Short Communication

Vitamin D receptor polymorphisms and prognosis of patients with epithelial ovarian cancer

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BACKGROUND: Recently, the vitamin D receptor (VDR) polymorphism FokI was shown to be associated with susceptibility to ovarian cancer. We aimed to examine whether VDR FokI polymorphisms influence the survival of patients with epithelial ovarian cancer (EOC).

METHODS: VDR polymorphisms from FokI in 101 patients with EOC were genotyped by sequencing. Overall survival was compared between FokI single nucleotide polymorphism using Kaplan–Meier survival curves with log-rank tests and the Cox proportional hazard model adjusted for ages, stages, histology, and existence of residual tumour.

RESULTS: The FokI C/C genotypes were associated with better prognosis compared with the C/T and T/T genotypes (log-rank test: \( P = 0.008 \); adjusted hazard ratio, 0.18; 95%CI 0.05–0.61; \( P = 0.006 \)).

CONCLUSIONS: These results suggest that the VDR polymorphisms from the FokI genotype may be associated with improved prognosis of patients with EOC.

Keywords: prognosis; single nuclear polymorphisms; SNPs; biomarker

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MATERIALS AND METHODS

Patients

This prospective cohort hospital-based study to detect important gene mutations in ovarian cancer was approved by the Ethics Committee for Biomedical Research of the Jikei Institutional Review Board, Jikei University School of Medicine, Tokyo, Japan. As post-hoc analysis, we performed to re-examine VDR FokI polymorphisms in this cohort. Between September 1996 and January 2009, approximately 600 patients with EOC took surgery. Of these, approximately 300 had Paclitaxel–Carboplatin treatment as a first line chemotherapy. Of these, approximately 250 patients provided informed consent. Of these, approximately 200 samples showed more than 80% of cancer cells. Of these, 125 samples were enriched DNA by laser capture micro-dissection or trimming with needle. Of these, 24 were not selected from this study because DNA specimens were not available. Thus, 101 patients with EOC were included in this study: Clinical information was abstracted from clinical and surgical charts. Postoperative stages were determined by International Federation of Gynecology and Obstetrics (FIGO) classification (Kikkawa et al., 2006). Patients were periodically (every 0.5–2 months) examined on an outpatient basis to make sure they had not relapsed.

Samples

In all cases, tumour samples from the primary site, but not metastatic sites, were stored at −80°C after excision. Cancer tissue was divided into two specimens: one for pathological
confirmation, in which the sample was composed of more than 80% cancer cells, and the other for DNA extraction. Peripheral blood samples were collected during the preoperative period from 10 of the same patient’s group to confirm whether alteration of sequence was due to either genomic polymorphisms or somatic mutations.

Polymorphisms of the VDR gene

DNA was extracted from the fresh frozen tumour and peripheral blood samples using QIAcube (Qiagen, Tokyo, Japan) following the manufacturer’s protocol. DNA fragments of FokI were amplified by polymerase chain reaction (PCR) using the following primers (forward/reverse): ctccgaaggcactgtgctcaggcct/atggaaa-cacctgtctctctcc; sequence, gcctgctgctgggagata; parameters: denaturation at 98°C for 1 min, followed by 30 cycles at 98°C for 10 s, annealing at 68°C for 4 min, and the stopping reaction at 16°C.

The PCR products were incubated with rApid alkaline phosphatase (Roche Diagnostics, Mannheim, Germany) and Exonuclease I (New England BioLabs, Ipswich, MA, USA) at 37°C for 30 min, then 80°C for 15 min. Using Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Tokyo, Japan), the aliquots were incubated under the following conditions: 96°C for 5 min; 25 cycles at 96°C for 10 s, 50°C for 5 s, and 60°C for 2 min. Treated PCR products were sequenced with the ABI PRISM 3700 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequencing was confirmed with independent duplicate analyses in 20 samples for quality control to find no discrepancy. Genotyping success rate was 69%, although genotyping success rate improved to 100% by repeated measure. Genotyping was performed by ST and CN, who were blinded to clinical information.

Statistical analysis

The analysis of variance and $\chi^2$ test were used to evaluate differences in patients’ characteristics stratified by VDR genotypes. Overall survival curves were drawn using the Kaplan–Meier method and compared by VDR polymorphisms using log-rank tests. Cox proportional hazard models were fitted for multivariate method and compared by VDR polymorphisms using log-rank test. Overall survival curves were drawn using the Kaplan–Meier method and compared by VDR polymorphisms using log-rank tests. Cox proportional hazard models with adjustment for postoperative stages, histology, existence of residual tumour and ages (Table 2). Without multivariate analysis, patients with FokI C/C genotype, stage I disease, or without residual tumour after surgery did show a significantly reduced crude hazard ratio. Even with multivariate analysis, patients with the FokI C/C genotype showed significantly worse survival compared to patients with C/T or T/T genotypes (Figure 1B). When cancer stages were restricted to stage I, there was no significant difference between C/C and C/T plus T/T genotypes (Figure 1B). When cancer stages were restricted to stages III–IV, around 53% of the patients had advanced stages of disease (stages III–IV). We confirmed that the polymorphism FokI was common between the tumour and peripheral blood samples obtained from the same 10 patients, suggesting genomic alterations, but not somatic mutations. Genotype frequencies of the VDR polymorphisms based on tumour samples were as follows: FokI C/C, 35%; C/T, 55%; and T/T, 10%. Patients’ characteristics were not significantly different among FokI genotypes (Table 1).

