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Frequent *CCNE1* amplification in endometrial intraepithelial carcinoma and uterine serous carcinoma

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Uterine serous carcinoma accounts for only 10% of all uterine epithelial cancers, but is the leading cause of death among them. The pathogenesis of this aggressive neoplasm has been largely elusive until recently, when comprehensive genome-wide analyses of uterine serous carcinoma have been performed. Among amplified cancer-related genes, *CCNE1*, encoding for cyclin E1, is frequently amplified in uterine serous carcinoma. In the current study we applied fluorescence *in situ* hybridization (FISH) to determine *CCNE1* copy number in uterine serous carcinoma and concurrent endometrial intraepithelial carcinoma, the noninvasive component of uterine serous carcinoma, and the results were correlated with clinicopathological and molecular features. We found that 20 (45%) of 44 uterine serous carcinomas and 11 (41%) of 27 endometrial intraepithelial carcinomas showed *CCNE1* amplification. Overall, we found high concordance in *CCNE1* copy number in concurrent uterine serous carcinoma and endometrial intraepithelial carcinoma pairs (*P*-value = 0.0003). No correlation was observed between *CCNE1* copy number and clinicopathological features, as well as common mutations previously reported in uterine serous carcinoma. In summary, we confirm that amplification of *CCNE1* is a frequent molecular genetic change in uterine serous carcinoma. Moreover, the identification of *CCNE1* amplification in many endometrial intraepithelial carcinomas suggests that this genetic event occurs early during tumor progression.
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Uterine serous carcinoma accounts for only 10% of all uterine corpus epithelial cancers, but is the leading cause of endometrial cancer-related death.[1,2] The pathogenesis of this aggressive neoplasm has been largely elusive until recently, when comprehensive genome-wide analyses of uterine serous carcinoma were performed.[3–6] Whole-exome sequencing revealed that *TP53*, *PIK3CA*, *FBXW7* and *PPP2R1A* are the most commonly mutated genes in uterine serous carcinoma.[3] Furthermore, we applied SNP arrays for copy number analysis in 23 uterine serous carcinomas, and identified high levels of amplification of *CCNE1,* encoding for cyclin E1, in 26.1% of the cases.[3] This result was subsequently validated by other investigators including those from The Cancer Genome Atlas consortium.[4,5]

Cyclin E1 is a nuclear protein critically involved in the regulation of cell cycle by promoting the transition from G1 phase to S phase.[7] In normal cells, cyclin E1 is expressed when the cells undergo DNA synthesis, and once the task is completed, cyclin E1 expression decreases. One of the mechanisms responsible for cyclin E1 degradation is mediated by ubiquitination through the SCF–Fbxw7 protein complex.[8] Aberrant accumulation of cyclin E1 is common in a variety of carcinomas of breast, cervix, colorectum, stomach, and ovary,[9,10] and as a result, a high level of cyclin E1 shortenings G1 phase, expedites G1/S transition and facilitates cellular proliferation. Interestingly, somatic mutations of *FBXW7,* a gene encoding Fbxw7, are frequently mutated in uterine serous carcinomas including those uterine serous carcinomas without *CCNE1* amplification.[3–5] Defective Fbxw7 is unable to promote cyclin E1 degradation and, consequently, mutated Fbxw7 indirectly enhances cyclin E1 expression.

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Thus, approximately half of uterine serous carcinomas had either a molecular genetic alteration in *FBXW7* or *CCNE1* amplification that may contribute to cyclin E1 overexpression.[3,4] These observations underscore the role of cyclin E–FBXW7 pathway in the development of uterine serous carcinoma.

In the current study, we applied fluorescence *in situ* hybridization (FISH) to determine *CCNE1* copy number at a single-cell resolution in uterine serous carcinoma and concurrent endometrial intraepithelial carcinoma. We also correlated the findings with other molecular alterations and clinicopathological features. Our results not only confirm the frequent amplification of *CCNE1* in uterine serous carcinoma but also provide new evidence that increased *CCNE1* copy number has occurred in the noninvasive stage of uterine serous carcinoma progression, suggesting that *CCNE1* amplification represents one of the early molecular events in the pathogenesis of uterine serous carcinoma.

