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Molecular alterations in endometrial and ovarian clear cell carcinomas: clinical impacts of telomerase reverse transcriptase promoter mutation

Hsien-Neng Huang[1,2,7], Ying-Cheng Chiang[3,4,7], Wen-Fang Cheng[3,4,5], Chi-An Chen[3], Ming-Chieh Lin[6] and Kuan-Ting Kuo[1,6]

[1]Department of Pathology, College of Medicine, Graduate Institute of Pathology, National Taiwan University, Taipei, Taiwan; [2]Department of Pathology, National Taiwan University Hospital Hsin-Chu Branch, Hsinchu, Taiwan; [3]Department of Obstetrics and Gynecology, College of Medicine, National Taiwan University, Taipei, Taiwan; [4]Graduate Institute of Clinical Medicine, College of Medicine, National Taiwan University, Taipei, Taiwan; [5]Graduate Institute of Oncology, College of Medicine, National Taiwan University, Taipei, Taiwan and [6]Department of Pathology, College of Medicine, National Taiwan University Hospital, National Taiwan University, Taipei, Taiwan

Recently, mutations of *telomerase reverse transcriptase* (*TERT*) promoter were found in several types of cancer. A few reports demonstrate *TERT* promoter mutations in ovarian clear cell carcinomas but endometrial clear cell carcinoma has not been studied. The aims of this study were to compare differences of molecular alterations and clinical factors, and identify their prognostic impact in endometrial and ovarian clear cell carcinomas. We evaluated mutations of the *TERT* promoter and *PIK3CA*, expression of ARID1A, and other clinicopathological factors in 56 ovarian and 14 endometrial clear cell carcinomas. We found that *TERT* promoter mutations were present in 21% (3/14) of endometrial clear cell carcinomas and 16% (9/56) of ovarian clear cell carcinomas. Compared with ovarian clear cell carcinomas, endometrial clear cell carcinomas showed older mean patient age ($P<0.001$), preserved ARID1A immunoreactivity ($P=0.017$) and infrequent *PIK3CA* mutation ($P=0.025$). In ovarian clear cell carcinomas, *TERT* promoter mutations were correlated with patient age >45 ($P=0.045$) and preserved ARID1A expression ($P=0.003$). In cases of endometrial clear cell carcinoma, *TERT* promoter mutations were not statistically associated with any other clinicopathological factors. In ovarian clear cell carcinoma patients with early FIGO stage (stages I and II), *TERT* promoter mutation was an independent prognostic factor and correlated with a shorter disease-free survival and overall survival ($P=0.015$ and 0.009, respectively). In recurrent ovarian clear cell carcinoma patients with early FIGO stage, *TERT* promoter mutations were associated with early relapse within 6 months ($P=0.018$). We concluded that *TERT* promoter mutations were present in endometrial and ovarian clear cell carcinomas. Distinct molecular alteration patterns in endometrial and ovarian clear cell carcinomas implied different processes of tumorigenesis in these morphologically similar tumors. In ovarian clear cell carcinoma of early FIGO stage, patients with *TERT* promoter mutation require close follow-up during the initial 6 months following chemotherapy.
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Ovarian clear cell carcinoma represents 5–25% of all epithelial ovarian carcinomas with geographic variation.[1] Compared with high-grade serous adenocarcinomas, ovarian clear cell carcinomas are characterized by a higher incidence among Asians, younger patient age and early tumor staging at presentation, association with endometriosis, higher frequencies of AT-rich interactive domain 1 A (*ARID1A*) mutation and phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (*PIK3CA*) mutation, and higher resistance to first-line platinum and taxane-based chemotherapy.[2–4]

Correspondence: Dr K-T Kuo, MD, Department of Pathology, College of Medicine, National Taiwan University Hospital, National Taiwan University, Taiwan, 3rd floor, No. 7, Chung Shan South Road, Taipei 10001, Taiwan.
E-mail: pathologykimo@gmail.com
[7]These authors contributed equally to this work.
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Primary endometrial clear cell carcinoma (which has similar histologic characteristics to ovarian clear cell carcinoma) is a rare histologic type that accounts for less than 3% of all endometrial carcinomas.[5,6] Interobserver reproducibility of endometrial clear cell carcinoma was poor,[7,8] and a panel of hepatocyte nuclear factor-1β (HNF-1β) and estrogen receptor (ER) immunohistochemistry was suggested for differentiation of endometrial clear cell carcinoma from endometrioid and serous carcinoma.[9] Owing to the rarity of endometrial clear cell carcinoma, information on the molecular characterization of this tumor is limited. Previous studies demonstrated that loss of ARID1A expression occurred in 22.7%[10] to 40%[11] of endometrial clear cell carcinomas but mutations of the *TP53* or *PTEN* gene were uncommon.[12]

