Quantitative study on the correlation between p53 gene mutation and its expression in endometrial carcinoma cell lines

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Summary

Mutant p53 protein is not degradated but accumulates in the nuclei. However, the relation between p53 gene mutation and quantity of p53 protein has not been clarified yet in endometrial carcinoma. We investigated the correlation between p53 gene mutation and its protein expression quantitatively using 11 cell lines of endometrial adenocarcinoma, endometrioid type and two serous-type cell lines. To examine p53 mutation, PCR-SSCP analysis in exon 5 to 8 and direct sequence were carried out. p53 expression was determined by immunocytochemistry and immunoblotting. The percentage of positive staining in the nuclei by immunocytochemistry was calculated as a labeling index (LI). The amount of p53 detected by immunoblotting was expressed as a comparative value of Ishikawa cells. Point mutation of p53 gene was detected in four of 11 (36.4%) cell lines of endometrioid adenocarcinoma, and all two of serous adenocarcinoma. There was a significant positive correlation between p53 LI and p53 values. The LI and the values of p53 were significantly higher in the mutant group than the wild one, thus showing a quantitative correlation between p53 protein expression and p53 gene mutation in endometrial carcinoma cell lines. It is plausible that immunohistochemical analysis of p53 could be qualified to be a convenient indicator of p53 gene mutation on clinical materials, if p53 LI is more than 40 (M-SD in mutant p53).

Key words: p53; Endometrial carcinoma; Mutation; Immunoblotting; Immunocytochemistry; HEC-1-A; Ishikawa.

Introduction

Tumor suppressor gene p53 that localizes in the short arm of chromosome 17 [1] plays an important role in cellular events including cell cycle arrest, apoptosis induction and DNA repair. Alteration in the p53 gene is a common feature of many solid cancers, and is considered to be a critical step in tumorigenesis. Most mutations of p53 genes in solid tumors occur in the hotspots within conserved areas from exon 5 to 8 [2]. It is said that mutant p53 protein is not degradated by murine double minute 2 (MDM2) that is upregulated by wild p53 and also promotes its degradation. Therefore, the half-life of p53 produced by the mutant gene is much longer than that by the wild gene, and p53 accumulates in the nuclei of cells [3, 4].

In endometrial carcinoma, p53 gene mutation and its overexpression is correlated with clinicopathological factors such as histological grade and stage, and is confirmed to be an indicator of prognosis [5-10]. The relation between p53 overexpresion by immunohistochemistry and p53 gene mutation in endometrial carcinoma has been reported in the literature [10-12]. Kohler *et al.* reported that all of five cases with p53 overexpression were found to have single point mutations either in exons 6-8 of the p53 gene, and all of the three cases that did not demonstrate p53 overexpression preserved wild type sequence of p53 gene [8]. In contrast, p53 gene mutation was not detected in 18 cases of endometrial carcinoma, although immunohistochemical staining was positive for p53 [13]. However, the relationship between p53 gene mutation and p53 LI by immunocytochemistry or its quantity by immunoblotting has not been investigated in detail in endometrial carcinoma yet. In this study, we investigated quantitative correlations between the gene mutation and protein level in endometrial carcinoma cell lines in vitro by using immunoblotting and immunocytochemistry.

Materials and Methods

Cells and cellular preparation

Thirteen cell lines derived from endometrial carcinoma [14], including 11 with endometrioid adenocarcinoma and two with serous adenocarcinoma (Table 1) are the materials of the study. All cell lines were cultured in a monolayer with Eagle's minimum essential medium supplemented by 15% fetal bovine serum in a 5% CO_2 and air atmosphere. The cells grown in a full sheet were washed with phosphate buffered- saline (PBS, 0.0075M, pH7.4) three times, and harvested after being treated with 0.1% tripsin and 0.02% ethylendiaminetetraacetic acid • 2Na • $2H_2O$ (EDTA). One part of the collected cells was cultured in Lab-tek chambers for immunocytochemistry, and rest of them were prepared for DNA analysis or immunoblotting analysis.

