Analysis of Fluorescence *In Situ* Hybridization, mtDNA quantification, and mtDNA sequence for the detection of early bladder cancer

Jong-Ha Yoo

Department of Medicine

The Graduate School, Yonsei University

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Directed by Professor Jong Rak Choi

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Jong-Ha Yoo

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This certifies that the Doctoral Dissertation of Jong-Ha Yoo is approved.

Thesis Supervisor : Jong Rak Choi

Myung-Geun Shin: Thesis Committee Member#1

Nam Hoon Cho: Thesis Committee Member#2

Myoung Hee Kim: Thesis Committee Member#3

Young Deuk Choi: Thesis Committee Member#4

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It was a great accomplishment of mine that I could broaden my academic knowledge and upgrade my experimental skill while completing the thesis. Based on it, I would like to contribute to the advance of the field of cancer-related molecular pathology.

Jong-Ha Yoo

<TABLE OF CONTENTS>

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Analysis of Fluorescence *In Situ* Hybridization, mtDNA quantification, and mtDNA sequence for the detection of early bladder cancer

Jong-Ha Yoo

Department of Medicine
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We designed this study to test the sensitivities of cytology, the NMP-22 assay, and FISH in the early detection of urothelial carcinoma, and to identify mtDNA alterations in urinary epithelial cells. We collected 41 urine samples and 26 corresponding peripheral blood samples from patients with clinically suspected urothelial carcinoma. The FISH and NMP22 assays detected 92.1% of the cancers and cytology detected 60.5%. In the low grade group, NMP-22 and FISH analyses were more sensitive than cytology, but in the high grade group, all three methods showed about 90% sensitivity. Overall, the FISH and NMP-22, or FISH and cytology assays combined detected 97.4% of cancers, while cytology with NMP-22

detected 92.1%. In the low grade group, the sensitivity of the three methods combined was above 80%, but in high grade group, the combined sensitivity was about 100%. In the mtDNA control region, we detected characteristic heteroplasmic mtDNA substitution mutations in one patient and mtDNA length heteroplasmic mutation in 303 polyC or 16184 poly C in twenty patients. Overall, urothelial carcinoma-specific mtDNA mutations were observed in 20 of the 26 patients (76.9%). The average mtDNA copy numbers in urine samples and corresponding peripheral blood samples (83.45 ± 60.36 and 39.0 ± 24.38, respectively), differed significantly ($P < 0.001$). The mtDNA copy numbers in the urine samples from patients with high grade and low grade tumors (81.83 ± 67.78 and 86.49 ± 46.69, respectively) did not differ significantly ($P = 0.589$). In conclusion, the FISH assay showed the highest sensitivity for detecting low grade urothelial carcinoma, and mtDNA copy numbers in urine samples were higher than those in the corresponding peripheral blood samples. The frequency of mtDNA mutations in the D-loop region in patients with cancer was about 80% in our study. This report further supports the significance of genetic alteration in urothelial carcinoma and the clinical utility of the FISH, mtDNA quantitation PCR, mtDNA sequencing, and capillary electrophoresis for this purpose.

Key words: bladder cancer, Fluorescence *In Situ* Hybridization (FISH), mtDNA copy number, mtDNA sequence

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I. INTRODUCTION

Bladder cancer is the ninth most common cancer worldwide and accounts for 3.3% of all malignancies. In Korea, it is the most common urinary tract cancer and ranks sixth in incidence among cancers in men and 16th in women.[1-3]

The detection of bladder cancer noninvasively, although desirable,

presents challenges. Cystoscopy involves discomfort for the patient and potential risks such as urethral and bladder neck stricture.[4] In addition, cytology, the highly specific standard method for detection and monitoring,[5] is relatively insensitive for detection.[6-10] Cytology is least sensitive for low grade bladder tumors, but suboptimal even for high grade tumors. The drawbacks of cytology and the extensive training required to interpret it have spurred development of alternative urinary tests for bladder cancer. A number of these tests are based on the detection of antigens (BTA-STAT, NMP-22 and fibrin degradation products) present at increased levels in the urine of patients with bladder cancer.[11-14] Many of these tests show significantly higher sensitivity, but lower specificity than cytology.[15-17]

Other tests such as digital image analysis,[18] fluorescence *in situ* hybridization (FISH)[19-21] and microsatellite analysis[22] are based on the detection of exfoliated urothelial cells with genetic alterations, such as aneuploidy or loss of heterozygosity, consistent with a diagnosis of bladder cancer. FISH uses fluorescently labeled centromeric and locus-specific DNA probes to detect urinary cells with chromosomal gains or losses.[19-21, 23, 24] The finding of such cells is consistent with a diagnosis of bladder cancer, or less likely, other genitourinary cancers. Halling et al.[24] proposed a multi-target FISH approach, using probes to the centromeres of chromosomes 3, 7, 17 and 9p21 (*p16/CDKN2A* gene). The FISH assay

reportedly shows high specificity and sensitivity in the detection of bladder cancer.[19, 24, 25]

Most studies on the molecular genetics of bladder cancer focus on changes in genomic DNA (oncogenes and tumor suppressor genes such as *c-H-ras, c-erb-B2, p53,* and *Rb*)[26, 27] and subsequent cellular events (DNA repair, genomic instability, and changes in gene expression, signal transduction and apoptosis). Mitochondrial function and mtDNA attract less interest in bladder cancer.

Mitochondria perform the essential work of oxidative metabolism and ATP synthesis in all animal cells, and participate in the control of apoptosis. A role for mitochondria in neoplastic transformation and promotion is predicted, because many tumor cells show impairment in energy metabolism and high rates of glycolysis.[28] Abnormalities in mitochondrial structure and aberrant expression of respiratory enzymes in tumor cells may result from defects in mtDNA.[29] Mitochondria are semi-autonomous organelles with their own genome.[30] The double-stranded circular mtDNA contains 37 genes, which encode the 13 polypeptides of oxidative phosphorylation, 22 tRNAs and two rRNAs. Expression of the entire genome is needed to maintain mitochondrial functions, and small changes in mtDNA cause significant impairment.