Telomeres are located at the ends of eukaryotic chromosomes and are crucial for chromosomal integrity and avoidance of cell death or senescence. Telomerase is a ribonucleoprotein and can add TTAGGG hexamers to the ends of chromosomes.[13] Telomerase activity is generally higher in cancer cells than normal cells and is required to maintain tumor growth.[13] Telomerase reverse transcriptase (TERT) is a catalytic subunit of telomerase,[14] and increased activity of TERT is present in most cancers, including ovarian cancer.[14,15] Mutations of *TERT* promoter contribute to a mechanism that allows for increased telomerase activity and is found in melanomas,[16,17] thyroid carcinomas,[18,19] myxoid liposarcomas, hepatocellular carcinomas, urothelial carcinomas, squamous cell carcinomas of the tongue, medulloblastomas, and gliomas.[20] Most *TERT* promoter mutations in malignancies are located at two hotspots, $-124C>T$ and $-146C>T$.[20] The mutations of the *TERT* promoter in these two hotspots lead to the creation of E-twenty-six/ternary complex factor-binding motifs and a subsequent increase in transcription.[16,17]

Only two previous reports have studied *TERT* promoter mutations in cancers of the gynecologic tract.[20,21] Killela *et al*[20] described the presence of *TERT* promoter mutations in endometrial, ovarian, and cervical cancer. Wu *et al*[21] reported that ovarian clear cell carcinoma was the predominant histologic type of gynecological malignancy that harbored mutations of the *TERT* promoter. However, *TERT* promoter mutations of endometrial clear cell carcinoma have not been studied. Molecular genetic differences between endometrial and ovarian clear cell carcinoma are also still unclear. Therefore, we analyzed mutations of the *TERT* promoter and *PIK3CA*, expression of ARID1A, and clinical information in endometrial and ovarian clear cell carcinomas. Then we evaluated the molecular and clinical differences in endometrial and ovarian clear cell carcinomas, relationships between *TERT* promoter mutations, and other clinicopathological factors, and prognostic impacts of these clinicopathological factors in ovarian clear cell carcinomas.

Materials and methods

Patients and Tissue Materials

Seventy formalin-fixed, paraffin-embedded tissue specimens (56 ovarian and 14 endometrial clear cell carcinomas) were obtained from the archives of 1995–2011, at the Department of Pathology of National Taiwan University Hospital. Some cases of ovarian clear cell carcinoma were also reported in our previous study.[22] All patients underwent operations including total hysterectomy with bilateral salpingo-oophorectomy, at least, and no concurrent endometrial and ovarian clear cell carcinomas were found. All patients with ovarian clear cell carcinoma underwent debulking surgery. The specimens were diagnosed as pure ovarian or endometrial clear cell carcinoma according to the World Health Organization classification.[23] Except for pure and typical morphology, cases of endometrial clear cell carcinoma in this study were diffusely positive for the HNF-1β immunostain but negative or focally positive ($<10\%$) for ER immunostain (Figure 1).

Immunohistochemistry

Immunohistochemical staining of ARID1A was performed with protocols as previously described.[22] The ARID1A immunoreactivities were divided into either undetectable or positive (weakly or strongly) for nuclear staining, and stromal cells were used as an internal positive control.[24]