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Table 1. — *p53 gene mutation, p53 LI and value by immunoblotting in endometrial carcinoma cell lines.*

Cell line	Origin[a]	PCR-SSCP-direct sequence exon	codon	mutation	amino acid	LI [%][b]	Mean ± SD	Immunoblotting	Mean value ± SD
HEC-1-A	G2	7	248	CGG-CAG	Arg-Gln	63.4	67.9 ± 25.5[c]	6.0 ± 1.7	3.3 ± 1.7[d]
HEC-59	G2	8	273	CGT-CAT	Arg-His	48.8		1.8 ± 0.6	
HEC-116	G2	7	248	CGG-CAG	Arg-Gln	45.7		2.3 ± 0.4	
HEC-251	G3	5	154	GGC-GAC	Gly-Asp	49.7		2.0 ± 0.6	
HEC-180	Serous	6	195	ATC-AAC	Ile-Asn	100.0		3.5 ± 0.2	
HEC-155	Serous	8	286	GAA-AAA	Glu-Lys	99.7		4.2 ± 0.3	
Ishikawa	G1	wild				34.9	22.6 ± 12.5[c]	1	0.7 ± 0.4[d]
HEC-6	AA	wild				28.5		1.1 ± 0.2	
HEC-88	AA	wild				1.2		0.3 ± 0.0	
HEC-108	G3	wild				33.1		1.1 ± 0.3	
HEC-151	G2	wild				17.5		0.3 ± 0.1	
HEC-265	G1	wild				20.4		0.6 ± 0.2	
HEC-50B	G3	6 (int6)		42 bp deletion		0		0	

a: histologic type and grade of original tumor.
G1: endometrioid adenocarcinoma, grade 1, G2: endometrioid adenocarcinoma, grade 2.
G2: endometrioid adenocarcinoma, grade 3, AA: adenoacanthoma, Serous; serous adenocarcinoma.
b: LI (labeling index): percentage of positive cells for p53 examined by immunocytochemistry.
c, d: p = 0.002 (Mann-Whitney U-test).

DNA extraction and polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis

DNA of endometrial carcinoma cells was extracted by a proteinase K digestion and phenol chloroform method. Oligonucleotide primer pairs at exons 5-8 of p53 gene were as follows [15]:
exon 5 (sense, antisense): 5'-TGTTCACTTGTGCCCTGACT-3', 5'-CAGCCCTGTCGTCTCTCCAG-3';
exon6:5'-TGGTTGCCCAGGGTCCCCAG-3', 5'-GGAGGGC-CACTGACAACCA-3';
exon7:5'-CTTACCACAGGTCTCCCCAA-3', 5'-AGGGG-TCAGCGGCAAGCAGA-3';
exon 8:5'-TTGGGAGTAGATGGAGCCT-3', 5'-AGTGTTA-GACTGGAAACTTT-3'.

The 5'-ends of primers (100 pmol) were labeled with [γ-^{32}P]ATP (50 pmol, 7000 Ci/mmol) and T4 polynucleotide kinase (5U) in 10 µl of 50 mM Tris-HCl (pH 8.3), 10 mM $MgCl_2$ and 5 mM dithiothreitol at 37°C for 30 minutes. PCR was carried out in a total volume of 10 µl containing 100 ng of DNA template at final concentrations of 10 mM Tris-HCl (pH8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% gelatin, 0.2% formamide, 0.5 mM labeled primers and 1 unit of Taq polymerase (Takara, Japan). The mixture for PCR was overlaid with one drop of light mineral oil and placed in a Program Control System PC-700 (ASTEC), and then, subjected to one cycle at 95°C for three minutes and 35 cycles of amplification at 95°C for 30 seconds, followed by annealing at either temperature 55°C (exon 7, 8) or 60°C (exon 5, 6) for 30 seconds and processing for extention at 72°C for one minute. Following the last cycle, tubes were incubated further for five minutes at 72°C. After PCR-amplification, the mixture was heated at 95°C for five minutes with 10 µl formamide dye mixture (95% formamide, 20mM EDTA, 0.05% xylene cyanol and 0.05% bromophenol blue). Then, 2.5 µl of the preparation was applied to 5% polyacrylamide gel containing 0.5 x Tris-borate, EDTA buffer with 5% glycerol. Electophoresis was performed at 40 watts for three hours cooling with a fan. The gel was dried at 80°C for 45 minutes and exposed to Kodak XAR film at room temperature for 15 minutes to 24 hours with an intensifying screen. As a normal control, DNA extracted from lymphocytes of a normal woman whose menstrual cycle was regular was used.

