19q12 amplified and non-amplified subsets of high grade serous ovarian cancer with overexpression of cyclin E1 differ in their molecular drivers and clinical outcomes

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Abstract:

Objectives: Readily apparent cyclin E1 expression occurs in 50% of HGSOC, but only half are linked to 19q12 locus amplification. The amplified /cyclin E1^{hi} subset has intact *BRCA1/2*, unfavorable outcome, and is potentially therapeutically targetable. We studied whether non-amplified /cyclin E1^{hi} HGSOC has similar characteristics. We also assessed the expression of cyclin E1 degradation-associated proteins, FBXW7 and USP28, as potential drivers of high cyclin E1 expression in both subsets.

Methods: 262 HGSOC cases were analyzed by *in situ* hybridization for 19q12 locus amplification and immunohistochemistry for cyclin E1, URI1 (another protein encoded by the 19q12 locus), FBXW7 and USP28 expression. Tumors were classified by 19q12 amplification status and correlated to cyclin E1 and URI1 expression, *BRCA1/2* germline mutation, FBXW7 and USP28 expression, and clinical outcomes. Additionally, we assessed the relative genomic instability of amplified/cyclin E1^{hi} and non-amplified/cyclin E1^{hi} groups of HGSOC datasets from The Cancer Genome Atlas.

Results: Of the 82 cyclin E1^{hi} cases, 43 (52%) were amplified and 39 (48%) were non-amplified. Unlike amplified tumors, non-amplified/cyclin E1^{hi} tumor status was not mutually exclusive with *gBRCA1/2* mutation. The non-amplified/cyclin E1^{hi} group had significantly increased USP28, while the amplified/cyclin E1^{hi} cancers had significantly lower FBXW7 expression consistent with a role for both in stabilizing cyclin E1. Notably, only the amplified/cyclin E1^{hi} subset was associated with genomic instability and had a worse outcome than non-amplified/cyclin E1^{hi} group.

Conclusions: Amplified/cyclin E1^{hi} and non-amplified/cyclin E1^{hi} tumors have different pathological and biological characteristics and clinical outcomes indicating that they are separate subsets of cyclin E1^{hi} HGSOC.

Keywords: HGSOC; Cyclin E1; 19q12; CCNE1; FBXW7; DDR

INTRODUCTION

Amplification of *CCNE1* is one of very few molecular therapeutic targets in high grade serous ovarian cancer (HGSOC), occurring in 15-30% of HGSOC cases [1, 2]. This subset typically has intact homologous recombination repair pathways (HR), as *CCNE1* amplification is mutually exclusive with germline *BRCA1* or *BRCA2* mutation [2]. *CCNE1*-amplified tumors respond poorly to platinum therapy and have limited responsiveness to PARP inhibitors [2, 3], which has focused efforts on developing targeted therapy for this particular subgroup [4].

CCNE1 is located on chromosome 19q12, within a 0.2Mb cluster of five tightly co-regulated genes, including *C19orf2* (*URI1*) [5]. Cyclin E1, the cell cycle protein encoded by *CCNE1*, is a master regulator of progression through G_1/S phase and centrosome duplication. Overexpression of cyclin E1 drives cell cycle progression and correlates with platinum-resistance [5, 6]. In ovarian cancer cells, suppression of *CCNE1* results in G_1 arrest, reduced cell viability and apoptosis but only when cells are 19q12 amplified. This suggests that cyclin E1 is the main driver oncogene of 19q12 amplification [5], although there is evidence that URI1-amplified ovarian cancer cells can be dependent on URI1 function for their survival [7].

High cyclin E1 expression occurs in up to 50% of HGSOC cases [8]. In both HGSOC and other tumor types it is apparent that high expression can occur in the absence of 19q12 amplification [8-11]. To date the studies on high cyclin E1 expression as a molecular subtype of HGSOC have been almost exclusively in the context of *CCNE1* amplification, and it is not known whether tumors with cyclin $E1^{hi}$ in the absence of 19q12 amplification have the same clinical and biological features. Understanding the subsets of cyclin E1 over expressing tumors that may be targeted is important as clinical trials advance [12], especially given that *in vitro* studies suggest a requirement for *CCNE1* amplification for therapeutic efficacy [5, 13].

Apart from amplification, disruption of degradation is one of the major mechanisms driving high cyclin E1 protein in cancer. In normal cell cycles the cyclin E1 protein is proteosomally degraded during the cell cycle [14]. The FBXW7 subunit of the SCF^{FBXW7} ubiquitin ligase complex binds and ubiquitinates phosphorylated cyclin E1 during S phase, marking it for degradation [15, 16]. Loss-of-function *FBXW7* mutations lead to cyclin E1 stabilization but are infrequent in HGSOC [17]. However, FBXW7 can be inactivated by other mechanisms such as antagonism by the deubiquitinase USP28. This occurs through two mechanisms, loss of USP28 leads to FBXW7 autoubiquitination and degradation, or high USP28 will deubiquitinate and stabilize FBXW7-target proteins, including cyclin E1 [18-20].