DNA Extraction and Mutation Analysis

DNA extraction, polymerase chain reaction (PCR) amplification for sequencing of the *TERT* promoter and the *PIK3CA* gene were performed in all cases by protocols as previously described.[3,16] Tumor components from formalin-fixed and paraffin-embedded tissue blocks were manually dissected from 10-μm sections. Genomic DNA of tumor tissue was extracted using the QIAmp DNA FFPE tissue kit (Qiagen, CA). The primer sets used for *PIK3CA* were listed as follows: 5′-TCAGCAGTTACTATTCTGTGACTGG-3′ (forward primer) and 5′-GTAAAACGACGGCCAG TTGCTGAGATCAGCCAAATTCA-3′ (reverse primer) for exon 9, as well as 5′-GTAAAACGACGGCCAG TGACATTTGAGCAAAGACCTGAAG-3′ (forward primer) and 5′-TGGATTGTGCAATTCCTATGC-3′ (reverse primer) for exon 20.[3] Two primer sets were used for *TERT* promoter.[16] Primer set no. 1 was 5′-ACGAACGTGGCCAGCGGCAG-3′ (forward primer) and 5′-CTGGCGTCCCTGCACCCTGG-3′ (reverse primer) and the product was 474 bps in length. Primer set no. 2 was employed in cases where amplification of the fragment failed. The primer set no. 2 was 5′-CAGCGCTGCCTGAAACTC-3′ (forward primer) and 5′-GTCCTGCCCCTTCACCTT-3′ (reverse primer).

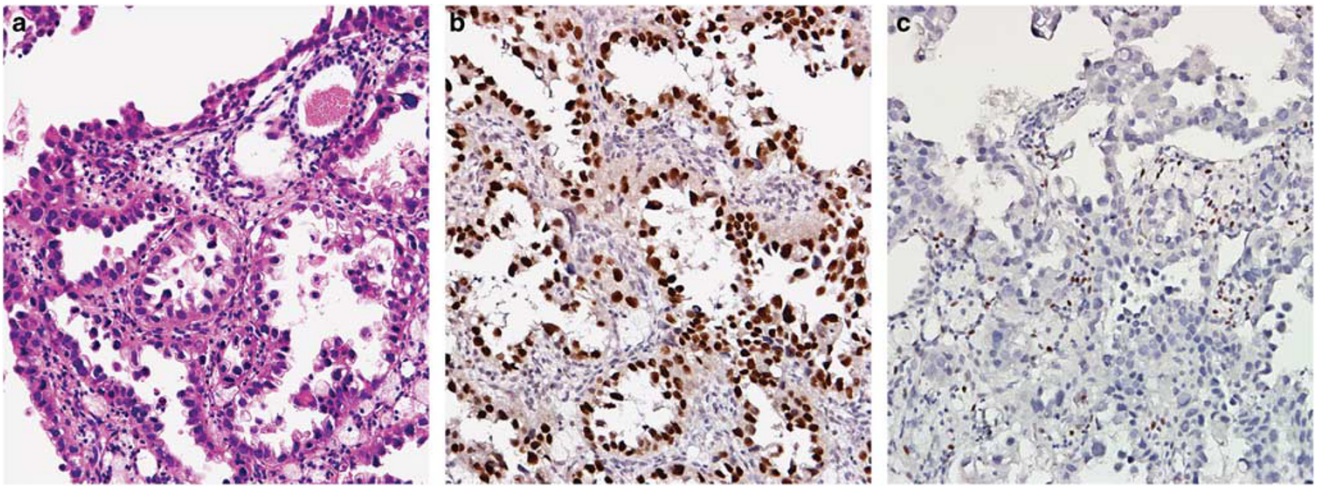

Figure 1 Representative hematoxylin and eosin-stained sections (**a**) and immunohistochemistry for HNF-1β (**b**) and ER (**c**) of endometrial clear cell carcinoma used in the study.