Sequence analysis

Positive samples showing aberrant mobility shifts or bands by PCR-SSCP analysis were amplified as described above and loaded onto a 2% agarose gel. The amplified bands were cut and purified with SUPREC-01 (TAKARA, Japan). The purified DNAs were reamplified by asymmertrical PCR with a reverse primer and sequenced with 5'-end labeled forward primer using the Sequence-Pro Kit (TOYOBO, Japan) according to the manufacturer's instructions. Gel electrophoresis and autoradiography were then performed as described above or sequence analysis was carried out using DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech, Japan) and ABI Prism 377 auto sequencer (Perkin-Elmer, USA), according to the manufacturer's instructions.

Immunocytochemistry

Immunocytochemistry was performed on 95% ethanol-fixed cells that had been cultured in a full sheet in a Lub-tek chamber,

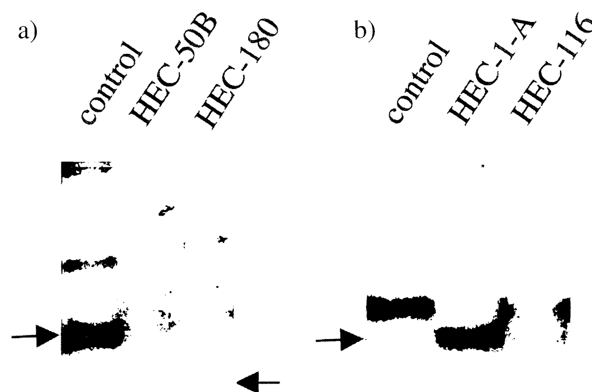

Figure 1. — p53 PCR-SSCP in endometrial carcinoma cell lines. HEC-50B and HEC-180 showing aberrant mobility shift (arrows) in exon 6 (a), and HEC-1-A and HEC-116 showing aberrant mobility shift (arrows) in exon 7 (b).