Unlike nuclear DNA, mtDNA is not protected by histones and is more susceptible to damage by chemical carcinogens and activated

metabolites that covalently bind DNA, and inhibit transcription and translation.[31-33] The mtDNA may also be more susceptible to damage by reactive oxygen species (ROS) released in respiration.[34] This damage would be especially high in tumor cells, which are often deficient in superoxide dismutase activity.[35] mtDNA has a lower DNA repair capacity than the genomic DNA and therefore suffers a higher rate of mutation.[36-38] The mitochondrion lacks proofreading exonuclease activity, and replicates its genome with low fidelity.[39] Because the mtDNA genes lack introns, mutations in coding sequences may directly alter amino acid sequences and protein structures.[40] mtDNA mutations may intensify oxidative stress, which leads to further mutation and promotes carcinogenesis.[41]

Somatic mutations in mtDNA are detected in cancers of diverse human tissues, including the esophagus,[42] stomach,[43] liver,[41] colon and rectum,[44] kidney,[45] bladder,[8, 27] prostate,[46] ovary,[37] breast,[41] head and neck,[47] lung,[48] thyroid gland,[45] and brain.[41, 49] These mitochondrial defects occur largely as point mutations, single base deletions and insertions in the non-coding D-loop region, and in coding regions for protein components of oxidative phosphorylation. An increase in mtDNA content of head, neck, and prostate cancers may represent a compensatory response to a decline in respiratory function.[47, 50]

Few studies have focused on mtDNA mutations and copy numbers in human bladder cancer. To clarify the carcinogenic significance of such

defects, we screened urine samples and corresponding peripheral blood samples for bladder cancer-specific mtDNA mutations. We also compared the sensitivities of the cytological, NMP-22, and FISH assays for the detection of bladder cancer in urine samples.

II. MATERIALS AND METHODS

We obtained 41 urine samples and 26 matched peripheral blood samples from patients with clinically suspected urothelial carcinoma (Table 1). Transurethral resections (TURB) were performed, and these were classified as positive for urothelial carcinoma in 38 patients (93%) and negative for urothelial carcinoma in 3 patients (7%). The tumor grade was low in 11 patients (29.0%), high in 23 patients (60.5%), and carcinoma *in situ* (CIS) in 4 patients (10.5%). The Institutional Review Board approved this study and all patients gave their written informed consent.

Table 1. Assayed results in individual patients with clinically suspected bladder cancer

No.	Ag/ sex	Grade	Cyto -logy	NMP-22*	FISH	Heteroplasmic mutation			mtDNA copy number**	
						Stat-us	Type	Loca-tion	Urine	Blood
1	31/ M	Low	+	19.95	+	-	NA	NA	59.69	14.57
2	67/ M	High	+	30.08	+	+	Length	303 poly C	18.99	12.53
3	54/ F	High	+	297.89	+	+	Length	303 poly C	2.01	28.88
4	64/ M	Low	-	40.95	+	+	Length	303 poly C	42.38	14.4
5	80/ F	Low	-	1.39	+	NC			NC	NC
6	73/ M	High	+	131.62	+	-	NA	NA	15.66	16.89
7	55/ M	Carcinoma *in situ*	+	66.20	+	+	Length	303 poly C	185.28	39.90
8	58/ M	Low	+	102.87	+	-	NA	NA	164.64	109.18
9	75/ M	Low	+	66.97	+	NC			NC	NC
10	53/ F	High	+	141.28	+	NC			NC	NC
11	70/ M	High	+	236.04	+	-	NA	NA	77.22	53.22
12	50/ M	Low	+	50.19	+	+	Length	16184 poly C	149.48	78.34
13	50/ M	Benign	-	14.01	+	NC			NC	NC
14	54/ F	High	+	168.27	+	+	Length	303/ 16184 poly C	205.42	54.2
15	47/ F	Carcinoma *in situ*	-	33.14	+	NC			NC	NC
16	66/ M	High	+	76.66	+	+	Length	303 poly C	54.17	47.04
17	82/ M	High	+	195.63	+	NC			NC	NC
18	76/ M	Low	+	20.40	+	+	Length	303 poly C	69.42	48.33
19	39/ M	Benign	-	38.52	+	NC			NC	NC
20	79/ M	High	+	37.86	+	NC			NC	NC
21	68/ M	High	+	63.24	+	+	Length	303 poly C	53.34	59.22
22	51/ M	High	-	17.68	+	+	Length	303 poly C	161.47	69.20
23	66/ M	High	+	531.64	+	NC			NC	NC
24	63/ F	High	-	8.74	+	+	Length	303 poly C	99.00	60.87
25	68/ M	High	+	464.36	+	+	Length	303 poly C	76.54	14.97

(To be continued)

Table 1. Assayed results in individual patients with clinically suspected bladder cancer

No.	Age /sex	Grade	Cyto -logy	NMP-22*	FISH	Heteroplasmic mutation			mtDNA copy number**	
						Stat-us	Type	Loca-tion	Urine	Blood
26	82/M	High	+	270.93	+	+	Length	16184 poly C	193.62	56.53
27	76/M	High	+	118.16	+	+	Length	303 poly C	6.04	20.40
28	70/M	High	+	24.42	+	NC			NC	NC
29	21/M	Low	-	8.15	-	-	NA	NA	23.66	20.45
30	53/M	Benign	-	59.13	-	NC			NC	NC
31	78/M	High	+	238.96	+	NC			NC	NC
32	74/F	High	+	33.89	+	NC			NC	NC
33	81/M	High	+	31.88	-	NC			NC	NC
34	64/M	Carcinoma *in situ*	+	562.87	+	+	Length	303 poly C	109.75	31.22
35	70/M	High	+	33.68	+	NC			NC	NC
36	38/M	High	-	15.43	+	+	Length	303/16184 poly C	27.79	51.59
37	61/M	High	+	587.88	-	-	NA	NA	73.60	17.19
38	52/M	Carcinoma *in situ*	+	313.57	+	+	Length	303 poly C	31.24	15.93
39	45/F	Low	+	21.45	+	+	Length	303 poly C	106.24	15.49
40	70/M	Low	+	21.1	+	+	Length	303 poly C	78.15	42.90
41	74/M	Low	-	25.82	+	+	Substitu-tion/ Length	D-loop/ 303 poly C	84.79	27.22
Mean				103.17					83.45	39.0
SD				128.48					60.36	24.38
P									<0.001	

Abbreviations: *, reported as U/ml (reference range: 0-9.9); **, reported as mtDNA/nDNA x 100; Length, Length heteroplasmic mtDNA mutation in the control region; NA, not applicable; NC, no check; SD, standard deviation; P, P-value from non-parametric Mann-Whitney test, statistical significant difference between urine and blood samples.