Materials and methods

Case Selection

A total of 44 uterine serous carcinomas and 27 concurrent endometrial intraepithelial carcinomas were retrieved from the archival file at the Department of Pathology, the Johns Hopkins Hospital (Baltimore, MD). All the available slides were reviewed to confirm the diagnosis by two pathologists (EK and IMS). Both clinicopathological features and molecular characteristics were recorded.[3] This study was approved by the Institutional Review Board of the Johns Hopkins Medical Institutions.

Fluorescence *In Situ* Hybridization

Two-color FISH assay was used to measure the gene copy number of *CCNE1* in endometrial intraepithelial carcinoma and uterine serous carcinoma. Briefly, 4-μm thick sections were incubated at 62 °C for 30 min, deparaffinized in xylene, hydrated through graded ethanol, and placed in deionized water. The sections were incubated in Trilogy™ (cat# CMX833, Cell Marque, Austin, TX), at 88 °C for 40 min and then washed in 2X Aniara saline-sodium citrate (SSC). The slides were then placed in a denaturation solution (70% formamide/2X SCC) at 75 °C for 5 min, rinsed in 2X SSC and allowed to cool down to room temperature for 5 min. The slides were then dehydrated through graded ethanol, and dried in an oven at 62 °C for 2 min. FISH probes for *CCNE1* and CEP19, the centromeric region of chromosome 19 (cat# FG0013, Abnova Corp, Taipei, Taiwan) were applied to the slides according to the vendor's instructions. Denaturation was accomplished by incubating the slides at 80 °C for 15 min. Hybridization was performed at 37 °C for 20–24 h. The slides were washed in 1.5 mol/l urea in 0.2X SSC for 20 min, followed by washing in 2X SSC at room temperature for 2 min. The slides were drained, dehydrated through graded ethanol, air-dried, mounted with ProLong Gold Antifade Reagent with DAPI (cat# P-36931, Invitrogen, Eugene, Oregon, USA) and imaged.[11,12]

Three images from each lesion were acquired at 400X magnification using a Nikon 50i epifluorescence microscope equipped with fluorescence excitation/emission filters for different fluorophores (Omega Optical). Grayscale images were captured using the Nikon NIS-Elements software and an attached Photometrics CoolSNAP EZ digital camera. For presentation and measurement purposes, images were pseudo-colored and merged. We analyzed the *CCNE1* copy number per cell in at least 50 non-overlapping nuclei. The *CCNE1* gene copy number was classified into five FISH strata as previously described.[13] Specifically we defined: (1) *CCNE1* amplification as the presence of either loose or tight *CCNE1* cluster, or the ratio of *CCNE1* to centromeric probe (CEP19) ≥ 2 in more than 20% of the analyzed tumor cells, (2) high polisomy with ≥ 4 copies in $\geq 40\%$ cells, (3) low polisomy with ≥ 4 copies in 10–40% cells, (4) trisomy with ≥ 3 copies in $\geq 10\%$ cells and ≥ 4 copies in $< 10\%$ cells, (5) disomy with 3–4 copies in $< 10\%$ cells. *CCNE1* amplification was considered to have increased gene copy number (FISH positive), and the categories from disomy to low polysomy were considered not to have increased gene copy number (FISH negative).

Statistical Analysis

Comparisons of FISH outcomes between matched endometrial intraepithelial carcinoma and uterine serous carcinoma were obtained using the one-tailed Wilcoxon signed-rank test. A simple linear regression model was used to determine the relationship of the ratio of *CCNE1*/CEP19 FISH signals between uterine serous carcinoma and endometrial intraepithelial carcinoma. R^2 value was calculated. Comparisons of FISH outcome with clinicopathological and molecular data were performed using the two-tailed unpaired Fisher's exact test. Kaplan–Meier survival plot was generated to compare overall survival between patients with different *CCNE1* FISH status, and difference between survival curves were estimated using the long-rank test. *P*-values of 0.05 or less were considered statistically significant. Statistical analysis was carried out using the GraphPad Prism software version 5.0 (GraphPad Software, San Diego, CA, USA).

