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Mutational Analysis of *KRAS*, *BRAF*, and *TP53* Genes of Ovarian Serous Carcinomas in Korean Women

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Purpose: To assess the prevalence of *KRAS*, *BRAF*, and *TP53* mutations in cases of low-grade and high-grade serous carcinomas and to evaluate the clinical outcomes of these morphologically distinct carcinomas. **Materials and Methods:** Patients with primary invasive serous carcinomas were classified according to the universal grading system. Grade 2 serous tumors were excluded. A total of 100 patients were included for clinical evaluation. Thirty-seven patients, including 20 with low-grade and 17 with high-grade carcinomas, were selected for mutational analysis. **Results:** The low-grade carcinoma group was characterized by young age and premenopausal period compared with the high-grade carcinoma group, but there were no statistically significant differences in stage, metastasis of lymph node and residual disease. There were no statistically significant differences in survival rates, however, the low-grade carcinoma group showed a trend for improved progression-free survival compared with the high-grade carcinoma group of early stage ($p = 0.064$). Mutations in *KRAS* and *BRAF* were found in 6 (30%) and 2 (10%) patients in the low-grade carcinoma group, respectively, however, they were not found in the high-grade carcinoma group. *KRAS* and *BRAF* mutations were mutually exclusive, and both mutations were observed in 40% (8/20). The frequency of *TP53* mutations in low-grade and high-grade carcinoma groups were found in 20% (4/20) and 70.6% (12/17), respectively ($p = 0.009$). **Conclusion:** Low-grade serous carcinoma shows mutation pattern different from that with high-grade carcinoma. As there were no significant differences in stage distribution and survival, especially in advanced stage, we suggest that more studies are needed to segregate these patients into distinct disease entities.

Key Words : Ovary, serous carcinoma, grade, mutation

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INTRODUCTION

Ovarian carcinoma is the most lethal gynecologic malignancy.[1,2] There are several morphological subtypes of ovarian carcinoma and serous type is the most common. The molecular tumorigenesis leading to this heterogeneous group of ovarian cancers is mostly unknown because of the lack of a universal tumor progression model. However, based on several clinicopathologic and molecular genetic studies, a dualistic model of ovarian serous carcinogenesis was recently proposed and is now gaining widespread acceptance.[2-4] It is presumed that low-grade serous carcinoma develops in a stepwise manner such as an adenoma-carcinoma sequence in which a benign serous cystadenoma develops into a usual serous borderline tumor, a noninvasive micropapillary serous carcinoma, and finally to a low-grade serous carcinoma.[2] By contrast, the more common high-grade serous carcinoma develops in a *de novo* manner directly from ovarian surface epithelium or the epithelium of cortical inclusion cysts.[3]

KRAS and its effector *BRAF* are members of the Ras/Raf/MEK/extracellular signal-regulated kinase/mitogen-activated protein kinase pathway, which is a well-characterized signaling mechanism that mediates cellular responses to growth signals.[5] In this pathway,

RAS oncogenes play a pivotal role in tumorigenesis. *RAS* mutation was first reported in malignant melanoma, lung, and papillary thyroid carcinoma.[6-9] In non-invasive and invasive ovarian carcinoma, mutation of *KRAS* and *BRAF* has previously been described with *KRAS* mutation which was mostly detected in mucinous ovarian tumor and in serous borderline tumors, but not in invasive serous carcinoma.[2-4,10-15] *TP53* is a tumorsuppressor gene that is located on the short arm of chromosome 17 and encodes a 53-kDa nuclear protein that is involved in the regulation of cell growth. Mutation of the *TP53* gene is the most common mutation in human cancers.[16] It has been suggested that p53 protein alterations due to missense mutations and loss of the p53 protein by nonsense or frame-shift mutations might play important roles in clonal expansion of neoplastic cells.[17] Recently, the dualistic pathway was supported by molecular genetic differences in the frequency of *KRAS*, *BRAF*, and *TP53* mutations between low-grade and high-grade serous carcinomas, with *KRAS* and *BRAF* mutations being more frequent and *TP53* mutations being less common in low-grade serous carcinomas.[2,4,14,18]