1. FISH

Urine preparation

Urine specimens for FISH were processed the same day, usually within two to six hours of receipt. The volume of urine used for FISH was about 50 ml. Urinary epithelial cells were sedimented at 600 x g for 10 minutes and resuspended in 15 ml hypotonic solution (0.075 M. potassium chloride) for 10 minutes. The cells were then sedimented again at 600 x g for 10 minutes and resuspended in 10 ml 3:1 methanol:glacial acetic acid. This procedure was repeated two more times and the final cell pellet was resuspended in 50 to 200 ml (depending on cell pellet size) of the residual 3:1 methanol:acetic acid.

Slide preparation

We placed 3-, 10- and 30-ul samples of the cell suspension in separate 0.6 cm wells of a 12-well slide. These amounts of cell suspension ensured that most samples had at least one well with the appropriate density of cells. If the density of the cells was too high or low, a corrected concentration of cell suspension was applied to a fourth well.

FISH

The probe mix consisted of directly labeled probes to the

peri-centromeric regions of chromosomes 3 (CEP3), 7 (CEP7) and 17 (CEP17), and to band 9p21 at locus (LSI 9p21). The CEP3, CEP7, CEP17 and LSI 9p21 probes were labeled with Texas red, spectrum green, spectrum aqua and spectrum gold fluorophores, respectively. These probes were selected from a set of 9 (centromeric probes to 9, 7, 17, 3, 8, 11, 18, Y and a locus-specific probe to 9p21) that were initially evaluated for their sensitivity in detection of neoplastic cells in the urine of patients with biopsy-confirmed urothelial carcinoma.[51] The 9p21 probe was also used because it hybridizes to the site of P16 (CDKN2A), a gene frequently inactivated early in urothelial carcinogenesis.[52, 53] Slides were incubated in 2X saline/sodium citrate (SSC) at 37℃ for 10 minutes; 0.5 mg/ml pepsin, pH 1.0, at 37℃ for 13 minutes; phosphate buffered saline at room temperature for five minutes; 1% formaldehyde at room temperature for five minutes; and phosphate buffered saline at room temperature for five minutes. The slides were then placed in 70%, 85% and 100% ethanol for one minute each and denatured in 2X SSC/70% formamide at 73℃ for five minutes. The FISH probe mix (1 ul probe mix, 8 ul hybridization mix and 1 ul water) was denatured at 73℃ for five minutes. Denatured slides were rehydrated in 70%, 85% and 100% ethanol for one minute each, and dried, and then 3 ul of the denatured probe were placed in each of the three wells containing specimen. The slide was cover-slipped, sealed with rubber cement and incubated at 37℃ overnight in a humidified chamber. The

slides were washed in 0.4X SSC/0.3% NP-40 at 73°C for two minutes and rinsed in 2X SSC/0.1% NP-40 at room temperature. Then 10 ul of DAPI II counterstain were placed in each well and the slide was cover-slipped.

Interpretation of FISH results

Each patient specimen slide was first scanned for cells with abnormal-appearing nuclei (large, irregular shape, etc.) since these are most likely to have experienced some sort of cancer-associated genetic change. The DNA probe profile (the number of probe signals of each color) was then recorded to determine the copy number of chromosomes 3, 7, or 17, and to test for deletion of the 9p21 locus. If no nuclei appeared abnormal, the probe signal patterns of the remaining cells were reviewed to identify cancer-associated changes in the absence of morphological change. The finding of five or more urinary cells with gains of two or more chromosomes, or 10 or more cells with gains of a single chromosome (for example, trisomy 7) on the slide was scored as positive for urothelial carcinoma. Homozygous deletion of 9p21 in more than 20% of the epithelial cells was also considered a positive result.

2. Cytology

A bladder wash sample was obtained during cystoscopy by rinsing the bladder at least twice with 50 ml of saline solution. The cytology specimens were obtained on the same day that the specimen was collected for FISH.

3. Nuclear matrix protein (NMP)-22 assay

NMP-22 assay was performed using an automated chemiluminescent assay system (IMMULITE, Diagnostic Products Corp., Los Angeles, USA).

4. PCR amplification and sequencing of the mtDNA control region

Total leukocytes from the peripheral blood samples and urothelial cells were separated by density gradient centrifugation and washed twice in PBS. The number of cells suspended in PBS was adjusted to 1 x 10^7 cells/ml, and total DNA was extracted with an AccuPrep Genomic DNA Extraction Kit (Bioneer, Daejon, Korea). The extracted DNA was resuspended in TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) and photometrically quantified. To amplify and sequence the control region (nucleotides 16024 to 16569 and 1 to 576), we used a set of designated primer pairs and PCR

conditions based on a published protocol.[54] Each amplified mtDNA product was purified with an AccuPrep PCR Purification Kit (Bioneer) and sequenced with the BigDye Terminator v3.1 Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and the ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The sequencing primers used for each mtDNA product are listed in Table 2. The experimentally determined mtDNA sequences were compared to the Revised Cambridge Reference Sequence (RCRS) (http://www.mitomap.org/mitomap/mitoseq.html) with the Blast2 program (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html) and the database search tool, MitoAnalyzer (http://www.cstl.nist.gov/biotech/strbase/mitoanalyzer.html, 2001), to identify polymorphisms and mutations differing from the RCRS. To exclude potential artifacts, PCR amplifications from the original cell lysates were replicated one or two additional times, and then resequenced.

Table 2. Primer sets for the PCR and direct sequencing of the mtDNA control region

Product size	mtDNA segment	Primer sequence (5' to 3') for PCR		Sequencing primers (5' to 3')	
1.12 kb	Control region (np 16,024-16,569; np 1-576)	F15971	TTAACTCCACCA-TTAGCACC	F15971	TTAACTCCACCATTAG-CACC
				R48	GCATGGAGAGCTCCC-GTGAGTGG
		R611	CAGTGTATTGCT-TTGAGGAGG	F15	CACCCTATTAACCACT-CACG
				R611	CAGTGTATTGCTTTGA-GGAGG

Abbreviations: np, nucleotide position; F, forward primer; R, reverse primer.