Results

CCNE1 Copy Number Analysis in Concurrent Uterine Serous Carcinoma and Endometrial Intraepithelial Carcinoma

Gene copy number of *CCNE1* was determined in 44 uterine serous carcinomas and 27 concurrent

endometrial intraepithelial carcinomas using two-color FISH assay. Overall, the frequency of copy number gain in *CCNE1* (FISH positive: amplification; Figures 1 and 2) was similar between uterine serous carcinoma (20 of 44, 45%) and endometrial intraepithelial carcinoma (11 of 27, 41%) (Table 1). Notably, the level of *CCNE1* copy number gain in amplified samples was remarkably high (*CCNE1*/CEP19 ratio 1.3–13.2, median 3.8, Figure 2).

The concordance of *CCNE1* copy number abnormalities between uterine serous carcinoma and endometrial intraepithelial carcinoma from the same case was high (25 of 27, 93%; *P*-value = 0.0003). Fifteen (56%) of 25 matched uterine serous carcinoma and endometrial intraepithelial carcinoma pairs were FISH negative in both lesions, whereas 10 (37%) matched lesions showed *CCNE1* amplification in both tumor lesions (Table 2). Discordance in *CCNE1* copy number status was detected in only two (7%) cases; one of these uterine serous carcinomas had a gain in copy number while endometrial intraepithelial carcinoma was FISH negative, and vice versa in the remaining case. Linear regression also showed a significant correlation of *CCNE1*/CEP19 ratio between uterine serous carcinoma and endometrial intraepithelial carcinoma with a R^2 of 0.900 (Figure 3).

We also observed that three (7%) uterine serous carcinomas showed intratumoral heterogeneity as manifested by regional difference in *CCNE1* copy number alteration, because there were tumor areas with focal *CCNE1* amplification that were adjacent to tumor cells without amplification.

CCNE1 Copy Number and Somatic Gene Mutations

Based on exome sequencing, we have recently reported *TP53*, *FBXW7, PIK3CA,* and *PPP2R1A* as the most commonly mutated genes in uterine serous carcinoma.[3] Information regarding the presence of somatic mutations of these four genes was available in 40 cases. We identified *TP53*, *FBXW7, PIK3CA,* and *PPP2R1A* somatic mutations in 32 (80%), 11 (28%), 6 (15%), and 6 (15%) of 40 cases, respectively.[3] There was no correlation between the presence of any of these mutations and *CCNE1* gene amplification (*P*-values > 0.05, Table 3).

CCNE1 Copy Number and Clinicopathological Characteristics

Clinical information was available in 44 cases. No evidence of correlation was observed between *CCNE1* copy number and clinicopathological features, including age, race, clinical, and pathological stage, overall survival, and angiolymphatic invasion.

Discussion

Recent DNA copy number analyses using SNP arrays have demonstrated that *CCNE1* is one of the most commonly amplified genes in uterine serous carcinoma as it occurs in 23–48% of uterine serous carcinomas.[3–5] However, the prevalence of *CCNE1* amplification has not been reported at the tissue level and it is unclear if *CCNE1* amplification occurs during early stages of tumor development. In the current study we performed two-color FISH assay on 44 uterine serous carcinomas and detected *CCNE1* amplification in 45% of them. Furthermore, we also found that *CCNE1* was amplified in endometrial intraepithelial carcinoma, the noninvasive component of uterine serous carcinoma, indicating that DNA copy number gain of *CCNE1* has occurred at early stages of tumor development. The above results should have several biological and clinical implications.