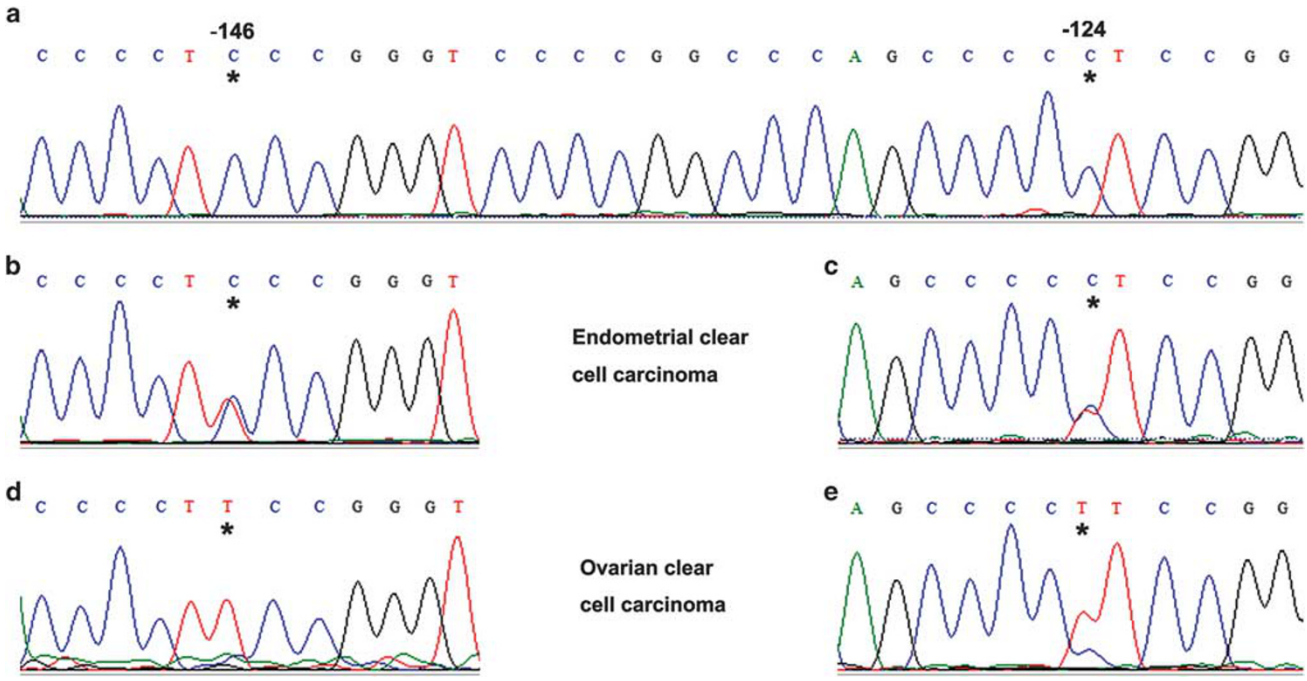

Figure 2 Representative chromatograms of wild-type *TERT* promoter (**a**) and mutational status of endometrial (**b**, **c**) and ovarian (**d**, **e**) clear cell carcinomas at two different hotspots (highlighted by asterisks), $-124C>T$ and $-146C>T$.

Sanger DNA sequencing was performed using ABI 3730 DNA Analyzer (Life Technologies, NY).

Statistical Analysis

The statistical analyses were conducted using PASW Statistics (IBM Corporation, Armonk, NY, USA). Comparison of molecular alterations in endometrial and ovarian clear cell carcinomas, as well as associations between *TERT* promoter mutation and other clinicopathological factors, were evaluated using the χ^2 test or Fisher's exact test. Mann–Whitney *U*-test was used to assess the differences in age between patients with endometrial and ovarian clear cell carcinomas. The influences of clinicopathological parameters on disease-free survival and overall survival were analyzed by Cox proportional hazard model. *P*-values from Wald's statistic were recorded. The Kaplan–Meier survival analysis with log-rank significance test was used to estimate the probabilities of survival. The cutoff of significance level was 0.05.

Table 1 Comparison of clinicopathological factors between endometrial and ovarian clear cell carcinomas

	Endometrial (n = *14*)	*Ovarian* (n = *56*)	P-*value*
Mean age	71 Years	48 Years	<0.001
Loss of ARID1A expression	3 (21%)	32 (57%)	0.017
TERT promoter mutation	3 (21%)	9 (16%)	0.695
PIK3CA mutation	1 (7%)	23 (41%)	0.025

Results

The mean ages of endometrial and ovarian clear cell carcinoma patients were 71 and 48 years old, respectively. The median follow-up period for patients of ovarian clear cell carcinomas was 31 months (1–207 months). Prognostic factors of endometrial clear cell carcinomas were not evaluated because of the limited number of cases included.

TERT promoter mutations were present in 16% (9/56) of ovarian clear cell carcinomas and 21% (3/14) of endometrial clear cell carcinomas. In ovarian clear cell carcinomas with *TERT* promoter mutations, eight cases were −124C>T and one case was −146C>T. In endometrial clear cell carcinomas, two cases were −146C>T and one case was −124C>T (Figure 2).

