Mutational Analysis of β -Catenin Gene in Japanese Ovarian Carcinomas: Frequent Mutations in Endometrioid Carcinomas

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To investigate the contribution of the β -catenin gene to the development of ovarian carcinomas, mutational analysis of exon 3 of the β -catenin gene was conducted. We analyzed 61 primary ovarian carcinomas, consisting of 49 non-endometrioid-type and 12 endometrioid-type tumors, for genetic alteration of the β -catenin gene. Five carcinomas showed β -catenin mutations (S37C, T41I, T41A), including 4 (33%) of 12 endometrioid-type tumors and 1 (14%) of 7 mucinous-type tumors. All of these mutations altered at the serine/threonine residues that are potential sites of GSK3- β phosphorylation. We detected no carcinomas with interstitial deletion involving exon 3 of β -catenin. Furthermore, we immunohistochemically studied 27 of the 61 ovarian carcinomas for which tissue samples were available for examination. All 4 cases exhibited mutations in exon 3 of β -catenin, including a mucinous carcinoma. Our results suggested that β -catenin gene mutation at potential GSK3- β phosphorylation sites results in accumulation of β -catenin protein within the cells and its translocation to nuclei. Accumulated β -catenin protein may be involved in the development of endometrioid-type ovarian carcinomas, and some mucinous-type ovarian carcinomas.

Key words: β -Catenin — Human ovarian carcinoma — β -Catenin-Tcf pathway

Ovarian carcinoma is a common gynecologic malignancy in Japan,¹⁾ as well as among Caucasian women. Like many other solid tumors, ovarian carcinoma may develop through a multi-step process involving activation of oncogenes and inactivation of tumor suppressor genes, but the molecular events underlying tumor development and/or progression of ovarian carcinoma are not yet clearly understood. However, the frequency of mutation in the *p53* tumor suppressor gene in ovarian carcinomas has been reported to be 40–50%.^{2,3)} Although alterations of *k-ras* oncogene in ovarian carcinoma have also been reported, the frequency of such mutations appears to be low.³⁾ The *BRCA1* gene, which is related to susceptibility to breast/ovarian cancer, was located on chromosome 17q by genetic linkage analysis and identified by positional cloning methods.⁴⁾ *BRCA1*-gene mutations occur in approximately 5% of patients with ovarian cancer diagnosed before the age of 70.⁵⁾ Furthermore, *PTEN/MMAC1* gene has recently been identified as a tumor suppressor gene, and has been shown to be a site of mutation in about 20% of endometrioid ovarian carcinomas.⁶⁾ How-

ever, this mutation has also been seen infrequently in other types of ovarian carcinoma.

Recent reports have demonstrated that β -catenin plays a role in the wingless/Wnt signal transduction pathway,⁷⁾ and oncogenic mutations of β -catenin gene were detected in several types of cancers,^{8–13)} such as colorectal cancer, melanoma, medulloblastoma, uterine endometrial cancer, and hepatocellular carcinoma. APC protein binds to β -catenin, promoting its degradation and inactivation.^{14–16)} APC works with GSK3- β to regulate β -catenin through potential phosphorylation of three serine and one threonine residues.^{3,17)} β -catenin gene mutation stabilizes β -catenin itself and causes it to accumulate within the cell and to translocate to nuclei, through inhibition of its phosphorylation. Mutated β -catenin, in association with members of the Tcf-Lef family, functions as a transcriptional factor, providing transfer cell proliferation signals to the nucleus.^{18,19)} In a Western population, Palacios and Gamallo¹⁰⁾ found β -catenin gene mutations in 3 endometrioid lesions, upon screening of 40 primary epithelial ovarian tumors.

This report describes the first extensive screening for β -catenin gene mutations in ovarian carcinomas in Japan, to determine the role of β -catenin mutation in the development and/or progression of ovarian carcinomas. Sixty-one primary ovarian carcinomas were examined for oncogenic

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Abbreviations: LOH, loss of heterozygosity; SSCP, single strand confirmation polymorphism; APC, adenomatous polyposis coli.

mutations of the *β-catenin* gene, and 27 of them were screened for immunoexpression of the protein.