RESULTS

Patients’ characteristics

Patients ranged in age from 42–64 years. When classified under FIGO staging, around 53% of the patients had advanced stages of disease (stages III–IV). We confirmed that the polymorphism FokI was common between the tumour and peripheral blood samples obtained from the same 10 patients, suggesting genomic alterations, but not somatic mutations. Genotype frequencies of the VDR polymorphisms based on tumour samples were as follows: FokI C/C, 35%; C/T, 55%; and T/T, 10%. Patients’ characteristics were not significantly different among FokI genotypes (Table 1).

Cox proportional hazard models with multivariate adjustment

Cox proportional hazard models were computed to determine the significance of FokI with adjustment for postoperative stages, histology, existence of residual tumour and ages (Table 2). Without multivariate analysis, patients with FokI C/C genotype, stage I disease, or without residual tumour after surgery did show a significantly reduced crude hazard ratio. Even with multivariate analysis, patients with the FokI C/C genotype showed significantly worse survival compared to patients with C/T or T/T genotypes (Figure 1B). When cancer stages were restricted to stages III–IV, around 53% of the patients had advanced stages of disease (stages III–IV). We confirmed that the polymorphism FokI was common between the tumour and peripheral blood samples obtained from the same 10 patients, suggesting genomic alterations, but not somatic mutations. Genotype frequencies of the VDR polymorphisms based on tumour samples were as follows: FokI C/C, 35%; C/T, 55%; and T/T, 10%. Patients’ characteristics were not significantly different among FokI genotypes (Table 1).

Table 1 Patients’ characteristics stratified by FokI genotype

| Variable number (%) | Total 101 | Fok I C/C 35 (35) | Fok I C/T 56 (55) | Fok I T/T 10 (10) | P value |
|---------------------|-----------|------------------|-----------------|------------------|---------|
| Age, year (mean ± s.d.) | 53.1 ± 10.6 | 52.0 ± 8.8 | 54.6 ± 12.1 | 48.1 ± 5.6 | 0.18 a |
| Histological type; Number (%) | | | | | 0.54 b |
| Endometrioid | 21 | 9 (43) | 11 (52) | 1 (5) | |
| Clear cell | 35 | 10 (29) | 22 (63) | 3 (9) | |
| Serous | 38 | 12 (32) | 20 (53) | 6 (16) | |
| Mucinous | 6 | 4 (67) | 2 (33) | 0 | |
| Mixed mesodermal | 1 | 0 | 1 | 0 | |
| FIGO staging | | | | | 0.36 b |
| I (A, B, C) | 41 | 15 (36) | 22 (54) | 4 (10) | |
| II (A, B, C) | 6 | 1 (17) | 3 (50) | 2 (33) | |
| III (A, B, C) | 44 | 16 (36) | 26 (59) | 2 (5) | |
| IV | 10 | 3 (30) | 5 (50) | 2 (20) | |
| Residual tumour b | | | | | 0.43 b |
| < 2 cm | 69 | 25 (36) | 36 (52) | 8 (12) | |
| 2 cm | 12 | 3 (25) | 7 (58) | 2 (17) | |
| 2 cm | 20 | 7 (35) | 13 (65) | 0 (0) | |

Abbreviation: FIGO = Federation of Gynecology and Obstetrics. ANOVA, $\chi^2$ test. *Existence and size of residual tumour after surgery.
DISCUSSION

In this study, we found that the VDR FokI C/C genotype was associated with a better overall survival rate in patients with EOC than a combination of FokI C/T and FokI T/T, even after adjustment for cancer stage, histology, existence of residual tumour and age. Arai et al. (1997) showed that compared with the FokI T/T genotype, FokI C/C had 1.7-fold greater function of vitamin D-dependent transcriptional activation of a reporter construct under the control of a vitamin D response element in transfected HeLa cells. Similarly, Colin et al. (2000) showed a significantly lower (50%) effective dose of 1,25-(OH)2D3 in the FokI C/C genotype than in FokI C/T genotype. By switching from ATG (FokI T) to ACG (FokI C) polymorphism, the first potential start site moves to the 3' direction, resulting in proteins that are three amino acids shorter and more functional (Gross et al., 1996). Thus, patients with FokI C/C may react more to vitamin D, resulting in a higher survival rate.