5. Determination of mtDNA length heteroplasmies in the control region

mtDNA minisatellites located in the mtDNA non-coding hypervariable regions (HV) 1 and 2. Amplified products were separated according to size by capillary electrophoresis, as previously described,[54] to detect length heteroplasmic mutations of 16184 poly C (^{16184}CCCCCTCCCC^{16193}, 5CT4C), 303 poly C (^{303}CCCCCCCTCCCCC^{315}, 7CT5C), and 514 (CA) repeat (^{514}CACACACACA^{523}, $(CA)_5$ repeats).

6. Quantification PCR for the determination of the mtDNA copy number

A highly conserved region of the mtDNA genome that codes for the *CYTB* gene (np 14,909–15,396; 488 bp) was selected to determine the number of mtDNA per cell. Quantitative PCR from original samples was independently repeated to exclude potential artifacts. To generate a standard curve for quantification of mtDNA, the purified PCR product of the *CYTB* gene was inserted into the pGEM-T easy vector and used to transform E. coli JM 109 (Promega, Madison, WI, USA) for production of recombinant plasmids. A 250-ul mix containing 12.5 ul of 2 x Quantitect SYBR green PCR master mix (Qiagen, Valencia, CA, USA), 400 uM *CYTB* primers F14909 (5'-TACTCACCAGACGCCTCAACCG-3') and R15396

(5'-TTATCGGAATGGGAGGTGATTC-3'), and 400 uM each of *β-Actin* forward primer 5'-ACCCACACTGTGCCCATCTAC-3' and reverse primer 5'-TCGGTGAGGATCTTCATGAGGTA-3' were used for PCR with the Rotor-Gene real-time centrifugal DNA amplification system (Corbett Research, Sydney, Australia). To amplify mitochondrial regions, 500 pg of DNA were used, and 10 ng were used to amplify *β-Actin*. Primers were chosen to avoid amplification of genomic pseudogenes homologous to mitochondrial genes, and this was confirmed for all primers by BLAST results (http://www.ncbi.nlm.nih.gov/BLAST/). For PCR, hot start reactions at 50℃ for 2 min and at 95℃ for 15 min were followed by 35 cycles of 20s at 94℃, 30s at 56℃, 30s at 72℃ and a melting reaction with a decrease of 1℃ per cycle between 72℃ and 92℃. The mtDNA copy number was calculated using the following formula: mtDNA/nDNA ratios x 100.

7. Statistical analysis

The sensitivities of the various tests were determined from the results for 41 patients with clinically suspected urothelial carcinoma. The non-parametric Mann-Whitney test was used to compare mtDNA copy numbers between specimens (urine and peripheral blood cells) and

between tumor grades (low grade and high grade). A *P*-value less than 0.05 was considered statistically significant.

III. RESULTS

We determined the histological grade, bladder wash cytology, NMP22 result, FISH, heteroplasmic mutation of the mtDNA control region, and mtDNA copy number for the 41 patients with clinically suspected urothelial carcinoma (Table 1). Of the 41 patients, three were negative for cancer. Heteroplasmic mutations in the mtDNA control region and mtDNA copy numbers were analyzed in 26 of these patients. Representative examples of positive FISH results from patients with biopsy-proven urothelial carcinoma are shown in Figure 1.

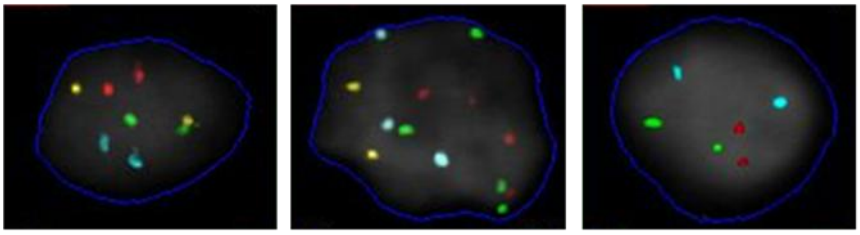

Figure 1. (A) Normal FISH result. Each probe signal (CEP3-red, CEP7-green, CEP17-aqua, LSI 9p21-gold) is shown in two copies. (B) Abnormal cells exhibit multiple copies of CEP3, CEP7, and CEP17. (C) Abnormal cells exhibit loss of LSI 9p21.

1. Overall and grade sensitivity

Overall, the FISH and NMP22 assays individually detected 92.1% of biopsy-confirmed cancers and cytology detected 60.5% (Table 3). In the low grade group, NMP-22 and FISH analyses were more sensitive than cytology, but in the high grade group, the sensitivities of these three analyses were all about 90%.

Overall, the FISH and NMP-22, or FISH and cytological assays detected 97.4% of confirmed cancers, and cytology and NMP-22 combined detected 92.1% (Table 4). In the low grade group, the combined sensitivity was above 80%, but in the high grade group, the combined sensitivity was about 100%.

Of the 37 patients with positive FISH results, two patients (5.4%) were determined to have no cancer. We concluded that chronic nonspecific inflammation and erosion with hemorrhage caused these false positives.

Table 3. Overall and grade sensitivities of tests

	No./Total No. (%)		
	Cytology	**NMP-22**	**FISH**
Grade sensitivity:			
Low	**4/11 (36.4)**	**9/11 (81.8)**	**10/11 (91.0)**
High	**20/23 (87.0)**	**22/23 (95.6)**	**21/23 (91.3)**
Carcinoma *in situ*	**3/4 (75.0)**	**4/4 (100)**	**4/4 (100)**
Overall sensitivity	**23/38 (60.5)**	**35/38 (92.1)**	**35/38 (92.1)**

Table 4. Overall and grade combined sensitivities of tests

	No./Total No. (%)		
	Cytology and FISH	**NMP-22 and cytology**	**FISH and NMP-22**
Grade sensitivity:			
Low	**10/11 (91.0)**	**9/11 (81.8)**	**10/11 (91.0)**
High	**23/23 (100)**	**22/23 (95.6)**	**23/23 (100)**
Carcinoma *in situ*	**4/4 (100)**	**4/4 (100)**	**4/4 (100)**
Overall sensitivity	**37/38 (97.4)**	**35/38 (92.1)**	**37/38 (97.4)**

2. mtDNA sequence alterations

In our direct seqeuncing of the mtDNA control region in urothelial and peripheral blood cells, we observed characteristic heteroplasmic mtDNA substitution mutations in one patient (patient no. 41, low grade, Fig. 2).