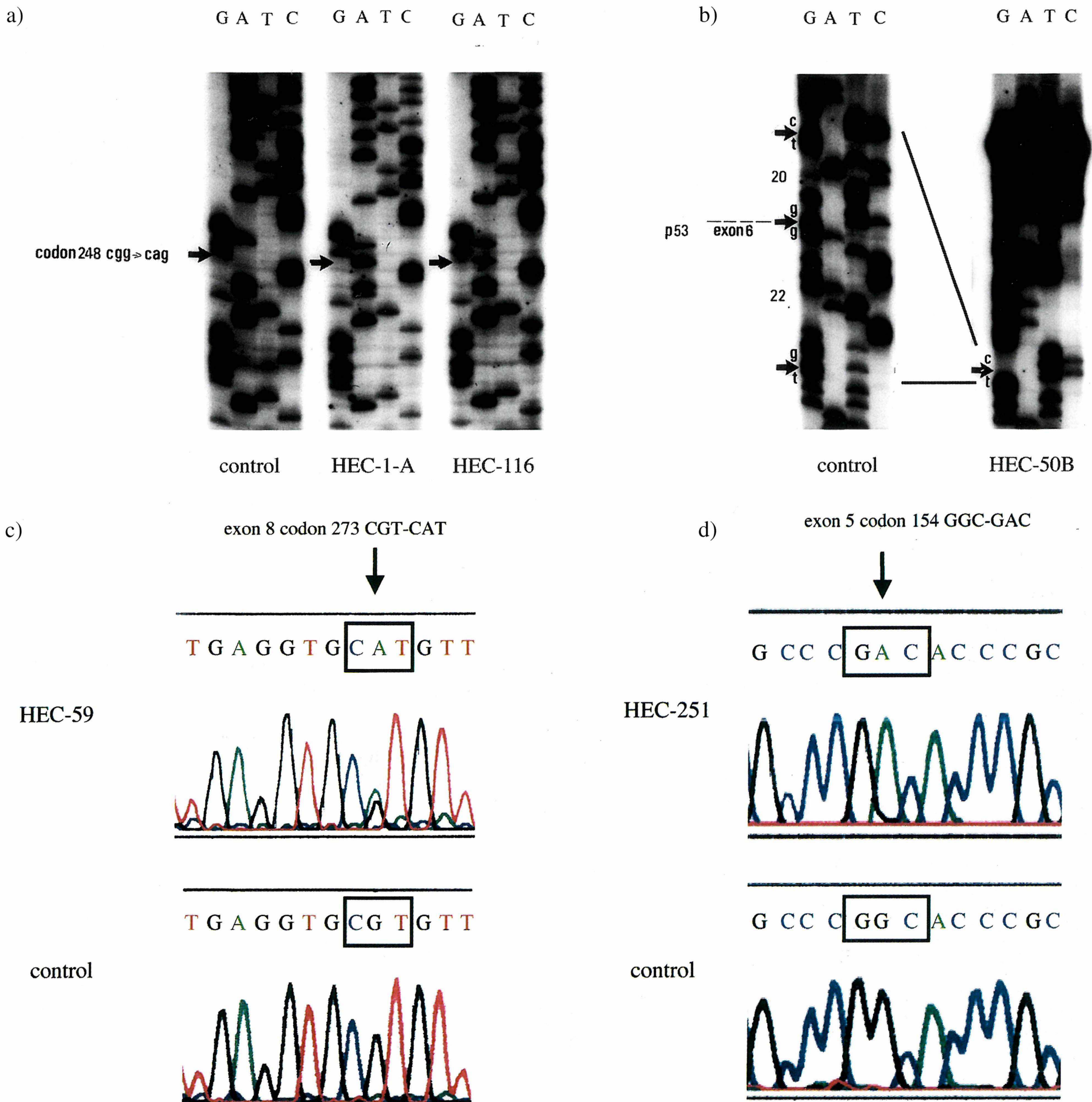

Figure 2. — p53 sequence analysis in endometrial carcinoma cell lines detecting point mutation (CGG to CAG) at codon 248 in exon 7 in HEC-1-A and HEC-116 (a), 42bp deletion in exon 6 to intron 6 of HEC-50B (b), point mutation (CGT to CAT) at codon 273 in exon 8 in HEC-59 and point mutation (GGC to GAC) at codon 154 in exon 5 in HEC-251.

using a labeled streptavidin-biotin method (DAKO, Japan). In brief, the specimens were incubated with 0.3% H_2O_2 and then 2% normal swine serum. Subsequently, they were incubated with anti-p53 mouse monoclonal antibody (DO7, Novocastra, UK, 1:80 dilution) overnight at 4°C, rinsed with PBS and incubated with biotinylated 2nd antibody for 30 min. The procedure was followed by being reacted in streptavidin conjucated horseradish peroxidase and diaminobenzidine. Nuclear counterstaining was done using Mayer's haematoxylin solution. As negative controls, cells were stained by replacing the primary antibody with mouse serum. The labeling index (LI) was expressed as the percentage of positive staining nuclei, after calculating 1000-1800 cells per cell line in high power fields of a microscope.

Immunoblotting

Endometrial carcinoma cells were harvested by dispersing the cells with 0.1% tripsin and 0.02% EDTA, and washed and centrifugated three times with cold PBS. Then, the cells were lysed in Laemmli's sample buffer using handy micro-homogenizer (Nition Irikakikai seisakusho, Japan). Ten µl (2 µg/µl) of the lysates were heated at 100°C for three minutes and loaded onto 10% sodium lauryl sulfate-polyacrylamide gel. Subse-