Comparisons of clinicopathological factors of endometrial and ovarian clear cell carcinomas are shown in Table 1. Compared with ovarian clear cell carcinomas, endometrial clear cell carcinomas were characterized by older mean patient age ($P<0.001$), preserved ARID1A immunoreactivity ($P=0.017$) and infrequent *PIK3CA* mutation ($P=0.025$).

In endometrial clear cell carcinomas, *TERT* promoter mutations were not correlated with age ($P=0.209$), loss of ARID1A expression ($P=0.547$), or *PIK3CA* mutation ($P=1.000$). Associations between *TERT* promoter mutations and other clinicopathological factors in ovarian clear cell carcinomas are listed in Table 2. In ovarian clear cell carcinomas, *TERT* promoter mutations were correlated to patient age >45 years ($P=0.045$) and preserved ARID1A expression ($P=0.003$). In early-stage ovarian clear cell carcinomas (FIGO stage I and II), *TERT* promoter mutations were associated with early relapse within 6 months after chemotherapy (100% *vs* 11%, $P=0.018$), but not correlated to other clinicopathological factors. In advanced-stage ovarian clear cell carcinomas (FIGO stage III and IV), *TERT* promoter mutations were related to preserved ARID1A expression ($P=0.036$), but not associated with other clinicopathological factors.

The prognostic effects of clinicopathological factors are summarized in Table 3. The FIGO stage was the only prognostic factor for ovarian clear cell carcinoma ($P<0.001$). For ovarian clear cell carcinoma patients in the early stages, *TERT* promoter mutations were the only prognostic factor and correlated with shorter disease-free survival ($P=0.030$) and overall survival ($P=0.023$). Kaplan–Meier analysis also revealed that DFS disease-free survival and overall survival were shorter among the *TERT* promoter mutated patients of the early stages ($P=0.015$ in disease-free survival and 0.009 in overall survival, respectively; Figure 3). In ovarian clear cell carcinoma with advanced staging, FIGO stage IV was the only prognostic factor for ovarian clear cell carcinoma ($P=0.030$ for disease-free survival and 0.002 for overall survival).

Table 2 Correlations between *TERT* promoter mutation and clinicopathological factors in ovarian clear cell carcinomas

		TERT *promoter No. (%)*		
All patients (n = *56*)	*No.*	*Mutant* n = *9 (16)*	*Wild-type* n = *47 (84)*	P-*value*
Age				0.045
≤45	18	0	18 (38)	
>45	38	9 (100)	29 (62)	
FIGO stage				1.000
I + II	34	6 (67)	28 (60)	
III + IV	22	3 (33)	19 (40)	
Relapse[a]				0.458
Present	25	5 (63)	20 (45)	
Absent	27	3 (38)	24 (55)	
Relapse period				0.133
<6 Months	11	4 (80)	7 (35)	
≥6 Months	14	1 (20)	13 (65)	
ARID1A expression				0.003
Negative	32	1 (11)	31 (66)	
Positive	24	8 (89)	16 (34)	
PIK3CA *mutation*				0.074
Mutant	22	1 (11)	21 (45)	
Wild type	34	8 (89)	26 (55)	

[a]Not assessed in four cases.

Discussion

This is the first report on the *TERT* promoter mutations in endometrial clear cell carcinomas. We found that *TERT* promoter mutations were present in endometrial clear cell carcinomas with similar prevalence and hotspots as in ovarian clear cell carcinomas. In the study by Killela *et al*,[20] *TERT* promoter mutations were present in two of nineteen endometrial carcinomas without specifying histologic type. However, all of the endometrial carcinomas, including 24 endometrioid adenocarcinomas and 12 serous adenocarcinomas, were absent for *TERT* promoter mutation in the study by Wu *et al*.[21] Combined with the findings of ours, Killela, and

Table 3 Univariate analysis of survival in ovarian clear cell carcinomas of all (a), early-stage (b), and advanced-stage (c) patients