A capillary electrophoresis showed 303 poly C, 16184 poly C and 514 CA repeats length heteroplasmy in analyzed 26 patients. Length heteroplasmy for 303 poly C displayed ten types of patterns: 7CT6C + 8CT6C (38.5%), 15C + 16C + 17C (19.2%), 15C + 16C + 17C + 18C (11.5%), 8CT6C + 9CT6C (7.7%), 8CT6C (3.8%), 9CT6C + 10CT6C (3.8%), 15C + 16C (3.8%), 14C + 15C + 16C (3.8%), 16C + 17C + 18C (3.8%), and 14C + 15C + 16C + 17C (3.8%) (Table 5 and Fig. 3). Length heteroplasmy for 514 CA repeats showed 5 (CA) repeats (61.5%), 4 (CA) repeats (34.6%), and 7 (CA) repeats (3.8%) (Table 5). Length heteroplasmy for 16184 poly C showed five types of patterns: 5CT4C (65.4%), 9C + 10C + 11C + 12C (19.2%), 8C + 9C + 10C + 11C (7.7%), 6CT5C (3.8%), and 9C + 10C (3.8%) (Table 5 and Fig. 4). Specimens that showed dissimilarities in length heteroplasmy between patient peripheral blood and urine were 76.9% (20 cases) for 303 poly C or 16184 poly C, 69.2% (18 cases) for 303 poly C, 15.4% (4 cases) for 16184 poly C, and 0% (0 cases) for 514 CA repeats (Tables 1 and 5).

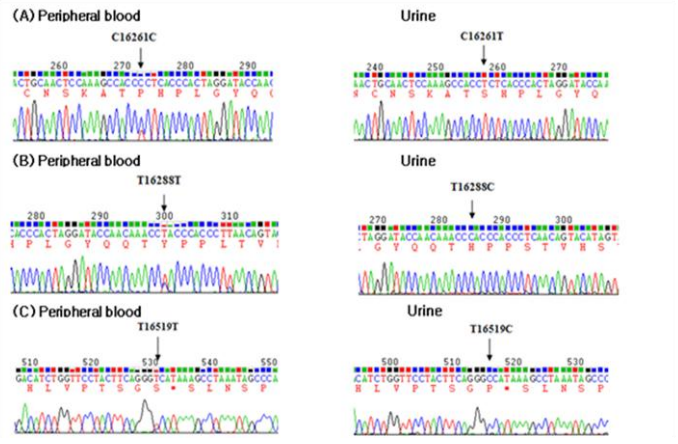

Figure 2. Sequencing chromatogram of mtDNA mutations. Heteroplasmic mtDNA mutations were observed in control region from patient No. 41. These heteroplasmic mutations were not found in the corresponding peripheral blood samples.

Table 5. mtDNA length heteroplasmic mutations in urine and corresponding blood samples

Patient No.	Heteroplasmic mutation	Specimen	Markers for mtDNA length heteroplasmies		
			303 polyC ract	16184 polyC tract	514 CA repeat
1	-	Urine	9CT6C + 10CT6C	5CT4C	$(CA)_5$ repeat
		PB	9CT6C + 10CT6C	5CT4C	$(CA)_5$ repeat
2	+	Urine	15C, 16C, 17C, 18C	5CT4C	$(CA)_4$ repeat
		PB	16C, 17C, 18C, 19C	5CT4C	$(CA)_4$ repeat
3	+	Urine	7CT6C + 8CT6C	9C, 10C, 11C, 12C	$(CA)_4$ repeat
		PB	7CT6C	9C, 10C, 11C, 12C	$(CA)_4$ repeat
4	+	Urine	7CT6C + 8CT6C	9C, 10C, 11C, 12C	$(CA)_5$ repeat
		PB	7CT6C	9C, 10C, 11C, 12C	$(CA)_5$ repeat
6	-	Urine	7CT6C + 8CT6C	5CT4C	$(CA)_5$ repeat
		PB	7CT6C + 8CT6C	5CT4C	$(CA)_5$ repeat
7	+	Urine	14C, 15C, 16C, 17C	5CT4C	$(CA)_4$ repeat
		PB	15C, 16C, 17C	5CT4C	$(CA)_4$ repeat
8	-	Urine	7CT6C + 8CT6C	5CT4C	$(CA)_4$ repeat
		PB	7CT6C + 8CT6C	5CT4C	$(CA)_4$ repeat
11	-	Urine	7CT6C + 8CT6C	5CT4C	$(CA)_5$ repeat
		PB	7CT6C + 8CT6C	5CT4C	$(CA)_5$ repeat
12	+	Urine	16C, 17C, 18C	8C, 9C, 10C, 11C	$(CA)_4$ repeat
		PB	16C, 17C, 18C	5CT4C	$(CA)_4$ repeat
14	+	Urine	15C, 16C, 17C	8C, 9C, 10C, 11C	$(CA)_4$ repeat
		PB	16C, 17C, 18C	9C, 10C, 11C, 12C	$(CA)_4$ repeat

(To be continued)

Table 5. mtDNA length heteroplasmic mutations in urine and corresponding blood samples