Like ovarian high-grade serous carcinoma, uterine serous carcinoma is characterized by frequent *CCNE1* amplification in addition to *TP53* mutations in the majority of cases.[3,4] Because both uterine serous carcinoma and ovarian high-grade serous carcinoma develop along the type II pathway in endometrial and ovarian cancers, respectively, this finding raises a possibility that both *TP53* mutation and *CCNE1* amplification are the defining features of type II tumors arising from gynecological organs. In fact, based on The Cancer Genome Atlas endome-

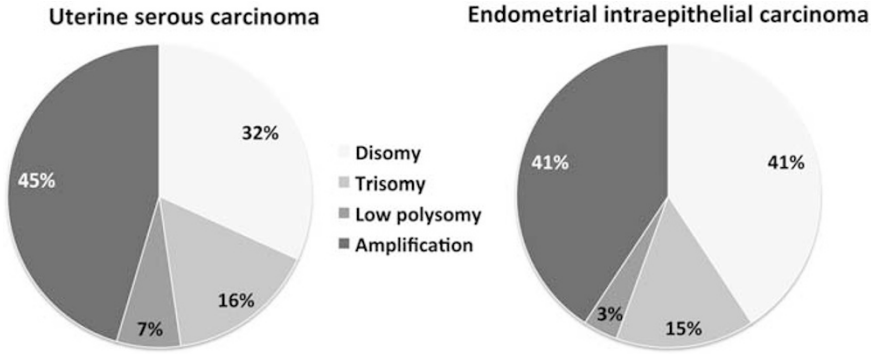

Figure 1 Summary of percentage of cases showing different *CCNE1* copy number changes. A total of 44 primary uterine serous carcinomas and 27 endometrial intraepithelial carcinomas were analyzed by fluorescence *in situ* hybridization.