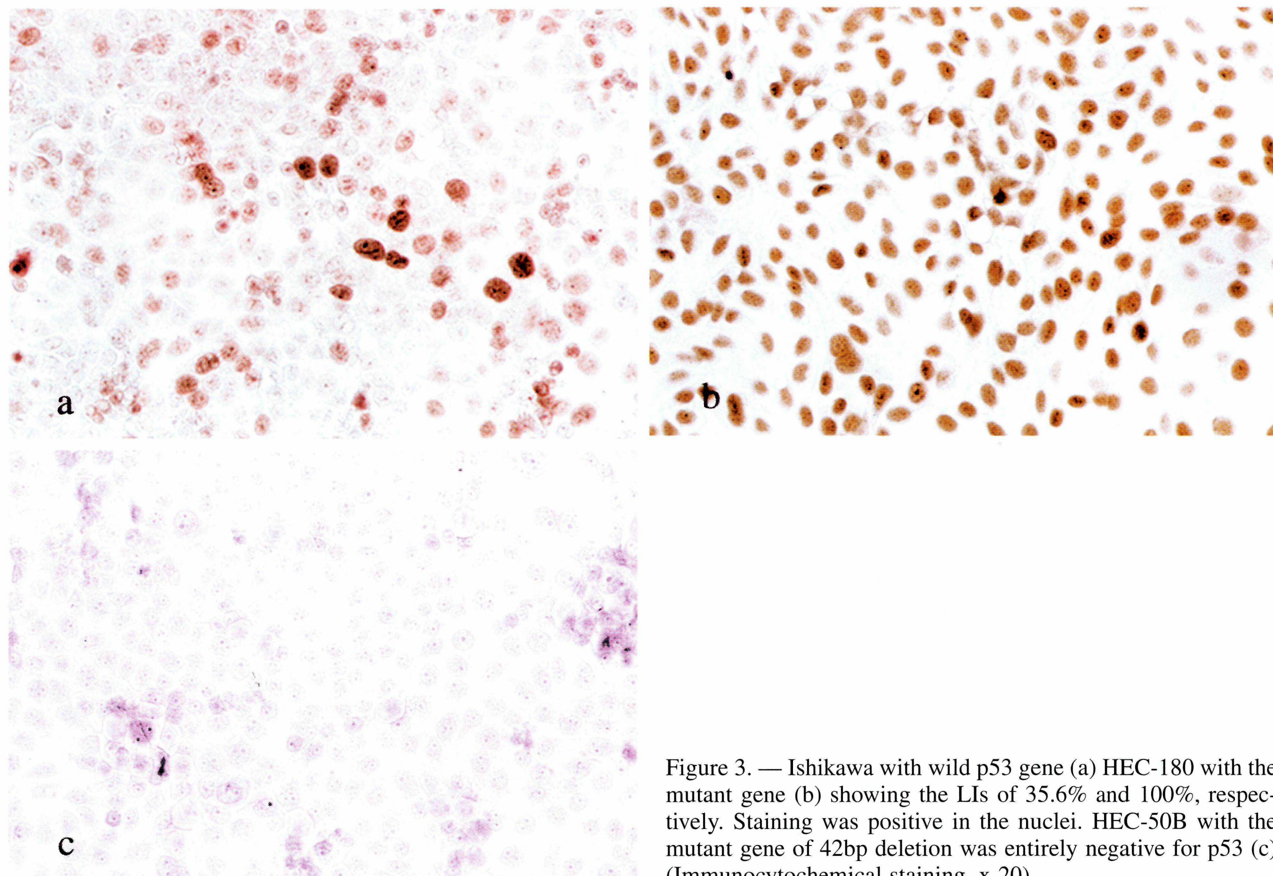

Figure 3. — Ishikawa with wild p53 gene (a) HEC-180 with the mutant gene (b) showing the LIs of 35.6% and 100%, respectively. Staining was positive in the nuclei. HEC-50B with the mutant gene of 42bp deletion was entirely negative for p53 (c) (Immunocytochemical staining, x 20).

quently, they were transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore, USA). The membrane was incubated with Block Ace (Dainihon Seiyaku, Japan) and then with DO-7 (1:1000 dilution) for one hour, and washed with PBS containing 0.3% Tween 20. This was followed by incubation with horseradish peroxidase-linked mouse IgG, (1:2000 dilution) for one hour, and washed with PBS containing 0.3% Tween 20. A band was detected being exposed on a X-ray film by an enhanced chemiluminescence technique (Amersham Pharmacia Biotech, Japan). The p53 protein was measured by densitometry using NIH (National Institute of Health) Image, and the amount was expressed as a relative value based on the