MATERIALS AND METHODS

Preparation of DNA samples Materials used in this study were obtained during the course of surgical treatments at Sapporo Medical University and Kushiro City Hospital. We examined 50 fresh, frozen samples of various types of ovarian carcinomas (35 serous type, 7 mucinous type, 7 clear cell type, 1 endometrioid type), and 11 paraffin-embedded samples of endometrioid-type ovarian carcinomas.

DNA was extracted from fresh frozen samples according to routine methods,²⁰⁾ and the samples were extracted from paraffin-embedded samples using the DEXPAT kit (TaKaRa, Ohtsu) system, according to the manufacturer's directions.

Histological diagnosis of each tumor was made according to the WHO classification,²¹⁾ and the clinical stage was determined in accordance with the criteria of the International Federation of Gynecology and Obstetrics.²²⁾

LOH analysis Matched samples of normal and tumor genomic DNA of case T-32 were analyzed for LOH with a microsatellite marker mapped in the *β-catenin* region (D3S2968). Products of PCR experiments were subjected to electrophoresis, followed by staining the gel with SYBR Green I (FMC Bio Products, Rockland, ME).²³⁾

PCR-SSCP and sequence analysis All samples were screened for genetic alterations by SSCP analysis of the *β-catenin* exon 3, using PCR primers G-F and G-R.²⁴⁾

About 50 ng of each of the genomic DNAs was amplified by PCR in a 20 μ l reaction mixture, containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of dNTP, 0.4 mM of primer (G-F and G-R), and 0.5 units of recombinant *Taq* DNA polymerase (TaKaRa). Two microliters of each of 11 crude extracts was also amplified by PCR using the same mixture. Mixtures were heated to 94°C for 2 min, and then cycled 35 times; each cycle consisted of denaturation at 94°C for 30 s, anneal-

ing at 55°C for 30 s, and strand elongation at 72°C for 30 s. PCR products were denatured and subjected to electrophoresis in 12% polyacrylamide gel (ratio of acrylamide/bis-acrylamide, 39:1) with 10% glycerol at 17°C. After electrophoresis, gels were stained with SYBR Green II (FMC Bio Products).

The nucleotide sequence of aberrant PCR products was directly determined using an Applied Biosystems model 377 DNA sequencer (Perkin-Elmer Cetus, Norwalk, CT) with PCR primer and a Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems, Foster City, CA). Each mutation was verified in both the sense and antisense directions.

Analysis of rearrangement We performed PCR experiments using genomic DNA with primer pairs of C-F and C-R²⁴⁾ corresponding to the nucleotide sequences in exons 2 and 4, to detect genomic rearrangements involving exon 3. The PCR products were electrophoresed in a 1.5% agarose gel and visualized by staining with ethidium bromide.

Immunohistochemical staining for *β-catenin* Immunostaining for *β-catenin* was performed using a *β-catenin* monoclonal antibody (Transduction Laboratories, Lexington, KY).¹²⁾

RESULTS

Mutational analysis of *β-catenin* Exon 3 of the *β-catenin* gene from 61 primary ovarian carcinomas was screened by PCR-SSCP, because it encodes the regulatory domain of *β-catenin*, which has previously been found to contain activating mutations. Five (8%) of 61 primary ovarian carcinomas tested showed aberrant migration by PCR-SSCP analysis, including 4 (33%) of 12 endometrioid carcinomas and 1 (14%) of 7 mucinous carcinomas, and these 5 carcinomas were further analyzed. Nucleotide sequencing analysis revealed that these 5 tumors (T-32, T-70, T-76, T-79 and T-82) contained missense mutations that altered potential GSK3- β phosphorylation sites (S37C, T41I, T41A, T41A, and S37C, respectively; Table

Table I. Genetic and Immunohistochemical Study of *β-Catenin* in Ovarian Carcinomas