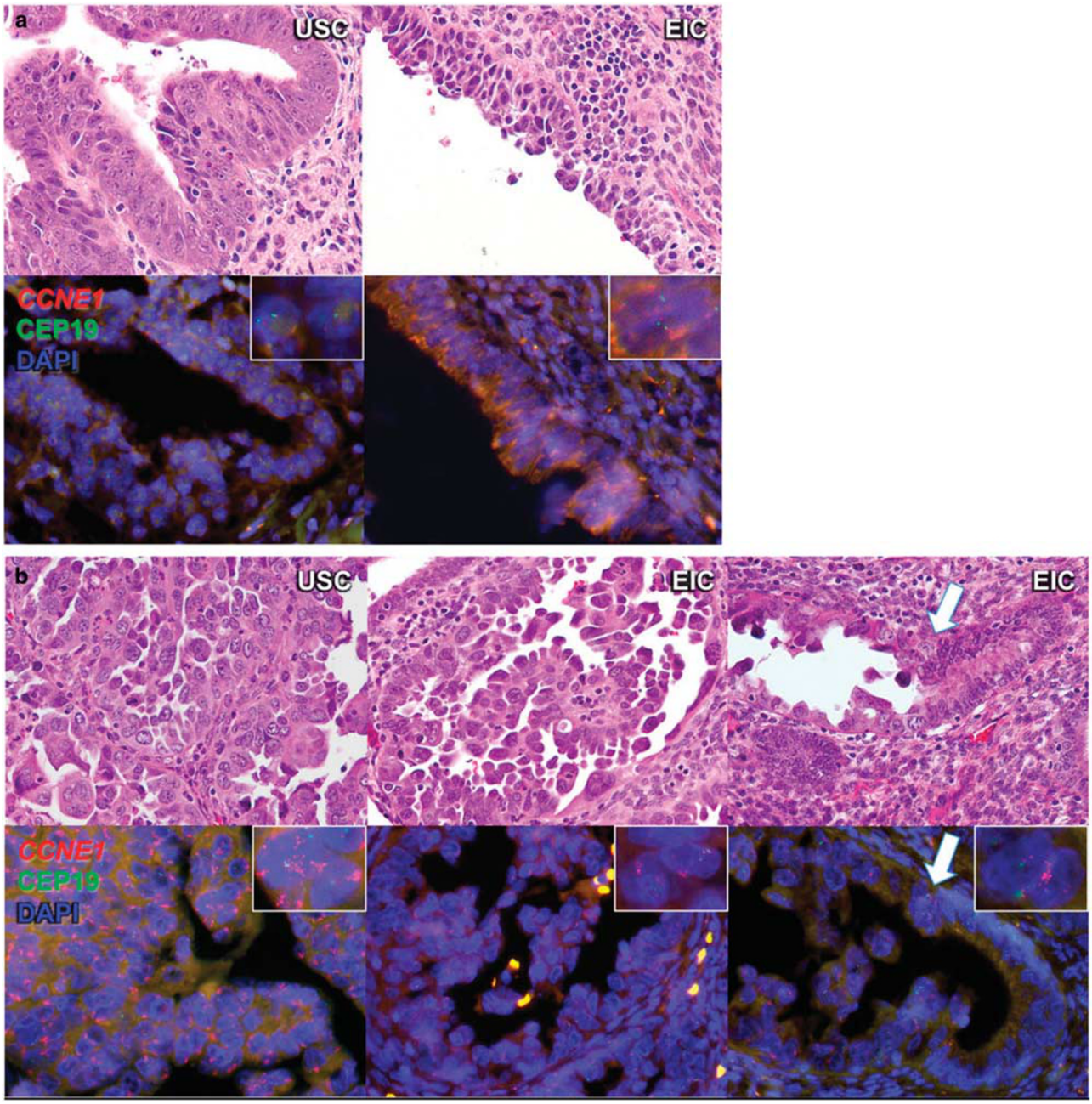

Figure 2 Examples of uterine serous carcinoma (USC) and concurrent endometrial intraepithelial carcinoma (EIC) (hematoxylin-eosin staining, top panels) with corresponding *CCNE1* status by fluorescence *in situ* hybridization (FISH) (bottom panels). (**a**) Both cancer components present disomy for *CCNE1* (red) as compared with centromeric probe, CEP19 (green). (**b**) FISH demonstrates *CCNE1* amplification in both uterine serous carcinoma and endometrial intraepithelial carcinoma components in another case. The arrow indicates the junction between normal-appearing endometrial epithelium and *CCNE1*-amplified endometrial intraepithelial carcinoma. Inserts: higher magnification.

trial cancer data set, *CCNE1* amplification and *TP53* mutations are specifically identified in 'serous-like' carcinomas (P-value<0.0001).[5] As compared with type I neoplasms, type II carcinomas are highly aggressive with worse overall survival. Previous studies have shown that type II cancers, including uterine serous carcinoma and ovarian high-grade serous carcinoma, are characterized by prominent DNA copy number alterations, which reflect a history of chromosomal instability.[3] Interestingly, *CCNE1* amplification and *TP53* mutations have been known to participate in promoting chromosomal instability, a feature essential for tumor development in many types of human cancer.[14–17]

In addition to the roles in chromosomal instability, cyclin E1 upregulation is critical for cellular

Table 1 *CCNE1* copy number by fluorescence *in situ* hybridization (FISH) in 44 primary uterine serous carcinomas and 27 endometrial intraepithelial carcinomas

Copy number category	*Uterine serous carcinoma,* n *(%)*	*Endometrial intraepithelial carcinoma,* n *(%)*
FISH negative	24 (55)	16 (59)
Disomy	14 (32)	11 (41)
Trisomy	7 (16)	4 (15)
Low polysomy	3 (7)	1 (3)
FISH Positive	20 (45)	11 (41)
Amplification	20 (45)	11 (41)
Total	44 (100)	27 (100)

Abbreviation: n, number of cases.

Table 2 Correlation between *CCNE1* copy number by fluorescence *in situ* hybridization (FISH) in 27 primary uterine serous carcinomas and concurrent endometrial intraepithelial carcinomas

Copy number category	*Uterine serous carcinoma,* n *(%)*	*Endometrial intraepithelial carcinoma,* n *(%)*
FISH negative	16 (59)	16 (59)
Disomy	10 (37)	11 (41)
Trisomy	4 (15)	4 (15)
Low polysomy	2 (7)	1 (3)
FISH Positive	11 (41)	11 (41)
Amplification	11 (41)	11 (41)
Total	27 (100)	27 (100)

Abbreviation: n, number of cases.

proliferation. For example, in breast and ovarian cancers, *in vitro* silencing of *CCNE1* suppresses cellular growth selectively in cells harboring *CCNE1* amplification.[10,18,19] On the other hand, ectopic expression of *CCNE1* increases cellular proliferation in ovarian cancer cell lines.[18] Moreover, these data support the driver role of *CCNE1* amplification in cancers as cyclin E1 mediates G1/S transition through the activation of CDK2. Interestingly, the survival of breast cancer cells harboring *CCNE1* gene amplification depends on CDK2 expression and kinase activity.[18] Consequently, *CCNE1* amplification is a potential biomarker of sensitivity to CDK2 inhibitors.