Patient No.	Heteroplasmic mutation	Specimen	Markers for mtDNA length heteroplasmies		
			303 polyC tract	16184 polyC tract	514 CA repeat
16	+	Urine	15C, 16C, 17C, 18C	9C, 10C, 11C, 12C	$(CA)_7$ repeat
		PB	16C, 17C, 18C	9C, 10C, 11C, 12C	$(CA)_7$ repeat
18	+	Urine	15C, 16C	5CT4C	$(CA)_4$ repeat
		PB	15C, 16C, 17C	5CT4C	$(CA)_4$ repeat
21	+	Urine	15C, 16C, 17C	9C, 10C, 11C, 12C	$(CA)_5$ repeat
		PB	15C, 16C, 17C, 18C	9C, 10C, 11C, 12C	$(CA)_5$ repeat
22	+	Urine	15C, 16C, 17C	5CT4C	$(CA)_4$ repeat
		PB	8CT6C + 9CT6C	5CT4C	$(CA)_4$ repeat
24	+	Urine	15C, 16C, 17C	5CT4C	$(CA)_5$ repeat
		PB	15C, 16C, 17C, 18C	5CT4C	$(CA)_5$ repeat
25	+	Urine	7CT6C + 8CT6C	5CT4C	$(CA)_5$ repeat
		PB	8CT6C + 9CT6C	5CT4C	$(CA)_5$ repeat
26	+	Urine	7CT6C + 8CT6C	9C, 10C	$(CA)_5$ repeat
		PB	7CT6C + 8CT6C	9C, 10C, 11C, 12C	$(CA)_5$ repeat
27	+	Urine	14C, 15C, 16C	5CT4C	$(CA)_5$ repeat
		PB	8CT6C + 9CT6C	5CT4C	$(CA)_5$ repeat
29	-	Urine	7CT6C + 8CT6C	6CT5C	$(CA)_5$ repeat
		PB	7CT6C + 8CT6C	6CT5C	$(CA)_5$ repeat
34	+	Urine	7CT6C + 8CT6C	5CT4C	$(CA)_5$ repeat
		PB	14C, 15C, 16C	5CT4C	$(CA)_5$ repeat

(To be continued)

Table 5. mtDNA length heteroplasmic mutations in urine and corresponding blood samples

Patient No.	Heteroplasmic mutation	Specimen	Markers for mtDNA length heteroplasmies		
			303 polyC ract	16184 polyC tract	514 CA repeat
36	+	Urine	15C, 16C, 17C, 18C	9C, 10C, 11C, 12C	$(CA)_5$ repeat
		PB	15C, 16C, 17C	10C, 11C, 12C	$(CA)_5$ repeat
37	-	Urine	8CT6C + 9CT6C	5CT4C	$(CA)_5$ repeat
		PB	8CT6C + 9CT6C	5CT4C	$(CA)_5$ repeat
38	+	Urine	8CT6C	5CT4C	$(CA)_5$ repeat
		PB	7CT6C + 8CT6C	5CT4C	$(CA)_5$ repeat
39	+	Urine	8CT6C + 9CT6C	5CT4C	$(CA)_5$ repeat
		PB	8CT6C	5CT4C	$(CA)_5$ repeat
40	+	Urine	15C, 16C, 17C	5CT4C	$(CA)_4$ repeat
		PB	8CT6C + 9CT6C	5CT4C	$(CA)_4$ repeat
41	+	Urine	7CT6C + 8CT6C	5CT4C	$(CA)_5$ repeat
		PB	7CT6C	5CT4C	$(CA)_5$ repeat

Abbreviations: PB, peripheral blood; 303 polyC tract, ^{303}CCCCCCCTCCCCC^{315}; 16184 polyC tract, ^{16184}CCCCCTCCCC^{16193}; 514 CA repeat, ^{514}CACACACACA^{523}.

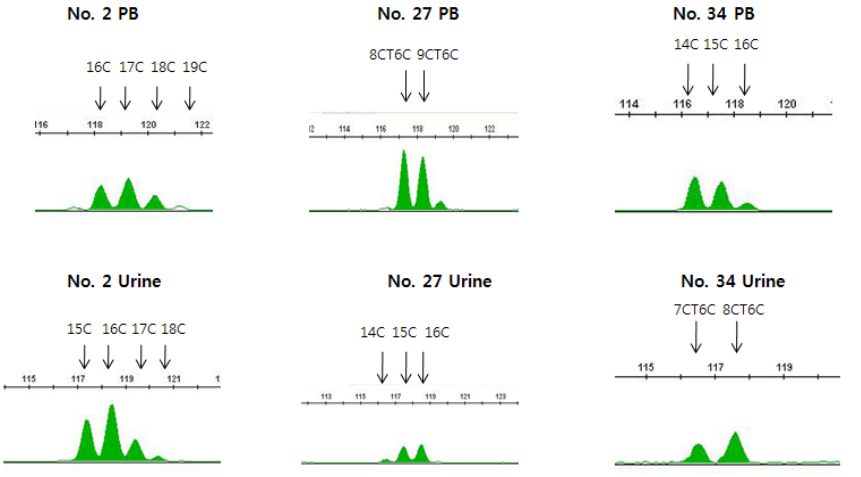

Figure 3. mtDNA length heteroplasmic mutations in the 303 polyC tract. A gene scan analysis of the polyC tract at nucleotide position (np) 303-315 demonstrated typical length heteroplasmic mutations from patient no. 2, 27, and 34, respectively.

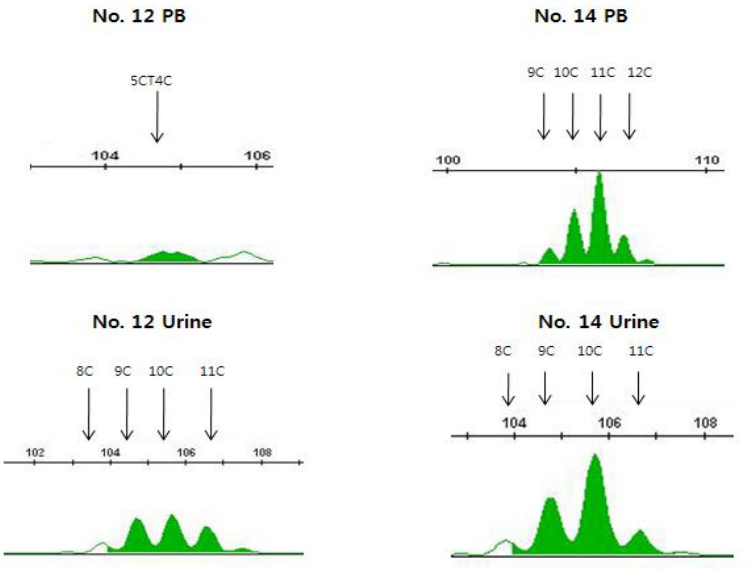

Figure 4. mtDNA length heteroplasmic mutations in the 16184 polyC tract. A gene scan analysis of the polyC tract at nucleotide position (np) 16184-16193 demonstrated typical length heteroplasmic mutations from patient no. 12 and 14, respectively.