Despite increasing acceptance of the dualistic model, relatively few studies have attempted to provide evidence that supports or refutes this carcinogenesis model. Determination of the molecular pathways involved in the development of ovarian serous carcinoma would markedly improve our understanding of its pathogenesis, thereby providing a basis for the development of new diagnostic tests and therapeutic strategies. Therefore, the aim of this study was to analyze the molecular genetic changes, including *KRAS*, *BRAF*, and *TP53* mutations, in Korean patients with ovarian serous carcinoma and to evaluate the clinical outcomes in these 2 morphologically distinct ovarian serous carcinomas.

MATERIALS AND METHODS

Patient population and clinical data

The surgical pathology files of the Department of Obstetrics and Gynecology, University of Ulsan, the Asan Medical Center, during the period between January 1996 and August 2003 were searched for all cases of ovarian tumors diagnosed as serous carcinomas. Of 164 cases identified, 104 cases with available slides were enrolled for evaluation. The medical records, including clinical outcomes, were reviewed. All patients underwent surgery, and operative reports were reviewed. The following information was taken from the medical records: age, serum CA-125 level at the time of diagnosis, International Federation of Gynecology and Obstetrics (FIGO) stage, dimension of residual tumor, clinical status at completion of primary chemotherapy, date of last contact or death, and date of disease progression. The debulking operation was defined as optimal if the largest tumor mass remaining was less than 2 cm in diameter.

Histological evaluation

All slides were reviewed by a gynecologic pathologist. Cases were classified according to the universal grading system.[19,20] Architectural grade, nuclear grade, and mitotic count were scored each. Final grading was made as follows; total scores of 3-5 for Grade 1, total scores of 6-7 for Grade 2, and total scores of 8-9 for Grade 3. In this study, Grade 2 carcinomas were excluded in order to minimize difference of reproducibility. A total of 100 patients with low-grade (Grade 1) or high-grade (Grade 3) carcinoma were compared and analyzed.

Tissue section and tumor DNA samples

Paraffin-embedded tissues from 20 low-grade serous carcinomas and 17 high-grade serous carcinomas were used for molecular genetic analysis. Of low-grade serous carcinoma specimens, three were stage I, one was stage II, 13 were stage III, and three were stage IV. In high-grade serous carcinoma group, one with stage I, 1 with stage II, 14 with stage III and 1 with stage IV were included. The histological region of interest was identified on a hematoxylin-eosin-stained section, which was marked and cut into 3-6 serial sections. DNA was purified from each section and the *KRAS*, *BRAF*, and *TP53* genes were assessed for mutations using digital PCR-based techniques.

Amplification of *KRAS*, *BRAF* and *T53* genes

Fifty nanograms of DNA were amplified in a 20 μL reaction solution containing 2 μL of 10X buffer (Roche, Mannheim, Germany), 1.7-2.5 mmol/L $MgCl_2$, 0.3 μM each of primer pair (primer sequences) (Table 1), 250 μM deoxynucleotide

Table 1. Primers Used for Amplification and Sequencing of *KRAS*, *BRAF*, and *TP53* Genes

Amplified fragment	Primer sequence
KRAS	
Exon 2	F: 5'-TGACATGTTCTAATATAGTCAC R: 5'-ACAAGATTTACCTCTATTGTTG
BRAF	
Exon 15	F: 5'-AGCAGCATCTCAGGGCCA R: 5'-ATGCTTGCTCTGATAGGAAAATGA
TP53	
Exon 5	F: 5'-TGCCGTCTTCCAGTTGCTTTATC R: 5'-GCCAGACCTAAGAGCAATCAGTGAG
Exon 6	F: 5'-GGGGCTGGAGAGACGACA R: 5'-TGGGGTTATAGGGAGGTCAAA
Exon 7	F: 5'-GGGCGACAGAGCGAGATTC R: 5'-GGGGTCAGAGGCAAGCAGAG
Exon 8	F: 5'-GGAGTAGATGGAGCCTGGTTTTTTA R: 5'-GGCAAGGAAAGGTGATAAAAGTG
Exon 9	F: 5'-AAGCAAGCAGGACAAGAAGC R: 5'-AAGAAAACGGCATTTTGAGTG

