsy sample was found to harbor non-epidermal cells <20%, such as inflammatory cells.

Because of a limited amount of genomic DNA from the punch-biopsy skin, 21 paired genomic DNAs were initially examined for LOH using a total of 17 microsatellite markers from 11 chromosomal arms, 3p, 4p, 4q, 5q, 7p, 8p, 9p, 11p, 13q, 17p, and 18q. The intensity ratios of most of the heterozygous psoriatic lesions relative to that of blood DNA, i.e., the difference in the allelic signals between the normal and lesional DNAs, clustered less than 1.5 fold representing the baseline level of PCR noise. Psoriatic lesions with a relative allelic ratio greater than 1.5 fold were observed in the markers on chromosome 3p, 8p, and 9p, D3S1478, ANK1, D8S7, D9S157, and D9S199, which is indicative of the allelic imbalance responsible for the LOH. Of the markers associated with frequent LOHs, the ANK1 and D8S7 markers showed up as ghost bands, which may not have been reproduced in repeated PCRs. Ghost bands were considered to be nonspecific findings with genomic sequence redundancy, suboptimal PCR condition, or unstable nature of microsatellite marker (Fig. 2).

The heterozygous cases with separate alleles exhibited stable allelic ratios, whereas the heterozygous cases with overlapped allelic bands revealed a borderline level of a relative allelic ratio. When two intimate allelic bands differed in size by one repeat unit, such as in cases 10 (1.54) and 16 (1.50), the D9S199 markers were scored as being a borderline LOH (Fig. 2). To improve the feasibility of the allelic imbalance, two alleles differing in size by more than one repeat unit were defined as the informative case for LOH analysis.