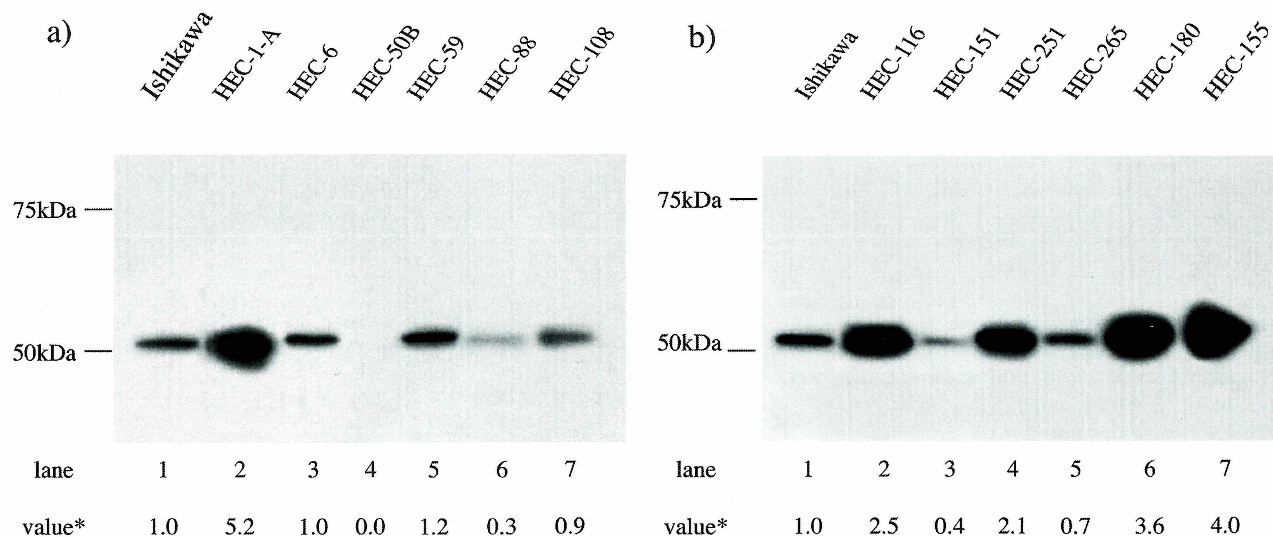

Figure 4. — Immunoblotting analysis of p53 in 13 endometrial carcinoma cell lines showing individual patterns (a, b).
*value: Values of densitometric analysis are expressed based on that of Ishikawa cells.

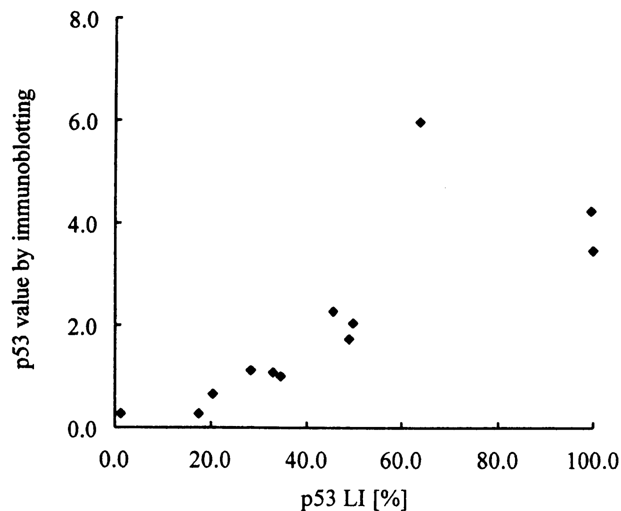

Figure 5. — The correlation between p53 LI by immunocytochemistry and p53 values by immunoblotting in 12 endometrial carcinoma cell lines (Spearman's rank correlation test; p = 0.0022, r_s = 0.923).

amount of Ishikawa cells in the same membrane which had been realized to have wild type p53 gene. One value corresponds to the p53 in 2.0 µg protein of Ishikawa cells. Immunoblotting analysis was performed in triplicate and the values were expressed as mean ± standard deviation (M ± SD).

Results

p53 gene mutation

Mutation of p53 gene was detected in five of 11 (45.5%) cell lines of endometrioid adenocarcinoma, and all two of serous adenocarcinoma. HEC-50B showed 42 base pair (bp) deletions in exon 6 to intron 6 (Figures 1, 2), and the other six cell lines revealed missense point mutation (transition in 5, transversion in 1).