Like ovarian high-grade serous carcinomas of which many may develop from serous tubal intraepithelial carcinoma, uterine serous carcinoma frequently co-exists with endometrial intraepithelial carcinoma, the noninvasive component, which has been thought as a precursor lesion of uterine serous carcinoma.[3,20,21] *CCNE1* amplification in many endometrial intraepithelial carcinomas suggests that this genetic event occurs early during tumor progression of uterine serous carcinoma. Thus, both *TP53* mutation and *CCNE1* amplification are associated with early tumor development, as in ovarian high-grade serous carcinoma.[20,22] Since *TP53* mutation and cyclin E1 upregulation are cardinal features of endometrial intraepithelial carcinoma, it would be of great interest to determine if both molecular changes lead to tumorigenesis in endometrial epithelial cells in animal models.

Finally, the lack of an association between *CCNE1* amplification and clinical features may indicate that *CCNE1* amplification is important during very early stages of tumor development and thus does not impact on the aggressive behavior of uterine serous carcinoma. Similarly, the findings from The Cancer Genome Atlas did not show an association between *CCNE1* amplification and overall survival in endometrial serous-like carcinomas.[5] Alternatively, it may be due to a limited number of cases analyzed in this study.

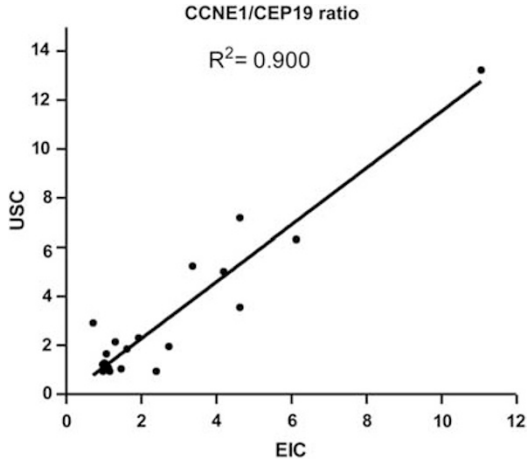

Figure 3 Correlation of *CCNE1* copy number between uterine serous carcinoma (USC) and endometrial intraepithelial carcinoma (EIC) from the same patients. The *CCNE1* copy number was presented as the ratio of *CCNE1* to CEP19, the centromeric region, and linear regression was used to model the relationship of the *CCNE1* copy number between uterine serous carcinoma and endometrial intraepithelial carcinoma. Each dot represents an individual case.

Table 3 Correlation between *CCNE1* amplification by fluorescence *in situ* hybridization in 40 primary uterine serous carcinomas and somatic mutations in *FBXW7, PIK3CA, PPP2R1A* and *TP53*

	Total	*FBXW7 mutation,* n *(%)*[a]	*PIK3CA mutation,* n *(%)*[a]	*PPP2R1A mutation,* n *(%)*[a]	*TP53 mutation,* n *(%)*[a]
CCNE1 amplified	17	6 (35)	2 (12)	3 (18)	15 (88)
CCNE1 nonamplified	23	5 (22)	4 (17)	3 (13)	17 (74)
Total	40	11 (28)	6 (15)	6 (15)	32 (80)

Abbreviation: n, number of cases mutated.
[a]Percentage of mutations in *CCNE1* amplified, non-amplified and total sample groups.

In conclusion, our FISH analysis provides cogent evidence that *CCNE1* amplification is a common molecular genetic alteration in uterine serous carcinoma and endometrial intraepithelial carcinoma. This finding validates the previous observation from genome-wide analysis based on comprehensive DNA copy number changes. Further studies are required to better delineate the clinical and biological impact of *CCNE1* amplification on overall survival and therapy response in uterine serous carcinoma patients.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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