In this study we address the prevalence of high cyclin E1 expression in HGSOC and its relationship to 19q12 amplification, *BRCA1/2* status, genomic stability and patient outcome. We address for the first time the expression of cyclin E1 degradation-associated proteins in relation to cyclin E1 expression with or without *CCNE1* amplification. These investigations reveal that amplified/cyclin $E1^{hi}$ and non-amplified/cyclin $E1^{hi}$ are non-equivalent subsets of HGSOC, with distinct associations to *BRCA* status, FBXW7 and USP28 proteins, genomic instability and patient outcome.

METHODS

Tumor samples

Seven tissue microarrays (TMA) representing 341 cases of HGSOC were provided by the Australian Ovarian Cancer Study (AOCS), of which 262 cases were assessable in terms of sufficient tumor tissue and staining quality for immunohistochemistry (IHC) and *in situ* hybridization (ISH). The World Health Organization criteria were used to histologically classify ovarian cancers as HGSOC [21]. The International Federation of Gynecology and Obstetrics (FIGO)/American Joint Committee on Cancer staging system was used for tumor staging [22]. Ethics

board approval was obtained at all institutions for patient recruitment, sample collection and research studies. Written informed consent was obtained from all participants for participation in research studies.

Dual-Colour ISH assay for detection of the 19q12 locus amplification status

A pre-diluted ready to use 19q12 DNP ISH probe that covers the coding sequences of the *CCNE1* and *URI1* genes [9] and an insulin receptor (INSR) DIG ISH probe a surrogate reference for diploid copy number located on chromosome 19p13.2, were provided by Ventana Medical Systems (Tucson, AZ, USA). The assay was optimized for use on the Ventana ULTRA^{TM} platform. Assessable cases were those with interpretable black (19q12) and red (INSR) signals in normal and malignant cells, at least 50 assessable malignant cells, and minimal background staining. Amplification status at the 19q12 locus was determined by dividing the average 19q12 copy number by the average INSR copy number for 50 tumor cells per core. Tumors with a 19q12: INSR ratio ≥ 3 and / or 19q12 locus copy number ≥ 6 were considered amplified (assessment criteria and staining technique are detailed in Supplementary methods, and Supplementary Figure S1A-F).

Cyclin E1, URI1, FBXW7 and USP28 Immunohistochemistry

Cyclin E1 (clone sc-247-HE12) (Santa Cruz Biotechnology, CA) 1:100, and URI1 (clone 1-21) (Ventana) mouse and rabbit monoclonal antibodies (pre-diluted), FBXW7 rabbit monoclonal 1:25, which detects the three isoforms α,β and γ (SP-237) (Spring Bioscience, CA) and the USP28 rabbit polyclonal (HPA006778) 1:50 (Sigma Aldrich) antibodies were optimized using the Ventana Bench Mark ULTRA^{TM} automated staining platform and the Optiview^{TM} Detection kit. Cyclin E1, FBXW7 and USP28, were assessed based on nuclear staining whereas URI1 expression was assessed by cytoplasmic staining. Positive controls were included in each cycle from HGSOC for cyclin E1 and URI1, uterine cervix for FBXW7, and colorectal cancer for USP28. The expression of each of the proteins was assessed using a 0

to 3+ intensity score. A semi quantitative H score was obtained by adding 3 times the percentage of strongly staining (3+) cells plus 2 times the percentage of moderately staining (2+) cells plus 1 times the percentage of weakly staining (1+) cells, giving a range of 0 to 300. FBXW7 staining was almost exclusive of the nuclear membrane. Heterogeneous expression was captured using the semi-quantitative H score (Supplementary Methods, Supplementary Figure S1H-I).

For both ISH and IHC, the tumors were scored by a trained and qualified observer blinded to each of the IHC results, *BRCA1/2* status and clinical outcomes. Categorization of ambiguous cases was confirmed by a pathologist.

Statistics

Statistical analysis was performed using Prism Software™ version 7. We determined the frequency and correlation of *19q12* amplification and cyclin E1 expression across our cohort using protein and copy number assessments from consecutive sections of TMAs. We developed optimized cut-offs for amplified versus non-amplified and high versus low expressers using Receiver Operator Curve (ROC) that reflected the highest sensitivity and specificity of gene/protein expression correlation (Supplementary methods, Supplementary Figure S1F-G) as well as the best correlation with outcome. Kaplan Meier curves were used to plot the progression free survival (PFS) and overall survival (OS).