Immunocytochemical analysis of p53

Immunoreaction of p53 was located in the nuclei of the carcinoma cells (Figures 3-a, b). HEC-50B was entirely negative for p53 (Figure 3-c). Except for HEC-50B, average LI of the cell lines with mutant p53 was 67.9 ± 25.5% (M ± SD), whereas that with wild type was 22.6 ± 12.5%. p53 LI was significantly higher in endometrial carcinoma cells with mutant p53 than those with the wild one (Mann-Whitney U-test, p = 0.002, t-test, p = 0.005, Table 1).

Immunoblotting analysis of p53

Expression patterns of p53 in 11 cell lines of endometrioid adenocarcinoma and two of serous adenocarcinoma are shown in Figure 4 and these immunoblotting values are shown in Table 1. HEC-50B was only negative for p53. Except for HEC-50B, the average value of the cells with mutant p53 was 3.3 ± 1.7, whereas that with wild type was 0.7 ± 0.4. The values of p53 expression were significantly higher in mutant cells than in wild ones (Mann-Whitney U-test, p = 0.002, t-test, p = 0.011, Table 1). There was a significant quantitative correlation between p53 LI by immunocytochemistry and the p53 value by immunoblotting (Spearman's rank correlation test, $p < 0.001$, r_s = 0.923, Figure 5).

Discussion

In the present paper, p53 gene mutation was realized in five of 11 cell lines of endometrioid adenocarcinoma, and all two of serous adenocarcinoma. All mutations were in the sequence specific DNA binding domain. Six of seven (85.7%) gene mutations were one-point mutations, and the mutation in HEC-50B was deletion in exon 6 to intron 6. p53 in HEC-50B was not entirely detected by either immunocytochemistry or immunoblotting. The hot spot of p53 gene point mutation in endometrial carcinoma was reported in codon 248 [2], in which two of seven (28.6%) mutations were located in this study. In the study done by using lung cancer cell lines [16], all of the high p53 expressers had missense mutations in exons 5-8, whereas low p53 expressers showed deletion, splicing, nonsense, and missense outside of exons 5-8. From these data, Bodner *et al.* [16] concluded that p53 expression in tumor cells is dependent upon the type of mutation of the p53 tumor-suppressor gene. In the present study on endometrial carcinoma cells, mutations found to show high p53 expression were missens in all six cell lines.

Except for HEC-50B, the average LI of the endometrial carcinoma cells with mutant p53 was significantly higher than that with wild type. The average p53 value of the cells with mutant p53 measured by immunoblotting was also significantly higher than that with wild one. p53 LI by immunocytochemistry was correlated significantly with the value by immunoblotting, and was higher in the endometrial carcinoma cells with mutant type than those with the wild type. More than 40% (M-SD) of LI by immunocytochemistry and more than 1.5 (M-SD) of p53 value by immunoblotting seemed to indicate p53 gene mutations. In addition, as p53 LI by immunocytochemistry was correlated with p53 values by immunoblotting, it is suggested that immunohistochemical analysis of p53 on clinical materials could be a good and convenient indicator of p53 gene mutations, if appropriate LI is adopted for cut-off.

p53 overexpression by immunohistochemistry in endometrial carcinoma has been compared with p53 gene mutation [8, 10, 13]. However, the results are not conclusive. Stewart *et al.* [13] reported by using a microdissection technique that p53 gene mutation was not detected although immunohistochemical staining was positive for p53. This p53 gene analysis, however, was performed microdissecting positive areas that were mostly focal in staining, of which cases were seemingly in those with low p53 LI.

In ovarian carcinoma a significant correlation between positive immunohistochemical staining p53 gene muta-

tion has been reported which was defined as positive when and the positive ratio was greater than 5% [17]. However, a false-positive discordance rate (positive immunostaining and no mutation) was recorded in 20.5%. In our study, the average LI of p53 in endometrial carcinoma cells without p53 mutations was 22.6 ± 12.5%. Therefore, at least in endometrial carcinomas the 5% cut-off level seems to be too low. We clarified the quantitative correlation between p53 and p53 gene mutations in the cell lines of endometrial carcinoma. More than 40% of p53 LI in endometrial carcinoma by immunohistochemistry seems to be an indicator for p53 gene mutations by in vitro studies.

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References

[1] Miller C., Mohandas T., Wolf D., Prokocimer M., Rotter V., Koeffler H. P.: "Human p53 gene localized to short arm of chromosome 17". *Nature,* 1986, *319*, 783.