F, forward primer; R, reverse primer.

triphosphate, and 2.5 units of DNA polymerase (Roche). The Polymerase Chain Reaction (PCR) program for *KRAS* amplification involved a single denaturation step at 95°C for 5 minutes, followed by 35 cycles of 30 seconds at 95°C (denaturation), 30 seconds at 55°C (annealing), and 40 seconds at 72°C (extension), followed by 10 minutes at 72°C Amplifications of the *BRAF* and *TP53* genes involved a 5-minute initial denaturation step at 94°C, followed by 30 cycles of 1 minute at 94°C, 1 minute at 59°C, and 1 minute at 72°C, and a 10-minute final extension step at 72°C. PCR products were then 2% gel-purified with a QIAgen gel extraction kit (Qiagen).

Direct sequencing

DNA templates were processed for the DNA sequencing reaction using the ABI-PRISM BigDye Terminator version 3.1 (Applied Biosystems, Foster, CA, USA) with both forward and reverse sequence-specific primers. Twenty nanograms of purified PCR products were added to 20 μL of sequencing reaction solution containing 8 μL of BigDye Terminator v3.1 and 0.1 μM of the same PCR primer. Sequencing reactions involved 25 cycles of 10 seconds at 96°C, 5 seconds at 50°C, and 4 minutes at 60°C Sequence data were generated with the ABI PRISM 3100 DNA Analyzer (Applied Biosystems). Sequences were analyzed by Sequencer 3.1.1 software (Applied Biosystems) to compare genetic variations.

Statistical analysis

SPSS 10.0 statistical software (Superior Performance Software System, SPSS for Windows, Microsoft, Chicago, IL, USA) was used for all statistical calculations. Categorical variables were compared using the Chi-square and Fisher's exact tests. Mean, median, and standard deviation were calculated for continuous variables. Progression-free survival and overall survival times were estimated using the method of Kaplan and Meier. The log-rank test was used to compare differences between survival curves. p values of < 0.05 were considered statistically significant.

RESULTS

Clinical findings

In this study, 27 low-grade and 73 high-grade serous carcinomas were selected. The mean age at diagnosis was lower in patients with low-grade carcinoma than in those with high-grade carcinoma (48.7 ± 15.0 years vs. 55.2 ± 10.7 years, $p = 0.045$), and there were more premenopausal women in the low-grade carcinoma group ($p = 0.041$). Serum CA-125 concentration, positive peritoneal cytology, lymph node metastasis, optimality of debulking surgery, and adjuvant chemotherapy were similar in 2 groups (Table 2). There was no

Table 2. Characteristics of Patients with Ovarian Serous Carcinomas

	Low-grade serous carcinoma (n = 27)	High-grade serous carcinoma (n = 73)	*p* value
Median age (yrs)	44 (26 - 77)	54 (35 - 75)	0.043
Premenopause	16 (59.3%)	26 (35.6%)	0.041
Median CA-125 (U/mL) (range)	379 (9.4 - 27,900)	1,225 (28.8 - 22,400)	0.501
FIGO Stage			0.191
I	6 (22.2%)	6 (8.2%)	
II	1 (3.7%)	2 (2.7%)	
III	17 (63.0%)	58 (79.5%)	
IV	3 (11.1%)	7 (9.6%)	
Cytology			
Adenocarcinoma	15 (55.6%)	54 (74.0%)	0.098
Lymph-node metastasis	10 (52.6%)	38 (71.7%)	0.161
Residual disease			0.633
< 2 cm	20 (74.1%)	50 (68.5%)	
≥ 2 cm	7 (25.9%)	23 (31.5%)	
Adjuvant chemotherapy	25 (92.6%)	66 (90.4%)	1.000