RESULTS

Patient demographics

Clinical data from 262 HGSOC cases from patients enrolled in the AOCS is provided in Table 1. The age of the patients ranged between 30.2 and 80 years, with a median age of 60.1 years. The median PFS from the time of diagnosis was 14.20 months, and the median OS was 40.14 months. One case was lacking PFS and OS data. Twenty six cases were untested for *BRCA1/2* (10%) and 236 cases had documented germ-line *BRCA1* and *BRCA2* status, 41 (15.6%) with *BRCA1* mutation, 25 (9.5%) with *BRCA2* mutation and 170 (64.9%) being wild type for both.

High cyclin E1 expression occurs independently of 19q12 amplification

19q12 (*CCNE1*) amplification and cyclin E1 expression were assessable in 262 cases. Overall, 20.2% (n=53) of the cohort had 19q12 amplification, 13% (n=34) had low copy number gain and the remaining 66.8% (n=175) were disomic (Figure 1A). Eighty one percent (43/53) of 19q12 amplified cases had high level cyclin E1 immunostaining. Only a moderate correlation was found between 19q12 (*CCNE1*) copy number and cyclin E1 expression ($r=0.46$, $p<0.0001$, Figure 1A) because a third of the high expressers did not demonstrate any copy number gain of *CCNE1* and 12.1% (n=10) had low copy number gain (Figure 1B). Conversely 18.9% (n=10) of amplified samples had low levels of cyclin E1 immunostaining. Therefore, while *CCNE1* amplification is consistently associated with cyclin E1 overexpression, almost half of tumors with high levels of cyclin E1 protein overexpression appears to be due to non-amplification-dependent mechanisms.

19q12 amplification is homogenous within HGSOC samples

Clonal variation within solid cancers is common including subclonal involvement of driver mutations [23]. We determined the clonal status of *19q12* amplification to assist in validating it as a therapeutic target, and also assess whether tumors could

be considered simply as amplified or non-amplified. Comparing locus copy number values in 153 cases with duplicate tissue cores revealed a highly significant correlation between the locus copy numbers (r=0.94, p<0.0001) (Figure 1C). Next, we examined full-face tissue sections from multiple tumor samples of 5 amplified and 2 non-amplified cases with high cyclin E1 expression. Sections from each were stained with the 19q12 probe and the locus copy number was assessed in 50 cells in three representative regions from each tumor section. We found that amplification was consistently high (≥6 *19q12* copy number) in the full-face sections from those samples identified as having 19q12 amplified TMA cores, and that these tumors also had invariably high cyclin E1 expression (H score range: 160-270). An example of a 19q12 amplified cyclin $E1^{Hi}$ tumor is shown in Figure 1 for IHC and ISH (Figure 1D), TMA cores (Figure 1E) and full-face sections (Figure 1F), and four further cases in Supplementary Figure S2A. Conversely, the two cases identified as non-amplified cyclin $E1^{Hi}$ from TMA cores had low amplification in all regions of the full-face tissue sections (<6 *19q12* copy number) and high H scores in all regions (H score range: 150-210) (Supplementary Figure S2B). These findings are consistent with previous reports of *CCNE1* amplification being an early event in the genesis of HGSOC [24, 25], and a prior observation of homogenous 19q12 amplification through an ovarian tumor [26].

Low FBXW7 and high USP28 may augment high cyclin E1 expression in amplified/cyclin $E1^{hi}$ and non-amplified/cyclin $E1^{hi}$ cases, respectively

To explore potential mechanisms underlying high cyclin E1 expression in non-amplified tumors, we investigated whether cyclin E1 overexpression was related to impaired proteasomal degradation of cyclin E1 due to loss of FBXW7 expression and/or change in USP28 expression. We correlated cyclin E1, FBXW7 and USP28 expression with 19q12 amplification status. Both FBXW7 and USP28 expression ranged between 0 and 300 and the median H scores of 40 and 110, respectively,

were used as a cut-off for high and low expression levels. Exhaustion of tissue cores led to unequal numbers of assessable cases for each antibody.

Loss of FBXW7 leads to stabilization of cyclin E1, and, notably, 31% (n=13/36) of the amplified high cyclin E1 expresser cases had no apparent FBXW7 expression (Figure 2A). Examples of the expression levels of FBXW7 observed in different cases of HGSOC are shown in Figure 2B. In tumors with high levels of cyclin E1 (n=75), low FBXW7 was positively associated with 19q12 amplification (p=0.010, Fisher Exact Test), suggesting that *CCNE1* amplification also requires reduced cyclin E1 turnover to maintain high protein levels to exert its oncogenic effect. In contrast, no significant difference in FBXW7 expression was observed in the group with low cyclin E1 expression, regardless of 19q12 copy number (p=0.745, n=162) (Figure 2C).

USP28 can induce cyclin E1 stabilization either through its absence, which leads to autoubiquitination and degradation of FBXW7, or through its overexpression, which leads to deubiquitination and stabilisation of cyclin E1 [20]. We examined our cohort for loss of USP28 expression. Absence of USP28 protein was found in only 6 cases and is therefore unlikely to impact on FBXW7 or cyclin E1 expression.