[2] Greenblatt M.S., Bennett W.P., Hollstein M., Harris C.C.: "Mutations in the p53 tumor suppressor gene: Clues to cancer etiology and molecular pathogenesis". *Cancer Res.,* 1994, *54*, 4855.

[3] Ashcroft M., Vousden K.H.: "Regulation of p53 stability". *Oncogene,* 1999, *18*, 7637.

[4] Kubbutat M. H. G., Vousden K. H.: "Keeping an old friend under control: Regulation of p53 stability". *Mol. Med. Today,* 1998, *4,* 250.

[5] Athanassiadou P., Petrakakou E., Liossi A., Nakopoulou L., Zerva C., Dimopoulos A., Athanassiades P.: "Prognostic Significance of p53, bcl-2, and EGFR in Carcinoma of the Endometrium". *Acta cytol.,* 1999, *43,* 1039.

[6] Reinartz J.J., George E., Lindgren B.R., Niehans G.A.: "Expression of p53, transforming growth factor alpha, epidermal growth factor receptor, and correlation with survival and known predictors of survival". *Human Pathol.,* 1994, *25,* 1075.

[7] Enomoto T., Fujita M., Inoue M., Rice J. M., Nakajima R., Tanizawa O., Nomura T.: "Alterations of the p53 tumor suppressor gene and its association with activation of the c-K-ras-2 protooncogene in premalignant and malignant lesions of the human uterin endometrium". *Cancer Res.,* 1993, *53,* 1883.

[8] Kohler M. F., Berchuck A., Davidoff A. M., Humphrey P. A., Dodge R. K., Iglehart J. D. *et al.*: "Overexpression and mutation of p53 in endometrial carcinoma". *Cancer Res.,* 1992, *52,* 1622.

[9] Salvesen H.B., Iversen O.E., Akslen L.A.: "Prognostic significance of angiogenesis and Ki-67, p53, and p21 expression: A population-based endometrial carcinoma study". *J. Clin. Oncol.,* 1999, *17,* 1382.

[10] Lax S.F., Kendall B., Tashiro H., Slebos R. J. C., Ellenson L. H.: "The frequency of p53, K-ras mutations, and microsatellite instability differs in uterine endometrioid and serous carcinoma evidence of distinct molecular genetic pathways". *Cancer,* 2000, *88,* 814.

[11] Ito K., Sasano H., Matunaga G., Sato S., Yajima A., Nasim S., Garret C. T.: "Correlations between p21 expression and clinicopathological findings, p53 gene and patients with endometrial carcinoma". *J. Pathol.,* 1997, *183,* 318.

[12] Niwa K., Murase T., Morishita S., Hashimoto M., Itoh N., Tamaya T.: "p53 overexpression and mutation in endometrial carcinoma: inverted relation with estrogen and progesterone receptor status". *Cancer Detec. Prev.,* 1999, *23,* 147.

[13] Stewart R. L., Royds J. A., Burton J.L., Heatley M. K., Wells M.: "Direct sequencing of the p53 gene shows absence of mutations in endometrioid endometrial adenocarcinomas expressing p53 protein". *Histopathology*, 1998, *33,* 440.

[14] Kuramoto H., Nishida M., Morisawa T., Hamano M., Hata H., Kato Y. *et al.*: "Establishment and characterization of human endometrial cancer cell lines". *Annals N. Y. Acad. Sci.,* 1991, *622,* 402.

[15] Uchida T., Wada C., Shitara T., Egawa S., Koshiba, K.: "Infrequent involvement of p53 gene mutations in the tumorigenesis of Japanese prostate cancer". *Br. J. Cancer,* 1993, *68,* 751.

[16] Bodner S. M., Minna J. D., Jensen S. M, D'Amico D., Carbone D., Mitsudomi T. *et al.*: "Expression of mutant p53 protein in lung cancer correlates with the class of p53 gene mutation". *Oncogene,* 1992, *7,* 746.

[17] Shahin M. S., Hughes J. H., Sood A. K., Buller R. E.: "The prognostic significance of p53 tumor suppressor gene alterations in ovarian carcinoma". *Cancer,* 2000, *89,* 2006.

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