Table 3. *KRAS*, *BRAF*, and *TP53* Mutations of Low-Grade and High-Grade Ovarian Serous Carcinoma

Tumor	No. of cases	*KRAS*	*BRAF*	*TP53*
High-grade serous carcinoma	17	0	0	12 (70.6%)
Low-grade serous carcinoma	20	6 (30%)	2 (10%)	4 (20.0%)

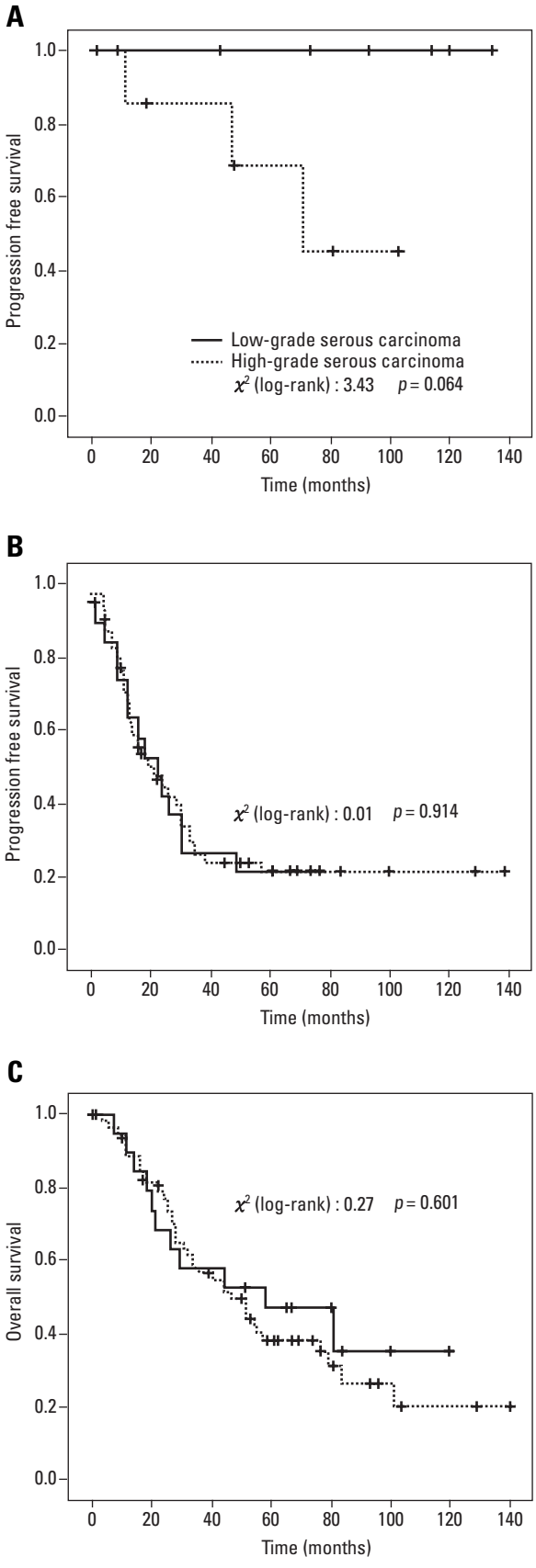

Fig. 1. (A) progression-free survival in early stage, (B) progression-free survival in advanced stage, and (C) overall survival of the patients with advanced stage.

significant difference in Federation of Gynaecology and Obstetrics (FIGO) stage, however, early stage including stage I or II accounted for larger portion in low grade group (25.9% vs. 11.0%, $p = 0.110$). During the median follow-up of 67 months for the low-grade group and 45 months for the high-grade group, there were 11 (40.7%) tumor-related deaths in patients with low-grade carcinoma and 40 (54.8%) deaths in patients with high-grade carcinoma ($p = 0.262$). There were no statistically significant differences in progression-free and overall survival rates according to FIGO stage between the two groups, but the low-grade carcinoma group in early stage (FIGO stage I and II) showed a trend for improved 5-year progression-free survival, compared with the high-grade carcinoma group (100% vs. 68.6%, $p = 0.064$) (Fig. 1).