(a) *All patients* (n = *56*)	*No.*	*Disease-free survival HR (95% CI)*	P-*value*	*Overall survival HR (95% CI)*	P-*value*
Age			0.129		0.128
≤45	18	1.0		1.0	
>45	38	2.34 (0.78–7.01)		2.33 (0.78–6.91)	
FIGO stage			<0.001		<0.001
I + II	34	1.0		1.0	
III + IV	22	5.84 (2.29–14.86)		5.07 (2.04–12.64)	
TERT *promoter*			0.123		0.224
Mutant	9	1.0		1.0	
Wild type	47	0.45 (0.17–1.24)		0.54 (0.20–1.47)	
ARID1A expression			0.729		0.665
Negative	32	1.0		1.0	
Positive	24	0.86 (0.36–2.03)		0.83 (0.35–1.95)	
PIK3CA *mutation*			0.159		0.264
Mutant	22	1.0		1.0	
Wild type	34	1.98 (0.77–5.10)		1.65 (0.68–4.00)	

(b) *Early-stage patients* (n = *34*)	*No.*	*Disease-free survival HR (95% CI)*	P-*value*	*Overall survival HR (95% CI)*	P-*value*
Age			0.226		0.245
≤45	9	1.0		1.0	
>45	25	47.53 (0.09–246.09)		39.99 (0.08–297.64)	
FIGO stage			0.058		0.211
I	29	1.0		1.0	
II	5	4.20 (0.96–18.46)		2.61 (0.58–11.69)	
TERT *promoter*			0.030		0.023
Mutant	6	1.0		1.0	
Wild type	28	0.19 (0.04–0.85)		0.16 (0.03–0.78)	
ARID1A expression			0.950		0.915
Negative	18	1.0		1.0	
Positive	16	0.96 (0.24–3.89)		1.08 (0.27–4.32)	
PIK3CA *mutation*			0.304		0.294
Mutant	15	1.0		1.0	
Wild type	19	2.37 (0.46–12.27)		2.41 (0.47–12.45)	

(c) *Advanced-stage patients* (n = *22*)	*No.*	*Disease-free survival HR (95% CI)*	P-*value*	*Overall survival HR (95% CI)*	P-*value*
Age			0.335		0.303
≤45	9	1.0		1.0	
>45	13	1.77 (0.56–5.65)		1.84 (0.58–5.89)	
FIGO stage			0.030		0.002
III	18	1.0		1.0	
IV	4	3.84 (1.14–12.95)		9.08 (2.19–37.65)	
TERT *promoter*			0.754		0.832
Mutant	3	1.0		1.0	
Wild type	19	0.79 (0.18–3.54)		0.85 (0.19–3.86)	
ARID1A expression			0.895		0.960
Negative	14	1.0		1.0	
Positive	8	1.08 (0.36–3.25)		0.97 (0.32–2.92)	
PIK3CA *mutation*			0.582		0.791
Mutant	7	1.0		1.0	
Wild type	15	1.39 (0.43–4.44)		0.85 (0.26–2.83)	

Abbreviations: CI, Confidence interval; HR, hazard ratio.