The remainder of cases showed a wide range of USP28 staining, and there was a moderate but significant positive correlation between USP28 expression and cyclin E1 across these cases (Figure 3A). Examples of USP28 expression are shown in Figure 3B. Among tumors with high levels of cyclin E1 (n=76), USP28 was significantly higher in non-amplified/cyclin $E1^{hi}$ compared to amplified cases/cyclin $E1^{hi}$ (p=0.009, Fisher Exact Test). Similar to FBXW7, no significant difference in USP28 expression was observed in the group of low cyclin E1 expressers, whether amplified or not (p>0.999, n=165) (Figure 3C).

We also examined TCGA ovarian cancer dataset for gene amplification and protein expression finding that amplification of USP28 had a non-significant trend to be mutually exclusive with *CCNE1* amplification (p=0.14) (Figure 3D). We analyzed HGSOC cases from the TCGA that were diploid at the 19q12 locus or displayed low level gain, and found a non-significant trend for high cyclin E1 protein expression to be associated with increased copy number of USP28 (p=0.07, Mann-Whitney test; Figure 3-E). Together with the findings in our cohort, these data suggest that reduced protein turnover may contribute to high cyclin E1 expression in the non-amplified cases, possibly by USP28-mediated functional deactivation of FBXW7.

BRCA1/2 germline mutations are significantly more prevalent in non-amplified/cyclin E1^{hi} cases than in amplified cases/cyclin E1^{hi}

We, and others [27, 28] (Figure 4A) had previously noted the mutual exclusivity of *CCNE1* amplification with mutation in either *BRCA1* or *BRCA2* (*BRCA1/2*), arising due to synthetic lethality associated with these events [29]. Mutual exclusivity therefore provides a measure by which we could indirectly evaluate the effect of cyclin E1 over expression in the absence of amplification. We related *BRCA1* and *BRCA2* germline mutation status to cyclin E1 protein and *19q12* locus copy number in 236 samples where complete information was available.

The overall distribution and correlation of cases by their germline *BRCA1* and *BRCA2* status versus their 19q12: INSR ratio and cyclin E1 protein expression (n=236) is shown in Figure 4B. Regardless of cyclin E1 expression, the amplified cohort had significantly fewer cases with *BRCA1/2* mutations than the non-amplified cohort (p=0.005, Fisher's Exact Test) (Figure 4C). When further subdivided by protein and copy number grouped by amplification, a significantly higher rate of *BRCA1/2* mutation was observed in non-amplified/cyclin E1^{hi} (9 out of 37 (24.3%) of cases) compared to amplified cases/cyclin E1^{hi} (2 out of 34 (5.9%) of cases) (p=0.050, Fisher's Exact Test) (Figure 4D). No significant difference in *BRCA1/2*

mutation rate was observed in non-amplified/cyclin E1^{lo} compared to amplified/cyclin E1^{lo} (Figure 4E). Thus, the mutual exclusivity between *CCNE1* amplification and *BRCA1/2* does not appear to extend to non-amplified/cyclin E1^{hi} tumors.

High expression of cyclin E1 is associated with poorer survival when accompanied by 19q12 amplification

As a further measure of the impact of cyclin E1 over expression in the absence of amplification, we evaluated patient outcome in amplified and non-amplified subjects. Subjects were divided into four groups according to locus copy number (amplified or non-amplified) and cyclin E1 expression status (high or low). Patients with amplified/cyclin E1^{hi} tumors had significantly worse OS when compared to the non-amplified/cyclin E1^{lo} group (median 28.57 vs. 45.6 months, HR 0.6, 95% CI, 0.43 to 0.91, p=0.0202). Although amplified/cyclin E1^{hi} cases had shorter OS compared to non-amplified/cyclin E1^{hi} cases, the difference was not statistically significant (median 28.6 vs. 43.3 months, HR 0.7, 95% CI, 0.41 to 1.07, p=0.298). Moreover, within the amplified subset, amplified/cyclin E1^{hi} had a shorter OS when compared to the amplified/cyclin E1^{lo} (median 28.57 vs. 66.34 months) however due to the small number of amplified low expressers, the study was not powered to give a statistical significance (p=0.20). Additionally, for the non-amplified cases, non-amplified/cyclin E1^{hi} tumors had no significant impact on OS compared to non-amplified/cyclin E1^{lo} tumors (median 43.33 vs. 45.6 months, HR 0.9, 95% CI, 0.64 to 1.41, p=0.40). However, when the four groups compared the OS was not significantly different (p=0.0955). Neither locus copy number or expression status appeared to have an impact on PFS (Supplementary Figure S3A). These findings imply that for cyclin E1, both gene amplification and high protein expression are associated with poor outcome and that the clinical behaviour of tumors showing high expression of cyclin E1 without amplification is unlikely to be similar to those with amplification.