KRAS

We found that 6 (30%) low-grade serous carcinomas contained activating *KRAS* mutations at codon 12 (mutations of GGT to GAT), but these mutations were not found in high-grade serous carcinomas (Table 3, Fig. 2).

BRAF

BRAF mutations were not found in any of the high-grade serous carcinomas, whereas the *BRAF* mutation was identified only in low-grade serous carcinomas. Two cases (10%) of low-grade carcinoma contained a mutation in exon 15, and codon 600 was affected (V600E) (Fig. 2). *KRAS* and *BRAF* mutations were mutually exclusive.

TP53

TP53 mutations were found in 4 of the 20 (20.0%) cases of low-grade serous carcinoma. Of these four mutations, three were missense mutations and 1 was a mutation that affected the splicing site. By contrast, mutations were found in 12 of the 17 (70.6%) cases of high-grade serous carcinoma, 6 of which were missense mutations, 2 were nonsense mutations, and 4 were deletion mutations (Fig. 3). The frequency of *TP53* mutations was significantly higher in high-grade than in low-grade serous carcinomas ($p = 0.009$).

DISCUSSION

The importance of stage and residual disease status as prognostic factors in ovarian serous carcinoma is universally accepted,[21] but the significance of histologic grade has been questioned due to the lack of a uniform grading system and poor reproducibility. Universal grading system has been used with good reproducibility, and its prognostic use in cases of ovarian epithelial tumors has been statistically supported.[19, 22] In the present study, the patients with grade 1 and grade 3 except grade 2 were selected to evaluate the clinical outcomes and prognostic role of these 2, morphologically distinct, ovarian

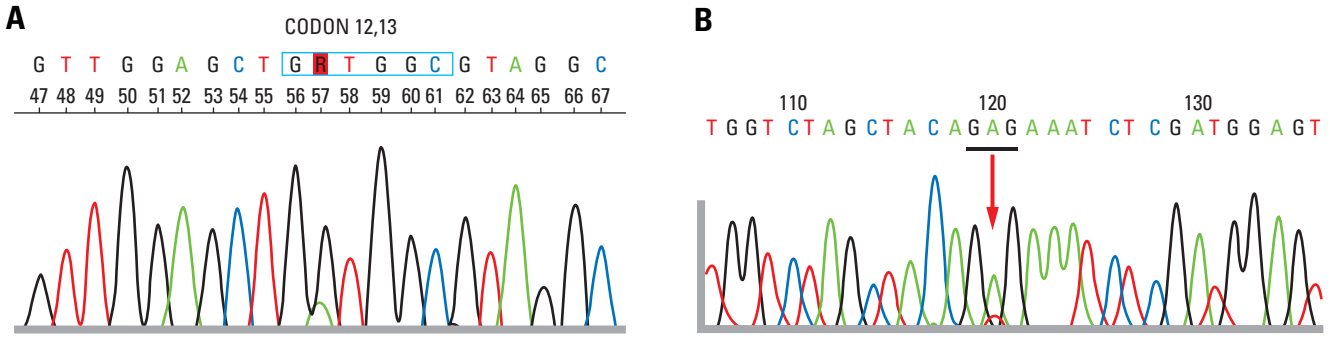

Fig. 2. (A) A point mutation of the *KRAS* gene in low-grade serous carcinoma in codon 12, GGT → GAT. (B) A *BRAF* mutation in a low-grade serous carcinoma in exon 15, position 600, substitution of A for T, GTG → GAG.