PCR noise and artifacts in intact and poor quality DNAs

Of the 11 chromosomal arms examined, 3p, 8p, and 9p

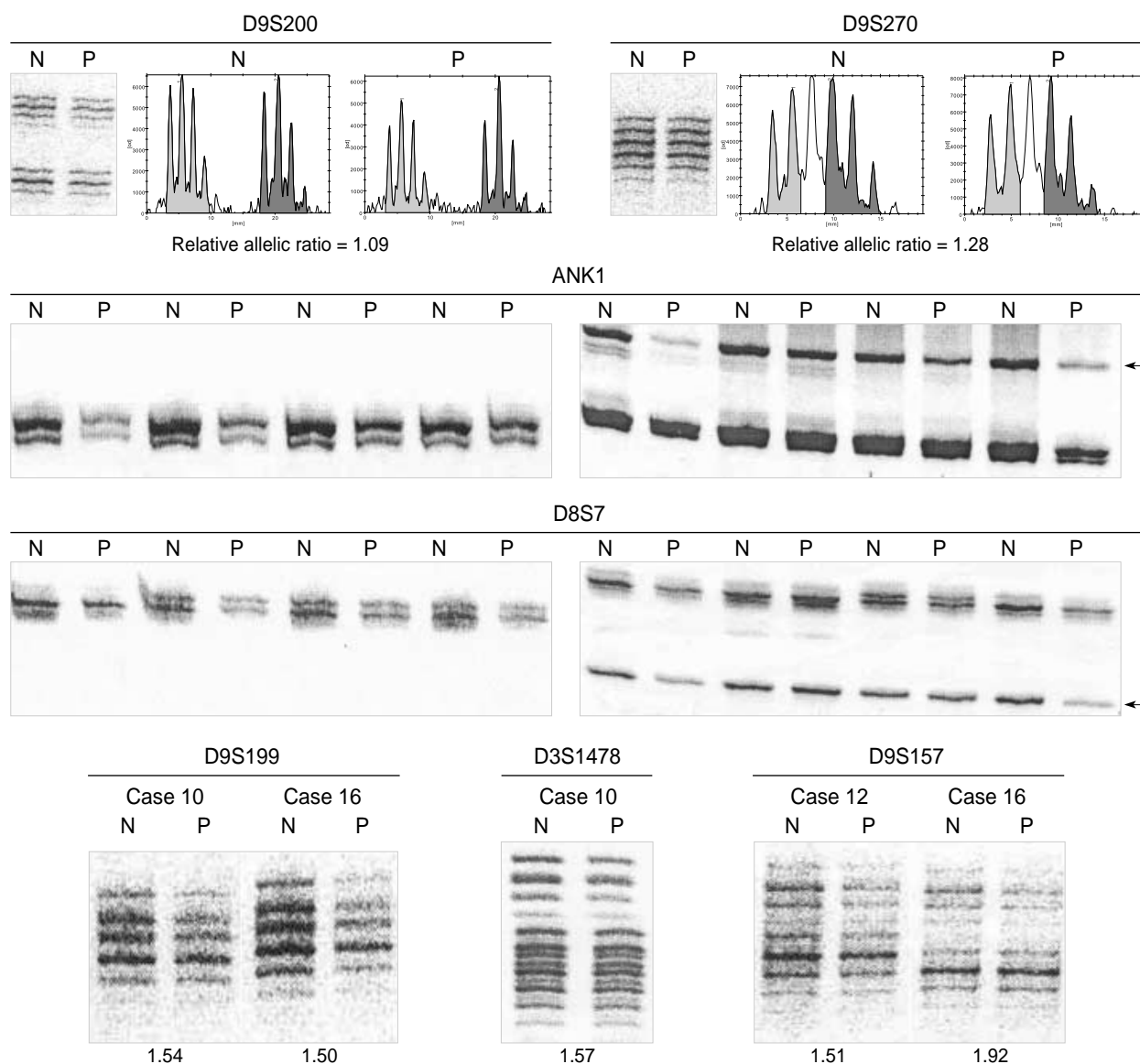


Fig. 2. Measurement of the difference in the allelic intensity between psoriatic DNA (P) and normal DNA (N). The allelic ratio of psoriatic DNA relative to that of normal DNA is shown below the paired lanes. A peak area including both the main and shadow bands was measured for a single allelic intensity. As shown in D9S200 marker, typical heterozygosity exhibits two separate allelic bands accompanying the satellite bands. In D9S270 marker, the cases with two alleles in proximity where the shadow bands from both alleles co-migrated, the overlapping peaks (open area) were subtracted from the allelic intensity measurements. Both the ANK1 and D8S7 markers show allelic band artifacts (indicated by arrows) that were not observed in other PCRs. Borderline-level LOHs in juxtaposed heterozygous alleles at D9S199 were due to the excessive co-migration of the main and satellite bands between the two alleles. Three cases (cases 10, 12 and 16) with diminished upper alleles at D3S1478 and D9S157 appeared to be caused by equivocal amplifications of the upper and low alleles.

showing a LOH or LOH artifacts were further examined in the psoriatic lesions and colorectal cancers to compare the relative allelic ratios between the frozen skin tissue (intact DNA) and the formalin-fixed tissue (poor quality DNA). The additional markers on the three chromosomal arms, D3S1597, D3S1611, D8S261, D8S262, D8S503, D8S552, D8S1734, D9S200, D9S270, and D9S288 were used in the 15 psoriatic lesions and 33 colorectal cancers because of the small DNA amounts. There were 242 and 275 informative

heterozygous markers in the psoriasis and colorectal cancer patients, respectively.

Of the 242 heterozygous markers from the psoriatic DNAs with two separate alleles, 239 (99%) demonstrated balanced allelic ratios distributed in a narrow range of <1.50 (Fig. 3). The remaining 3 markers were defined as being LOH-positive based on the relative allelic ratio values, 1.57 (case 10), 1.51 (case 12), and 1.92 (case 16), which were indicative of the diminished upper allelic signals (Fig. 2). The infrequent