Table 2  Cox proportional hazard models

| Variable                  | Single-variate analyses | Multivariate analysis |
|---------------------------|-------------------------|----------------------|
|                           | Crude HR                | 95% CI               | P value | AHR       | 95% CI | P value |
| FokI C/C: 66              | 0.26                    | 0.09 – 0.76          | 0.014   | 0.18      | 0.05 – 0.61 | 0.006 |
| FokI C/T or T/T: 35       | Reference               |                      |         | Reference |          |        |
| Stage I: 41               | 0.05                    | 0.005 – 0.42         | 0.006   | 0.02      | 0.002 – 0.28 | 0.004 |
| Stage II: 6               | 0.78                    | 0.14 – 4.32          | 0.78    | 0.76      | 0.89 – 6.54 | 0.81  |
| Stage III: 44             | 1.32                    | 0.45 – 3.86          | 0.62    | 1.09      | 0.33 – 3.61 | 0.89  |
| Stage IV: 10              | Reference               |                      |         | Reference |          |        |
| Histology                 |                         |                      |         |           |          |        |
| Endometrioid: 21          | 0.17                    | 0.02 – 1.71          | 0.13    | 0.05      | 0.03 – 0.95 | 0.04  |
| Clear Cell: 35            | 0.51                    | 0.06 – 4.04          | 0.52    | 0.53      | 0.05 – 5.16 | 0.58  |
| Serous: 38                | 0.60                    | 0.08 – 4.70          | 0.63    | 0.08      | 0.07 – 0.88 | 0.04  |
| Mucinous: 6               | Reference               |                      |         | Reference |          |        |
| Mixed mesodermal: 1       | 0.27                    | 0.02 – 4.31          | 0.35    | 0.09      | 0.04 – 2.10 | 0.13  |
| Residual tumour           |                         |                      |         |           |          |        |
| None: 69                  | Reference               |                      |         | Reference |          |        |
| <2 cm: 12                 | 5.67                    | 1.84 – 17.43         | <0.001  | 1.73      | 0.39 – 7.69 | 0.47  |
| ≥2 cm: 20                 | 11.19                   | 4.36 – 28.72         | <0.001  | 4.93      | 1.31 – 18.53 | 0.02  |
| Age                       | 1.06                    | 1.01 – 1.10          | 0.005   | 1.01      | 0.98 – 1.05 | 0.49  |

Abbreviations: HR = hazard ratio; CI = confidence interval; AHR = adjusted hazard ratio. Adjusted for postoperative stages, postoperative chemotherapy, histology (endometrioid, clear cell, serous, mucinous, mixed mesodermal), and residual tumour (none, <2 cm, ≥2 cm).

better prognostic markers: AHR, 0.18; 95% CI, 0.05 to 0.61; P = 0.006.
Higher serum levels of 25-hydroxyvitamin D (25(OH)D) can be associated with better outcome in EOC, as reported in early stages of breast, colon, and lung cancer (Zhou et al., 2007; Ng et al., 2008; Goodwin et al., 2009). Moreover, ecological studies showed that solar ultraviolet B irradiance was inversely associated with incidence rates of ovarian cancer (Garland et al., 2006) as well as survival of patients with ovarian cancer (Grant, 2006), implying a link between ultraviolet B irradiance, vitamin D, and ovarian cancer both in susceptibility and survival. A plausible explanation for why increased sun exposure and higher circulating levels of vitamin D are associated with a decreased risk of deadly cancers is as follows (Holick, 2007). Epithelial cells convert the primary circulating form of vitamin D, (25(OH)D), to its active form, 1,25-dihydroxyvitamin D, inside the cells, which bind VDR in their nucleus to regulate a variety of genes. These genes help prevent malignant transformation by keeping cellular proliferation and differentiation within normal ranges. In turn, if a cell becomes malignant, 1,25-dihydroxyvitamin D can induce apoptosis and prevent angiogenesis, thereby reducing the potential for the malignant cell to survive. Vitamin D3 derivative was shown to induce apoptosis of ovarian cells but not of primary fibroblasts and to suppress ovarian tumour growth in a mice model (Zhang et al., 2005; Lange et al., 2009).

There are several limitations to our study. Overall, the sample size is not large enough to detect significant differences in patients stratified by histology or stages. We did not use restriction fragment length polymorphism analysis and directly sequenced PCR fragments to avoid misclassification as much as possible and to compensate for the disadvantage of the small sample size. Moreover, cases were not consecutively selected, which may cause selection bias. This study was performed as post-hoc analysis of prospective cohort hospital-based study to detect important gene mutations in ovarian cancer. Thus, serum 25(OH)D was not measured, vitamin D and its synergistic effect with VDR polymorphism were not evaluated. There are other kinds of VDR polymorphisms, such as Cdx-2 and BsmI, related to disease risks that have been reported in previous articles, and we need to expand our range of analysis in the future.

In conclusion, there are still significant associations between better prognosis of patients with EOC and the FokI C/C genotype.

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Conflict of interest

The authors declare no conflict of interest.

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