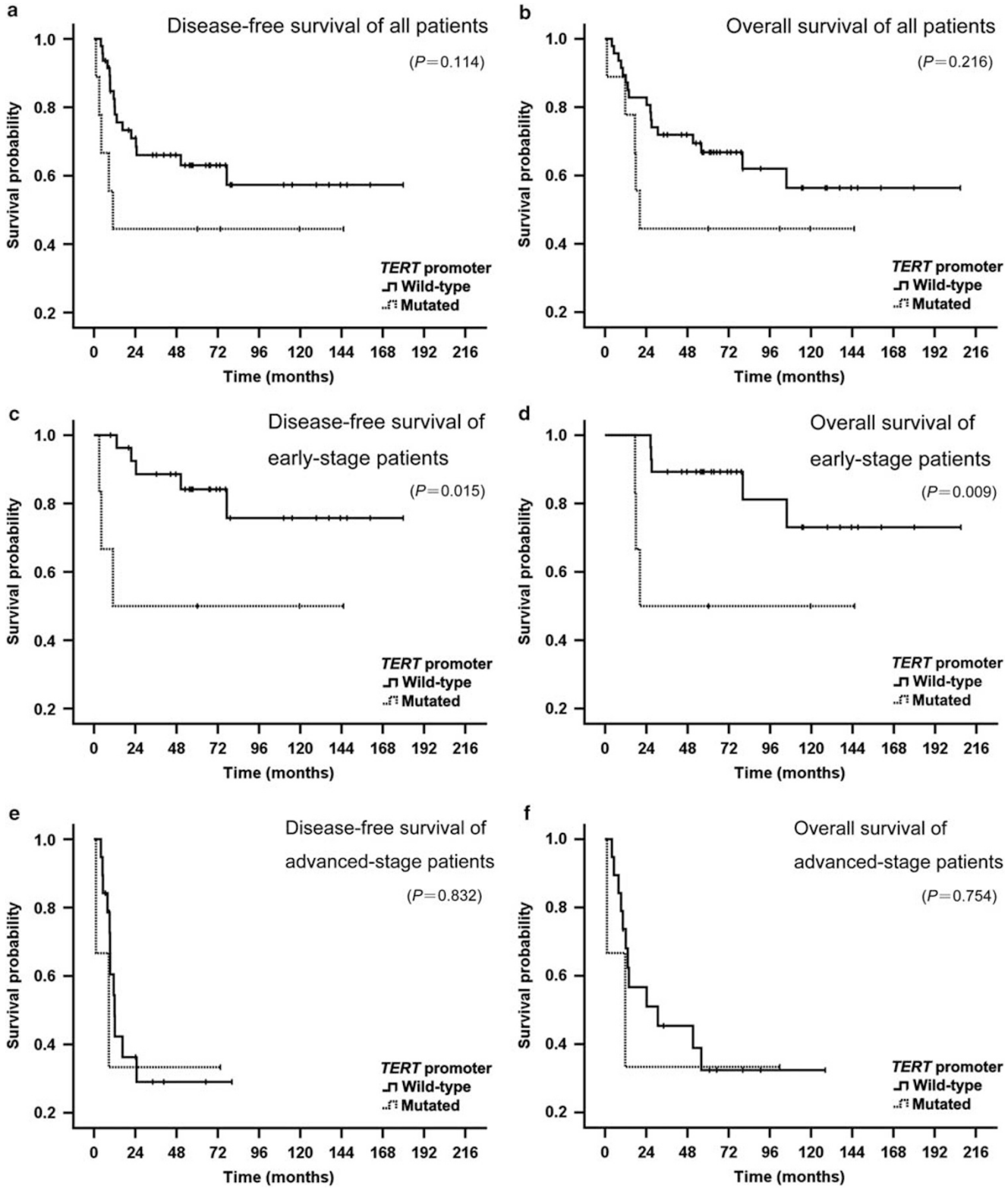

Figure 3 (**a**) Relationship between disease-free survival and *TERT* promoter mutation in patients with ovarian clear cell carcinoma. (**b**) Relationship between overall survival and *TERT* promoter mutation in patients with ovarian clear cell carcinoma. (**c**) Relationship between disease-free survival and *TERT* promoter mutation in patients with early-stage ovarian clear cell carcinoma. (**d**) Relationship between overall survival and *TERT* promoter mutation in patients with early-stage ovarian clear cell carcinoma. (**e**) Relationship between disease-free survival and *TERT* promoter mutation in patients with advanced-stage ovarian clear cell carcinoma. (**f**) Relationship between overall survival and *TERT* promoter mutation in patients with advanced-stage ovarian clear cell carcinoma.

Wu, we suspect that in carcinomas of endometrial origin, clear cell carcinoma is the predominant histologic type that harbors *TERT* promoter mutations. Further studies are needed to clarify the relationship between *TERT* promoter mutations and histologic type of endometrial carcinomas.