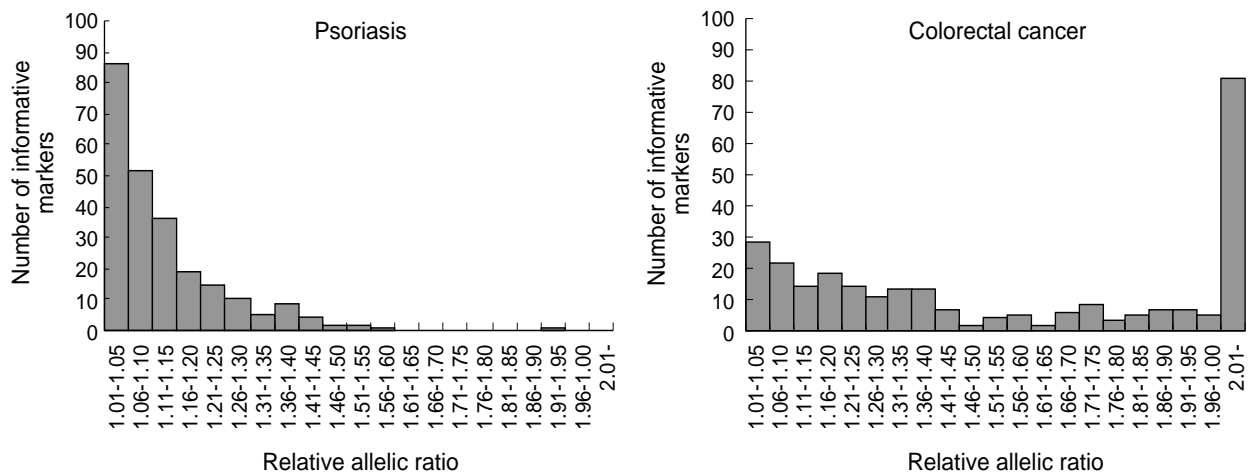


Fig. 3. Distribution of the informative heterozygous cases according to the relative allelic ratio in psoriasis and colorectal cancer. The background relative ratios of the wild-type alleles indicative of PCR noise of microsatellite sequences cluster in a range from 1.0 to 1.5 fold in psoriasis. Most of the unilateral signal alterations that were indicative of a LOH are greater than a cut-off point 1.5 in colorectal cancer.

upper allelic losses were unlikely to represent unilateral chromosomal loss, because the upper allelic signals tended to be equivocally diminished in relation to the lower allele, resulting in false-positive LOH (Fig. 2). The colorectal cancer DNAs demonstrated a broad range of relative allelic ratios, including high-level allelic imbalances greater than 1.5 fold, which similarly occurred at both the upper and lower alleles (Fig. 3). Especially, a set of the stable microsatellite markers identified a few borderline-level allelic ratios (1.8%, 5 of 275 informative markers) ranging from 1.5 to 1.6. These findings suggest that the stable marker set provided the reliable LOH results from variable genomic DNAs and that infrequent upper allelic loss in psoriatic lesions is most likely to be a false-positive LOH (Table 2).

DISCUSSION

The initial LOH studies on colorectal cancer suggest a genetic mechanism that underlies tumorigenesis through the inactivation of the tumor suppressor genes (19). Since the initial colorectal cancer studies employed genomic DNA hybridization with restriction fragment length polymorphism (RFLP) probes (9, 10), a laborious technique requiring a large amount of genomic DNA, PCR-based LOH tests for the purpose of diagnosis with the use of a number of microsatellite markers and the availability of formalin-fixed pathologic samples are of particular interest (14). Microsatellites are highly polymorphic repetitive nucleotides that are scattered throughout the genome. They are inherited stably, are unique to each individual and have a low inherent mutation rate (15, 20). However, recent assessments on the reliability of PCR-based microsatellite analysis have shown common artifacts leading to equivocal findings concerning the LOH profile in

Table 2. The results of microsatellite analysis of colorectal cancer (N=33)

Chromosome	Marker	Size (bp)	Location	Heterozygosity (%)	LOH* (%)
3p	D3S1298	194-220	24.2-22	72.4	10.3
	D3S1478	109-152	21.3-21.2	78.1	18.3
	D3S1597	80-100	pter-24.2	83.3	20
	D3S1611	277-288	24.2-22	63.6	12.1
8p	D8S503	240	22	37.5	55.6
	D8S261	128-144	22	30.3	41.2
	D8S262	114-128	23	66.7	45
	D8S552	180	22	70.4	55.6
	D8S1734	100-120	22-21.3	51.6	38.7
9p	D9S157	133-149	23-22	66.7	29.2
	D9S199	144-164	23	71.9	24.2
	D9S200	107-127	21-12	69.7	38.5
	D9S270	97	21	63.6	15.2
	D9S288	120-140	pter-22	73.3	31.3

*LOH: Loss of heterozygosity.

the formalin-fixed pathologic tissues with poor DNA quality (16-18). In the present study using intact genomic DNA from frozen tissues, common artifacts in the PCR-based microsatellite analysis were likely to be related to an admixture of inherent nonspecific allelic bands caused by the unstable nature of the microsatellite sequences.