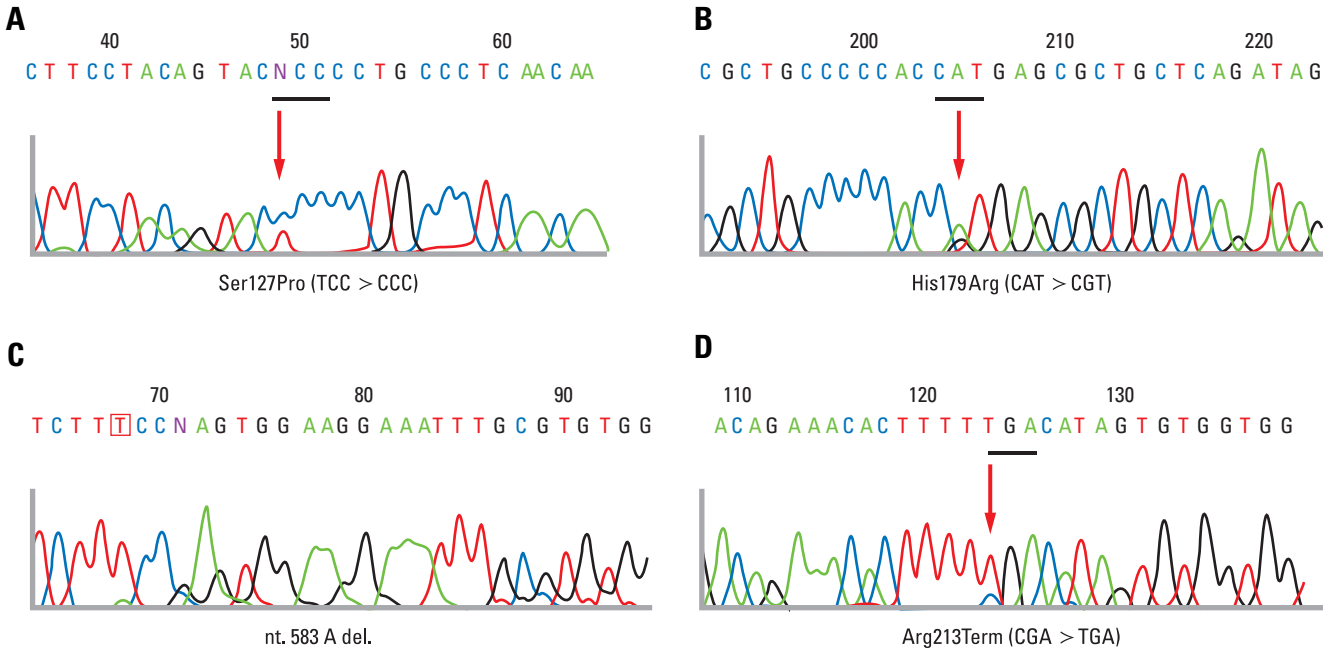

Fig. 3. Chromatograms of *TP53* nucleotide sequences. Nucleotide sequences determined by chromatograms are shown for different types of *TP53* mutations. (A)Ser127Pro (TCC > CCC). (B) His179Arg(CAT > CGT). (C) nt. 583 A del. (D) Arg213Term (CGA > TGA).