We performed a similar analysis using the TCGA ovarian cancer dataset, but instead comparing overall survival of subsets of patients defined by high or low *CCNE1* mRNA expression, and *CCNE1* gene amplification. The cutoff for high *CCNE1* expression was a z score of 0.7 based on the data distribution (Supplementary Figure S3B), and the tumors were classified as amplified or non-amplified cases using the GISTIC prediction used by TCGA. Generally, the OS of *CCNE1*^{hi} patients was not significantly different from that of *CCNE1*^{lo} patients (P=0.3365; Figure 4G). When we further divided these patients into groups with tumors that were amplified or not amplified, we found that the OS was not significantly different when comparing the 4 groups (P=0.0575). However, both patients with non-amplified/*CCNE1*^{lo} tumors and patients with non-amplified/*CCNE1*^{hi} tumors had significantly better survival compared to those with amplified/*CCNE1*^{hi} tumors (P=0.0125; P=0.0240 respectively, Figure 4H, Supplementary Figure S3C).

High cyclin E1 is associated with an index of chromosomal instability (CIN25) in 19q12 amplified cancers

HGSOC is characterized by its high genomic instability and cyclin E1 is a known driver of genomic instability, and in particular, chromosomal instability (CIN) [30]. The mutual exclusivity between *BRCA1/2* mutation and *CCNE1* amplification is thought to be due synthetic lethality arising from the failure to repair the excessive genomic instability associated with high cyclin E1 expression [31]. We assessed how cyclin E1 expression relates to genomic instability in HGSOC, and whether there is a difference between amplified/cyclin E1^{hi} HGSOC and non-amplified/cyclin E1^{hi} HGSOC. To do this we used the CIN25 gene signature [32]. CIN25 is the sum expression of 25 genes that correlate most highly with functional aneuploidy in tumors, and it is a significant predictor of clinical outcome in cancer [32].

Gene and protein expression data were obtained from TCGA for the Ovarian Serous Cystadenocarcinoma dataset, and a CIN25 signature was determined (see Supplementary Methods). We found that overall cyclin E1 protein had only a slight correlation to the ovarian CIN25 signature (Pearson's correlation=0.2164, p<0.0001) (Figure 5A). Tumors classified as 19q12 amplified by GISTIC had a moderate correlation with CIN25 (r=0.4221, p=0.0004) whereas non-amplified tumors only had a weak correlation (r=0.1772, p=0.0033). We then used Fisher Exact test to determine if there was a correlation between 19q12 amplification, cyclin E1 protein expression and CIN25. For this analysis, we classified tumors as cyclin $E1^{Hi}$ if they had a Z score >0 and high CIN25 for a Z score >0, (Supplementary Figure 3D). Overall, high cyclin E1 protein was significantly correlated with high CIN25 (p=0.0028), but this relationship only remained significant for 19q12 amplified cancers (p=0.0028) and not for non-amplified cancers (p=0.0810). Thus, the expression of cyclin E1 protein has a stronger association with chromosomal instability in amplified/cyclin $E1^{hi}$ cancers than in non-amplified tumors/cyclin $E1^{hi}$.

URI1, as another driver of 19q12 amplification

Given the previous suggested role of *URI1* in HGSOC [7] and its co-localization with *CCNE1*, we sought to investigate *URI1* as another driver for 19q12 amplification. We tested URI1 expression correlation to 19q12 amplification, cyclin E1 expression, clinical outcome and *BRCA1/2* status in both amplified and non-amplified subsets. URI1 data are provided in Supplementary Figure S4. Similarly to cyclin E1, URI1 expression correlated to 19q12 amplification (r=0.232 p=0.0005) (Supplementary Figure S4A), and representative URI1 IHC images are provided in Supplementary Figure S4B. URI1 was high in the majority of 19q12 amplified cancers (39/46; 84.8%), and high URI1 expression was also noted in (80/176; 45.5%) of tumors without 19q12 amplification (Supplementary Figure S4C).

Amplified/URI1^{hi} tumors also were depleted for germline *BRCA1/2* mutations. When grouped by amplification, a significantly higher rate of *BRCA1/2* mutation was noted in non-amplified/URI1^{hi} (19 out of 74 (25.7%) of cases) compared to amplified//URI1^{hi} cases (2 out of 34 (5.9%) of cases) (p=0.003, Fisher's Exact) while no significant difference was noted in non-amplified/URI1^{lo} compared to amplified/URI1^{lo} (Supplementary Figure S4D and E).