Zachos et al. observed frequent LOH on multiple chromosomes in psoriasis patients (8): chromosome 3p24-p21.2 (D3S1478, D3S1298; 57%), 7p15-q22 (D7S478, D7S519; 28%), and 8p21.1-p11.2 and 8p23 (ANK1, D8S7; 50%). They suggested that certain candidate genes related to cell cycle regulation were located in these particular chromosomes regions, which is similar to that observed in neoplastic disorders (21-23), and that LOH contributed to the development of human psoriatic lesions. In this study using a set of stable

microsatellite markers, a few LOHs were identified on 11 chromosomes tested in psoriasis, whereas colorectal cancers had multiple LOHs involving an extensive chromosomal region including the tumor suppressor gene. Nonspecific alleles in chromosome 8p that were not reproducible in repeated PCR were equivocally co-migrated with the reproducible true alleles, which could potentially be misinterpreted as heterozygous microsatellite alleles and LOH. These PCR artifacts in the microsatellite markers on chromosome 8p were the same microsatellite markers that were reported to demonstrate frequent LOH in the previous study (8). Our results suggest that the previous LOH profiles observed in psoriatic lesions might originate from PCR artifacts associated with unstable microsatellite markers.

Happle suggested that LOH was unlikely to be a causative genetic event for a psoriatic lesion of the common type, because symmetric involvement of psoriasis was considered to be a manifestation that originated from a germ cell and would be difficult to be explained by a LOH occurring in the somatic cells (15, 24). According to the 'two hits' theory, two alleles of the tumor suppressor genes are functionally inactivated by two rate-limiting alterations, a point mutation in the nucleotide sequences and a unilateral chromosomal loss targeting a specific genetic locus (25). The tumor-specific chromosomal loss that is never observed in a normal tissue is thought to confer a selective growth advantage for clonal expansion of a single somatic cell. Therefore, a LOH is unlikely to be responsible for psoriasis manifesting as a symmetric involvement.

Demands for standard guidelines for reliable LOH data are increasing because ambiguous allelic ratios may be encountered during PCR-based microsatellite analysis. This study found that intimately juxtaposed heterozygous alleles were an important source of a borderline level of a LOH, which were defined as being non-informative for a LOH. Heterozygous psoriatic cases with two separate alleles demonstrated borderline-level LOHs in 3 cases, which were likely to be PCR artifacts when compared with the frequent multiple LOHs observed in colorectal cancer. Three LOH-positive psoriasis cases with a diminished signal in each chromosomal arm were distinguishable from the concurrent LOHs involving multiple markers on a given chromosomal arm in colorectal cancer. The three psoriatic cases with a single LOH appeared to be caused by equivocal amplifications of the upper and low alleles by chance. It has been reported that a true LOH representing a chromosomal loss, which is a major event in malignant lesions, involves a total or an extensive chromosomal arm, rather than an interstitial region of the chromosome (26). Therefore, a solo LOH of psoriasis appears to be a false positive result in association with an imbalanced heterozygous amplification of wild-type alleles.

Various DNA quantities and qualities of the microdissected formalin-fixed paraffin-embedded tissue and normal cell contamination in the microdissected tumor tissue were con-

sidered to be the sources of erroneous allelic imbalances including a borderline or incomplete LOH. In this study, the unstable nature of the microsatellite markers selected, rather than genomic DNA sampling, is likely to facilitate the common PCR artifacts. A set of stable microsatellite markers exhibited a universal cut-off point (1.5) for a LOH in both frozen psoriatic lesions and formalin-fixed colorectal cancer tissues, thus providing the best discrimination between normal PCR noise and a LOH. Our results indicate that psoriasis is not associated with a LOH, which must be scored with careful attention concerning the false-positive results caused by error-prone microsatellite markers.

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