3. mtDNA copy number

Average mtDNA copy numbers in urine and peripheral blood cells (83.45 ± 60.36 and 39.0 ± 24.38, respectively) differed significantly (P < 0.001) in 21 of the 26 patients (80.8%) (Table 1, Figure 5). Average mtDNA copy numbers in the urine samples from patients with high grade and low grade tumors (81.83 ± 67.78 and 86.49 ± 46.69, respectively) did not differ significantly (P = 0.589) (Figure 6).

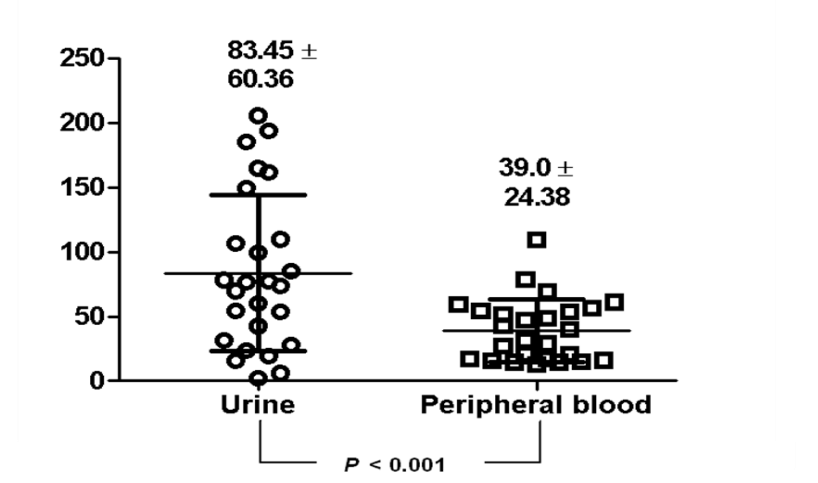

Figure 5. Result of mtDNA copy number in patients with bladder cancer (n=26). Data were presented as mean ± SD. The non-parametric Mann-Whitney test was used for the determination of statistical difference in the mtDNA copy number between two specimens (urine and peripheral blood cells).

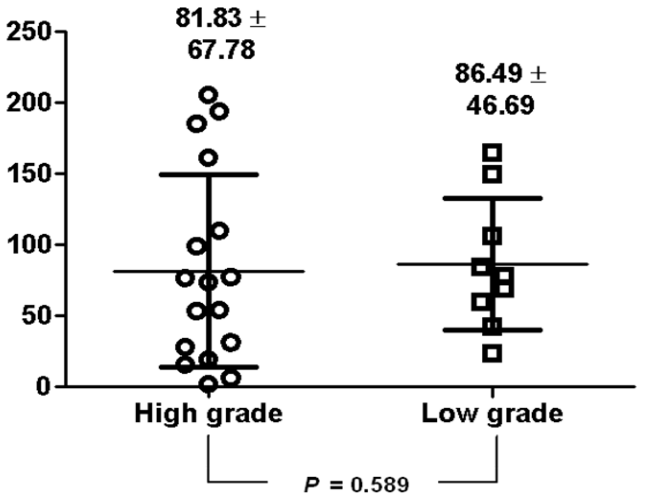

Figure 6. Result of mtDNA copy number in patients with bladder cancer by histological grade (n=26). Data were presented as mean ± SD. The non-parametric Mann-Whitney test was used for the determination of statistical difference in the mtDNA copy number between two grade groups (low grade and high grade).

4. Combined analysis of FISH and mtDNA alteration

FISH and mtDNA alteration were analyzed in 26 patients with urothelial carcinoma (Table 6). Of the 26 patients, twenty-three were positive for both analysis, two were only positive for mtDNA alteration, and one was only positive for FISH analysis.

Table 6. Combined analysis of FISH and mtDNA alteration

Total (n=26)		mtDNA alterations	
		Positive	Negative
FISH analysis	Positive	23	1
	Negative	2	0

mtDNA alterations mean increased mtDNA copy number in urine or heteroplasmic mtDNA mutation.

IV. DISCUSSION

In this small prospective study, the FISH assay showed the highest sensitivity in detecting urothelial carcinoma. Of the low grade tumors, FISH detected 91.0%, while cytology detected only 36.4%. Highly sensitive tests for urothelial carcinoma could reduce risk for progression to potentially fatal muscle-invasive disease by reducing the proportion of false negatives.[24]

Urothelial carcinoma continues to be associated with at least modest morbidity and mortality despite advances in the treatment of superficial disease with intravesical immunotherapy, and advanced disease with systemic chemotherapy. Delay in diagnosis is common and adversely affects patient outcome. The report of Messing et al. of absent late stage disease in patients diagnosed with a screening test indicates that early diagnosis and treatment do improve outcome.[55]

Despite the long-established use of cytology to diagnose urothelial carcinoma, Halling et al. found that published values for the sensitivity of this method range from 11 to 60%.[24] Cytology alone cannot reliably exclude cancer.

The United States Food and Drug Administration (FDA) has approved a nuclear matrix protein (NMP-22) test kit, an enzyme-linked immunoassay for this nuclear mitotic apparatus protein in voided urine, to

detect occult cells or rapidly recurring bladder cancer after transuretheral resection.[56] The sensitivity of NMP22 ranges from 48% to 90%, while specificity ranges from 85% to 87%.[57] In this study we found sensitivity greater than 90% for the NMP-22 assay (Table 3), and higher than that of all other assays for high grade tumors. Unfortunately, this test gave a positive result in three patients without evidence of urothelial carcinoma. The low specificity of the NMP-22 assay could lead to unnecessary cystoscopic examinations or other invasive procedures in patients with false-positive results.

The mtDNA is potentially exposed to intense oxidative stress from reactive oxygen species (ROS) released through electron transfer. Alterations of mtDNA may further increase ROS production, causing cellular injury and inflammation, as well as apoptosis, which promote carcinogenesis.[41, 58] These susceptibilities led us to search for mitochondrial alterations in urinary epithelial cells from patients with urothelial carcinoma, using peripheral blood cell mitochondria for comparison.

