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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 *Fok*I was shown to be associated with susceptibility to ovarian cancer. We aimed to examine whether VDR *Fok*I polymorphisms influence the survivals of patients with epithelial ovarian cancer (EOC).

METHODS: VDR polymorphisms from *Fok*I in 101 patients with EOC were genotyped by sequencing. Overall survival was compared between *Fok*I 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 *Fok*I 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 *Fok*I genotype may be associated with improved prognosis of patients with EOC.

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Recently, a meta-analysis showed that patients with one of single nuclear polymorphisms vitamin D receptor (VDR), the *Fok*I (rs2228570) TT genotype, had a significantly higher risk for developing ovarian cancer as well as prostate, breast, skin, non-Hodgkin lymphoma, and colorectal cancer compared with its CC genotype (Raimondi *et al*, 2009). Moreover, combined results from four case–control studies showed almost the same results regarding enhanced susceptibility to ovarian cancer (Tworoger *et al*, 2009). However, the risk of developing ovarian cancer based on *Fok*I genotype is minor (Lurie *et al*, 2007), and study findings are controversial (Clendenen *et al*, 2008).

Recently, Heist *et al* (2008) showed that the T allele of VDR *Fok*I polymorphisms was associated with significantly worse survival in patients with advanced non-small cell lung cancer, which is the first report to show a relationship between VDR polymorphisms and prognosis of patients with cancer. Although some articles show an association between *Fok*I polymorphisms and susceptibility to epithelial ovarian cancer (EOC), no report has shown a relationship between *Fok*I polymorphisms and survival of patients with EOC. Therefore, we investigated whether VDR *Fok*I polymorphisms influenced the prognosis of patients with EOC.

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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 *Fok*I 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 *Fok*I were amplified by polymerase chain reaction (PCR) using the following primers (forward/reverse): ctccgaaggcactgtgctcaggcct/atggaaa-caccttgcttcttctccctc; sequence, ggcctgggccctggggagat; 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 χ^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 analysis using postoperative stages (stages I–IV), histology (endometrioid, clear cell, serous mucinous, mixed mesodermal), residual tumour (none, <2 cm, $\geqslant 2$ cm), and ages. Adjusted hazard ratios (AHR) and 95% confidence intervals (CI) were computed. All statistical analyses were performed using STATA 9.1 (STATA Corp., College Station, TX, USA). $P<0.05$ was considered statistically significant.

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 *Fok*I 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: *Fok*I C/C, 35%; C/T, 55%; and TT, 10%. Patients' characteristics were not significantly different among *Fok*I genotypes (Table 1).

*Fok*I polymorphisms and overall survival

All 101 patients were followed for a median of 85 months; 45 (45%) relapsed and 28 (28%) died of EOC. Kaplan–Meier survival curves showed that the *Fok*I C/C genotype was associated with a better prognosis. At 30 months after surgery, 90% of patients with *Fok*I C/C genotype were still alive; in contrast, only 66% of patients with C/T and T/T were alive (Figure 1A). When cancer stage was 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 II–IV, 84% of patients with *Fok*I C/C were still alive; in contrast, only 50% of patients with C/T and T/T were still alive (Figure 1C).

Cox proportional hazard models with multivariate adjustment

Cox proportional hazard models were computed to determine the significance of *Fok*I with adjustment for postoperative stages, histology, existence of residual tumour and ages (Table 2). Without multivariate analysis, patients with *Fok*I 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 *Fok*I C/C genotype showed significantly

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[c]					0.43[b]
None	69	25 (36)	36 (52)	8 (12)	
<2 cm	12	3 (25)	7 (58)	2 (17)	
$\geqslant 2$ cm	20	7 (35)	13 (65)	0 (0)	

Abbreviation: FIGO = Federation of Gynecology and Obstetrics. [a]ANOVA. [b]χ^2 test. [c]Existence and size of residual tumour after surgery.

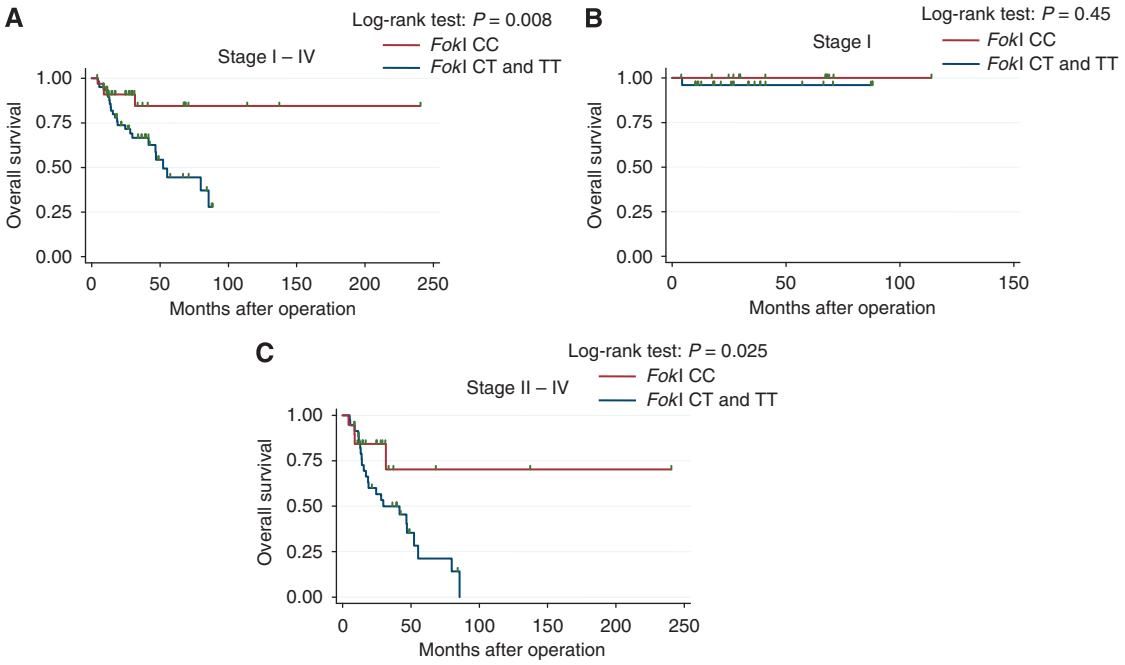

Figure 1 Kaplan–Meier curves of overall survival by (**A**) stages I–IV, (**B**) stage I, (**C**) stages II–IV.

Table 2 Cox proportional hazard models

	Single-variate analyses			Multivariate analysis		
Variable	**Crude HR**	**95% CI**	***P* value**	**AHR**	**95% CI**	***P* value**
*Fok*I C/C: 66	0.26	0.09–0.76	0.014	0.18	0.05–0.61	0.006
*Fok*I 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	0.003	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; $P = 0.006$.

DISCUSSION

In this study, we found that the VDR *Fok*I C/C genotype was associated with a better overall survival rate in patients with EOC than a combination of *Fok*I C/T and *Fok*I T/T, even after adjustment for cancer stage, histology, existence of residual tumour and age. Arai *et al* (1997) showed that compared with the *Fok*I T/T genotype, *Fok*I 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-$(OH)_2D_3$ in the FokI C/C genotype than in *Fok*I C/T genotype. By switching from ATG (*Fok*I T) to ACG (*Fok*I 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 *Fok*I C/C may react more to vitamin D, resulting in a higher survival rate.

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-dihydoroxyvitamin 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 *Bsm*I, 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 were significant associations between better prognosis of patients with EOC and the *Fok*I C/C genotype.

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Conflict of interest

The authors declare no conflict of interest.

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