Tumor	Histology	Age	Affected codon	Mutation	Allelic status	IHC ^{a)}
T32	Mucinous	43	37	TCT(Ser) $\rightarrow$ TGT(Cys)	LOH	(+) ^{b)}
T70	Endometrioid	60	41	ACC(Thr) $\rightarrow$ ATC(Ile)	Heterozygous	n.a. ^{c)}
T76	Endometrioid	47	41	ACC(Thr) $\rightarrow$ GCC(Ala)	Heterozygous	(+)
T79	Endometrioid	58	41	ACC(Thr) $\rightarrow$ GCC(Ala)	Heterozygous	(+)
T82	Endometrioid	41	37	TCT(Ser) $\rightarrow$ TGT(Cys)	Heterozygous	(+)

a) Immunohistochemical staining by anti-*β-catenin* antibody.

b) Nuclear and cytoplasmic expression of *β-catenin* protein.

c) Not applicable.

Fig. 1. A. β -Catenin mutations in ovarian tumors. Arrows indicate nucleotide change (a–b): a, TCT-to-TGT change at codon 37 in T-32; b, ACC-to-ATC change at codon 41 in T-70. B. Representative example of LOH on D3S2968 locus in T-32. T and N: paired DNA samples isolated from tumor and normal tissue. Tumor samples show loss of one allele when compared to the normal heterozygote.

I). Analysis of genomic DNA derived from normal tissue accompanying the 5 tumors with β -catenin mutation revealed the normal sequence (data not shown) that was characterized as being somatic in nature.

All these mutations occurred at potential serine/threonine residues in exon 3, which are essential phosphorylation sites for GSK3- β . The mutations have been reported in some cancers. Nucleotide sequence analysis of the 4 endometrioid-type ovarian carcinomas showed that only 1 of the 2 β -catenin alleles was affected, but the status of the mucinous ovarian carcinoma was unclear (Fig. 1A(a)). To clarify its mutational status as either homozygous or heterozygous with LOH, we performed LOH analysis using a dinucleotide repeat marker (D3S2968) located on the β -catenin gene locus.^{25, 26} This mucinous ovarian carcinoma (T-32) showed LOH on this locus (Fig. 1B), suggesting a loss of one allele in the tumor.

The average onset age of carcinomas with β -catenin mutation was quite low, 49.8 years, compared with the onset age of carcinomas with normal β -catenin, which was 57.4 years. However, the difference between the two groups was not significant. No correlation was found between the presence of β -catenin mutation and other clinical parameters, such as clinical stage.

Analysis of genomic rearrangement In order to detect genomic rearrangement involving exon 3, we performed a PCR experiment on this exon and the entire intronic sequences from genomic DNAs of 50 fresh frozen sam-

ples. We detected no aberrant band smaller than the normal 931-bp product.

Immunohistochemical study Because paraffin-block samples were available for 16 of the 49 non-endometrioid-type tumors, and 11 of the 12 endometrioid-type tumors, immunohistochemical staining of these 27 carcinomas was performed using an anti- β -catenin antibody, to analyze the localization and accumulation of β -catenin in the cells. In 1 mucinous (T-32) and 3 endometrioid (T-76, T-79, T-82) carcinomas, we detected a larger amount of β -catenin protein in cytoplasm and nuclei of cancer cells (Fig. 2); the other 23 cases showed intense membranous β -catenin expression. All 4 of these cases had missense mutations in exon 3 of β -catenin (Table I). One case (T-70) with mutation of β -catenin gene mutation was unavailable for immunohistochemical testing.

DISCUSSION

We describe here the results of the first extensive screening for β -catenin gene mutations in ovarian carcinomas in Japan.

Five (8%) of 61 ovarian carcinomas had genetic alterations in exon 3 of β -catenin; 4 (33%) of 12 endometrioid carcinomas and 1 (14%) of 7 mucinous carcinomas had a β -catenin mutation. Four (15%) of 27 ovarian carcinomas showed accumulation of this protein in the cell nuclei and cytoplasm; these 4 cases had missense mutations in exon

Fig. 2. Immunohistochemical analysis of T-32 by anti- β -catenin antibody. Strong staining of β -catenin is detected in cytoplasm and nuclei of cells.