In affected tissues of patients with inherited mitochondrial disorders, mutations are found mainly in the tRNA genes and coding regions of mtDNA.[59] The noncoding control region (D-loop) contains the elements that regulate mtDNA production and the highly variable (HV) region, a 'hot spot' for somatic mutation in various human cancers.[60] The mtDNA mutations detected in lesions but not in normal tissues can be

classified as pathological heteroplasmic mtDNA mutations.[61] These cancer-specific mutations may impair mitochondrial function, including the apoptotic signaling system associated with cytochrome c release.[62] In the present study, we observed urothelial carcinoma-specific mtDNA heteroplasmic mutations in 20 of the 26 patients (76.9%). The published mutation rate of the D-loop region of mtDNA in patients with urothelial carcinoma is about 80%.[40]

Mitochondria have their own independent genome, but the nuclear genome controls its replication and degradation.[61] Genomic alterations in diseased tissue will therefore perturb the mitochondrial function, and conversely, mtDNA dysfunction by any cause (e.g., ROS) may impair signaling between mitochondria and the nucleus.[62] In this study, an increase in mtDNA copy number and heteroplasmic mtDNA mutation represent mtDNA alteration, and a positive FISH result represents genomic alteration.

In neurodegenerative disease and hepatocellular carcinoma, mtDNA copy numbers may decline in affected tissues,[60, 63] the apparent consequence of mutation near the replication origin. mtDNA depletion may in turn induce a deficiency in oxidative phosphorylation and enhance production of ROS, and even render cancer cells more resistant to apoptosis or chemotherapy.[43] On the other hand, mtDNA copy numbers may actually increase when aging or certain cancers block ATP synthesis.[47,

[50, 64] This may represent a compensatory response to reduced respiratory function. In this study, mtDNA copy numbers in urine samples of patients with urothelial carcinoma were approximately three times higher than those in the corresponding peripheral blood samples. In patients with advanced head and neck cancers, Jiang et al.[47] also found increased salivary mtDNA. In our study, however, average mtDNA copy numbers in samples from patients with high grade and low grade tumors did not differ significantly. Migaldi et al.[65] reported that DNA microsatellite alterations in patients with urothelial cancer was not associated with tumor grade nor with tumor stage. Unfortunately, we didn't study mtDNA copy number and mtDNA sequence in patients with benign urothelial disease. Further study will be required to clinical application of mtDNA alteration.

In summary, the FISH assay showed the highest sensitivity for detecting low grade urothelial carcionoma. The frequency of heteroplasmic mtDNA mutations in the D-loop region in patients with cancer was about 80% in our study. The mtDNA copy numbers in urinary cell samples were higher than copy numbers in the corresponding peripheral blood samples. How these tests should be used to diagnose and manage urothelial carcinoma requires further study. A complete analysis of the mtDNA will be required to characterize new heteroplasmic mutations, determine their frequency, and establish their biological and prognostic significance.

V. CONCLUSION

The findings of this study provided experimental evidence that the FISH assay showed the highest sensitivity for detecting low grade urothelial carcinoma. heteroplasmic mtDNA mutations in urine frequently occur in the mtDNA control region as base substitution and length heteroplasmy. These mtDNA alterations might further impair a respiratory chain defect and increase the mtDNA copy number to compensate for the deficiency in ATP. This report further supports the significance of genetic alteration in urothelial carcinoma and the clinical utility of the FISH, mtDNA quantitation real-time PCR, mtDNA sequencing, and capillary electrophoresis for this purpose.

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ABSTRACT(IN KOREAN)

조기 방광암 검출을 위한 형광직접보합법, 사립체 핵산 정량 및 염기서열 분석

< 지도교수 최 종 락 >

연세대학교 대학원 의학과

유 종 하

본 연구는 방광암의 조기진단을 위해 요세포검사, NMP-22검사, 형광직접보합법의 민감도 비교와, 사립체 핵산의 이상을 분석하였다. 임상적으로 방광암이 의심되는 41명의 환자를 대상으로 소변검체와 그 중 26명의 말초혈액검체를 채취하였다. 형광직접보합법과 NMP-22검사의 민감도는 92.1%이었으며 요세포 검사의 민감도는 60.5%이었다. 낮은 병기의 환자군에서 NMP-22검사와 형광직접보합법의 민감도는 요세포검사보다

높았으나, 높은 병기의 환자군에서는 세 가지 방법 모두 약 90%의 민감도를 나타냈다. 포괄적으로, 형광직접보합법과 NMP-22검사 또는 형광직접보합법과 요세포검사를 결합하였을 경우 97.4%의 민감도를 보였으나 요세포검사와 NMP-22검사를 결합하였을 경우 민감도는 92.1%이었다. 낮은 병기의 환자군에서 세 가지 검사법을 결합할 경우 민감도는 약 90%이나, 높은 병기의 환자군에서는 약 100%이었다. 사립체 핵산의 조절부위인 D-loop 유전자위치에서, 특징적인 사립체 핵산 염기 치환 변이가 한 명의 환자에서 관찰되었으며, 20명의 환자에서 303 poly C tract과 16184 poly C tract에서 사립체 핵산의 length heteroplasmy가 관찰되었다. 즉, 방광암 특이 사립체 핵산 변이는 26명의 대상환자중 20명의 환자에서 검출되었다(76.9%). 소변검체와 말초혈액검체에서의 사립체 핵산 정량값은 통계학적으로 유의한 차이가 있었다(83.45 ± 60.36 과 39.0 ± 24.38, $P < 0.001$). 낮은 병기와 높은 병기의 환자군에 따른 소변검체의 사랍체 핵산 정량값은 통계학적으로 유의한 차이가 없었다(81.83 ± 67.78 and 86.49 ± 46.69, $P = 0.589$). 결론적으로 형광직접보합법은 조기 방광암 검출에 높은

민감도를 나타냈으며, D-loop유전자위치의 사립체 핵산 변이의 빈도는 약 80%정도 이었다. 사립체 핵산 정량값은 말초혈액검체에 비해 소변검체에서 높게 관찰되었다. 본 연구는 형광직접보합법, 사립체 핵산 정량분석을 위한 실시간 중합효소반응, 사립체 핵산 변이검출을 위한 염기서열분석 및 모세관 전기영동 분석을 통해 방광암에서의 유전적 변화의 중요성을 증명하였다.

핵심되는 말: 방광암, 형광직접보합법, 사립체 핵산 정량, 사립체 핵산 변이