serous carcinomas. We found that histologic grade did not have a prognostic role in each FIGO stage, nevertheless, our findings suggested clinical differences between low-grade serous carcinoma and high-grade serous carcinoma. In our study population, the age range for patients with low-grade serous carcinoma was rather large, but the median age of 44 years was much younger than that of patients with high-grade serous carcinoma (54 years) and there were more premenopausal women in the low-grade carcinoma group. These findings were similar to a previous report from M. D. Anderson that the median age at diagnosis was 43 years[23] and the median age of women with epithelial ovarian cancer (approximately 90% of which are high grade carcinoma) was 59-60 years.[24,25] In addition, low-grade serous tumors were diagnosed in more early stage (stage I and II). There were no statistically significant differences in prognosis according to FIGO stage between the two groups, but the low-grade carcinoma group showed a trend for improved progression-free survival compared with the high-grade carcinoma group of early stage. Our results add to previous studies that low-grade serous carcinoma is slow-glowing indolent neoplasm, but shows different feature from previous study that low-grade carcinoma is characterized by prolonged survival in advanced stage.[23,26] However, these data indicate the possibility that low-grade and high-grade serous carcinomas are two different tumor types, each with their own pathogenesis rather than different grades of the same neoplasm.

In a previous mutation study, *KRAS* mutations were observed in approximately 50% of serous tumors with low malignant potential and low-grade serous carcinomas, however, no *KRAS* mutations were found in high-grade serous carcinomas.[2] Subsequent studies confirmed these previous results and found

either *BRAF* or *KRAS* mutations in 68% of low-grade serous carcinomas and in 61% of serous tumors of low malignant potential, but in none of the high-grade serous carcinomas.[4] Furthermore, none of the low-grade tumors contained mutations in both *KRAS* and *BRAF*, indicating that these mutations are mutually exclusive. It has also been suggested that analysis of the other components of this pathway will reveal other mutations in the 40% of cases without *KRAS* and *BRAF* mutation. As alternate pathways, *TP53* and PTEN/PIK3CA mutations were suggested.[1] The main role of PTEN is a phosphatase activity, antagonizing the PI3 kinase/AKT pathway; that is, by keeping ART inactive and protecting p53 from degradation.[27] In the present study, *KRAS* mutations were found in 30%, *BRAF* mutations were found in 10%, and *KRAS* or *BRAF* mutations were found in 40% of low-grade serous carcinomas, whereas no *BRAF* or *KRAS* mutations were found in high-grade serous carcinoma. However, our study reported lower frequencies of *KRAS* or *BRAF* mutations than previous studies, and we suggest that the number of carcinomas due to mutation of another pathway-activating factor might be greater in Korean individuals.

Mutational alteration of the *TP53* gene was reported in 30-80% of cases of epithelial ovarian cancer,[16,28,29] but few studies directly compared the expression of *TP53* in low-grade and high-grade serous carcinomas. A recent study found functional *TP53* mutations in only one of 12 (8.3%) low-grade serous carcinomas and in 30 of 59 (50.8%) high-grade serous carcinomas.[13] The present study found *TP53* mutations in 20.0% of low-grade serous carcinomas and 70.6% of high-grade serous carcinomas, which is significantly greater than the incidence of low-grade serous carcinoma, but similar to the incidence of high-grade serous carcinoma previously reported. In this study, we found slightly increased incidences of *TP53* mutation in both groups compared with the previous study. However, based on the finding that there were *TP53* mutations without combined *KRAS* or *BRAF* mutations in 20.0% of low-grade serous carcinomas, the possibility that they are involved in progression of low-grade to high-grade serous carcinomas cannot be excluded. In addition, the possibility that other pathways are involved should also be considered. Shih et al.[11] described a micropapillary pattern simulating low-grade serous carcinoma which have had *TP53* mutations and lacked mutations of *KRAS* or *BRAF*. These tumors displaying a micropapillary architecture developed from a subset of low-grade serous carcinomas that lacked mutation of *KRAS* and BRAF and subsequently acquired a TP mutation, which in turn might increase the level of nuclear atypia.[30] We suggest that the above described suggestions might be able to explain higher frequency of *TP53* mutation in Korean women.

In conclusion, our findings support those of earlier clinical studies that low-grade serous carcinomas seem to have age distribution and mutational pattern distinct from high-grade serous carcinomas. However, there were no significant differences in stage distribution and survival, especially in advanced stage, between 2 groups, we therefore suggest that more studies are needed to segregate these patients into distinct disease entities.

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