3 of β -catenin. The frequency of β -catenin mutations at phosphorylation sites in Japanese endometrioid ovarian carcinomas was 33%. Palacios and Gamallo¹⁰ found β -catenin mutations in 50% of endometrioid ovarian carcinomas, and in no non-endometrioid ovarian carcinomas, in Spain. Our findings indicate that β -catenin mutations at serine/threonine residues that are potential GSK3- β phosphorylation sites resulted in accumulation and translocation of cytoplasmic β -catenin in endometrioid ovarian carcinomas. Three mutations of β -catenin in our cases occurred at serine/threonine residues in exon 3 of this gene.

Furthermore, 2 mutations (S37C, T41A) reported by Palacios and Gamallo,¹⁰ and 1 mutation (T41I) in uterine endometrial carcinoma¹² have also been identified. The onset age of carcinomas with β -catenin mutations might be younger than that of carcinomas with normal β -catenin, although the difference was not statistically significant.

A similar tendency was seen in uterine endometrioid carcinoma.²⁷ It has been observed that genetic events associated with carcinogenesis of uterine endometrial carcinoma are similar to those of endometrioid ovarian carcinoma, involving RER²⁸ and PTEN.⁶ However, the frequency of β -catenin mutation in endometrioid ovarian

carcinomas (33%, 4 of 12) tends to be higher than that in uterine endometrial carcinomas²⁷ (14%, 5 of 35), so β -catenin mutation may contribute more frequently to the development of endometrioid ovarian carcinoma than it does to that of uterine endometrial carcinoma.

In non-endometrioid ovarian carcinomas, we detected β -catenin gene mutation and nuclear expression of this protein in 1 (14%) of 7 mucinous ovarian carcinomas; this tumor showed no normal allele. Previous reports of β -catenin mutations indicated somatic and heterozygous status as oncogenic changes, and LOH on the β -catenin locus was not associated with accumulation of β -catenin in cancer cell cytoplasm and nuclei.²⁹ Sato *et al.*³⁰ reported that about 20% of ovarian carcinomas have LOH on a chromosome arm (3p21.3-3p23) within the β -catenin locus. Therefore, LOH on the β -catenin locus in mucinous carcinoma with this gene mutation may not be implicated in the accumulation of β -catenin protein in the cancer cells, and the significance of biallelic alteration of β -catenin is not clear at present. However, our results do not completely exclude the possibility that up-regulation and translocation of β -catenin protein in mucinous carcinoma are associated with not only β -catenin gene mutation at potential phosphorylation sites, but also LOH on the locus that includes β -catenin. In contrast, interstitial

deletions, which were detected in about 3% of sporadic colorectal cancers,²⁴⁾ and in about 3% of sporadic hepatocellular cancers,¹³⁾ were not detected in 50 ovarian carcinomas. However, this testing included only 1 endometrioid carcinoma, so in order to clarify the frequency of this deletion, we must perform this analysis on a greater number of endometrioid carcinomas. Our immunohistochemical analysis of β -catenin indicates that these mutations are responsible for the up-regulation of cytoplasmic β -catenin and its distribution in the nuclei of cancer cells.

The above results suggest that mutations at serine/threonine residues that are potential GSK3- β phosphoryla-

tion sites altered β -catenin's stability. Accumulation of mutant β -catenin could contribute to the development of a subset of ovarian carcinomas: perhaps 1/3 of endometrioid type, and a portion of mucinous type.