Although endometrial and ovarian cell carcinomas shared similar histologic features and a HNF-1$\beta+$/ER$-$ immunoprofile, there were statistically significant differences in other clinical data and molecular genetic alterations in this study. We observed that endometrial clear cell carcinomas tended to have patients who were older at presentation, preserved ARID1A expression, and had wild-type *PIK3CA* gene. These may reflect the different tumor origins and distinct microenvironments during tumor development. Ovarian clear cell carcinoma is often associated with endometriosis. Endometriosis induces a unique microenvironment of long-term blood accumulation with high concentrations of iron and oxidative stress,[25] which is not encountered in endometrial clear cell carcinoma. Except for ultraviolet radiation, oxidative stress is the other cause of mutagenesis in forms of C>T or CC>TT substitutions.[26] This accounts for the possible role of endometriosis-induced oxidative stress in *TERT* promoter mutations of ovarian clear cell carcinomas. Since endometriosis-induced oxidative stress is not present in the endometrium, characteristics of *TERT* promoter mutations may be different in endometrial and ovarian clear cell carcinomas. In our study, *TERT* promoter mutations were present in endometrial and ovarian clear cell carcinomas with similar frequencies. However, we observed that mutations at -124C>T were less frequent in endometrial clear cell carcinomas (33% of all *TERT* promoter mutations) than in ovarian clear cell carcinomas (89%). In the study by Wu *et al*[21], 90% of *TERT* promoter mutations in ovarian clear cell carcinomas occurred at -124C>T. A larger cohort of endometrial clear cell carcinoma is essential to clarify whether -146C>T is the most common type of *TERT* promoter mutation in this histologic type.

In this study, we observed that *TERT* promoter mutations were associated with older patient age (>45 years old) in ovarian clear cell carcinomas. The correlation between *TERT* promoter mutations and older patient age has been shown in glioblastoma, medulloblastoma,[20] papillary thyroid carcinoma (>45 years old),[27] and urothelial carcinoma of the urinary bladder (>50 years old).[28] In contrast, a previous study by Wu *et al* showed that no relationship could be found between patient age and *TERT* promoter mutations.[21] This discrepancy may be the result of different statistical methodologies and different patient cohorts. In the present study, we used Fisher's exact test and a criterion of 45 years old for patient stratification. As the prevalence of ovarian clear cell carcinoma varies in different races,[29] different study cohorts may also account for the disparity between the results of Wu and ours.

In ovarian clear cell carcinomas, we found that *TERT* promoter mutations were mutually exclusive with loss of ARID1A expression ($P=0.003$) or *PIK3CA* mutations ($P=0.074$). The result is similar to that of Wu *et al*.[21] This finding implies independent routes of tumorigenesis between ovarian clear cell carcinomas with mutated *TERT* promoter and ARID1A or *PIK3CA* alterations. More studies are necessary to confirm the relationship between *TERT* promoter mutations and other molecular alterations in endometrial clear cell carcinomas.

In primary glioblastomas,[20] differentiated thyroid carcinomas[30] and urothelial carcinomas of the urinary bladder,[28] *TERT* promoter mutation was an indicator of poor clinical outcome. In ovarian clear cell carcinomas, Widschwendter *et al*[15] and Wu *et al*[21] reported that TERT expression and *TERT* promoter mutation revealed no prognostic impact. Our study also demonstrated a similar result in ovarian clear cell carcinomas. In the present study, we further found that in early-stage patients, *TERT* promoter mutations indicated a likelihood of early relapse within 6 months after chemotherapy and a significantly poorer prognosis. According to the National Comprehensive Cancer Network (NCCN) guidelines, ovarian clear cell carcinomas that recur within 6 months of finishing chemotherapy are viewed as chemotherapy-resistant and should be considered for other therapeutic regimens.[31] Therefore, our study suggests that early-stage patients of ovarian clear cell carcinoma with *TERT* promoter mutations deserve a closer follow-up in the first 6 months after chemotherapy. In previous studies, molecular alterations in ovarian clear cell carcinomas, including *ARID1A*, *PIK3CA*, and *ZNF217*, were either not considered a prognostic factor or signified different conclusions.[22,24,32–37] Our study is the first report on the prognostic impact of *TERT* promoter mutations in early-stage ovarian clear cell carcinomas. *TERT* promoter mutations may have a role in the development of chemoresistance, but more in-depth investigations are necessary.

In conclusion, this is the first report demonstrating *TERT* promoter mutations in endometrial clear cell carcinomas. Although endometrial and ovarian clear cell carcinomas share similar morphological features and some immunoprofiles, there are molecular genetic differences in *PIK3CA* mutation and ARID1A expression, reflecting their different routes of tumorigenesis and distinct microenvironments. In early-stage ovarian clear cell carcinomas, *TERT* promoter mutation correlates with early relapse and is a potential molecular genetic change involved with chemoresistance of tumor cells.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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