The majority of 19q12-amplified cases co-expressed both URI1 and cyclin E1. Similar to *19q12*-amplified tumors, amplified/URI1^{hi} patients had the worst outcome (p=0.0021) (Supplementary Figure S4F). The clinical impact of cases that were *URI1*-amplified but not *CCNE1*-amplified was not assessable as the dual ISH probe covers both *CCNE1* and *URI1* genes and the majority of amplified cases expressed both proteins. This finding is in agreement with TCGA data where out of 316 cases of HGSOC, none of the cases were *URI1* amplified but not *CCNE1* amplified, while only 9 out of 64 (14%) cases were *CCNE*1 but not *URI1* amplified (Figure 4A). The relative contribution of *URI1* and *CCNE1* to the behavior of 19q12-amplified tumors is difficult to untangle due to the closely correlated nature of amplification of the two genes. A moderate correlation between cyclin E1 and URI1 protein expression irrespective of 19q12 amplification suggests that these proteins could act in concert to drive HGSOC growth and survival (Supplementary Figure S4G).

DISCUSSION

CCNE1 amplification currently defines the largest molecular subset of HR-proficient HGSOC. *BRCA1* and *BRCA2* HR-deficient HGSOC is the subject of much interest due to the therapeutic efficacy of PARP inhibitors, but no equivalent has been developed for HR-proficient HGSOC. CDK2 inhibitors are an attractive potential treatment for cyclin E1 overexpressing tumors and show promising efficacy in *CCNE1* amplified cell lines and xenografts [33, 34], especially in combination with AKT inhibitors [4]. It is not known whether this approach will be limited to tumors with *CCNE1* amplification, and for this reason we have defined the characteristics of HGSOC cancers with high cyclin E1 in the absence of gene amplification.

Non-amplified/cyclin $E1^{hi}$ tumors make up 33-47% of all cyclin $E1^{hi}$ HGSOC, and three orthogonal findings suggest that non-amplified/cyclin $E1^{hi}$ HGSOC has distinct molecular attributes. First, the almost complete mutual exclusivity of *CCNE1* amplification and germline *BRCA1/2* mutation does not extend to non-amplified/cyclin $E1^{hi}$ tumors as non-amplified/cyclin $E1^{hi}$ cancers more frequently exhibit *BRCA1* or *BRCA2* mutations. Second, evidence of CIN was more frequent in *CCNE1* amplified tumors. Finally, the clinical outcome of patients with 19q12 amplification was worse than those with cyclin E1 over expression in the absence of amplification.

The regulatory systems that control the degradation of cyclin E1 are often disrupted in cancer, and in HGSOC we find that different mechanisms may stabilize cyclin E1 depending on the amplification status of *CCNE1*. Low or absent FBXW7 expression is more common in 19q12 amplified/cyclin $E1^{hi}$ cases, and high expression of its antagonist, USP28, is common to non-amplified cases. The low FBXW7 expression in amplified/cyclin $E1^{hi}$ subset suggests that the proteosomal degradation of cyclin E1 needs to be impaired in order to sustain high cyclin E1 levels. Since somatic inactivating FBXW7 mutations are uncommon in ovarian cancer, absent or reduced

FBXW7 expression may result from silencing of FBXW7, for instance, by promoter hypermethylation [17-19]. Notably, loss of either p53 or p21 allows persistent high levels of cyclin E1, resulting from FBXW7 loss, to continuously drive genome instability [6, 35] and FBXW7 has been demonstrated to drive chromosomal instability via upregulation of cyclin E1 [36].

We speculate that persistently high level cyclin E1 expression, caused by *CCNE1* amplification, during S phase, potentially exacerbated by epigenetic silencing of FBXW7, results in oncogene – induced replication stress. This aberration generates chromosomal instability that leads to tumors with inherently poor clinical outcomes. Therefore, a successful replication stress response would be required to maintain replication competency and would therefore depend upon various intact DNA repair mechanisms (DDR), including the Ataxia-Telangiectasia mutated and Rad 3-related (ATR)-CHK1-WEE1 and HR pathways [37, 38]. This presumably accounts for the mutual exclusivity of *CCNE1* amplification and *BRCA1* and *BRCA2* mutations and the consequent resistance of *CCNE1* amplified tumors to platinum chemotherapy. Accordingly, it is not unreasonable to speculate that the amplified subset is likely to respond to DDR inhibition through CHEK1 and/or Wee1 inhibitors.

In non-amplified//cyclin E1^{hi} cancers we observe high levels of USP28 rather than decreased FBXW7. We speculate that this is because these cells do not have the observed genomic instability that is associated with 19q12 amplification in our analysis of the correlation of cyclin E1 expression to CIN25 in the TCGA HGSOC cohort. In unstressed cells, USP28 forms a complex with FBXW7 and antagonizes substrate ubiquitination, however, after DNA damage this complex dissociates promoting FBXW7-dependent substrate degradation [20]. Accordingly, the co-expression of cyclin E1 and USP28 in the 19q12 non-amplified HGSOC suggests that USP28 overexpression maintains persistently high cyclin E1 levels during S phase in the absence of replication stress and DNA damage associated with

CCNE1 amplification.