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REFERENCES

- 1) Takeda, T., Sagae, S., Koizumi, M., Terasawa, K., Ishioka, S., Takashima, S. and Kudo, R. Multiple primary malignancies in patients with gynecologic cancer. *Int. J. Gynecol. Cancer*, **5**, 34–39 (1995).
- 2) Kihana, T., Tsuda, H., Teshima, S., Okada, S., Matsuura, S. and Hirohashi, S. High incidence of p53 gene mutation in human ovarian cancer and its association with nuclear accumulation of p53 protein and tumor DNA aneuploidy. *Jpn. J. Cancer Res.*, **83**, 978–984 (1992).
- 3) Teneriello, M. G., Ebina, M., Linnoila, R. I., Henry, M., Nash, J. D., Park, R. C. and Birrer, M. J. P53 and K-ras gene mutations in epithelial ovarian neoplasms. *Cancer Res.*, **53**, 3103–3108 (1993).
- 4) Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P.A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L. M., Ding, W., Bell, R., Rosenthal, J., Hussey, C., Tran, T., McClure, M., Frye, C., Hattier, T., Phelps, R., Haugen-Strano, A., Katcher, H., Yakumo, K., Gholami, Z., Shaffer, D., Stone, S., Bayer, S., Wray, C., Bodgen, R., Dayananth, P., Ward, J., Tonin, P., Narod, S., Bristow, P. P., Norris, F. H., Helvering, L., Morrison, P., Rostock, P., Lai, M., Barrett, J. C., Lewis, C., Nauhausen, S., Cannon-Albright, L., Goldgar, D., Wiseman, R., Kamb, A. and Skolnik, M. H. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science*, **266**, 66–71 (1994).
- 5) Stratton, J. F., Gayther, S. A., Russel, P., Dearden, J., Gore, M., Blake, P., Easton, D. and Ponder, B. A. Contribution of BRCA1 mutations to ovarian cancer. *N. Engl. J. Med.*, **336**, 1125–1130 (1997).
- 6) Obata, K., Morland, S. J., Watson, R. H., Hitchcock, A., Chenevix-Trench, G., Thomas, E. J. and Campbell, I. G. Frequent PTEN/MMAC1 mutations in endometrioid but not serous or mucinous epithelial ovarian tumors. *Cancer Res.*, **58**, 2095–2097 (1998).
- 7) Gumbiner, B. M. Signal transduction by β -catenin. *Curr. Opin. Cell Biol.*, **7**, 634–640 (1995).
- 8) Sparks, A. B., Morin, P. J., Vogelstein, B. and Kinzler, K. W. Mutational analysis of the APC/ β -catenin/Tcf pathway in colorectal cancer. *Cancer Res.*, **58**, 1130–1134 (1998).
- 9) Rubinfeld, B., Robbins, P., El-Gamli, M., Albert, I., Porfiri, E. and Polakis, P. Stabilization of β -catenin by genetic defects in melanoma cell lines. *Science*, **275**, 1790–1792 (1997).
- 10) Palacios, J. and Gamallo, C. Mutations in the β -catenin gene (CTNNB1) in endometrioid ovarian carcinomas. *Cancer Res.*, **58**, 1344–1347 (1998).
- 11) Zurawel, R. H., Chiappa, S. A., Allen, C. and Raffel, C. Sporadic medulloblastomas contain oncogenic β -catenin mutations. *Cancer Res.*, **58**, 896–899 (1998).
- 12) Fukuchi, T., Sakamoto, M., Tsuda, H., Maruyama, K., Nozawa, S. and Hirohashi, S. β -Catenin mutation in carcinoma of the uterine endometrium. *Cancer Res.*, **58**, 3526–3528 (1998).
- 13) Miyoshi, Y., Iwao, K., Nagasawa, Y., Aihara, T., Sasaki, Y., Imaoka, S., Murata, M., Shimao, T. and Nakamura, Y. Activation of the β -catenin gene in primary hepatocellular carcinomas by somatic alterations involving exon 3. *Cancer Res.*, **58**, 2524–2527 (1998).
- 14) Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B. and Clevers, H. Constitutive transcriptional activation by a β -catenin-Tcf complex in APC^{-/-} colon carcinoma. *Science*, **275**, 1784–1787 (1997).
- 15) Rubinfeld, B., Souza, B., Albert, I., Munemitsu, S. and Polakis, P. The APC protein and E-cadherin form similar but independent complexes with α -catenin, β -catenin, and plakoglobin. *J. Biol. Chem.*, **270**, 5549–5555 (1995).
- 16) Munemitsu, S., Albert, I., Souza, B., Rubinfeld, B. and Polakis, P. Regulation of intracellular β -catenin levels by the adenomatous polyposis coli (APC) tumor suppressor protein. *Proc. Natl. Acad. Sci. USA*, **92**, 3046–3050 (1995).
- 17) Rubinfeld, B., Albert, I., Porfiri, E., Fiol, C., Munemitsu, S. and Polakis, P. Binding of GSK3- β to the APC- β -catenin complex and regulation of complex assembly. *Science*, **272**, 1023–1025 (1996).
- 18) Molenaar, M., Wetering, M., Oosterwegel, M., Peterson-Madulo, J., Goldsave, S., Kornek, V., Roose, J., Destree, O. and Clevers, H. Xtc3 transcription factor