In this study, we have shown, for the first time, significant biological and clinical differences between 19q12 amplified and non-amplified HGSOC cases that overexpress cyclin E1. These observations have implications for choice of therapy for each of these subsets. First, some non-amplified/cyclin $E1^{hi}$ HGSOC have HR deficiencies that may make them responsive to platinum based therapies and / or PARP inhibitors. This may explain the failure of cyclin E1 expression to predict taxane-platinum chemoresistance, when not characterized by 19q12 amplification status [39]. Non-amplified cases could also potentially be treated with protease inhibitors that specifically target deubiquitinases such as USP28 [40], but this is dependent on showing that this HGSOC subset is reliant on cyclin E1 expression. By contrast, the amplified subset could feasibly respond to CHEK1 and/or Wee1 inhibitors as they are likely to become dependent upon the ATM-ATR-CHEK1-Wee1 pathway. These distinct therapeutic options depend on the molecular attributes of each cyclin $E1^{hi}$ subset, and thus indicate caution in combining all cyclin $E1^{hi}$ HGSOC patients into clinical trials without a careful consideration of *CCNE1* amplification status.

Conflict of interest statement:

We make the disclosure that Dr. Waring and Dr. Aziz received non-financial support from Ventana Medical Systems, who provided reagents to perform the project and Professor David Bowtell receives funding from AstraZeneca Australia and Roche-Genentech to support research outside the submitted work. Dr Waring was an employee of Roche during part of this study.

Authors' contribution:

Study conception and design: P.W., D.B., D.E., G.A.; Acquisition of data: D.A., AOCS, N.D., C.E.C.; Analysis and interpretation of data: D.A., P.W., D.E., C.E.C., R.H.; Drafting of manuscript: D.A., P.W., C.E.C.; Critical revision: D.A., C.E.C., P.W., D.B., D.E.

FIGURE AND TABLE LEGENDS

TABLE 1: AOCS cohort patients' demographics

Figure 1:**19q12 (*CCNE1*) amplification correlates with cyclin E1 expression and the amplification is homogenous. A:** The distribution of cyclin E1 by 19q12: INSR ratio. **B:** Segregation of high versus low cyclin E1 expressers by 19q12 amplification status. **C:** Concordance of 19q12: INSR ratios between duplicate cores for 153 cases. r = Spearman coefficient, p = probability. **D:** Microscopic images of 19q12 ISH (lower) and cyclin E1 IHC (upper) on two cores from the left ovary and omentum from case #1973. Scale bar is 250 μm. **E:** 19q12 copy number and cyclin E1 H scores for TMAs from case #1973. **F**: 19q12 copy number and cyclin E1 H scores for different regions per block for multiple FFPE blocks/sites of representative case #1973 (Dotted line represents 19q12 copy number amplification threshold; n = 50 cells per region).

Figure 2: **FBXW7 expression is low or absent in amplified/cyclin E1^{hi} cases** **A:** Association of FBXW7 expression with cyclin E1 in all, amplified and non-amplified cases. Dashed lines on each axis represent the cutoff between high and low cyclin E1 (x-axis) and high and low FBXW7 expression (y-axis). **B:** Microscopic images of various expression of FBXW7 in different cases of HGSOC. Scale bar is100 μm. **C:** Association of FBXW7 high and low expression by 19q12 status and cyclin E1 high (left) and low (right) expression. p = probability (Fisher Exact test).

Figure 3: **USP28 expression is high in non-amplified/cyclin E1^{hi} cases** **A:** Association of USP28 expression with cyclin E1 in all, amplified and non-amplified cases. Dashed lines on each axis represent the cut-off between high and low cyclin E1 (x-axis) and high and low USP28 expression (y-axis) **B:** Microscopic images of

various expression of USP28 in different cases of HGSOC. Scale bar is100 μm. **C:** Association of USP28 high and low expression by 19q12 status and cyclin E1 high (left) and low (right) expression. p = probability (Fisher Exact test) **D:** Cyclin E1 amplification tends to be mutually exclusive with *USP28* amplification in HGSOC from TCGA datasets. **E.** *USP28* copy number gain has a non-significant trend for association with high cyclin E1 protein expression in the HGSOC TCGA dataset, P-value for two-sided Mann-Whitney test, error bars indicate 95% confidence interval.

Figure 4: Mutual exclusivity of 19q12 amplification, cyclin E1 expression and germline *BRCA1/2* mutation and the prognostic impact of 19q12 amplification and cyclin E1 expression.

A: TCGA oncoprint from 316 HGSOC cases showing mutual exclusivity of *CCNE1* and *URI1* copy number alterations from *BRCA1* and *BRCA2* mutation.

B: The distribution of *BRCA1/2* mutations against cyclin E1 (n=236) IHC H scores and 19q12: INSR ISH ratio (Dashed lines represent high cyclin E1 expression threshold and 19q12: INSR amplification threshold). **C-E:** The correlation of *BRCA1/2* mutation status vs 19q12 amplification status in the entire cohort Cyclin $E1^{hi}$, Cyclin $E1^{lo}$ cohorts. p = probability (Fisher Exact test). **F:** Kaplan Meier curves of overall survival for allocated subgroups of HGSOC, according to cyclin E1 expression status and amplification status. **G:** Kaplan Meier curves of overall survival for subgroups of HGSOC defined as *CCNE1* low (z-score<0.7) and *CCNE1* mRNA high (z-score>0.7) (n=530). **H:** Kaplan Meier curves of overall survival according to amplification status and *CCNE1* mRNA (n=530).

Figure 5: High cyclin E1 is associated with an index of chromosomal instability (CIN25) in 19q12 amplified cancers

A: Association between CIN25 and cyclin E1 expression across TCGA HGSOC cases. r = Pearson's coefficient, p = probability. **B:** High CIN25 (CIN25 >0) is

correlated with high cyclin E1 expression (cyclin E1 z-score>0) across 340 TCGA HGSOC cases. **C&D:** High cyclin E1 is correlated with high CIN25 in *CCNE1* amplified cases (C), but not in *CCNE1* non-amplified cases (D). p = probability (Fisher Exact test).

Supplementary table 1: Distribution of raw scores for INSR (< and ≥ 3) and 19q12 (<4, 4-6 and ≥6) by amplification status.

Supplementary Figure S1: Assessment of 19q12 status and determination of assay cut points.

A: Examples of dual ISH signal scoring (Black: 19q12 (*CCNE1)*, Red: *INSR*, adapted from Ventana Inform *Her2* Dual cocktail ISH assay guidelines. Small size dots=one copy, medium size dots=cluster of 6 copies, large size dots =cluster of 12 copies. **B-D:** Histogram distribution of **(B)** 19q12 and **(C)** INSR average copy number counts and **(D)** 19q12: INSR ratio (n=262). **E:** Correlation of 19q12: INSR ratio (ISH) with *CCNE1* copy number (qPCR) (n=59). r = Spearman coefficient, p = probability. **F&G** ROC analysis to determine the cutoffs of 19q12: INSR ratio (ISH) by cyclin E1 and URI1 expression H score **(F)** and of cyclin E1 and URI1 expression by 19q12: INSR ratio **(G)**. Circles and squares represent cut off points at highest sensitivity and specificity. **H&I:** Distribution of H scores for cyclin E1 **(H)** (n=262) and URI1 **(I)** (n=222).

Supplementary Figure S2: Homogenous 19q12 amplification and cyclin E1 expression in different sections of HGSOC tumors

19q12 copy number and cyclin E1 H scores for TMAs and different regions per FFPE block in individual tumors **A.** Matched TMAs and tissue blocks from 4 examples of 19q12 amplified cyclin $E1^{Hi}$ tumors. **B.** Matched TMAs and tissue blocks from 2

examples of 19q12 non- amplified cyclin E1^{Hi} tumors. (Dotted line represents 19q12 copy number amplification threshold; n = 50 cells per region).

Supplementary Figure S3: Effect of 19q12 copy number, cyclin E1 and *CCNE1* expression on progression free survival and overall survival in HGSOC.

A. Kaplan Meier curves of progression free survival of subgroups of HGSOC, defined by cyclin E1 protein expression status and 19q12: INSR ratio. **B.** Distribution of *CCNE1* mRNA z-scores in the HGSOC TCGA cohort, n=530. **C.** Log rank tests to compare Kaplan-Meier curves of overall survival for subgroups of HGSOC patients with amplified or non-amplified *CCNE1* gene locus, and high or low *CCNE1*. **D.** Distribution of cyclin E1 protein z-scores in the HGSOC TCGA cohort, n=340.

Supplementary Figure S4: URI1 amplification and expression in HGSOC.

A: The distribution of URI1 expression by 19q12: INSR ratio. **B:** Examples of various expression of URI1 in different cases of HGSOC. Scale bar is 100 μm. **C:** Segregation of high versus low cyclin E1 and URI1 expressers by 19q12 amplification status. **D&E:** The distribution of *BRCA1/2* mutations against URI1 (n=202) IHC high **(D)** and low **(E)** scores and 19q12: INSR ISH ratio. **F:** Kaplan Meier curves of overall survival for URI1 by allocated subgroups of HGSOC, according to protein expression status and 19q12: INSR ratio**. (G):** Association of cyclin E1 expression with URI1 in all, amplified and non-amplified cases. Dashed lines on each axis represent the cutoff between high and low cyclin E1 (x-axis) and high and low URI1 expression (y-axis).

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