- mediates β -catenin-induced axis formation in *Xenopus* embryos. *Cell*, **86**, 391–399 (1996).
- 19) Behrens, J., Kries, J. P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R. and Birchmeier, W. Functional interaction of β -catenin with the transcriptional factor LEF-1. *Nature*, **382**, 638–642 (1996).
- 20) Sato, T., Tanigami, A., Yamakawa, K., Akiyama, F., Kasumi, F., Sakamoto, G. and Nakamura, Y. Allelotype of breast cancer: cumulative allele losses promote tumor progression in primary breast cancer. *Cancer Res.*, **50**, 7184–7189 (1990).
- 21) Scully, R. E., Bonfiglio, T. A., Kurman, R. J., Silverberg, S. G. and Wilkinson, E. J. Histological typing of female genital tract tumors. In “International Histological Classification of Tumors,” 2nd Ed., ed. World Health Organization (1996). Springer-Verlag.
- 22) FIGO Stages—1988 Revision. *Gynecol. Oncol.*
- 23) White, H. W. and Kusukawa, N. Agarose-based system for separation of short tandem repeat loci. *Biotechniques*, **22**, 976–980 (1997).
- 24) Iwao, K., Nakamori, S., Kameyama, S., Imaoka, S., Kinoshita, M., Fukui, T., Ishiguro, S., Nakamura, Y. and Miyoshi, Y. Activation of the β -catenin gene by interstitial deletions involving exon 3 in primary colorectal carcinomas without adenomatous polyposis coli mutations. *Cancer Res.*, **58**, 1021–1026 (1998).
- 25) Bailey, A., Norris, A. L., Leek, J. P., Clissold, P. M., Carr, I. M., Ogilvie, D. J., Morrison, J. F. J., Meredith, D. M. and Markham, A. F. Yeast artificial chromosome cloning of the beta-catenin locus on human chromosome 3p21-22. *Chromos. Res.*, **3**, 201–203 (1995).
- 26) Klaus, C., Liehr, T., Hulsken, J., Behrens, J., Birchmeier, W., Grzeschic, K. H. and Ballhausen, W. G. Localization of the human beta-catenin gene (CTNNB1) to 3p21: a region implicated in tumor development. *Genomics*, **23**, 272–274 (1994).
- 27) Kobayashi, K., Sagae, S., Nishioka, Y., Tokino, T. and Kudo, R. Mutations of the β -catenin gene in endometrial carcinomas. *Jpn. J. Cancer Res.*, **90**, 55–59 (1999).
- 28) Fujita, M., Enomoto, T., Yoshino, K., Nomura, T., Buzard, G. S. and Okudaira, Y. Microsatellite instability and alterations in the hMSH2 gene in human ovarian cancer. *Int. J. Cancer*, **64**, 361–366 (1995).
- 29) Kersemaekers, A. M. F., Hermans, J., Fleuren, G. J. and van de Vijver, M. J. Loss of heterozygosity for defined regions on chromosome 3, 11 and 17 in carcinomas of the uterine cervix. *Br. J. Cancer*, **77**, 192–200 (1998).
- 30) Sato, T., Saito, H., Morita, R., Koi, S., Lee, J. H. and Nakamura, Y. Allelotype of ovarian cancer. *Cancer Res.*, **51**, 5118–5122 (1991).